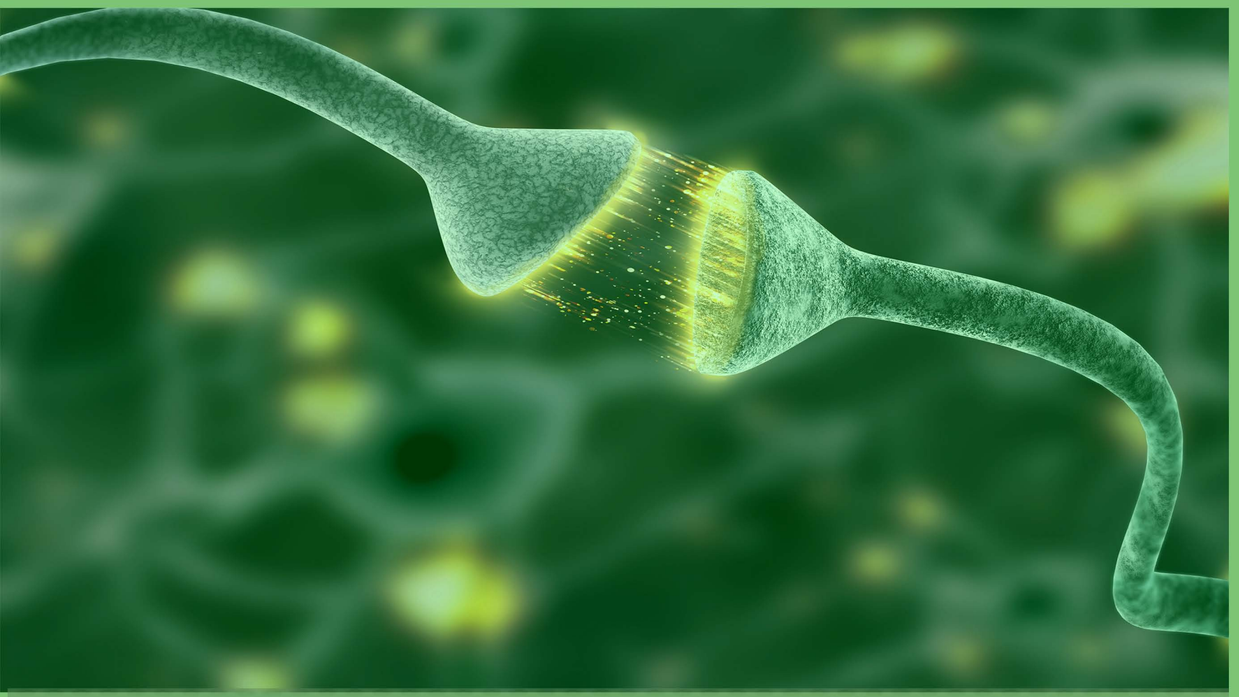
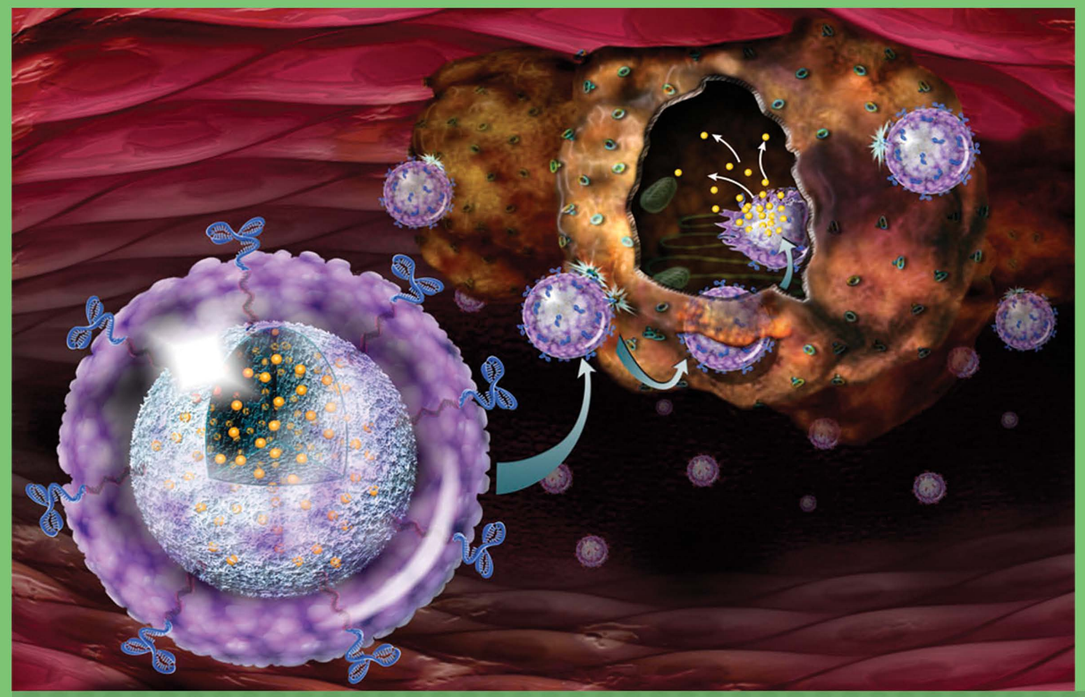


Ph.D. Thesis

SHILPA JOY

JULY 2013

# GABA AND 5-HT CHITOSAN NANOPARTICLES ENHANCED LIVER CELL PROLIFERATION AND NEURONAL SURVIVAL IN PARTIALLY HEPATECTOMISED RATS: $GABA_B$ AND $5\text{-}HT_{2A}$ RECEPTORS FUNCTIONAL REGULATION



*Ph.D. Thesis*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**THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF**

**DOCTOR OF PHILOSOPHY**

**IN**

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**OF**

**COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY**

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## *CERTIFICATE*

This is to certify that the thesis entitled **"GABA and 5-HT chitosan nanoparticles enhanced liver cell proliferation and neuronal survival in partially hepatectomised rats: $GABA_B$ and $5\text{-}HT_{2A}$ receptors functional regulation"** is a bonafide record of the research work carried out by **Mrs. Shilpa Joy,** under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree. All the relavent corrections and modifications suggested by the audience during the pre-synopsis seminar and recommentaions by the Doctoral Committee of the candidate has been incorporated in the thesis.

**Cochin – 682 022**
**JULY 15, 2013** **(C. S. Paulose)**

## DECLARATION

I hereby declare that the thesis entitled "**GABA and 5-HT chitosan nanoparticles enhanced liver cell proliferation and neuronal survival in partially hepatectomised rats: $GABA_B$ and 5-$HT_{2A}$ receptors functional regulation"** is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance of Dr. C. S. Paulose, Professor-Emeritus, UGC-BSR Faculty Fellow, Department of Biotechnology and no part thereof has been presented for the award of any other degree, diploma, associateship or other title or recognition from any University / Institution.

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*My tribute…..to a number of animals who have paid a price with their lives and suffering in the name of human protection. I pay my tribute to their sacrifice and pray that it is not in vain.*

*The woods are lovely, dark and deep.*

*But I have promises to keep, and miles to go before I sleep*

*Robert Frost*

*In all, the journey to my Ph.D. has been a long and challenging road. And I am thrilled it is done. But most of all, I am excited for what the future holds….*

***Shilpa Joy***

*Dedicated to my beloved family. . .*

## ABBREVIATIONS

| | |
|---|---|
| 5-HT | Serotonin |
| 5-HTT | Serotonin Transporter |
| AC | Adenylyl Cyclases |
| Bax | Bcl-2–associated X protein |
| BBB | Blood-brain barrier |
| BDNF | Brain-derived neurotrophic factor |
| BrdU | Bromodeoxyuridine |
| $Ca^{2+}$ | Calcium ions |
| cAMP | Cyclic adenosine monophosphate |
| Caspases | Cysteine-dependent aspartate-specific proteases |
| cGMP | Cyclic guanosine monophosphate |
| CNS | Central nervous system |
| CREB | Cyclic AMP binding protein |
| DAG | Diacylglycerol |
| ECD | Electrochemical detector |
| EDTA | Ethylene diamine tetra acetic acid |
| GABA | Gamma amino butyric acid |
| GAD | Glutamic acid decarboxylase |
| GDNF | Glial cell line-derived neurotrophic factor |
| $H_2O_2$ | Hydrogen peroxide |
| HGF | Hepatocyte growth factor |
| HPLC | High performance liquid chromatography |

| | |
|---|---|
| $IP_3$ | Inositol trisphosphate |
| IGF-I | Insulin like groth factor-1 |
| IP3R | Inositol trisphosphate receptor |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate |
| JNK | $NH_2$-terminal Jun kinases |
| MAPK | Mitogen-activated protein kinase |
| NF-κB | Nuclear factor-kappa B |
| NGF | Nerve growth factor |
| PBS | Phosphate buffered saline |
| PFA | Paraformaldehyde |
| PH | Partial hepatectomy |
| PI3 | Phosphatidyl inositol-3 |
| PI3-K | Phosphatidylinositol 3-kinase |
| PLC | Phospholipase C |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| ROS | Reactive oxygen species |
| SOD | Superoxide dismutase |
| TNF-α | Tumor necrosis factor- α |
| TPP | Trisodium penta polyphosphate |

# CONTENTS

# *Introduction*

Nanoparticulate drug delivery systems provide wide opportunities for solving problems associated with drug stability or disease states and create great expectations in the area of drug delivery (Bosselmann & Williams, 2012). Nanotechnology, in a simple way, explains the technology that deals with one billionth of a meter scale (Ochekpe, *et al.,* 2009). Fewer side effects, poor bioavailability, absorption at intestine, solubility, specific delivery to site of action with good pharmacological efficiency, slow release, degradation of drug and effective therapeutic outcome, are the major challenges faced by most of the drug delivery systems. To a great extent, biopolymer coated drug delivery systems coupled with nanotechnology alleviate the major drawbacks of the common delivery methods. Chitosan, deacetylated chitin, is a copolymer of β-(1, 4) linked glucosamine (deacetylated unit) and N- acetyl glucosamine (acetylated unit) (Radhakumary *et al.,* 2005). Chitosan is biodegradable, non-toxic and bio compatible. Owing to the removal of acetyl moieties that are present in the amine functional groups of chitin, chitosan is readily soluble in aqueous acidic solution. The solubilisation occurs through the protonation of amino groups on the C-2 position of D-glucosamine residues whereby polysaccharide is converted into polycation in acidic media. Chitosan interacts with many active compounds due to the presence of amine group in it. The presence of this active amine group in chitosan was exploited for the interaction with the active molecules in the present study. Nanoparticles of chitosan coupled drugs are utilized for drug delivery in eye, brain, liver, cancer tissues, treatment of spinal cord injury and infections (Sharma *et al*., 2007; Li, *et a.,* 2009; Paolicelli *et al.,* 2009; Cho *et al*., 2010). To deliver drugs directly to the intended site of action and to improve pharmacological efficiency by minimizing undesired side effects elsewhere in the body and decrease the long-term use of many drugs, polymeric drug delivery systems can be used (Thatte *et al.,* 2005).

Gamma amino butyric acid (GABA) is a non proteinaceous amino acid and is an important inhibitory neurotransmitter in the vertebrate central nervous system. Apart from the inhibitory role, it is reported that GABA involves in the cell proliferation in different regions of the body. The proliferative role of GABA was observed in the development of outer retina in rabbits (Messersmith & Redburn, 1993), TM3 Leydig cell multiplication in testis (Geigerseder *et al.*, 2004) and promotes neuronal cell growth, cell proliferation and migration **(**Ben-Yaakov & Golan, 2003). Baclofen, a GABA agonist, induced EGF mediated DNA synthesis in hepatocytes *in vitro*. There is an increase in hepatocyte proliferation through the activation of $GABA_B$ receptor. Also, it significantly reduces the TGF β1 suppression of EGF induced DNA synthesis. Thus the activation of GABA receptors, triggers DNA synthesis, mediated through the G protein, in primary cultures of rat hepatocytes (Biju *et al*., 2002). The expression of the stimulatory and inhibitory α-subunit of G protein coupled receptors to the effector targets like adenylate cyclase cause biphasic increase in hepatic cAMP. The cell proliferation is initialized by the activation of cAMP regulated transcription factors and the phosphorylation of cAMP regulatory element binding protein which influences the induction of cAMP inducible genes in the regenerating liver (Diehl & Rai, 1996). GABA is synthesized from glutamate, which is decarboxylated by glutamate decarboxylase (GAD). The major catabolic route for GABA is transamination with α- ketoglutarate catalyzed by GABA transaminase. Succinic semialdehyde formed in this reaction is rapidly oxidized to succinate by succinic semialdehyde dehydrogenase. This is the major pathway of GABA metabolism and is termed the GABA shunt (Masahito *et al*., 2001). The succinate so formed is utilized in citrate cycle and promotes the energy production.

Serotonin or 5-hydroxy tryptamine (5-HT) has been shown to be mitogenic in many cells other than neuronal, exerting its effect by its receptor-mediated second messenger pathways. One of the factors influencing the rate of dentate gyrus neurogenesis is serotonin (Djavadian, 2004). The $S_2$ receptor subtype of serotonin has been shown to have mediated cell growth in fibroblasts (Van Obberghen-Schilling *et al*., 1991). The serotonin $S_2$ receptor has been cloned

in the human liver and has been shown to have a high degree of homology with the $S_2$ receptors of rat and mouse liver (Bonahus *et al*., 1995). The $S_2$ receptors of serotonin are coupled to phospho- inositide turnover and diacylglycerol formation, which activates protein kinase C (PKC), an important second messenger for cell division (DeCourcelles *et al*., 1985). Serotonin S2 receptor–mediated activation of PKC has been shown to result in the phosphorylation of a 40-kD substrate protein of PKC in human platelets (DeCourcelles *et al*., 1984). Platelets are major carriers of serotonin in the blood. It was reported that serotonin can act as a potent hepatocyte co-mitogen and induce DNA synthesis in primary cultures of rat hepatocytes (Balasubramanian & Paulose, 1998).

In the present study, the synthesis of nanoparticles by GABA and 5-HT with chitosan and their delivery for active liver regeneration was emphasised. The liver regeneration in partially hepatectomised rats has contributed a powerful model for evaluating the hepatocyte proliferation *in vivo* (Michalopoulos & DeFrances, 1997). The liver is an important organ in the body that is responsible for various functions like bile production, storage and filtering of blood, metabolism of fats and sugars and thus making compounds which control blood volumes and clotting. Liver cell damage occurs due to several reasons. Liver's internal structure is distorted due to the scars formed as a result of alcohol induced cell death and inflammation (Lands, 1995; Maher & Friedman, 1995). One in twenty five deaths worldwide are directly related to alcohol consumption. An overdose of many drugs like acetaminophen causes severe liver injury which leads to liver failure. Liver cell destruction is also observed due to the attack of parasites like *Entamoeba histolytica* (Seydel & Stanley, 1998). Many hepatotoxic chemicals like dyes, preservatives and insecticides cause liver cell apoptosis (Fawthrop *et al*., 1991; Boobis *et al*., 1992). Wilson's disease (an inherited disorder of copper metabolism in which the liver, among other organs, becomes damaged) and Reye's syndrome (a combination of acute encephalopathy and fatty infiltration of the internal organs) also can be a cause for liver damage. The regeneration of damaged liver is considered as a solution for all disturbing factors of normal hepatocytes' functions. A two-third partial hepatectomy (PH) of rats induces the

remainder of the liver to undergo a synchronous first wave of DNA synthesis 24 hours post hepatectomy, followed by several rounds of mitosis (Higgins & Anderson, 1931; Harkness, 1957; Bucher, 1963; Grisham, 1964). The original DNA content of the regenerating liver remnant is normally restored within 96 h after PH (Russell & Bucher, 1983).

Hepatocyte growth factor (HGF) is an important growth factor which is expressed and elevated during liver regeneration in partially hepatectomised rats. It has been identified as a potent stimulator of hepatocyte growth and DNA synthesis (Su *et al.*, 2002). Thus, during liver cell proliferation after partial hepatectomy, the expression pattern of HGF gene represents the extent of liver regeneration. The DNA synthesis was quantified by the analysis of [$^3$H] thymidine uptake, thymidine kinase activity and BrdU labeling of the cells of regenerating liver. The extent of methylation on replicating DNA was also considered for comparing GABA and 5-HT chitosan nanoparticle induced liver regeneration with the regeneration without treatment. The second messengers inositol triphosphate ($IP_3$), 3'-5'-cyclic adenosine monophosphate (cAMP) and 3'-5'-cyclic guanosine monophosphate (cGMP) assays helped to study the $GABA_B$ and 5-$HT_{2A}$ mediated cell signalling during liver regeneration. The roles of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), tumour necrosis factor α (TNF-α) and Akt-1 and the apoptotic factors like caspase-8 and Bax in promoting hepatocyte proliferation was also emphasized in the present study.

Brain plays an important regulatory role in hepatic functions (Grady, 2005). It controls the animal's whole body functions, memory, attention, perceptual awareness, thought, language, and consciousness. In acute liver failure, high ammonia levels raise cerebral blood flow and increase inflammatory response that have been identified as major contributors to the development of hepatic encephalopathy (Mpabanzi & Jalan, 2012) and the related brain herniation, coma and brain swelling. This affects neurotransmitters levels and their receptors activation in brain. Auto regulation of blood flow in different brain regions is impaired and is associated with anaerobic glycolysis and oxidative stress. Inflammatory mediators like TNF-α also play an important role in regulation of

brain function by liver (Larsen & Wendon, 2002). So the regeneration of damaged liver restores the functions of brain regions. The liver is richly innervated and the central thyrotropin-releasing hormone, one of the important peptide transmitter substances, regulates hepatic proliferation through autonomic nervous system (Yoneda *et al.,* 1997). Extirpation of the brain cortex was shown to increase the rate of liver cell proliferation describing that the brain exerts an inhibitory function on liver cell division, growth and also transection of the spinal cord above the area innervating the liver resulted in decreased DNA synthesis (Vaptzarova & Popov, 1973).There were several reports highlighting the regulation of hepatic proliferation by the brain, but the role of neurotransmitters and their receptors in mediating neuronal survival during neurotransmitter conjugated chitosan nanoparticle induced liver regeneration was not studied.

GABA is reported to have an inhibitory effect on sympathetic outflow. Brain GABAergic changes are reported to regulate autonomic nerve function in rats (Martin & Haywood, 1998). GABA and GABA receptors were widely distributed in mammalian brain and are in high concentration in cortical, hippocampal, thalamic, basal ganglia and striatal structures. In animal models of liver injury, an increase in GABAergic tone has been demonstrated due to both an increase in GABA release and enhanced activation of the GABA receptor complex (Albrecht & Jones, 1999). The central serotonergic neurons participate in the regulation of sympathetic nerve discharge and have an inhibitory influence on central sympathetic pathways. 5-HT regulates cell proliferation, migration and maturation in a variety of cell types and alters the cytoskeleton of cells and thus influences the formation of cell contacts. 5-HT has been implicated more in behaviour, physiological mechanisms and disease processes than any other brain neurotransmitter. 5-$HT_{2A}$ receptor belongs to the super family of G-protein coupled receptors and mediates contractile responses of vascular, urinary, gastrointestinal and uterine smooth muscle preparations, platelet aggregation and increased capillary permeability in both rodent and human tissue.

CNS neurons are supported by several neurotrophic and transcription factors. Brain-derived neurotrophic factor (BDNF) is a potent trophic factor that

supports and promotes the survival and/or differentiation of GABAergic neurons *in vitro* (Mizuno *et al*., 1994; Ventimiglia *et al*., 1995). The cAMP response element-binding protein (CREB) is a transcription factor, which plays a pivotal role in neuroplasticity. It binds to certain DNA sequences called cAMP response elements (CRE) and thereby, increases or decreases the transcription of the downstream genes (Spaulding, 1993). PKB is another important regulator involved in neuroprotective effect against ischemic brain damage in genetically modified mice expressing an active form of PKB (known as Akt) in neuronal cells (Ohba *et al.*, 2004). Many molecules like TNF-α and NF-κB promotes neuronal survival and protects cells from neurotoxic insults (Brunet *et al*., 2001). All these factors regulate different aspects of neuronal and synaptic functions.

Present study also investigated the second messenger alterations by studying $IP_3$, cAMP and cGMP functional regulation and gene expression of phospholipase C (PLC) and cAMP regulatory element binding protein (CREB). The changes in gene expression of antioxidant enzyme like SOD were investigated. Gene expression studies of apoptotic factors like Bax, Caspase-8, NF-κB, TNF- α and Akt-1 were studied. The gene expression of neuronal survival factors like brain derived neurotrophic factor (BDNF) and glial cell line derived neurotrophic factor (GDNF) were also studied. Behavioural studies were planned to evaluate the locomotor function in control and experimental rats. Our present study on GABA and 5-HT chitosan nanoparticles induced active liver cell proliferation. This suggests promising therapeutic possibilities for the treatment of liver based diseases.

# OBJECTIVES OF THE PRESENT STUDY

In the present work we studied the potential of GABA and 5-HT chitosan nanoparticles treatment to enhance liver cell proliferation and reduce neuronal damage in partially hepatectomised rats. For achieving the aim, $GABA_B$ and $5\text{-}HT_{2A}$ receptors functional regulation, gene expression of growth factors, neuronal survival and apoptotic factors during GABA and 5-HT chitosan nanoparticles induced active liver regeneration in rats were studied. The objectives are

1. To synthesize and standardize GABA and 5-HT coupled chitosan nanoparticles, individually and in combination, for enhanced liver cell proliferation in partially hepatectomised rats.

2. To evaluate the interaction of GABA and 5-HT coupled chitosan nanoparticles with liver cells, *in vitro.*

3. To understand the mitogenic properties of GABA and 5-HT coupled chitosan nanoparticles, individually and in combination, by quantification of DNA and protein syntheses and confocal imaging of BrdU incorporation in regenerating liver.

4. To analyze the role of cell apoptotic factors like Bax, caspase-8 and antioxidant enzyme superoxide dismutase in liver cell proliferation.

5. To understand GABA and 5-HT signalling in the liver of partially hepatectomised rats by evaluating the level of second messengers- cAMP, $IP_3$ and expression of transcription factors- CREB, PLC, Akt-1, NF-κB and TNF-α.

6. To examine the alterations in brain GABA and 5-HT signalling in partially hepatectomised rats and treatment groups with GABA and 5-HT

chitosan nanoparticles, individually and in combination, through radio receptor studies and gene expression analysis of receptors.

7. To study the localization and expression of 5-$HT_{2A}$ and $GABA_B$ receptors in brain and liver sections using specific antibodies tagged with fluorescent dyes in confocal microscope.

8. To study neuronal survival in the brain regions by examining the gene expression status of Akt-1, NF-κB, TNF-α, BDNF, GDNF, IGF-1, Bax and caspase-8 of control and experimental rats.

9. To understand the locomotor deficits in partially hepatectomised rats by rotarod, grid walk and narrow beam walk tests.

# *Literature Review*

The ancient Greek myth of Titan Prometheus who received punishment for protecting mankind and deceiving Zeus is known to the scientific community who deal with hepatic diseases, mainly because Prometheus' liver was the target of torture. In Tityus myth, a Greek mythology, the vultures come to devour the giant's liver every new moon day in contrast the eagle came every other day to feed on Prometheus' liver (Tiniakos, *et al*., 2010). The myths of Prometheus and Tityus reveal only one aspect of liver regeneration, mainly the organ's ability to reconstitute its entire mass after partial removal. Twenty-seven centuries passed before the first experimental model of liver regeneration following partial hepatectomy (PH), the pioneering work of Higgins and Anderson was published in 1931 (Higgins & Anderson, 1931). They developed a simple surgical procedure in which two-thirds of a rat liver is removed. This model has been widely used to study mechanisms that govern liver regeneration after partial hepatectomy. The concepts of ancient mythographers were confirmed in observations that showed that liver mass increases a few hours after partial hepatectomy and reaches normal size within a few days (Higgins & Anderson, 1931).

Liver has the capacity to regulate its growth and size in response to the demands of the body on hepatic function. Liver regeneration after PH differs significantly from the wound healing processes present in other tissues: it is neither wound healing nor true regeneration but is a process of compensatory hyperplasia in which regrowth of the resected median and left lateral lobes does not occur. Regeneration is accomplished by cellular hyperplasia in the two remaining lobes and enlarges until their combined mass reaches that of the intact organ before PH (Fausto & Webber, 1994; Jin *et al*., 2012). From this sequence of events it is obvious that the control of liver growth, including both 'on' and 'off' signals is related to liver function rather than anatomy. It should also be noted that, in addition to the regenerative growth that compensates for the loss of tissue or cell death, hepatocytes respond to direct mitogenic agents (Ohmura, 1996).

Furthermore, certain chemicals that some humans are exposed to, such as lead nitrate, ethylene dibromide and agents that induce proliferation of peroxisomes in hepatocytes (including hypolipidemic drugs, phthalate esters and many industrial solvents) do not kill hepatocytes but directly cause proliferation of these cells *in vivo.* Carbon monoxide enhances early liver regeneration in mice after hepatectomy (Kuramitsu *et al*., 2011). There is a strong relationship between liver regeneration and angiogenesis and fibrosis. Losartan and Spironolactone directly induces angiogenesis and fibrogenesis in liver (Parlakgumus *et al*., 2013). Administration of these agents to rodents causes liver enlargement that is reversed when the chemicals are withdrawn (Kay & Fausto, 1997). Another recent report also supports the administration of proliferating agent like ursolic acid for enhancing liver regeneration (Jin *et al*., 2012).

The importance of the partial hepatectomy model is based on two popular aspects. *First*, the removal of the resected tissue is not associated with massive necrosis. The resected hepatic tissues are amenable to "clean" removal due to the multilobular structure of rat and mouse liver. Thus, regeneration of the residual lobes from its very beginning is mediated by processes relevant only to liver tissue and not to necrosis or acute inflammation. In contrast, models involving necrosis of lobular zones induced by toxins (eg, $CCl_4$), the events of first day after toxic injury are dominated by acute inflammation of the necrotic zones (Fujii *et al*., 2010). Polymorphonuclear leukocytes and macrophages infiltrate the necrotic area to remove dead hepatocytes. *Second*, because PH stimulates immediate initiation of regeneration without complications from inflammatory situations, and because PH can be performed in a few minutes, the regenerative phenomena can be precisely timed, with a reference (time 0) point from the time of the performance of PH. These two attributes of the model are the major reason for its usefulness, enhanced popularity and acceptance through the years by many investigators (Michalopoulos & DeFrances, 1997; Michalopoulos, 2007)

**Role of liver in the body**

The liver is our body's most important organ after the heart, performing many important functions including metabolism, detoxification and formation of important compounds including blood clotting factors. It also filters, regulates, and stores blood. Its functions include the processing of dietary amino acids, carbohydrates, lipids and vitamins; phagocytosis of particulate material in the portal circulation; synthesis of serum proteins; biotransformation of circulating metabolites; detoxification and excretion of endogenous waste products and pollutant xenobiotics into the bile (Crawford, 1994; Gueguen *et al*., 2006; Omiecinski *et al*., 2011). Its unique dual blood supply, which includes the portal venous system, makes the liver an intermediate filter for most of the venous drainage of the abdominal viscera (Wanless, 1999). These anatomical properties not only support the physiological functions of the liver but also make it vulnerable to a wide variety of metabolic, toxic, microbial, circulatory and neoplastic insults. Some of these insults cause primary hepatic diseases, such as viral hepatitis and hepatocellular carcinoma. Another important function of the liver is to manufacture bile, which plays a role in the digestion and absorption of many constituents in the normal diet. Bile, which is produced in the liver, is stored in the gall bladder, a specialized storage organ located just below the liver. The gall bladder empties bile into the intestine when food is ingested.

Stress, poor diet and over-medication are common problems in our "civilized" lifestyle. These lead to stress and functional damage to the liver. As a result, "sluggish" liver has become a common ailment. It affects memory, sleep, thyroid, body weight and other body functions. Both estrogen and androgen stimulate cell division. Elevated levels of these hormones lead to abnormal cell growth such as womens' uterine fibroids, ovarian cysts, endometriosis, breast cysts and breast cancer or mens' prostate enlargement and prostate cancer. Since the liver is the principal organ which removes these hormones, its failure to remove them efficiently often leads to their accumulation in the body and is a major cause of the above diseases. More often, however, hepatic involvement is secondary to

extra-hepatic disorders that include some of the most common diseases in humans, such as cardiac decompensation, disseminated cancer, alcoholism and extra-hepatic infections (Isselbacher & Podolsky, 1991). Inflammatory disorders of the liver dominate the clinical practice of hepatology, in part because nearly any insult to the liver can kill hepatocytes and induce the recruitment of inflammatory cells. Indeed, the liver is almost inevitably involved in blood-borne infections, whether systemic or arising within the abdomen (Crawford, 1994; Felmlee *et al*., 2013; Wong *et al*., 2013).

The liver also regulates body functions which affect emotional and mental activities. In a diseased condition, the liver's blood storage and regulatory functions are affected and bleeding or clots can result. When liver blood is deficient, nourishment to tendons and blood vessels is curtailed, the joints become stiff and muscles become spasmodic and numb. Blood deficiency in the liver even leads to stroke, dizziness, headaches, tinnitus, deafness, fainting or convulsion. When the liver blood is so deficient that it cannot nourish the eyes, night blindness or blurring results. If the liver is affected by stress or unhappy feelings, its vitality is repressed and the sides hurt and hiccups or hernia develops. The bowels become constipated and sleep is disturbed causing nightmares or insomnia. The structural position of the liver as a bridge between the returning blood from the digestive system and the lower part of the body to the heart makes the liver an important organ for the health of the heart. A weakened and swollen or congested liver can obstruct the venous blood flow to the heart causing heart palpitations or even heart attacks. In other words a healthy liver is essential for maintaining an adequate amount of blood flow to the heart and the heart can only pump the blood it receives (Neufeld, 1987).

According to Chinese medicine, the liver and kidneys are the organs that "age" us. That is why almost all longevity herbs used in Chinese medicine are liver and kidney tonics. Without a clean, efficient liver and healthy kidneys, blood is not filtered clean. "Dirty" blood, loaded with toxins or waste products, is heavier and more sluggish. This causes poor circulation and reduced capacity to

carry oxygen and nutrients. As a result, tissue and organ cells are undernourished. If this condition persists the cells will deteriorate and inevitably age. The eye and brain cells are especially affected because the blood has to flow against gravity to reach them. Malfunction of the liver and kidneys was mentioned as one of the causes leading to Alzheimer's disease. The brain is only 2% of our body weight, yet it needs 20% of our oxygen supply. If the toxin-loaded blood from a weak liver has limited capacity to carry oxygen, the brain cells are affected most.

The main unique cell types of the liver are hepatocytes, cholangiocytes (or biliary epithelial cells), Kupffer cells, hepatic stellate cells and sinusoidal endothelial cells. Hepatocytes are organized into cords that line an intricate, specialized capillary bed lined with fenestrated endothelial cells. The vascular network is organized into a system that allows for unidirectional flow from branches of the inflow vascular supply, the portal vein and the hepatic artery, through the sinusoids to the central veins, which coalesce into the hepatic vein and connect to the inferior vena cava. The portal vein and hepatic artery are found in cluster along with a collecting bile duct, collectively referred to as the 'portal triad' that is repeated throughout the liver tissue (Kang *et al*., 2012). With the onset of liver disease, the cells of the liver, called hepatocytes are injured or die. If the injury is mild and reversible, the cells regenerate and the patient is left with an entirely normal liver. This remarkable capacity of the liver to regenerate is a unique feature of the organ. When the injury is more severe or sustained, regeneration is incomplete or healing occurs with the development of fibrosis or scars, which can cause cirrhosis. Physicians ordinarily make a diagnosis of cirrhosis using some form of liver biopsy, permitting the identification of marked distortion of the normal architecture of the liver by scar formation and irregular incomplete areas of regeneration. The development of cirrhosis leads to the obstruction of normal blood flow through the liver, resulting in increased pressure in the veins throughout the abdomen (Burroughs, 2011). A consequence for patients is the development of enlarged blood vessels or varices, in the lower end of the oesophagus that can bleed and result in abnormal function of other organs

and even death. When cirrhosis develops, the normal functions of the liver are disrupted, leading to raised ammonia levels that can interfere with the normal function of the brain, producing coma. Decreased albumin levels contribute to fluid accumulation.

Liver regeneration is a compensatory hyperplasia and hypertrophy that occurs as a response to viral or toxic liver injury or hepatic resection (Taub, 2004). The capacity of adult liver cells, both parenchymal and non-parenchymal, to re-enter the cell cycle reflects the uniqueness of this process (Taub, 2004). Under normal conditions, only 0.5–1.0% of liver cells are regularly undergoing DNA replication (Cotran *et al*., 1994). However, upon stimulation, individual hepatocytes have a remarkable replicative capacity, as only a few hepatocytes are required to restore liver mass after profound injury (Taub, 2004). The ability of hepatocytes to undergo cellular growth and proliferation during regeneration, while continuing to carry out their metabolic tasks, makes possible a relatively rapid restoration of the delicate homeostatic equilibrium even after serious insult to the liver. Liver regeneration requires the activity of multiple signalling pathways, assuring synchronized proliferation of liver cells, protection from apoptotic signals, remodeling of extracellular matrix and restoration of lobular architecture (Fausto, 2001).

**Signalling molecules in liver regeneration**

Many molecules are involved in the liver regeneration after PH. Immediately upon removal of two-thirds of the liver in the standard PH model, the entire hepatic vascular influx is forced to perfuse through only one-third of the original capillary bed. Consequently, there is an increase in portal and capillary pressure, as well as an increased availability of circulating growth factors and hormones. Exogenously infused growth factors such as insulin, transforming growth factor-α (TGF-α) and HGF exhibited direct hepatotrophic effects, highlighting the importance of circulating growth factors in initiating hepatocyte growth and proliferation after a stimulus as well as maintenance of baseline liver size.

The molecular mechanisms of liver regeneration can be divided into two critical steps: the transition of the quiescent G0 phase hepatocyte into the cell cycle (priming phase) and progression beyond the restriction point in the G1 phase of the cycle (progression phase). These phases are under separate control; priming by the cytokines tumour necrosis factor (TNF) and interleukin-6 (IL-6), and cell cycle progression by the growth factors hepatocyte growth factor (HGF) and transforming growth factor (TGF)-α (Fausto, 2000). The priming phase does not lead to DNA replication unless the cells can progress through the cell cycle which is accomplished by growth factors.

Hepatocytes exhibit a mitogenic response to various growth factors and cytokines, such as, HGF, IGF-1, IL-6, TNF-*α*, EGF, TGF-*β* and PDGF (Michalopoulos & DeFrances, 1997; Matsuo *et al*, 2008; Kang *et al*., 2012; Nejak-Bowen *et al*., 2013). It is now well recognized that the adult liver contains a stem cell compartment that can be activated under conditions of severe liver injury to give rise to both hepatocytic and biliary epithelial cell (BEC) lineages (Evarts *et al*., 1987; Thorgeirsson *et al*., 1993; Thorgeirsson & Grisham, 2003; Duncan *et al*., 2009). Hepatic stem cells (HSCs) are thought to reside within the terminal bile ductules (Hering canals) located at the interface between parenchyma and biliary tracts. Upon activation, HSCs give rise to oval cells, which form a network of proliferating branching ducts that migrate into parenchyma, where they finally differentiate into hepatocytes (Theise *et al*., 1999; Paku *et al*., 2001; Riehle *et al*., 2011). Numerous molecular factors and cell types contribute to HSC activation either directly or indirectly (Roskams *et al*., 2010; Greenbaum *et al*., 2011; Tanaka *et al*., 2011). HGF is one of the complete mitogen of hepatocytes (induce DNA synthesis of primary hepatocytes in a chemically defined serum-free medium *in vitro* and liver enlargement when administered *in vivo*). It was one of the first isolated and studied circulating factors found to promote liver regeneration (Nakamura, *et al*, 1986, 1989; Zarnegar & Michalopoulos, 1989). HGF binds to and activates the tyrosine receptor kinase MET, a multifunctional receptor involved in a number of cellular processes, such as proliferation, growth, survival

and metabolism (Wang *et al*., 2002; Fafalios *et al*., 2011; Nejak-Bowen et al., 2013). Hu *et al*. reported that HGF/c-Met signalling promotes the activation and early expansion of oval cells after severe liver injury in an acetylaminofluorene/partial hepatectomy rat model (Hu *et al*., 1993). Recently HGF is considered as a therapeutic agent for liver cirrhosis and chronic hepatitis treatment (Mizuno & Nakamura, 2007). Supplementation with or reinduction of hepatocyte growth factor represents a new strategy for attenuating intractable liver diseases. The molecular events involved in liver regeneration are significantly influenced by the extent of resection, as massive (85%-90%) liver resection leads to suppression and delay of liver regeneration, compared to 70% partial hepatectomy (PH), because of suppressed and delayed induction of the regenerative genes TNF-α and IL-6 after 90% PH. Moreover, apoptosis rates are also elevated in 90% PH compared to 70% PH (Sowa, *et al*., 2008; Jia *et al*., 2013; Oguz *et al*., 2013). Several studies have shown that growth factors that promote liver regeneration (HGF and TGF-α) are up regulated in 70% PH, whereas no or only reduced induction occurs after 90% resection. These findings suggest that expression of the factors relevant to the regeneration of liver tissue is influenced by the extent of resection (Fausto, *et al*., 1995; Lowes, *et al*., 2003; Mangnall, *et al*., 2003; Sowa, *et al*., 2008).

These growth factors and cytokines lead to the subsequent activation of downstream transcription cascades, which effect the transition of the quiescent hepatocytes into the cell cycle and progression beyond the restriction point in the G1 phase (Michalopoulos & DeFrances, 1997). The cascades also result in the activation of transcription factors and signal transduction pathways, such as, NF-*κ*B, STAT3, MAPK/ERK, PI3K/Akt, AP-1, and CCAAT/enhancer-binding protein-*β*, which subsequently induce hepatocyte proliferation. Among these transcription factors and corresponding signal transductions, the TNF-*α*/NF-*κ*B, IL-6/STAT3, PI3K/Akt, and MAPK/ERK pathways are identified as the major cascades during the process of liver regeneration. $Ca^{2+}$ signalling also plays an important role in regulating the opening and closing of G-protein coupled receptors. The first calcium oscillations were reported in hepatocytes (Woods &

Cuthbertson, 1986). However, the functions of hepatocyte calcium signalling remain far less understood. In hepatocytes, as in most nonexcitable cells, $Ca^{2+}$ oscillations originate from the periodic opening of $Ca^{2+}$ channels located in the ER membrane, following activation of the phosphoinositide cascade. The binding of an agonist to a membrane-bound receptor activates the Gα-subunit of a G-protein complex coupled to the receptor. This activated G protein in turn stimulates phospholipase C (PLC) activity. The latter enzyme catalyzes the hydrolysis of the membrane-bound phosphatidyl-inositol bisphosphate (PIP2) into diacyl-glycerol and inositol trisphosphate (InsP3). $Ca^{2+}$ release from the internal stores is ensured by the InsP3R, a homotetramer that can bind up to 4 InsP3 molecules, forming a $Ca^{2+}$ channel (Taylor, 1998).

While cytokines are responsible for the passage of quiescent hepatocytes into the cell cycle (G0 to G1), cell cycle progression is then driven by growth factors, which over ride a restriction point in the late G1phase (Fausto, 2006). HGF and ligands of epidermal growth factor receptor (EGFR) are important growth factors that drive cell cycle progression during liver regeneration. Studies have shown that despite the expression of many mitogenic receptors, including receptors for platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), the only mitogens for hepatocytes are HGF and ligands of EGFR. The family of ligands that bind EGFR, in addition to EGF, includes TGF-α, heparin-binding EGF-like growth factor (HB-EGF) and amphiregulin (AR) (Michalopoulos, 1990; Fausto, 2006). Stimulation of the tyrosine kinase receptors for HGF and the EGF ligands activates numerous intracellular signalling pathways that regulate transcription factors involved in liver regeneration (Fausto, 2006). It is important to mention, with the possible exception of HGF, that complete elimination of a single growth factor does not entirely abrogate liver regeneration.

Reduction in apoptosis enhances regeneration of cells. The signals evoked by $IP_3$ mediated $Ca^{2+}$ ions in mitochondria triggers apoptosis. Phospholipase C (PLC) is the enzyme involved in the synthesis of $IP_3$ and thus the increased level of

$IP_3$ and phospholipase C result in enhanced apoptosis (Szalai, *et al.*, 1999; Lencesova & Krizanova, 2012; Lencesova *et al.*, 2013). The triggering of protein kinase C occurs through $IP_3$ mediated signalling pathway, which further leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB activates TNF-α mediated cell death (Su, 2002). Thus the suppression of NF-κB promotes liver regeneration.

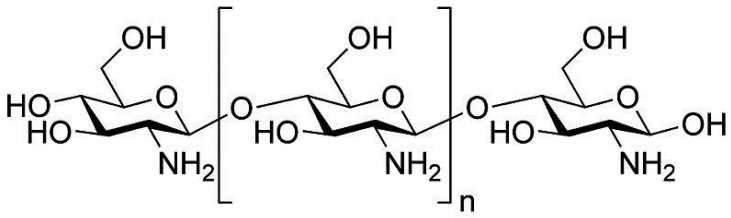
Animal body is exposed to exogenous and endogenous free radicals. Damage to liver cells automatically increases the free radical mediated stress. The cells necessitate oxygen to produce the energy. During mitochondrial respiration, the cells take in oxygen, burn it, release energy and free radicals are produced. Oxidative stress occurs when the antioxidant production is decreased or free radical production exceeds the body's ability to neutralize them. Oxidative stress is defined as a tissue injury induced by increase in reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$), superoxide anion $(O_2^{\bullet})^-$, and hydroxyl radical ($OH^{\bullet}$). ROS has been proposed to stimulate cell cycle progression as an intrinsic cellular signal (Burdon, 1995; Barrera, 2012). The reactive oxygen intermediates produced in mitochondria, peroxisomes and the cytosol are scavenged by cellular defending systems including enzymatic (eg. superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase and catalase) and nonenzymatic antioxidants (ex. glutathione G-SH, thioredoxin, lipoic acid, ubiquinol, albumin, uric acid, flavonoids, vitamins A, C and E ). Antioxidants are located in cell membranes, cytosol and in the blood plasma (Maritim *et al*., 2003). Regeneration of liver with maximum healthy cells is achieved with the combined effect of active cell proliferation and reduced injury mediated apoptosis.

**Chitosan, the best vehicle for drug delivery**

The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action. In most cases (conventional dosage forms), only a small amount of administered dose reaches the target site, while the majority of the drug distributes throughout the rest of the body in accordance with its physicochemical

and biochemical properties. Therefore, developing a drug delivery system that optimizes the pharmaceutical action of a drug while reducing its toxic side effects *in vivo* is a challenging task. A wide range of materials, such as natural or synthetic polymers, lipids, surfactants and dendrimers, have been employed as drug carriers (Duncan, 2003, 2006; Torchilin, 2008). Among these, polysaccharides have received increasing attention because of their outstanding physical and biological properties (Sampathkumar, 2005). Chitosan, a linear aminopolysaccharide composed of randomly distributed (1→4) linked D-glucosamine and N-acetyl-D-glucosamine units, is obtained by the deacetylation of chitin, a widespread natural polysaccharide found in the exoskeleton of crustaceans such as crab and shrimp (Liu *et al*., 2008). Since chitosan has entered the pharmaceutical arena in the early 1990s, it has inspired academic and industrial research teams to generate novel more effective therapeutic systems based on it. Apart from applications such as slimming, wound dressing, and tissue engineering, chitosan showed promising features as auxiliary agent in drug delivery. In contrast to all other biodegradable polymers having a monograph in a pharmacopoeia, chitosan is the only one exhibiting a cationic character rendering it unique among all others. This cationic character being based on its primary amino groups responsible for various properties and subsequently for its use in drug delivery systems (Liechty *et al*, 2010; Lallemand *et al*., 2012; Zhao *et al*., 2012; . Chitosan's properties also allow it to be used in transdermal drug delivery; it is mucoadhesive in nature, reactive (so it can be produced in many different forms) and most importantly, has a positive charge under acidic conditions. This positive charge comes from protonation of its free amino groups. Lack of a positive charge means chitosan is insoluble in neutral and basic environments. However, in acidic environments, protonation of the amino groups leads to an increase in solubility. The implications of this are very important to biomedical applications. This molecule will maintain its structure in a neutral environment, but will solubilize and degrade in an acidic environment. This means chitosan can be used to transport a drug to an acidic environment, where the chitosan packaging

will then degrade, releasing the drug to the desired environment. The protonated chitosan is broken down by lysozyme in the body to glucosamine (Baldrick, 2009) and the conjugate base of the acid (such as lactate or succinate) are substances naturally found in the body.




Chitosan's properties allow it to rapidly clot blood, and have recently gained approval in the United States and Europe for use in bandages and other hemostatic agents. Chitosan hemostatic products reduce blood loss in comparison to gauze dressings and increase patient survival (Pusateri *et al*., 2003).

The chemical modification of chitosan imparts amphiphilicity, which is an important characteristic for the formation of self-assembled nanoparticles, potentially suited for drug delivery applications. The hydrophobic cores of the nanoparticles could act as reservoirs or microcontainers for various bioactive substances. Because of their small size, nanoparticles can be administrated *via* the intravenous injection for targeted drug delivery. Conjugation of the targeting moieties to the surface of drug-loaded nanoparticles improves therapeutic efficiency of the drug (Dufes *et al*., 2004). Chitosan has been widely utilized as drug delivery systems for low molecular weight drugs, peptide and genes (Yoo *et al*., 2005; Amidi *et al*., 2006; Kim *et al*., 2008; Cavalli *et al*., 2012; Ramesan & Sharma, 2012). Chitosan nanoparticles are good for parenteral drug delivery. The susceptibility of chitosan to lysozyme makes it biodegradable (Nordtveit *et al*., 1994). Chitosan demonstrated a good retention in blood circulation and a slight accumulation in tissues, suggesting that chitosan is an effective carrier for drugs that are excreted rapidly (Onishi *et al*., 1996). The most promising drugs that have been extensively studied for delivery by this route are anticancer agents (Brasseur *et al*., 1980; Guan *et al*., 2012; Termsarasab *et al*., 2012). Delivery of antiinfectives such as antibacterial, antiviral, antifungal and antiparasitic drugs, is

another common use of nanoparticles (Bender *et al*., 1996; Page-Clisson *et al*., 1998; Soma *et al*., 2000). Chitosan is a best vehicle for ocular drug delivery. It was also extensively used for gene and vaccine delivery.

Chitosan nanoparticles are employed for slow and controlled release of drugs. Controlled release technology emerged during the 1980s as a commercially sound methodology. The achievement of predictable and reproducible release of an agent into a specific environment over an extended period of time has much significant merit. It creates a desired environment with optimal response, minimum side-effects and prolonged efficacy. Controlled-release dosage forms enhance the safety, efficacy and reliability of drug therapy (Elzoghby *et al*., 2012; Nair *et al*., 2013). They regulate the drug release rate and reduce the frequency of drug administration to encourage patients to comply with dosing instructions. Conventional dosage forms often lead to wide swings in serum drug concentrations. Most of the drug content is released soon after administration, causing drug levels in the body to rise rapidly, peak and then decline sharply.

Chitosan nanoparticles prepared by ionotropic gelation technique was first reported by Calvo *et al*., (1997) and has been widely examined and developed. The mechanism of chitosan nanoparticles formation is based on electrostatic interaction between amine group of chitosan and negatively charge group of polyanion such as tripolyphosphate. This technique offers a simple and mild preparation method in the aqueous environment. First, chitosan can be dissolved in acetic acid and then the polyanion is added. The nanoparticles were spontaneously formed under mechanical stirring at room temperature. The size and surface charge of particles can be modified by varying the ratio of chitosan and stabilizer.

**Role of serotonin and GABA in cell proliferation**

**Serotonin as a co-mitogen**

The morphogenic property of serotonin occurs in animal cells, in which serotonin alters the cytoskeleton of cells and thus influences the formation of contacts. In addition, serotonin regulates cell proliferation, migration and

maturation in a variety of cell types, including lung, kidney, endothelial cells, mast cells, neurons and astrocytes. In brain, serotonin has interactions with seven families of receptors, numbering at least 14 distinct proteins. 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors have opposing functions in a variety of cellular and behavioural processes. The 5-$HT_{1A}$ receptor develops early in the CNS and is associated with secretion of S-100β from astrocytes and reduction of cAMP levels in neurons. These actions provide intracellular stability for the cytoskeleton and result in cell differentiation and cessation of proliferation. Clinically, 5-$HT_{1A}$ receptor drugs decrease brain activity and act as anxiolytics. The 5-$HT_{2A}$ receptor develops more slowly and is associated with glycogenolysis in astrocytes and increased $Ca^{++}$ availability in neurons. These actions destabilize the internal cytoskeleton and result in cell proliferation, synaptogenesis and apoptosis. In humans, 5-$HT_{2A}$ receptor drugs produce hallucinations. The dynamic interactions between the 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors and the cytoskeleton may provide important insights into the etiology of brain disorders and provide novel strategies for their treatment (Azmitia, 2001).

For more than 50 years, 5-HT has been known to constrict blood vessels and induce shape changes in skeletal muscle (at both the light and electron microscope level) (O'Steen, 1967), platelets (Leven *et al.*, 1983), endothelial cells (Welles *et al.*, 1985) and fibroblast (Boswell *et al.*, 1992). In the periphery, 5-HT originates largely from mast cells, which can produce, release and re-uptake 5-HT. The released 5-HT, then act as a chemotactic, increase vascular permeability, vasodilatation and smooth muscle spasm (Metcalfe *et al.*, 1981). In addition to its role in morphological changes, 5-HT also has been shown to play a role in cell proliferation. Amongst the numerous 5-HT receptor families, the 5-$HT_2$ receptors are necessary for cell viability and proliferation (Collet, *et al*., 2008). 5-$HT_2$R is expressed at high levels in the hepato-gastrointestinal (GI) tract, including the liver (Nichols & Nichols, 2008). The physiological function of 5-$HT_2$R is still unclear and there are few studies of its downstream targets (Raymond, *et al*., 2001); however, 5-HT can initiate liver regeneration and mediate hepatocyte fibrosis. In cultured rat pulmonary artery smooth muscle cells (SMC), 5-HT

induces DNA synthesis and potentiates the mitogenic effect of platelet-derived growth factor (Eddahibi *et al.,* 1999). 5-HT effects on cell proliferation are involved with phosphorylation of GTPase-activating protein (GAP), an intermediate signal in 5-HT induced mitogenesis of SMC (Lee *et al.*, 1997). Earlier studies from our laboratory showed that 5-HT acting through specific receptor subtypes $5\text{-HT}_2$ (Balasubramaniam & Paulose, 1998) control cell proliferation and act as co-mitogens. Thus, there is evidence that 5-HT is involved in a variety of cellular processes involved in regulating metabolism, proliferation and morphology.

**GABA as a co-mitogen**

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the adult mammalian brain and is thought to be involved in cell proliferation, migration and in the promotion of cell survival. GABA is also found in many peripheral non neuronal tissues (Watanabe *et al*., 2002). GABA exerts its effects through $GABA_A$ and $GABA_B$ receptors; $GABA_A$ receptors (ionotropic) are coupled to chloride ion channels and $GABA_B$ receptors (metabotropic) are G protein-coupled receptors (GPCRs). GABA receptors are present in many peripheral non neuronal tissues, indicating that GABA exerts physiologic effects other than neurotransmitter effects in these tissues. 5-HT is involved in the development of Schwann cells in the peripheral nervous system (Magnaghi *et al*., 2004) and in the development of the palate (Ding *et al*., 2004), digestive tract (Gilon *et al*., 1987b; Wang *et al*., 2004), pancreas (Gilon *et al*., 1987a), liver (Minuk *et al*., 1993, 1997), osteoblasts (Fujimori *et al*., 2002), chondrocytes (Tamayama *et al*., 2005) and testicular cells (Geigerseder *et al*., 2004; Kanbara *et al*., 2005).

$GABA_B$ receptors belong to the GPCR family, members of which possess seven membrane-spanning domains. $GABA_B$ receptors are heterodimers of two receptor subunits, $GABA_B$ R1 and $GABA_B$ R2. Each subunit has an extracellular N-terminal domain and an intracellular C-terminal domain. The subunits interact

*via* intracellular coiled-coil domains near the C terminus. $GABA_B$ receptors are coupled to Gi/Go (Bolmann, 2000) and activation of $GABA_B$ receptors decreases cAMP accumulation by inhibiting the activity of adenylate cyclase. GPCR signal transduction pathways leading to cell proliferation predominantly involve receptor tyrosine kinases. Growth factors such as epidermal growth factor (EGF), transforming growth factor- α (TGF- α), and fibroblast growth factor (FGF) activate specific receptor tyrosine kinases, inducing proliferation and differentiation by various signal transduction pathways.

GABA, the main inhibitory neurotransmitter in the mature CNS, is implicated in playing a complex role during neurogenesis (Ben-Ari *et al.*, 1989; Baher *et al.*, 1996; Behar *et al.*, 2000; Haydar *et al.*, 2000). Through embryonic development, GABA was demonstrated as acting as a chemo-attractant and being involved in the regulation of progenitor cell proliferation. For example, GABA induces migration and motility of acutely dissociated embryonic cortical neurons (Baher *et al.*, 1996; Behar *et al.*, 2000). In addition, the neurotransmitters GABA and glutamate reportedly reduce the number of proliferating cells in dissociated or organotypic cultures of neocortex (LoTurco *et al.*, 1995). In contrast, GABA was shown to promote cell proliferation in cultures of cerebellar progenitors (Fiszman *et al.*, 1999). GABA also dramatically increases proliferation in the ventricular zone of the embryonic cerebrum in organotypic cultures by shortening the cell cycle. However, a reverse effect was observed in the subventricular zone (Haydar *et al.*, 2000). Thus, during embryonic neurogenesis, GABA emerges as an important signal for cell proliferation and migration, but its precise regulation is dependent on the region and cell type affected. Cellular response to GABA is mediated through its known receptors and the intracellular signals associated with them. The contribution of $GABA_A$ receptor to both chemo-attraction (Behar *et al.*, 2000) and cell proliferation (Haydar *et al.*, 2000) was indicated. However, in some aspects of cell motility there is an apparent involvement of GABA dependent G-protein indicating a role of $GABA_B$ receptor (Behar *et al.*, 2000). GABA acts as a trophic factor not solely during prenatal neurogenesis but also in the postnatal period in injured tissue. The effect of GABA involves stimulation of cell

proliferation and NGF secretion (Ben-Yaakov & Golan, 2003). We have previously shown that GABA acting through specific receptor subtypes $GABA_B$ (Biju *et al.*, 2002) control cell proliferation and act as co-mitogens.

Earlier reports from our laboratory explained the neuronal proliferative role of GABA and 5-HT in Parkinson's disease model (Kuruvilla *et al.,* 2013; Nandhu *et al*., 2011; Paul, et al., 2011) and spinal cord injured monoplegic rats (Romeo, *et al.,* 2013).

**Central nervous system and liver regeneration**

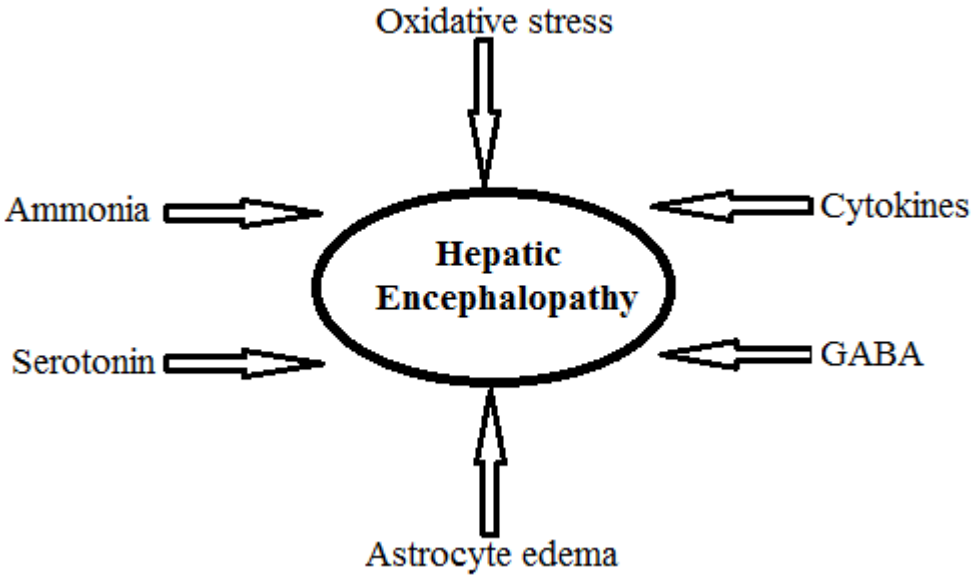
Synaptic defects undoubtedly contribute to the memory and cognitive defects that accompany neurodegeneration. However, the overwhelming feature of most neurodegenerative disorders is excessive neuronal cell death. The functional relationship between the liver and brain has been known for centuries. The neurotransmission in the brain is altered in liver diseases (Butterworth, 1995; Hala & Mahfouz, 2008). A spectrum of neuropsychiatric abnormalities in patients with liver dysfunction were observed and was characterized by intellectual impairments, personality changes and a depressed level of consciousness associated with multiple neurotransmitter systems, cerebral perfusion and astrocyte dysfunction (Avraham *et al*., 2009). Jain *et al*. (1991) reported an onset of mitochondrial damage in brain due to decreased synthesis of glutathione by damaged liver, which was the major glutathione synthesis site.

Cell death occurs by necrosis or apoptosis (Wyllie *et al.,* 1980; Martin, 2001; Kanduc *et al.,* 2002). These two mechanisms have distinct histologic and biochemical signatures. In necrosis, the stimulus of death (e.g., ischemia) is itself often the direct cause of the demise of the cell. In apoptosis, by contrast, the stimulus of death activates a cascade of events that orchestrate the destruction of the cell. Unlike necrosis, which is a pathologic process, apoptosis is part of normal development (physiologic apoptosis) however, it also occurs in a variety of diseases (aberrant apoptosis).

Apoptotic cell death also known as programmed cell death can be a feature of both acute and chronic neurologic diseases (Martin, 1999; Yuan, 2000; Martin, 2001). In apoptosis, a biochemical cascade activates proteases (Hala & Mahfouz, 2008*)* that destroy molecules that are required for cell survival and others that mediate a program of cell suicide. The major executioners in the apoptotic program are proteases known as caspases (cysteine-dependent, aspartate-specific proteases). Caspases directly and indirectly orchestrate the morphologic changes of the cell during apoptosis. Caspases exist as latent precursors, which, when activated, initiate the death program by destroying key components of the cellular infrastructure and activating factors that mediate damage to the cells. Caspases have been categorized into upstream initiators and downstream executioners.

Upstream caspases are subclassified into two groups, according to the molecules modulating their activation. Procaspases 1, 2, 4, 5, 9, 11, 12 and 13 have a long N-terminal prodomain called the caspase-recruiting domain (CARD). Caspases 8 and 10 have a long N-terminal prodomain called the death-effector domain (DED). A regulating molecule is required for specific binding to the CARD/DED domain, which results in caspase activation. Once upstream caspases are activated in an amplifying cascade, they activate the executioner caspases downstream (Hengartner, 2000; Shi, 2002; Bouchier-Hayes & Martin, 2002).

Liver dysfunction leads to impaired metabolism of several compounds, which enter brain vigorously. They impart several changes and disturbances in brain function and neuronal survival. Ultimately, hepatic encephalopathy (HE) results from functional disturbances of cells involved in neurotransmission. The neurologic impairment in HE is believed to be caused by multiple factors.




Previous hypotheses had attempted to explain cerebral damage by individual neurotoxic mediators or disruption of single neurotransmitters (Ozsoylu *et al*., 1985; Mullen, 1995; Cordoba *et al*., 1998; Haussinger *et al*., 2000; Blei, 2001; Yurdaydin, 2001; Ahbouche & Butterworth, 2004; Mullen, 2006; Cordoba & Blei, 2007). Several mechanisms, however, likely induce the observed abnormalities of neuronal and astrocytic function. Potential pathogenic factors include a direct neurotoxic effect of ammonia, oxidative stress caused by generation of reactive oxygen species, endogenous benzodiazepine-like ligands, subclinical intracellular astrocytic edema, GABA like molecules that act as GABA agonists, abnormal histamine and serotonin neurotransmission, endogenous opiates, neurosteroids, inflammatory cytokines, and potential manganese toxicity (Butterworth, 2006; Mullen, 2006; Cordoba & Blei, 2007). Hyperammonemia is directly neurotoxic and also sensitizes astrocytes and neurons to injury by other pathways and mediators (Zieve *et al*., 1987; Skowrońska & Albrecht, 2012). Presently many agents are potentially useful for HE treatment. The ketoanalogues of the branched-chain amino acids (ketoleucine, ketoisocaproate, and ketovaline) could reduce the ammonia pool by amination to the corresponding amino acids,

while increasing precursors for protein synthesis and inhibiting protein breakdown (Munoz & Walser, 1986a; 1986b). Following amination, the ketoanalogues can be effectively incorporated into biologically important proteins (Munoz & Walser, 1986b). Some clinical studies have indeed shown benefit for HE, but these molecules have not been further developed because of poor palatability (Herlong *et al*., 1980). Supplements with branched-chain amino acids have been extensively investigated as potential therapy for HE without proof of efficacy and branched-chain amino acid is currently rarely used. Benzoate is useful as an ammonia scavenger to treat HE (Sushma *et al*., 1982; Enns *et al*., 2007). It is approved as an ammonia reducing agent for urea cycle enzyme deficiency syndromes. It is currently under evaluation as therapy for HE. Opioid and benzodiazepine antagonists, such as naloxone and flumazenil, have been proposed as potential therapies for HE, but have too short-lived effects to be clinically useful (Barbaro *et al*., 1998). Further research is needed to ascertain the therapeutic implications of these multiple pathways for HE.Thus as a result of altered neurotransmission and signalling, entire body functions collapse. So there is a need for developing a therapeutically efficient system that favours enhanced liver cell proliferation coupled increased neuronal survival.

**GABA and 5-HT receptors**

**GABA Receptors**

Gamma aminobutyric acid was discovered over 40 years ago as a key inhibitory neurotransmitter in the brain (Bazemore *et al*., 1957; Krnjevic & Phillis, 1963). Since then, evidence has accumulated that this amino acid function as a neurotransmitter not only in the CNS but also in the peripheral nervous system, including the mesenteric plexus (Amenta, 1986), major pelvic ganglia (Akasu *et al*., 1999), sympathetic ganglia, encompassing the rat superior cervical ganglion (Kasa *et al*., 1988) and abdominal prevertebral ganglia (Parkman & Stapelfeldt, 1993). In the mammalian central nervous system, GABA is the most important inhibitory neurotransmitter occurring in 30-40% of all synapses. Three types of GABA receptors have been identified: $GABA_A$ and $GABA_C$ receptors are ligand-

gated $Cl^-$ channels, while $GABA_B$ receptors are G-protein coupled (Chebib & Johnston, 1999). $GABA_A$ receptors are ligand gated $Cl^-$ channels that consist of a heteromeric mixture of protein subunits forming a pentameric structure and $GABA_B$ receptors couple to $Ca^{2+}$ and $K^+$ channels *via* G-proteins and second messengers (Johnston, 1996). In the CNS, application of GABA reduces excitability by a combination of $GABA_A$ and $GABA_B$ receptor activation, leading to membrane re-polarization, reduced $Ca^{2+}$ influx and suppression of neurotransmitter release. $GABA_A$ receptors are composed of five subunits from seven different subunit families with multiple subtypes (α1–6, β1–3, γ1–3, δ, ε, θ and π) that form a ligand-gated chloride ion channel.

***$GABA_A$ Receptor:***

$GABA_A$ receptors are pentameric in structure, with the five subunits arranged like spokes of a wheel around a central $Cl^-$ selective pore (Barnard, 2001). Nineteen GABA receptor subunits have been cloned from rats, which include α1–6, β1–3, γ1–3, ρ1–3, δ, θ, ε and π (Whiting *et al*., 1999). The 19 subunits are encoded by 19 distinct genes. Each subunit has four transmembrane segments, with both the amino and carboxy termini located extracellularly. These extracellular segments form the recognition sites, two per channel, for GABA and also, in some channel types, the recognition site, one per channel, for benzodiazepine-like allosteric modulators. The genetic diversity of multiple $GABA_A$ receptor subunits permits the assembly of a vast number of receptor heteromeric isoforms. Apparently, the subunit composition determines the pharmacological profile of the resulting receptor subtypes (Barnard *et al*., 1998). Mechanisms that modulate the stability and function of postsynaptic $GABA_A$ receptor subtypes and that are implicated in functional plasticity of inhibitory transmission in the brain are of special interest (Luscher & Keller, 2004).

***$GABA_B$ Receptor:***

The $GABA_B$ receptor is part of the class C of GPCRs that also includes the mGlu, the $Ca^{2+}$-sensing and the sweet and umami taste receptors among others (Pin *et al.*, 2003). These receptors are dimers, either homodimers linked by a disulphide bond (mGlu and $Ca^{2+}$-sensing receptors) or heterodimers made of two similar, but distinct subunits (the $GABA_B$ and taste receptors). Indeed, the $GABA_B$ receptor was the first G protein coupled receptor to be identified that requires two distinct subunits to function: the $GABA_{B1}$ and $GABA_{B2}$ subunits (Jones *et al*., 1998). Although the $GABA_{B1}$ subunit was soon shown to bind all known $GABA_B$ ligands (both agonists and antagonists) this protein did not form a functional $GABA_B$ receptor when expressed alone (Kaupmann *et al*., 1997).

***$GABA_C$ Receptors:***

$GABA_C$ receptors, which are a subfamily of $GABA_A$ receptors, are members of the Cys-loop superfamily of ligand-gated ion channels (LGICs), an important group of receptors involved in rapid synaptic transmission and whose malfunction results in a variety of neurological disorders; hence, understanding their mechanism of action is of considerable pharmacological interest. $GABA_C$ receptors are mostly located in retinal neurons where they play a role in retinal signalling involved in diseases such as macromolecular degeneration (Bormann, 2000). The receptors are activated by the binding of GABA, the main inhibitory neurotransmitter in the central nervous system. $GABA_C$ receptors have distinct pharmacological properties from $GABA_A$ receptors, e.g., they are not inhibited by bicuculline, the classic $GABA_A$ receptor antagonist (Barnard *et al*., 1998; Chebib *et al*., 2000). Like all the LGICs belonging to the Cys-loop superfamily, $GABA_C$ receptors are composed of five subunits arranged in a pentagonal array around a central ion-permeant pore. Each subunit has an extracellular N-terminal domain (ECD), a transmembrane domain composed of four $\alpha$-helices and an intracellular domain. Three subunits ($\rho_{1–3}$) have been identified; these all form functional homomeric or heteromeric receptors (Enz, 2001).

**Serotonin Receptors**

5-HT receptors comprise a complex family. On the basis of their pharmacology, signal transduction mechanisms and molecular structure, more than a dozen types of 5-HT receptors have been identified (Hoyer *et al.,* 1994). Most of these receptors are coupled to various G proteins with the exception of the 5-$HT_3$ receptor, which is a ligand gated cation channel (Derkach *et al.,* 1989). Multiple 5-HT receptor subtypes are expressed in the cerebral cortex (Mengod *et al.,* 1996). In cerebral cortex, 5-$HT_3$ receptors are only expressed in inhibitory neurons (Morales & Bloom, 1997) whereas 5-$HT_{2A}$ receptors are heavily expressed in pyramidal cells and to a lesser extent in inhibitory neurons (Hamada *et al.,* 1998; Jakab & Goldman-Rakic 1998; Willins *et al.,* 1997). Since the 1960s, many experiments using *in vivo* microiontophoretic methods have characterized how 5-HT affects neuronal behaviour. The predominant effect of 5-HT on cerebral cortical pyramidal neurons is an inhibition of spontaneous spiking. (Jacobs & Azmitia, 1992). Intracellular studies in rat cortical slices suggested that 5-HT induces depolarization and action potential firing in pyramidal cells (Araneda & Andrade, 1991). Furthermore, Aghajanian & Marek (1997) reported that 5-HT enhances spontaneous excitatory postsynaptic currents (sEPSCs) without significantly changing spontaneous inhibitory postsynaptic currents (sIPSCs) in frontal pyramidal neurons. These *in vitro* results suggest that 5-HT is mainly excitatory in cortical neuronal circuitry. 5-HT and A-methyl-5-HT had no effect on sEPSCs in layer I neurons. Even though sampling bias might have contributed to this observation, the fact that activation of 5-$HT_{2A}$ receptors induced robust enhancement of sEPSCs in all pyramidal neurons tested suggests that this differential modulation of sEPSCs in the two cell types was real. 5-$HT_{2A}$ receptor expression is high in pyramidal neuron proximal apical dendrites and low in distal parts (Jakab & Goldman-Rakic, 1998; Willins *et al.,* 1997). It is possible that activation of dendritic 5-$HT_{2A}$ receptors induce dendritic transmitter release and/or release of retrograde messenger(s).

During brain development, serotonin provides essential neurotrophic signals (Justin *et al*., 2004; Anju & Paulose, 2011). 5-HT is known to play an important role in several physiological functions (Jackson & Paulose, 2000; Abraham *et al*., 2010). A root cause of sudden infant death syndrome (SIDS) is due to disturbances of serotonin levels in key pacemaker cells in the brain. Several subtypes of signal transducing 5-HT receptors have been characterized pharmacologically and cloned. Depending on their subtype, these receptors act on G-proteins and thereby activate phospholipase C or adenylate cyclase (Fanburg & Lee, 1997; Lee *et al*., 2009). By analogy with other signalling molecules, it is generally assumed that these receptors operate at the cell surface, without necessarily mediating the uptake of 5-HT. In addition, 5-HT is internalized into a variety of cell types, including platelets, neurons, mast cells, endothelial cells and smooth muscle cells, through an active transport mechanism that is powered by a transmembrane $Na^+/Cl^-$ gradient (Junod, 1972).

***5-HT$_2$ receptors***

This class has three subtypes 5-$HT_{2A}$, 5-$HT_{2B}$ and 5-$HT_{2C}$, showing 46-50 % structural homology, preferably linked to Gq11 protein and increasing inositol trisphosphate hydrolysis and intracellular $Ca^{2+}$ concentration. This is the main excitatory receptor subtype among the G-protein coupled receptors for serotonin, although 5-$HT_{2A}$ also has an inhibitory effect on certain areas such as the visual cortex and the orbitofrontal cortex (Hannon & Hoyer 2002).

***5-HT$_{2A}$ Receptors:***

The initial report of 5-$HT_{2A}$ receptor mRNA expression in the rat CNS was based on northern blots of RNA extracted from various regions of the CNS (Julius, *et al*., 1990). Further studies using *in situ* hybridization indicated high expression levels in layers 1, 4 and 5a of the cerebral cortex, the entorhinal and the piriform cortex. The olfactory bulb and some brain stem areas like the hypoglossal, pontine, motor trigeminal and facial nuclei, also showed expression. Intermediate expressing areas were the limbic system and basal ganglia. These

studies did not detect transcripts in the cerebellum, thalamic nuclei and found low expression in the hippocampus. The serotonin 2A receptor (5-$HT_{2A}$) has been implicated in mental disorders with complex etiologies that are still not clearly understood, in processes such as learning and memory and also in neurogenesis. There are a large number of drugs targeted to this receptor. Though the receptor has been studied largely in relation to its multiple functions in the CNS, high levels of receptor expression in other areas such as the intestine, platelets and endothelial cells suggest that it could play crucial roles in other aspects of physiology. GPCRs, including the 5-$HT_{2A}$ receptor, exhibit critical differences in aspects of functional regulation from those seen in conventionally studied model GPCRs such as the β-2-adrenergic receptor. The receptor also couples to a number of intracellular signalling cascades, making it an important receptor to study (Lee *et al*, 2009; Abraham *et al*., 2010; Anju & Paulose, 2011; Kuruvilla *et al*., 2013). Stimulation of the 5-$HT_{2A}$ receptor leads to the production of at least three distinct biochemical signals, $IP_3$/diacylglycerol, arachidonic acid (AA), 2-arachidonylglycerol (2-AG) and the relative activation of these pathways varies with the ligand used (Kurrasch-Orbaugh, *et al*., 2003).

### *5-$HT_{2B}$ receptors*

5-$HT_{2B}$ imunoreactivity was detected in the cerebellum, lateral septum, hypothalamus and medial part of the amygdala. (Cox *et al*., 1995; Schmuck *et al*., 1994). Moreover, activation of 5-$HT_{2B}$ receptor in mouse fibroblasts has mitogenic effect through the activation of MAP kinase (mitogen activated protein kinase) (Nebigil et al., 2000). Antagonists of 5-$HT_{2B}$ receptors (e.g. SB 200646) are relatively new and find clinical application in the treatment and prevention of migraine (Kennet *et al*., 1994). It appears that this receptor is also expressed in heart valves and is responsible for valvulopaties described in patients using preparations for reduction of the appetite containing dexfenfluramine (Bhattacharyya *et al*., 2009).

**Neuronal survival factors**

Neuronal viability is maintained through a complex interacting network of signalling pathways that can be perturbed in response to a multitude of cellular stresses. A shift in one or more of these signalling pathways can alter the fate of a neuron resulting in cell death or continued survival. The nature of the stresses affecting neurons, the duration of the stresses, the developmental stage of the neuron and a variety of other factors influence the signalling pathways that are ultimately affected. These diverse parameters also regulate the temporal response as well as the final disposition of the affected neurons.

**CREB**

CREB is a transcription factor involved in adult neurogenesis, learning and memory (Merz *et al*., 2011). CREB is involved in many functions in the nervous system, including neurogenesis and neuronal survival, development, differentiation, neuroprotection, axonal outgrowth and regeneration, synaptic plasticity (Mioduszewska *et al.,* 2003; Persengiev & Green, 2003; Dragunow, 2004; Barco & Kandel, 2006)**.** Genes whose transcription is regulated by CREB include: c-fos, BDNF, tyrosine hydroxylase and neuropeptides such as somatostatin, enkephalin, VGF and corticotropin-releasing hormone (Lauren, 2005). BDNF is a survival gene contains cAMP response element. It is a crucial neurotrophic factor and possess pro-survival and/or differentiation effects on several neuronal populations and synaptic plasticity (Thoenen, 2000).

CREB is a downstream target of cyclic AMP signalling (Fusco *et al*., 2012). Multiple lines of evidence define a role for CREB in proliferation and differentiation of certain cells and tissues (Heasley *et al.,* 1991; Spaulding, 1993; Iyengar, 1996). Disruption of CREB activity, using expression of a dominant-negative CREB slows neurite outgrowth and blocks adipocyte differentiation (Engelman *et al.,* 1998; Shimomura *et al.,* 1998).

**Akt**

Protein kinase B (PKB, also known as Akt) is an important regulator involved in several cellular functions including cell growth and apoptosis (Hanada *et al.*, 2004; Feng *et al*., 2010; Diez *et al*., 2012). Akt is a member of the serine/threonine kinase family (Alessi *et al.,* 1997). Akt is an important mediator of the physiological effects of several growth and survival factors and promotes cell survival through the inhibition of apoptosis (Downward, 1998; Datta *et al.,* 1999). Within the nucleus, Akt controls expression of genes involved in cell survival *via* the transcription factors Forkhead, NF-κB and CREB (Brunet *et al.,* 2001). In non-neuronal cells certain survival stimuli activate Akt independently of $PI_3$-kinase including agonists of the PKA pathway and increases cytoplasmic calcium levels (Moule *et al.,* 1997; Sable *et al.,* 1997; Yano *et al.,* 1998; Filippa *et al.,* 1999).

There are three distinct isoforms (a, b, g) that are widely expressed around the body, although PKBg (Akt3) is the major isoform expressed in neurons (Brodbeck *et al.,* 1999; Masure *et al.,* 1999). It is a member of the cAMP-dependent, cGMP-dependent and protein kinase C (AGC) family of protein kinases and is a common target of growth factor signalling pathways including insulin. Activation of Akt is required for the neuroprotective function of growth factors such as IGF1 (Cleveland-Donovan *et al*., 2010; Suzuki *et al*., 2013) and expression of wild type Akt protects neurons against toxin-induced death (Dudek *et al.,* 1997; Zhou *et al.,* 1998, 2000).

Observations over the past decade have identified the $PI_3K$-Akt pathway's importance in mediating survival in PC12 cells and cultured neurons from the peripheral and central nervous systems (Yao & Cooper, 1995; Ghosh & Greenberg, 1995; Crowder & Freeman, 1998). Neurotrophic factors such as NGF, BDNF, glial cell line-derived neurotrophic factor (GDNF) and IGF-1 activate the $PI_3$-Akt signalling cascade through corresponding receptor tyrosine kinases such as the high affinity neurotrophin receptors (Trk's) (Segal & Greenberg, 1996). After receptor dimerization, $PI_3K$ is recruited to the plasma membrane where its

catalytic subunit generates lipid second messengers, phosphoinositide phosphates ($PIP_2$, $PIP_3$), at the inner surface of the plasma membrane. Phosphoinositide-dependent protein kinase-1 (PDK1) then acts in concert with $PIP_2$ and $PIP_3$ to phosphorylate and activate Akt (Brunet *et al.,* 2001). Alternately Trk receptors stimulate $PI_3K$ *via* the Ras G-protein, IRS signalling and Gab-1, an adaptor protein which binds to Trk and directly stimulates $PI_3K$ (Holgado-Madruga *et al.,* 1997; Kauffmann-Zeh *et al.,* 1997). Studies have demonstrated downstream signalling effects that regulate cellular survival, proliferation and metabolism. For example, Akt phosphorylates and inactivates forkhead transcription factor like 1 (FKHRL1), a member of the family of Fork head transcriptional regulators. Inactivated FKHRL1 is unable to induce the expression of death genes in cerebellar granule neurons (Brunet *et al.,* 1999). In primary hippocampal neurons subjected to hypoxia or nitric oxide, p53 activation and p53-mediated Bax up regulation are also blocked by Akt signalling (Yamaguchi *et al.,* 2001). Akt activates the CREB and NF-κB, additional transcriptional regulators that promote neuronal survival (Maggirwar *et al.,* 1998; Bonni *et al.,* 1999). In addition Akt can directly inhibit the apoptotic machinery by phosphorylation at sites both upstream (BAD) and downstream (Caspase-9) of mitochondrial cytochrome C release (Datta *et al.,* 1997; Cardone *et al.,* 1998). Finally, there is evidence to support the role of Akt in promoting neuronal survival through metabolic effects by regulating glucose metabolism in neurons (Hetman *et al.,* 2000).

**BDNF**

Brain-derived neurotrophic factor (BDNF) is important in differentiation, survival and plasticity of the CNS. BDNF has been demonstrated to be important to the regulation of energy metabolism. Recent studies also have shown significant roles of BDNF in energy metabolism regulation. Effects of BDNF on energy metabolism have also been observed in human subjects. In a clinical case report, a patient with severe obesity carries a mutation in the BDNF receptor TrkB (Yeo *et al.,* 2004). These data suggest that BDNF is important to energy metabolism regulation. Hyperphagia and obesity occur in animal models with BDNF

deficiency (Lyons *et al.,* 1999; Kernie *et al.,* 2000; Rios *et al.,* 2001). Chronic ventricular or peripheral administration of BDNF decreases food intake and body weight gain and reverses the phenotype of obese and hyperphagic BDNF +/_ mice (Sauer *et al.,* 1993; Kernie *et al.,* 2000; Ono *et al.,* 2000) . BDNF is a potent trophic factor supports striatal cells and promotes survival and/or differentiation of GABAergic neurons *in vitro* (Mizuno *et al.,* 1994; Ventimiglia *et al.,* 1995). BDNF is a survival gene contains cAMP response element. It is a crucial neurotrophic factor and possess pro-survival and differentiation effects on several neuronal populations and synaptic plasticity (Thoenen, 2000).

**GDNF**

Many studies with *in vitro* and *in vivo* models have shown that Glial cell line-derived neurotrophic factor (GDNF) supports neuritic outgrowth or survival of mesencephalic dopaminergic neurons, cranial nerve and spinal cord motor neurons, brain stem noradrenergic neurons (Arenas *et al.,* 1995), basal forebrain cholinergic neurons, Purkinje cells and certain groups of dorsal ganglion and sympathetic neurons (Lin *et al.,* 1993; Kreiglestein *et al.,* 1995; Siegel & Chauhan, 2000). The binding of GDNF to GFRα receptors activates a transmembrane tyrosine kinase, c-Ret and induces further downstream signalling *via* multiple pathways including the MAP kinase pathway and phospolipase Cγ pathway. GDNF also induces responses through c-Ret-independent mechanisms such as the activation of Src family tyrosine kinases and interaction of the receptor complex with neural cell adhesion molecule (Sariola & Saarma, 2003; Chiocco *et al.*, 2007).

**NF-κB**

NF-κB is a redox-sensitive nuclear transcriptional factor and is an important regulator of antioxidant enzymes (Rahman & MacNee, 2000). NF-κB is a dimeric transcription factor composed of five members, p50, RelA/p65, c-Rel, RelB, and p52 that can diversely combine to form the active transcriptional dimer.

NF-κB is ubiquitously expressed in peripheral and brain cells and regulates the expression of a wide variety of genes involved in cell survival, growth, stress responses, immune and inflammatory processes (Baldwin, 1996; Shimada *et al.*, 2001; Weih & Caamañ, 2003). NF-κB controls the expression of genes that regulate a broad range of biological processes in the CNS such as synaptic plasticity, neurogenesis and differentiation (Ghosh & Hayden, 2008). NF-κB family members have been implicated in the development of the nervous system and plasticity of synapses (O'Neill & Kaltschmidt, 1997; Meffert *et al.*, 2003; O'Mahony *et al.*, 2006). NF-κB has also been related to ROS while certain NF-κB regulated genes play a major role in regulating the amount of ROS in the cell. ROS have various inhibitory or stimulatory roles in NF-κB signalling. NF-κB is persistently activated in cancer, chronic inflammation, neurodegenerative diseases, stress, stroke, trauma, heart disease and other disease conditions (Mémet, 2006; Xiao *et al.*, 2006). NF-κB is an important regulator in programmed cell death and play important roles in normal brain function and neurodegenerative disorders (Grilli & Memo, 1999; Denk *et al.*, 2000; Mattson & Meffer, 2006). In the CNS, NF-κB can play an anti-apoptotic or pro-apoptotic role in cell death and regulates the genes involved in neuronal death and survival (Kaltschmidt *et al.*, 2005).

**IGF-1**

IGF-1 is genetically related polypeptide similar to insulin with similar three-dimensional and primary structures. IGF-1 is synthesized primarily in the liver and also in the brain. Its synthesis is regulated by growth hormone, insulin and nutritional intake (Mathews *et al.*, 1988; Russell-Jones *et al.*, 1992; Thissen *et al.*, 1994). IGF-1 receptors are widely expressed in the brain and are localized preferably in neuron rich structures in many brain areas, such as the granule cell layers of the olfactory bulb, hippocampal formation and cerebral cortex. It has profound effects on the regulation of proliferation and differentiation of many cell types as well as metabolic effects, which are similar to those of insulin, including actions on glucose metabolism.

IGF-1 is involved in neuronal development, stimulates neurogenesis and synaptogenesis, facilitates oligodendrocyte development, promotes neuron and oligodendrocyte survival and stimulates myelination. All this speaks about a very important role it has in preserving the integrity of neuronal cells and in protecting the brain structures from damages and injury (D'Ercole *et al.*, 2002). The alterations of proteins, the components of brain insulin- and IGF-1-regulated signalling cascades, typical of diabetes mellitus, pre-diabetic states and are the causes of the diabetes associated neurodegenerative diseases.

**Second Messengers**

Neurons use many different second messengers as intracellular signals. These messengers differ in the mechanism by which they are produced and removed, as well as their downstream targets and effects. Second messenger systems are complexes of regulatory and catalytic proteins, which are activated by first messengers to form second messengers. Second messengers relay signals received at receptors on the cell surface to target molecules in the cytosol and/or nucleus. Three major classes of second messengers are (1) cyclic nucleotides (e.g., cAMP and cGMP), (2) inositol trisphosphate ($IP_3$) and diacylglycerol (DAG), (3) calcium ions ($Ca^{2+}$). The signal transduction in metabotrophic neurotransmitters occur through activation of second messengers, whereas ionotrophic neurotransmitters act through ligand gated ion channels. The changes in neurotransmitter level and its receptor should agree with a concomitant change in second messenger for effective signal transduction.

**cAMP**

cAMP is produced from ATP in response to a variety of extracellular signals such as hormones, growth factors and neurotransmitters. Cyclic AMP is produced when G-proteins activate adenylyl cyclase in the plasma membrane. This enzyme converts ATP into cAMP by removing two phosphate groups from the ATP. Elevated levels of cAMP in the cell lead to activation of different cAMP

targets. It was long thought that the only target of cAMP was the cAMP-dependent protein kinase (cAPK) which has become a model of protein kinase structure and regulation (Doskeland *et al*., 1993; Francis & Corbin, 1999; Canaves & Taylor, 2002). cAMP produced by adenylyl cyclase, activates PKA by binding to the regulatory subunits in ways that result in the release and nuclear translocation of active catalytic subunits (Meinkoth *et al.,* 1993). cAMP stimulates the proliferation of many cell types, but in some cases cAMP actually inhibit cellular proliferation (Pastan *et al.,* 1975; Bokoch, 1993; Dugan *et al.,* 1999; Hagemann & Rapp, 1999; Zwartkruis & Bos, 1999; Wang *et al.,* 2000).

**cGMP**

An essential element of the signalling cascade leading to synaptic plasticity is the intracellular second messenger molecule guanosine 3′,5′-cyclic monophosphate (cGMP). Cyclic GMP is similarly produced from GTP by the action of guanylyl cyclase. Once the intracellular concentration of cGMP is elevated, these nucleotides bind to the targets. The most common target of cGMP is cGMP-dependent protein kinase (PKG).The nitric oxide–cyclic guanosine 3',5' mono phosphate (NO-cGMP) pathway is a key player in a range of neuronal functions including neuroprotection and neurotoxicity (Weill & Green, 1984; Thippeswamy & Morris, 1997; Lipton *et al.,* 1994; Kim *et al.,* 1999; Thippeswamy *et al.,* 2001a; Fiscus, 2002; Nakamizo *et al.,* 2003; Duchen, 2004). The physiological functions of NO are mediated by activation of soluble guanylyl cyclases (sGC) which generates cGMP (Gibb *et al.,* 2003). Under pathological conditions such as cerebral ischemia, sGC-independent mechanism appears to be involved in the neurotoxic action of NO (Nelson *et al.,* 2003). Recent evidence suggests that cGMP-mediated actions of NO are protective to both neurons and glia (Thippeswamy & Morris, 1997; Thippeswamy *et al.,* 2001b, 2002; Andoh *et al.,* 2002; Ha *et al.,* 2003; Snyder & Kim, 2004). In addition, cAMP and cGMP can bind to certain ion channels, thereby influencing neuronal signalling.

**$IP_3$**

The cellular responses elicited by the interaction of many extracellular signalling molecules with their cell surface receptors are triggered by the rapid hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2). This reaction is catalyzed by phosphoinositide-specific phospholipase C (PLC) isozymes and results in the generation of two intracellular messengers, DAG and $IP_3$. These messengers then promote the activation of protein kinase C and the release of $Ca^{2+}$ from intracellular stores. $IP_3$ is further converted by the actions of several distinct kinases and phosphatases to a variety of inositol phosphates, some of which are also implicated in intracellular signalling. IP3 is a ubiquitous second messenger that functions by binding to receptors (IP3Rs) on the ER membrane to cause liberation of sequestered $Ca^{2+}$ (Berridge, 1998, 2002). The resultant cytosolic $Ca^{2+}$transients serve numerous signalling functions in neurons, including modulation of membrane excitability synaptic plasticity and gene expression (Fujii *et al.,* 2000; Miyata *et al.,* 2000; Nishiyama *et al.,* 2000; Mellstrom & Naranjo, 2001; Yamamoto *et al.,* 2002; Stutzmann *et al.,* 2003). The neuronal intracellular calcium has an important role in the regulation of synaptic plasticity (Barbara, 2002). Moreover, disruptions in this pathway are implicated in neurodegenerative disorders (Abe, 1997; Mattson *et al.,* 2000; LaFerla, 2002). Therefore, factors that modulate or disrupt IP3-mediated $Ca^{2+}$ signalling are expected to exert powerful physiological and possibly pathological effects on the nervous system. Numerous stimuli have been reported to activate the $IP_3$ pathway and receptor stimulation is commonly linked to phospholipase C activation, as with certain neurotransmitters, growth factors and hormones (Rana & Hokin, 1990).

Liver cell proliferation after partial hepatectomy is associated with the action of several growth factor, cytokine, neurotransmitter, second messengers and transcription factor. Both liver and brain are victims of the disturbed metabolic functions. The non metabolized compounds due to inefficiency of liver cells to process these metabolites enter brain and cause adverse effects like neuronal

apoptosis and imbalance in neurotransmissions. Thus a therapeutic agent that favours enhancement in liver cell proliferation along with neuronal maintenance gains immense importance. The present study focused on detailed relevance of GABA and 5-HT chitosan nanoparticles treatment in liver cell proliferation and neuronal survival during liver injury by giving emphasis on motor co-ordination, GABA and 5-HT receptors mediated signalling, expression of apoptotic factors and growth factors in both liver and brain.

# *Materials and Methods*

## CHEMICALS USED IN THE STUDY AND THEIR SOURCES

### Biochemicals

Serotonin creatinine sulfate, γ-aminobutyric acid, ketanserin, baclofen, bovine serum albumin fraction V, SOD, sodium octyl sulfonic acid, ethylene diamine tetra acetic acid (EDTA), Tris HCl, sucrose, magnesium chloride, calcium chloride, bromodeoxyuridine (BrdU) and paraformaldehyde (PFA) were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally from SRL, India. HPLC solvents were of HPLC grade obtained from SRL and MERCK, India. Tissue freezing medium Jung was purchased from Leica Microsystems Nussloch GmbH, Germany. Chitosan (MW-25KDa) was a gift from Central Institute of Fisheries Technology, Cochin, India.

### Radiochemicals

[Ethylene-$^{3}$H]-ketanserin hydrochloride (Sp. Activity 63.3Ci/mmol), [$^{3}$H] baclofen (Sp. Activity 42.9 Ci/mmol), [$^{3}$H] Gamma aminobutyric acid (Sp. Activity 76.2 Ci/mmol), [$^{3}$H] thymidine (Sp. Activity 18.0 Ci/mmol) and [$^{3}$H] leucine (Sp. Activity 63.0 Ci/mmol) were purchased from Amersham Bioscience, USA. [$^{3}$H] methyl *S*-adenosylmethionine (Sp. Activity 80 Ci/mmol, [$^{3}$H] $IP_3$, [$^{3}$H] cAMP and [$^{3}$H] cGMP kits were purchased from American Radiolabelled Chemicals, USA.

### Molecular Biology Chemicals

Tri-reagent kit was purchased from Sigma Chemical Co., St. Louis, USA. ABI PRISM High Capacity cDNA Archive kit, Primers, endogenous control (β-actin) and Taqman probes for Real-Time PCR were purchased from Applied Biosystems, Foster City, CA, USA. $GABA_B$ (Rn_00578911), $5HT_{2A}$ (Rn_01468302), CREB (Rn_00561126), phospholipase C (Rn_01647142), IGF-1 (Rn_99999087), SOD (Rn01477289), Bax (Rn_01480160) Akt-1 (Rn_00583646), NF-κB (Rn_01399583), TNF-α (Rn_99999017), Caspase-8 (Rn_00574069),

hepatocyte growth factor (Rn_00690368), Mat2A (Rn_01643368), BDNF (Rn_01484924), and GDNF (Rn_00569510) primers were used for the gene expression studies.

**Confocal Dyes**

Rat primary antibody for 5-$HT_{2A}$ (No: ab16028, Abcam), $GABA_B$ (No: ab68426), Bromo deoxyuridine (Cat. No. B8434, Sigma Aldrich, USA) and secondary antibody of Alexa Fluor 594 (No: A11012 and No: A11005, Invitrogen) were used for the immunohistochemistry studies using confocal microscope.

**ANIMALS**

Adult male Wistar rats of 250-300g body weight were purchased from Kerala Agriculture University, Mannuthy, India and Amrita Institute of Medical Sciences, Kochi, India and used for all experiments. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water *ad libitum*. Adequate measures were also taken to minimize pain and discomfort of the animals. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health and CPCSEA guidelines.

**PREPARATION OF GABA AND 5-HT CHITOSAN NANOPARTICLES**

The chitosan nanoparticles were prepared by ionic gelation method (Calvo *et al*., 1997). Chitosan was dissolved in 2% acetic acid to get chitosan solution of concentration 1mg/mL. Chitosan nanoparticles from 50 mL chitosan solution were precipitated by the addition of 33 mL of 1 mg / mL penta sodium tri polyphosphate (TPP) solution with rapid stirring. To incorporate GABA in to chitosan, a solution of concentration, 8.824 µg of GABA / mL of chitosan solution was prepared and the precipitation of GABA - chitosan nanoparticles were done by the above method. To incorporate serotonin creatinine sulphate (5-HT) to chitosan, 300 µg of 5-HT / mL of chitosan solution was prepared and the precipitation of 5-HT - chitosan nanoparticles were done. To prepare a

combination of GABA and 5-HT chitosan nanoparticles, 400 µg of 5-HT and 20 µg of GABA were dissolved in chitosan solution and the nanoparticles were precipitated by the addition of TPP. The precipitated nanoparticles were centrifuged for 20 minutes at 16,000xg. The pellet was washed thoroughly with distilled water and then resuspended in saline. The SEM image of the nanoparticles was taken with a magnification of 10000X by scanning electron microscope (JEOL Model JSM - 6390LV).

**FT-IR spectroscopy**

The FT-IR spectrum of chitosan, chitosan nanoparticles, GABA, GABA incorporated chitosan nanoparticles, 5-HT, 5-HT incorporated chitosan nanoparticles and GABA and 5-HT incorporated chitosan nanoparticles were taken using Fourier Transform Infra Red spectrometer (Thermo Nicolet, Avatar 370) with a spectral range of 4000-400 $cm^{-1}$.

**Determination of encapsulation efficiency and *in vitro* release studies**

The maximum encapsulation efficiency (Rao *et al*., 2010) of GABA with chitosan nanoparticles was obtained by giving emphasis to concentration of GABA added to the chitosan solution. The encapsulation efficiency was calculated by incorporating [$^{3}$H] GABA with chitosan and the radioactivity of the GABA, which was bound on the chitosan nanoparticles, were related to its concentration (Motulsky & Christopaulos, 2004; Shilpa *et al*., 2012). Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The same method is adopted for determining the encapsulation efficiency of GABA in GABA and 5-HT chitosan nanoparticles.

The maximum encapsulation efficiency of 5-HT in both 5-HT chitosan nanoparticles and GABA and 5-HT chitosan nanoparticles was obtained by considering the 5-HT concentration that was added to the chitosan solution. The concentration of 5-HT, which was bound to the nanoparticles, were found by HPLC with electrochemical detector (Waters, USA) fitted with CLS-ODS reverse

phase column of 5 µm particle size. After centrifugation of nanoparticle suspension, the supernatant with unbound 5-HT was filtered through 0.22 µm HPLC grade filters and injected to the column. The mobile phase consisted of 50mM sodium phosphate dibasic, 0.03M citric acid, 0.1 mM EDTA, 0.6 mM sodium octylsulfonate and 15% methanol. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.22 µm (Millipore) and degassed. A Waters model 515, Milford, USA, pump was used to deliver the solvent at a rate of 1 mL/minute. The 5-HT was identified by amperometric detection using an electrochemical detector (Waters model 2465) with a reduction potential of +0.80V. The peaks obtained were compared with standard creatinine sulphate and quantitatively estimated using an integrator (Empower software) interfaced detector.

% Encapsulation = (Concentration of GABA or 5-HT bound to chitosan nanoparticles / Concentration of GABA or 5-HT added initially) x 100.

In *in vitro* release studies, the different nanoparticles were suspended in the respective PBS solution, pH 7.4. All were gently stirred at different time intervals from 0 to 40 hours. The concentration of released GABA and 5-HT at each time from the nanoparticles was calculated using the above mentioned methods to get a release profile *in vitro*.

## CELL UPTAKE OF GABA AND 5-HT CHITOSAN NANOPARTICLES

### Preparation of FITC labeled chitosan nanoparticles

**C**hitosan solution (1mg/mL), of volume 50mL was prepared and the nanoparticles were precipitated by adding TPP. The nanoparticles were centrifuged and the pellet was resuspended in 5 mL DMSO and sonicated for 1 minute. Then a solution of 10 mg/ mL FITC in DMSO was added to the nanoparticle suspension. Stirred the solution gently and kept overnight at dark. After stirring, particles were washed with DMSO several times until the non conjugated FITC was eliminated completely (Min *et al*., 2004). The liver was perfused initially with $Ca^{2+}$ buffer, pH 7.4 (142 mM NaCl, 6.7 mM KCl, 10 mM HEPES and 5.5 mM NaOH) and then with collagenase buffer, pH 7.6 (67 mM

NaCl, 6.7 mM KCl, 100 mM HEPES, 4.76 mM $CaCl_2$. $2H_2O$, 0.66 mM NaOH and collagenase). The perfused liver was minced in PBS, pH 7.4 and kept for collagenase digestion. The cells were filtered and washed. Resuspended the cells in William's media and 150 µL of cell suspension (cell density of $1.6 \times 10^5$ cells/$cm^2$) was added to a four well glass slide. Then the cells were incubated in 5% $CO_2$ atmosphere for 24 hours at 37°C.

**Uptake of FITC labeled nanoparticles by liver cells**

50 µL of FITC labeled and unlabelled nanoparticles were added to the corresponding cell suspension in each well and incubated for 2 hours. After the incubation, the fluorescent images were captured using confocal microscope with an excitation at 488 nm (Yuqing *et al.*, 2009).

**EXPERIMENTAL DESIGN**

The experimental rats were divided into the following five groups

1. Sham operated control (C)
2. Partially hepatectomised group without any treatment (PHNT)
3. Partially hepatectomised group with GABA chitosan nanoparticle treatment (GCNP)
4. Partially hepatectomised group with 5-HT chitosan nanoparticle treatment (SCNP)
5. Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment (GSCNP)

Each group consisted of 4-6 animals.

Chitosan nanoparticles modified with drugs, could be recognized by their respective receptors on cells and were transferred to liver cells through receptor mediated endocytosis. This enhanced their ability to target to the liver, in which receptor mediated cell signalling was activated and enabled the longevity of these nanoparticles in the liver. In contrast, chitosan nanoparticles without modification, targeted sparely to the liver, and a large part of these nanoparticles were cleared

from the body in urine (Tian *et al*., 2010; Park *et al*., 2007). So giving importance to the above fact and based on our previous observations, a control group treated with chitosan nanoparticle alone was not included.

Two – thirds of the liver constituting the median and left lateral lobes were surgically excised under anaesthesia, following a 16 hour fast (Higgins & Anderson, 1931). Sham operations involved median excision of the body wall followed by all manipulations except removal of lobes. All rats were undergone surgeries between 7 and 9 A.M to avoid diurnal variations in responses.

After the surgical excision of median and left lateral lobes of liver, 1 mL of 30 µg/µL GABA chitosan nanoparticles, 5-HT chitosan nanoparticles and GABA and 5-HT chitosan nanoparticles suspended in saline were injected intra peritoneal to the respective rats.

**Sacrifice and tissue preparation**

All the molecular level changes in liver including the DNA synthesis prior to the first mitotic phase occur between 20 and 24 h post hepatectomy (Bucher, 1963). The assays and experiments performed during this period provided a significant comparison among the experimental groups. Thus the experimental rats were sacrificed by decapitation 24 hours post hepatectomy. The liver, corpus striatum, cerebral cortex and brain stem were dissected out quickly over ice according to the procedure of Glowinski & Iversen (1966). The blood samples were collected and plasma was separated by centrifugation. The tissue samples and plasma were kept at -80° C until assay. All animal care and procedures were in accordance with Institutional and National Institute of Health guidelines.

## EFFECT OF GABA AND 5-HT ENCAPSULATED CHITOSAN NANOPARTICLES ON DNA AND PROTEIN SYNTHESES IN LIVER CELLS

After partial hepatectomy and treatments, liver from all the experimental groups were perfused first with $Ca^{2+}$ buffer and then with collagenase buffer. Cell suspension of 150 µL (cell density of $1.6 \times 10^5$ cells/$cm^2$) was added to a four well

poly L-lysine coated glass slide. Then the cells were incubated for 24 hours at 37°C in 5% $CO_2$ atmosphere. Before incubation, [$^3$H] leucine of specific activity 63 Ci/mmol was added to one set of culture plates for all the five experimental groups to determine the protein synthesis and [$^3$H] thymidine of specific activity 18 Ci/mmol to the next set of plates to determine the measurement of DNA synthesis. All the experiments were done in triplicates. The cells were scrapped off from the culture plates and centrifuged at 2000xg for 20 minutes. The supernatant was discarded and the pellet was resuspended in 50 µL, 1M NaOH and kept overnight. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

DNA synthesis was further determined by analyzing the activity of thymidine kinase (TK) in all the experimental groups. A 10% liver homogenate was prepared in 50 mM Tris HCl buffer, pH 7.5. It was centrifuged at 36000xg for 30 minutes and the supernatant was collected. TK was assayed by determining the conversion of [$^3$H] thymidine in the presence of ATP to [$^3$H] thymidine monophosphate (TMP) by the binding of latter nucleotide to DEAE cellulose discs (Maliekal *et al*., 1997). The reaction mixture contained 5mM [$^3$H] thymidine (0.5µCi), 10mM ATP, 100 mM NaF, 10 mM $MgCl_2$, 0.1 M Tris- HCl buffer, pH 8.0 and the liver supernatant fraction. After incubation at 37ºC for 15 minutes the reaction mixture was spotted in DE 81 paper discs. The bound radioactivity of [$^3$H] thymidine monophosphate was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

**BEHAVIOURAL STUDIES**

Animals were observed everyday for any overt abnormal activity.

**Beam-walk test**

After three days post hepatectomy, the animals were tested for the balance and motor coordination on a narrow beam maze (Allbutt & Henderson, 2007). This has a smooth wooden narrow beam of 105cm long, 4cm in width and thickness of 3cm. The beam was elevated from the ground by 1m with additional

supports. It has a start platform of 20cm in dimension from the start of the beam and an end platform of 20cm dimension at the end of 105cm long beam. There was food on the end platform for the reward of the animals. The journey time between start and end goal was measured. The time was recorded when the animal placed a weight bearing step entirely over the start line. The stopwatch was then stopped when all four feet were placed entirely upon the finishing platform at the opposite end of the beam. The maximum time allowed for the task was 2 min.

**Rotarod Test:**

Rotarod has been used to evaluate motor coordination by testing the ability of rats to remain on revolving rod (Dunham & Miya, 1957). The apparatus has a horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70 cm long rod was divided into four sections by wooden partitions. The rod was placed at a height of 50 cm to discourage the animals to jump from the rotating rod. The rate of rotation was adjusted in such a manner that it allowed the normal rats to stay on it for five minutes. Each rat was given five trials before the actual reading was taken. The readings were taken at 10, 15 and 25 rpm after three days of hepatectomy in all groups of rats.

**Grid Walk Test**

Deficits in descending motor control were examined by assessing the ability to navigate across a 1 m long runway with irregularly assigned gaps (0.5–5 cm) between round metal bars. Crossing this runway requires that animals accurately place their limbs on the bars. In baseline training and postoperative testing, every animal had to cross the grid for at least three times. The number of footfalls (errors) was counted in each crossing for 3 minute and a mean error rate was calculated (Z'Graggen *et al.*, 1998).

## $GABA_B$ AND 5-$HT_{2A}$ RECEPTORS BINDING STUDIES USING [$^3$H] BACLOFEN AND [$^3$H] KETANSERIN

[$^3$H] Baclofen binding to $GABA_B$ receptor in the membrane preparations were assayed (Hills *et al*., 1987). Crude membrane preparation was suspended in 50 mM Tris sulphate buffer, pH 7.4 containing 2 mM $CaCl_2$ and 0.3 - 0.4 mg protein. In saturation binding experiments, 10-100nM of [$^3$H] baclofen was incubated with and without excess of 100 µM unlabelled baclofen. The incubations were carried out at 20ºC for 20 minutes. The binding reactions were terminated by centrifugation at 14000xg for 10 minutes. The dried pellet was resuspended and counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

[$^3$H] Ketanserin binding to 5-$HT_{2A}$ receptor in the crude synaptic membrane preparation was done according to the modified procedure of Leysen *et al*. (1982). Crude membrane preparation was suspended in 50 mM Tris sulphate buffer, pH 7.6 containing 0.3 - 0.4 mg protein. In saturation binding experiments, assays were done using different concentrations of 0.5-10nM of [$^3$H] ketanserin which was incubated with and without excess of unlabelled 10 µM ketanserin. Tubes were incubated at 37ºC for 15 minutes and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washings with 5.0 ml of ice cold 50 mM Tris sulphate buffer, pH 7.6. The bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

### Protein determination

Protein was measured by the method of Lowry *et al*., (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein which was read in a spectrophotometer (Shimadzu UV-1700) at 660nm.

## ANALYSIS OF THE RECEPTOR BINDING DATA

### Linear regression analysis for Scatchard plots

The data was analysed according to Scatchard (1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding ($B_{max}$) and equilibrium dissociation constant ($K_d$), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The $K_d$ is inversely related to receptor affinity.

## GENE EXPRESSION STUDIES IN LIVER AND DIFFERENT BRAIN REGIONS OF EXPERIMENTAL RATS

### Preparation of RNA

RNA was isolated from the liver and different brain regions - brain stem, corpus striatum and cerebral cortex of control and experimental rats using Tri reagent from Sigma Chemical Co., St. Louis, USA.

### Isolation of RNA

Tissue (25-50 mg) homogenates were made in 0.5mL Tri Reagent and was centrifuged at 12,000 x *g* for 10 minutes at 4°C. The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5 minutes. 100μL of chloroform was added to it, mixed vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. The tubes were then centrifuged at 12,000 x *g* for 15 minutes at 4°C. Three distinct phases appear after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and a colourless upper aqueous phase contained RNA. The upper aqueous phase was transferred to a fresh tube and 250 μL of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000 x *g* for 10 min at 4ºC. RNA precipitated as a

pellet on the sides and bottom of the tube. The supernatants were removed and the RNA pellet was washed with 500µL of 75% ethanol, vortexed and centrifuged at 12,000 x *g* for 5 min at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2µL of RNA was made up to 1mL and absorbance was measured at 260 nm and 280 nm in spectrophotometer (Shimadzu UV-1700). For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one absorbance $_{260}$ = 42µg.

**cDNA Synthesis**

Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit in 0.2mL microfuge tubes. The reaction mixture of 20µL contained 0.2µg total RNA, 10X RT buffer, 25X dNTP mixture, 10X Random primers, MultiScribe RT (50U/µL) and RNase free water. The cDNA synthesis reactions were carried out at 25°C for 10 minutes and 37°C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA designed using Primer Express Software Version (3.0).

**Real-Time PCR Assay**

Real Time PCR assays were performed in 96-well plates in ABI 7300 Real Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled TaqMan probe (designed by Applied Biosystems). Endogenous control, β-actin, was labelled with a reporter dye (VIC). All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labeled with FAM at the 5' end and a quencher (Minor Groove Binding Protein - MGB) at the 3' end. The real-time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

The TaqMan reaction mixture of 20μL contained 25ng of total RNA-derived cDNAs, 200nM each of the forward primer, reverse primer and TaqMan probes, endogenous control (β-actin) and 12.5μL of TaqMan 2X Universal PCR Master Mix (Applied Biosystems). The volume was made up with RNase free water. Each run contained both negative (no template) and positive controls.
The thermocycling profile conditions were as follows:

| | | |
|---|---|---|
| 50ºC -- 2 minutes | --- Activation | |
| 95ºC -- 10 minutes | --- Initial Denaturation | |
| 95ºC -- 15 seconds | --- Denaturation | 40 cycles |
| 50ºC -- 30 seconds | --- Annealing | |
| 60ºC -- 1 minute | --- Final Extension | |

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{-actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained ($2^{-\Delta\Delta CT}$).

**DETERMINATION OF SOD ACTIVITY**

The liver, brain stem, cerebral cortex and corpus striatum were homogenized in 0.1M potassium phosphate buffer, pH 7.8 and centrifuged at 100,000 x g for 60 min at 4°C. The supernatant corresponds to the cytosolic fraction containing CuZn-SOD. The pellets were resuspended in the buffer, freeze-thawed three times and centrifuged at 100,000 x g for 60 min at 4°C. The supernatant, the particulate fraction containing Mn-SOD, was mixed with the cytosolic fraction to obtain the total enzyme fraction. SOD was analyzed after inhibition by SOD of the pyrogallol autoxidation (Marklund & Marklund, 1974) at

pH 8.2 in the presence of EDTA. A 3ml assay mixture contained 0.2 mM pyrogallol, 1 mM EDTA and 50 mM Tris-HCl buffer. Pyrogallol autooxidation was monitored at 420 nm for 3 min in a spectrophotometer (Shimadzu UV-1700) with or without the enzyme. The inhibition of pyrogallol oxidation was linear with the activity of the enzyme present. Fifty percent inhibition/mg protein/min was taken as one unit of the enzyme activity.

**DNA METHYLATION STUDY**

The DNA was isolated from the liver of experimental rats using TRI reagent according to the procedure of Chomczynski (1993). DNA concentration was determined by ultraviolet spectrophotometry (UV-1700 Pharma Spec, Shimadzu) with absorbance at 260 and 280 nm. All DNA samples had 260 to 280 absorbance ratios ≥ 1.7. DNA methylation was determined by using the modified method of Balaghi and Wagner (1993), in which DNA is incubated with [$^3$H] methyl *S*-adenosylmethionine in the presence of the CpG Methyl transferase. The reaction mixture contained 0.25 µg DNA, 0.015 U CpG Methyl transferase enzyme (product no. M0226S; New England Biolabs, Beverly, MA), [$^3$H] methyl *S*-adenosylmethionine (80 Ci/mmol, American radiolabeled chemicals, Inc., Saint Louis, USA), 1.5 µl NEB buffer (New England Biolabs, Beverly, MA), and sterile-filtered water to a total reaction volume of 15 µl. The mixture was incubated at 30 °C for 1 h and placed on ice for 5 min. The reaction mixture were loaded onto a 2.5-cm, round, Whatman DE81 ion-exchange paper filter. The filter was washed successively 3 times with 7.5 ml of 0.5 M sodium phosphate buffer (pH 8.0), then with 1 ml 70% ethanol, and finally with 1mL 100% ethanol. The filter was dried at room temperature and the radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The ability of DNA to incorporate [$^3$H] methyl groups *in vitro* is inversely related to endogenous DNA methylation.

**cAMP CONTENT IN THE LIVER AND BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO***

Brain tissues -(cerebral cortex, brain stem and corpus striatum) and liver were homogenised in a polytron homogeniser with cold 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15min and the supernatant was transferred to fresh tubes for cAMP assay using [$^3$H] cAMP Biotrak Assay System kit.

**Principle of the assay**

cAMP assay kit was used. The assay is based on the competition between unlabelled cAMP and a fixed quantity of [$^3$H] cAMP for binding to a protein which has a high specificity and affinity for cAMP. The amount of labeled protein -cAMP complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. Measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated.

| | | | | |
|---|---|---|---|---|
| Free [$^3$H] cAMP | | | Bound [$^3$H] cAMP-binding protein | |
| | + | Binding protein | = | + |
| cAMP | | | | cAMP-binding protein |

Separation of the protein bound cAMP from unbound nucleotide is achieved by adsorption of the free nucleotide on to a coated charcoal followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve.

**Assay Protocol**

The tubes were placed on a water bath at 0°C. The assay mixture consisted of different concentrations of standard, [$^3$H] cAMP and binding protein in case of standards; buffer, [$^3$H] cAMP and binding protein for zero blank and unknown samples, [$^3$H] cAMP and binding protein for determination of unknown

samples. The mixture was incubated at 2°C for 2h. Cold charcoal reagent was added to the tubes and the tubes were immediately centrifuged at 12,000 x g for 2min at 2°C. Aliquots of the supernatant was immediately transferred to scintillation vials and mixed with cocktail-T and counted in a liquid scintillation counter (Wallac, 1409).

$C_o/C_x$ is plotted on the Y-axis against picomoles of inactive cAMP on the X- axis of a linear graph paper, where $C_o$ is the counts per minute bound in the absence of unlabelled cAMP and $C_x$ is the counts per minute bound in the presence of standard or unknown unlabelled cAMP. From the $C_o/C_x$ value for the sample, the number of picomoles of unknown cAMP was calculated.

## $IP_3$ CONTENT IN THE LIVER AND BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO*

Brain tissues-(cerebral cortex, brain stem and corpus striatum) and liver were homogenised in a polytron homogeniser in 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15 minutes and the supernatant was transferred to fresh tubes for $IP_3$ assay using [$^3$H] $IP_3$ Biotrak Assay System kit.

### Principle of the assay

The assay was based on competition between [$^3$H] $IP_3$ and unlabelled $IP_3$ in the standard or samples for binding to a binding protein prepared from bovine adrenal cortex. The bound $IP_3$ was then separated from the free $IP_3$ by centrifugation. The free $IP_3$ in the supernatant was then discarded by simple decantation, leaving the bound fraction adhering to the tube. Measurement of the radioactivity in the tube enables the amount of unlabelled $IP_3$ in the sample to be determined.

**Assay Protocol**

Standards, ranging from 0.19 to 25pmoles/tube, [$^3$H] $IP_3$ and binding protein were added together and the volume was made up to 100µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The tubes were then vortexed and incubated on ice for 15min and they were centrifuged at 2000 x g for 10min at 4°C. The supernatant was aspirated out and the pellet was resuspended in water and incubated at room temperature for 10min. The tubes were then vortexed and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with %B/Bo on the Y-axis and $IP_3$ concentration (pmoles/tube) on the X-axis of a semi-log graph paper. $\%B/B_o$ was calculated as:

$$\frac{\text{(Standard or sample cpm – NSB cpm)}}{\text{($B_0$ cpm – NSB cpm)}} \times 100$$

NSB- non specific binding and $B_0$ - zero binding. $IP_3$ concentration in the samples was determined by interpolation from the plotted standard curve.

**cGMP CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO***

Brain tissues- (cerebral cortex, brain stem and corpus striatum) were homogenised in a polytron homogeniser with cold 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15min and the supernatant was transferred to fresh tubes for cGMP assay using [$^3$H] cGMP Biotrak Assay System kit.

**Principle of the assay**

The assay is based on the competition between unlabelled cGMP and a fixed quantity of the [$^3$H] cGMP for binding to an antiserum, which has a high specificity and affinity for cGMP. The amount of [$^3$H] cGMP bound to the antiserum is inversely related to the amount of cGMP present in the assay sample. Measurement of the antibody bound radioactivity enables the amount of unlabelled cGMP in the sample to be calculated. Separation of the antibody bound cGMP from the unbound nucleotide was done by ammonium sulphate precipitation, followed by centrifugation. The precipitate which contains the antibody bound complex was dissolved in water and its activity was determined by liquid scintillation counting. The concentration of unlabelled cGMP in the sample was determined from a linear standard curve.

**Assay Protocol**

Standards, ranging from 0.5 to 4.0pmoles/tube, and [$^3$H] cGMP were added together and the volume was made up to 100µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The antiserum was added to all the assay tubes and then vortexed. The tubes were incubated for 90min at 2 - 8°C. Ammonium sulphate was added to all tubes, mixed and allowed to stand for 5min in ice bath. The tubes were centrifuged at 12000 x g for 2min at room temperature. The supernatant was aspirated out and the pellet was dissolved in water and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with $C_o/C_x$ on the Y-axis and cGMP concentration (pmoles/tube) on the X-axis of a linear graph paper. $C_o$- the cpm bound in the absence of unlabelled cGMP; $C_x$- the cpm bound in the presence of standard/unknown cGMP. cGMP concentration in the samples was determined by interpolation from the plotted standard curve.

## IMMUNOCYTOCHEMISTRY OF $GABA_B$ AND 5-$HT_{2A}$ RECEPTORS IN LIVER AND BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

The experimental rats were deeply anesthetized and was transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. After perfusion the liver from each experimental group was dissected out and fixed in 4% paraformaldehyde for 1 hour and then equilibrated with 30% sucrose solution in PBS (0.1 M). 10 μm liver sections were cut using Cryostat (Leica, CM1510 S). The sections were washed with PBS and then blocked for 1hour with PBS containing 5% normal goat serum and 0.1% triton X-100. The primary antibodies of $GABA_B$ (1:500 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) and 5-$HT_{2A}$ (1:1000 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) were added to the respective sections and incubated overnight at 4 °C. After overnight incubation, the liver slices were rinsed with PBS and then incubated with fluorescent labelled secondary antibody (Alexa Fluor 594, code-A11012) prepared in PBS with 5% normal goat serum and 0.1% triton X-100 at 1:1000 dilution. The sections were washed with PBS thoroughly and then observed and photographed using confocal imaging system (Leica SP 5). Quantification was done using Leica application suit advanced fluorescence (LASAF) software by considering the mean pixel intensity of the image. The fluorescence obtained depends on the number of receptors specific to the added primary antibody. The mean pixel intensity was directly related to the fluorescence emitted from the sections and calculated with the LASAF software. All the imaging parameters in the confocal imaging system like PMT, pinhole and zoom factor were kept same for imaging the sections of all experimental groups.

## IMMUNOCYTOCHEMISTRY OF BROMODEOXYURIDINE IN THE LIVER OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Liver cell replication was evaluated based on the incorporation of BrdU (Sigma-Aldrich, St. Louis, Mo), a thymidine analog that incorporates into DNA in

the S phase. All experimental groups of rats were intraperitoneally injected with BrdU, two hours prior to sacrifice with a dosage of 50 mg/kg body weight, dissolved in saline (Masson *et al.,* 2009). Anaesthetized animals were transcardially perfused with 4%paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4). After perfusion the liver was dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1M PBS. 6 µm sections were cut using Cryostat (Leica, CM1510 S). The sections were treated with PBST (PBS in 0.05% Triton X-100) for 20 min. Liver sections were blocked with 5% normal goat serum for 4 hours. The sections were then incubated overnight at 4 °C with mouse primary antibody for Bromo deoxyuridine (BrdU) (Cat. No. B8434, Sigma-aldrich, St. Louis, USA, 1: 500 dilution in a 1X PBS solution containing 5% normal goat serum). After overnight incubation the sections were washed with PBS and then incubated for 1 hour with secondary antibody conjugated with Alexa Fluor 594 (Cat. No- A11005, 1:500 dilution in a 1X PBS solution containing 5% normal goat serum). After the incubation, the sections were washed with PBS. Tap excess PBS off, the slides and mount cover glass with Prolong Gold anti-fade mounting media. The sections were observed and photographed using confocal imaging system (Leica SP 5). Quantification was done using 'Leica application suit advanced fluorescence (LASAF) software' by considering the mean pixel intensity of the image. The fluorescence obtained depends on the number of specific binding sites to the added primary antibody. The mean pixel intensity was directly related to the fluorescence emitted from the sections and calculated with the LASAF software. All the imaging parameters in the confocal imaging system like PMT, pinhole and zoom factor were kept same for imaging the sections of all experimental groups.

**STATISTICS**

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding

data were analysed using non-linear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). Empower software was used for HPLC analysis. Relative Quantification Software was used for analyzing Real-Time PCR results.

# *Results*

**Scanning electron microscopic images of GABA and 5-HT chitosan nanoparticles**

The nanoparticles were prepared by ionic gelation method using penta sodium tripolyphosphate (TPP) as a strong anion for precipitating nanoparticles. Spherical and aggregated particles of 80±6 nm were observed from S.E.M. photograph (Figure 1).

**FT-IR spectra of GABA, 5-HT and nanoparticles**

FT-IR spectra of chitosan, GABA, 5-HT, chitosan-TPP nanoparticles, GABA-chitosan nanoparticles, 5-HT chitosan nanoparticles and GABA and 5-HT chitosan nanoparticles were studied. The –NH bending peak was observed at 1642.18 $cm^{-1}$ in chitosan flakes. But when chitosan was precipitated as nanoparticles with the help of TPP, the –NH bending peak shifted to 1547.68 $cm^{-1}$ and a new peak 1639.12 $cm^{-1}$ appeared. This showed an interaction of phosphate groups of TPP with the amino groups of chitosan (Figure 2A, 2B).

The –NH stretching vibration at 3022.14-2618.33 $cm^{-1}$ of pure GABA was shifted to 3390.89-2970 $cm^{-1}$ in GABA- chitosan nanoparticles. This was due to the interaction of GABA with chitosan. C-H aliphatic stretching of GABA at 2957 $cm^{-1}$ and 2920 $cm^{-1}$ which was overlapping with -NH stretching bands changed its positions to 2977.26 and 2880.33 $cm^{-1}$. The weak assymetrical –NH bending in GABA at 1642 $cm^{-1}$ became broad and shoulder peaks were observed in GABA chitosan nanoparticles at 1500 $cm^{-1}$. The strong assymetrical $COO^-$ peak of GABA at 1596.11 $cm^{-1}$ disappeared due to the formation of salt by the interaction between carboxylate group of GABA and amino group of chitosan and thus a new shoulder peak at 1405.57 $cm^{-1}$ appeared. 1400.46 $cm^{-1}$ represented a weak $COO^-$ symmetrical peak and the influence of this was observed in GABA chitosan nanoparticles at 1405.57 $cm^{-1}$ (salt of carboxyl) (Figure 3A, 3B).

In 5-HT, the S-O bond was intense,broad and a short peak at 1230.44 $cm^{-1}$ was seen. –NH stretch at 3436 $cm^{-1}$ in chitosan nanoparticle was changed to

broadened peaks from 3464.72 $cm^{-1}$ to 3280 $cm^{-1}$ on binding with 5-HT. There was a shift in C-N stretch of amine in serotonin when the 5-HT chitosan nanoparticles were formed. In 5-HT chitosan nanoparticles, the S-O bond at 1080.79 $cm^{-1}$ and a short peak at1232.95 $cm^{-1}$ were observed. In the nanoparticle, a new peak at 1386.87 $cm^{-1}$ was formed which denotes the S-N interaction due to the reaction between sulphate ion in 5-HT and $NH_3^+$ ion in the chitosan (Figure 4A, 4B).

In GABA and 5-HT chitosan nanoparticles, new and modified peaks were observed due to the presence of GABA and 5-HT. In pure GABA, -CH aliphatic stretch at 2957 $cm^{-1}$ and 2920 $cm^{-1}$, which were overlapping with –NH stretching bands changed to 2924.8 and 2810 $cm^{-1}$ due to the interaction with chitosan. The peak at 1384.99 in chitosan nanoparticle, is a shoulder peak in the nanoparticles with GABA and 5-HT due to the formation of salt between GABA and chitosan. A peak at 1072.44 $cm^{-1}$, which denotes the S-O bond in the nanoparticles, shows the presence of 5-HT. A weak asymmetric –NH binding at 1642 $cm^{-1}$ in GABA became broad in GABA and 5-HT chitosan nanoparticles. The –NH bending at 1500 $cm^{-1}$ in non encapsulated chitosan nanoparticle was shifted to 1543.63 $cm^{-1}$ in 5-HT chitosan nanoparticles and also in GABA and 5-HT chitosan nanoparticles, which showed the interaction of 5-HT with chitosan nanoparticles (Figure 5).

**Encapsulation efficiency in binding GABA with chitosan nanoparticles**

The efficiency of interaction of GABA and 5-HT with chitosan was studied by varying concentrations of GABA and 5-HT. By changing the concentration of GABA from 0 to 35 µg and considering the chitosan solution of 1mg/mL, the maximum encapsulation of 93±8.3% was obtained at a concentration 8.824 µg of GABA / mL chitosan solution (Figure 6).

In 5-HT chitosan nanoparticles, changing the concentration of 5-HT from 0 to 400 µg/mL of chitosan solution (1mg/mL), a maximum encapsulation of 86±7.1% was obtained at a concentration 300 µg of 5-HT / mL chitosan solution (Figure 7).

Considering the encapsulation of 5-HT in GABA and 5-HT chitosan nanoparticles for a concentration range of 0-500 µg, a maximum encapsulation efficiency of 80±7.35% at 400 µg of 5-HT / mL chitosan solution was obtained. A maximum encapsulation efficiency of 71±7.2% was observed at 20µg GABA / mL chitosan solution when the concentration range was 0-35 µg GABA / mL of chitosan solution (Figure 8, 9).

### *In vitro* release of GABA and 5-HT from chitosan nanoparticles

From *in vitro* release studies of GABA, there was an initial burst release of 20% GABA in 0.5 hours, 87% in 20 hours and 94% in 30 hours from the nanoparticles in PBS (Figure 10).

From the *in vitro* release studies of 5-HT, there was an initial burst release of 28±2% in 0.5 hours and 96±8.6% in 30 hours from the 5-HT chitosan nanoparticles dispersed in PBS (Figure 11).

Initially 30±2% of 5-HT was released in 0.5 hours from GABA and 5-HT chitosan nanoparticles and the release continued up to 97±8.9% at 20 hours. From the GABA and 5-HT chitosan nanoparticles, there was an initial burst release of 22±2% GABA by 0.5 hours and 95±8.6% by 30 hours was observed (figure 12).

### Cell uptake study

Chitosan nanoparticles were labeled with FITC. The cultured hepatocytes were incubated in the presence of FITC labeled GABA and 5-HT chitosan nanoparticles and unlabelled nanoparticles for 2 hours. A negative control showing the auto fluorescence in the cell was obtained. The fluorescent image was observed in confocal microscope with an excitation at 488 nm. The interaction of FITC labeled nanoparticles with hepatocytes was observed. The binding of fluorescent labeled GABA and 5-HT chitosan nanoparticles on the surface of hepatocytes and the entry of nanoparticles in to the hepatocytes by phagocytosis led to the observation of fluorescence in the cell (Figure 13).

**Liver**

**[$^3$H] Thymidine and [$^3$H] leucine uptake studies**

Incorporation of [$^3$H] thymidine in to the replicating DNA was used as a biochemical marker to quantify DNA synthesis. There was no significant change in [$^3$H] Thymidine uptake by the rats treated with chitosan nanoparticles alone. [$^3$H] Thymidine uptake by PHNT was significantly increased ($p<0.01$) when compared to C. There was also a significant increase ($p<0.001$) in [$^3$H] thymidine uptake by hepatocytes of GCNP, SCNP and GSCNP groups when compared with C. Compared to PHNT, there was a significant increase in thymidine uptake by GCNP ($p<0.01$), SCNP ($p<0.05$) and GSCNP ($p<0.001$). There was a significant decrease in thymidine uptake by SCNP ($p<0.001$) and GSCNP ($p<0.01$) when compared to GCNP. In GSCNP group, a significant increase ($p<0.01$) in [$^3$H] thymidine uptake was observed when compared to SCNP (Table 1).

[$^3$H] leucine uptake studies conducted in hepatocytes helped to quantify protein synthesis *in vitro*. There was no significant difference in the leucine uptake by hepatocytes from CN and C. When compared to C, there was a significant increase in [$^3$H] leucine uptake by the hepatocytes isolated from PHNT ($p<0.05$), GCNP, SCNP ($p<0.01$) and GSCNP ($p<0.001$). The utilization of leucine in the media by hepatocytes from both GCNP and SCNP showed a significant increase ($p<0.01$) when compared to PHNT. A prominent increase ($p<0.001$) in [$^3$H] leucine uptake by GSCNP was observed when compared to PHNT. In GSCNP, there was a significant increase ($p<0.01$) in leucine uptake when compared to both GCNP and SCNP (Table 2).

**Thymidine kinase activity in the liver of experimental groups**

The enzyme thymidine kinase converts thymidine in to thymidine monophosphate, which is involved in the DNA synthesis. Thymidine kinase activity in all the experimental groups showed a significant increase ($p<0.001$) when compared with C. There was also a significant increase ($p<0.001$) in thymidine kinase activity in GCNP, SCNP and GSCNP when compared to PHNT.

A significant increase ($p<0.001$) in the enzyme activity was observed in SCNP and GSCNP when compared to GCNP. The $K_d$ value of the enzyme in all partially hepatectomised groups were decreased ($p<0.05$) when compared to C. There was no change in the enzyme activity in CN when compared to C (Table 3).

**BrdU incorporation study**

BrdU is a thymidine analog, that incorporates into DNA in the 'S' phase of cell cycle. Liver cell multiplication was evaluated based on the incorporation of BrdU. In the actively regenerating liver of all partially hepactomised rats, cell division was also fast when compared to C. In all the treatment groups, BrdU binding on the DNA was significantly increased ($p<0.001$) when compared to those with no treatment. The DNA synthesis and cell division in rats treated with GABA and 5-HT chitosan nanoparticles in combination was significantly increased ($p<0.001$) when compared to those treated with individual nanoparticles. This observation supported the therapeutic exploration of GABA and 5-HT encapsulated chitosan nanoparticles in the treatment of liver based diseases (Figure 14, Table 4).

**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the liver of experimental rats**

$GABA_B$ receptors subtypes mediated liver cell multiplication in partially hepatectomised rats was studied. $B_{max}$ represents the number of receptors and $K_d$ represents the affinity of receptors towards the ligand. $B_{max}$ of $GABA_B$ receptor binding showed a significant decrease ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The $B_{max}$ of $GABA_B$ receptor was significantly decreased in both GCNP and GSCNP ($p<0.001$) and SCNP ($p<0.01$) when compared to PHNT. The $B_{max}$ was significantly increased ($p<0.01$) in SCNP and decreased ($p<0.001$) in GSCNP when compared to GCNP. While comparing the receptor $B_{max}$ in GSCNP and SCNP, a significant decrease ($p<0.01$) in GABA and 5-HT chitosan nanoparticle treated group. The $K_d$ value of receptor binding for PHNT, GCNP and GSCNP showed a significant increase ($p<0.001$) with respect

to C. While comparing with PHNT, $K_d$ value of $GABA_B$ receptor binding in GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$). The receptor $K_d$ value in GSCNP showed a significant increase ($p<0.001$) when compared to GCNP and SCNP (Figure 15, Table 5).

**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the liver of experimental rats**

Gene expression study of $GABA_B$ receptor mRNA in the regenerating liver of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) when compared to C and also a significant decrease ($p<0.001$) in each of the nanoparticle treated groups when compared to PHNT. When compared to both GCNP and SCNP, a significant decrease ($p<0.001$) in $GABA_B$ receptor subunit expression was observed in GSCNP (figure 16, Table 6).

**Confocal microscopic imaging studies of $GABA_B$ receptors in the liver of experimental groups**

Confocal microscopic image of $GABA_B$ receptors in the regenerating liver of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) in the mean pixel intensity when compared to C and also a significant decrease ($p<0.001$) in each of the nanoparticle treated groups when compared to PHNT. When compared to both GCNP and SCNP, a significant decrease ($p<0.01$) in mean pixel intensity was observed in GSCNP (figure 17, Table 7).

**Scatchard analysis of [$^3$H] ketanserin binding against ketanserin to 5-$HT_{2A}$ receptors in the liver of experimental rats**

5-$HT_{2A}$ receptor subtypes mediated liver cell multiplication in partially hepatectomised rats was studied. $B_{max}$ of 5-$HT_{2A}$ receptor study showed a significant decrease ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The 5-$HT_{2A}$ receptor $B_{max}$ was significantly increased in GCNP ($p<0.05$) and in both SCNP and GSCNP ($p<0.001$) when compared to PHNT. The

receptor $B_{max}$ was significantly increased ($p<0.001$) in SCNP and GSCNP when compared to GCNP. In GSCNP, the receptor number was significantly increased ($p<0.01$) when compared to SCNP. The $K_d$ value of 5-$HT_{2A}$ receptors in PHNT, GCNP and GSCNP showed a significant increase ($p<0.001$) when compared to C. In SCNP group, $K_d$ value of 5-$HT_{2A}$ receptor showed a significant decrease ($p<0.001$) with that of PHNT. While comparing with GCNP, $K_d$ value of receptor binding in SCNP showed a significant decrease ($p<0.001$). The $K_d$ value was significantly increased ($p<0.001$) in GSCNP when compared to SCNP (Figure 18, Table 8).

**Real Time PCR amplification of 5-$HT_{2A}$ receptor subunit mRNA in the liver of experimental rats**

5-$HT_{2A}$ receptor expression was observed to be decreased significantly ($p<0.001$) in all the partially hepatectomised groups with or without nanoparticle treatment when compared to C. There was no significant change in the expression of the receptor in GCNP when compared to PHNT. Considering other groups, there was a significant increase in 5-$HT_{2A}$ receptor gene expression in SCNP ($p<0.01$) and GSCNP ($p<0.001$) when compared to PHNT. A significant decrease ($p<0.001$) in receptor subunit expression was observed in GSCNP when compared to GCNP and SCNP (Figure 19, Table 9).

**Confocal microscopic imaging studies of 5-$HT_{2A}$ receptors in the liver of experimental groups**

Confocal microscopic image of 5-$HT_{2A}$ receptors showed a significant decrease in mean pixel intensity in PHNT ($p<0.001$), GCNP ($p<0.001$), SCNP ($p<0.05$) and GSCNP ($p<0.05$) when compared to C. There was also a significant increase in mean pixel intensity of GCNP ($p<0.001$), SCNP ($p<0.001$) and GSCNP ($p<0.001$) when compared to PHNT where as a significant decrease ($p<0.01$) in mean pixel intensity was observed in GSCNP (figure 20, Table 10).

**cAMP content in the liver of experimental rats**

cAMP is involved in G protein coupled receptor mediated cell signalling during liver regeneration. cAMP content of the regenerating liver of PHNT was significantly decreased ($p<0.01$) compared to C. For groups GCNP, SCNP and GSCNP also, the cAMP content was significantly reduced ($p<0.001$) when compared to C. The cAMP content was significantly decreased in both GCNP and SCNP ($p<0.05$) and GSCNP ($p<0.01$) when compared to PHNT. In GSCNP, the cAMP content was significantly decreased ($p<0.001$) when compared to GCNP and SCNP (Figure 21).

**Real Time PCR amplification of CREB mRNA in the liver of experimental rats**

The gene expression of transcription factor CREB, which was activated by cAMP, involved in the signalling cascade of G protein coupled receptors showed a significant decrease ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The gene expression of CREB was decreased significantly ($p<0.001$) in GCNP, SCNP and GSCNP when compared to PHNT. The gene expression of CREB was significantly decreased in GSCNP ($p<0.001$) when compared to GCNP and SCNP (Figure 22, Table 11).

**$IP_3$ content in the liver of experimental rats**

$IP_3$ content of the regenerating liver of all partially hepatectomised rats showed a significant decrease ($p<0.001$) when compared to C. The $IP_3$ content in GCNP, SCNP and GSCNP was decreased significantly ($p<0.001$) when compared to PHNT. There was no significant change in $IP_3$ contents of nanoparticle treated groups (Figure 23).

**Real Time PCR amplification of phospholipase C mRNA in the liver of experimental rats**

Gene expression studies of phospholipase C (PLC) in the regenerating liver of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) when compared to sham operated control. A significant decrease in gene expression was observed in GCNP ($p<0.05$), SCNP ($p<0.01$) and GSCNP ($p<0.001$) when compared to PHNT. The PLC gene expression was also significantly reduced ($p<0.001$) in a combination of GABA and 5-HT chitosan nanoparticle treated group when compared to individually treated group (Figure 24, Table 12).

**Real Time PCR amplification of NF-κB mRNA in the liver of experimental rats**

The NF-κB expression studies in the regenerating liver of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) when compared to C. In all partially hepatectomised groups treated with respective nanoparticles, the gene expression was significantly decreased ($p<0.001$) when compared to that with no treatment. The NF-κB gene expression was also significantly decreased ($p<0.001$) in GSCNP when compared to both GCNP and SCNP (Figure 25, Table 13).

**Real Time PCR amplification of TNF-α mRNA in the liver of experimental rats**

The cytokines lead to the subsequent activation of downstream transcription cascades, which effect the transition of the quiescent hepatocytes to the G1 phase. TNF-$\alpha$ is one of the major molecule involved in the signal transduction for liver regeneration. The TNF-$\alpha$ mRNA expression in the liver of GCNP, SCNP and GSCNP was significantly decreased ($p<0.001$) when compared to C and PHNT. There was no significant variation in the TNF-$\alpha$ mRNA expression in GSCNP when compared to GCNP and SCNP (Figure 26, Table 14).

**Real Time PCR amplification of Akt-1 mRNA in the liver of experimental rats**

Akt-1 is also involved in the progression of signal cascades in liver cell proliferation. From our studies, there was a significant decrease ($p<0.001$) in the expression of Akt-1 mRNA in the liver of all partially hepatectomised experimental rats when compared to C. The gene expression was significantly down regulated ($p<0.001$) in all groups treated with nanoparticles when compared to group without treatment (Figure 27, Table 15).

**Real Time PCR amplification of SOD mRNA in the liver of experimental rats**

Superoxide dismutase is an antioxidant enzyme and its expression was directly dependant on the level of reactive oxygen species, which activates the apoptotic signalling. The SOD gene expression was significantly down regulated ($p<0.001$) in GCNP, SCNP and GSCNP when compared to C and PHNT. The gene expression of SOD in PHNT was significantly reduced ($p<0.001$) when compared to C. There was also a significant decrease ($p<0.001$) in SOD gene expression of GSCNP group when compared to GCNP and SCNP (figure 28, Table 16).

**Activity of SOD in the liver of experimental rats**

The concentration of antioxidant enzyme SOD depends on the formation of reactive oxygen species in the body. The reactive oxygen species levels were increased due to any kind of damage to the cells, which further led to an increase in the SOD activity. The SOD activity was significantly decreased ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. There was a significant decrease ($P<0.001$) in SOD activity in GCNP, SCNP and GSCNP when compared to PHNT. The enzyme activity was also reduced significantly ($p<0.001$) in GSCNP when compared to GCNP and SCNP (Table 17).

**Real Time PCR amplification of Bax mRNA in the liver of experimental rats**

Liver injury induces both apoptotic and proliferative signalling. Bax is the protein involved in the apoptosis of cells. The Bax expression was significantly down regulated ($p<0.001$) in all partially hepatectomised rats when compared to C. In all the treatment groups, the gene expression was significantly decreased ($p<0.001$) when compared to that with no treatment. The Bax expression was significantly reduced ($p<0.001$) in the rats treated with GABA and 5-HT chitosan nanoparticles when compared to the rats with individual nanoparticle treatment (Figure 29, Table 18).

**Real Time PCR amplification of caspase-8 mRNA in the liver of experimental rats**

Cell apoptosis is achieved by two major apoptotic pathways (extrinsic and intrinsic). The extrinsic pathway involves the binding of cytokines to death receptors, activation of caspase-8, and cleavage and activation of effector caspase-3. Caspase-8 is a key factor uniquely associated with this pathway. Caspase-8 expression in the PHNT, GCNP, SCNP and GSCNP was significantly decreased ($p<0.001$) when compared to C. There was also a significant decrease ($p<0.001$) in caspase-8 expression for GCNP, SCNP and GSCNP when compared to PHNT. The combination of GABA and 5-HT chitosan nanoparticles significantly reduced ($p<0.001$) caspase-8 expression in partially hepatectomised rats when compared to the individual treatment (Figure 30, Table 19).

**Real Time PCR amplification of MAT2A mRNA in the liver of experimental rats**

MAT2A, which catalyzes the formation of the methyl donor *S*-adenosylmethionine, helps in DNA methylation. Its gene expression was significantly down regulated ($p<0.001$) in GCNP, SCNP and GSCNP when compared to C and PHNT. In PHNT, the gene expression was significantly reduced ($p<0.001$) when compared to C. In rats with combination of GABA and 5-HT encapsulated chitosan nanoparticles treatment, the MAT2A gene expression

was significantly decreased ($p<0.001$) compared to individual treatment (Figure 31, Table 20).

**[$^3$H] methyl group incorporation on the liver DNA of experimental rats.**

Rapid DNA synthesis prior to mitosis resulted in decreased DNA methylation. Thus *in vitro* methyl group incorporation to the DNA, which was isolated from rapidly dividing cells, was observed to be high when compared to normal cells. The [$^3$H] methyl group incorporation in the DNA was significantly up regulated ($p<0.001$) in GCNP, SCNP and GSCNP when compared to C and PHNT. The incorporation in PHNT was significantly increased ($p<0.001$) when compared to C. The [$^3$H] methyl group incorporation on DNA was increased significantly ($p<0.001$) in GSCNP when compared to GCNP and SCNP. (Figure 32, Table 21).

**DNA content in the liver of experimental rats**

To support the experimental outcomes of methylation study, the DNA content was also increased significantly ($p<0.001$) in all nanoparticle treated group when compared to partially hepatectomised groups with no treatment and control. The DNA content was significantly increased ($p<0.001$) in GSCNP group when compared to GCNP and SCNP (Table 22).

**Real Time PCR amplification of hepatocyte growth factor mRNA in the liver of experimental rats**

Hepatocyte growth factor (HGF) is the most potent stimulator of hepatocyte growth and DNA synthesis identified. Thus increase in HGF gene expression was considered as a marker for liver regeneration. HGF gene expression was significantly up regulated ($p<0.001$) in GCNP, SCNP and GSCNP when compared to C and PHNT. The gene expression was also significantly increased ($p<0.001$) in GSCNP when compared to both GCNP and SCNP (Figure 33, Table 23).

**Real Time PCR amplification of Insulin like growth factor-1 mRNA in the liver of experimental rats**

Liver cell proliferation is initiated and progressed by the combined effect of growth factors and cytokines. The gene expression of IGF-1 mRNA was significantly increased ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The IGF-1 mRNA expression in all groups treated with nanoparticles showed a significant increase ($p<0.05$) in GCNP and SCNP and an increase ($p<0.001$) in GSCNP when compared to PHNT. There was also a significant increase ($p<0.001$) in the IGF-1 expression in GSCNP when compared to GCNP and SCNP. This showed an up regulation of growth factor gene expression in the proliferating liver cells that are achieved by the combined effect of GABA and 5-HT in the chitosan nanoparticles than individual treatment (Figure 34, Table 24).

**Real Time PCR amplification of hepatocyte growth factor mRNA in the liver of experimental rat on seventh day of post hepatectomy**

The level of HGF was elevated during hepatocyte proliferation. When the liver gains complete mass after regeneration, hepatocyte growth factor expression was decreased considerably. HGF expression was increased significantly ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The HGF expression in all nanoparticle treated rats were decreased significantly ($p<0.001$) when compared to PHNT. The gene expression in GSCNP was significantly decreased ($p<0.001$) and reversed to control when compared to individual treatment with GABA and 5-HT chitosan nanoparticles (Figure 35, Table 25).

**Cerebral cortex**

**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the cerebral cortex of experimental rats**

$B_{max}$ represents the number of receptors and $K_d$ denotes the affinity of receptors towards the ligand. $B_{max}$ and $K_d$ of $GABA_B$ receptors in GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) when compared to C and both GCNP and SCNP showed a significant increase ($p<0.001$) when compared to PHNT. $B_{max}$ and $K_d$ of $GABA_B$ receptors in PHNT showed a significant decrease ($p<0.001$) when compared to C. The receptor $B_{max}$ in GSCNP was significantly decreased when compared to GCNP ($p<0.01$) and SCNP ($p<0.001$). The $K_d$ value of receptor binding showed a significant decrease ($p<0.001$) when compared to groups with individual GABA and 5-HT chitosan nanoparticle treatment (Figure 36, Table 26).

**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the cerebral cortex of experimental rats**

Gene expression studies of $GABA_B$ receptor in the cerebral cortex was significantly decreased in PHNT ($p<0.01$), GCNP and SCNP ($p<0.001$) when compared to C. The receptor gene expression in SCNP and GCNP showed a significant decrease ($p<0.01$) when compared to PHNT. The receptor gene expression was significantly decreased in GSCNP when compared to PHNT ($p<0.001$) and both GCNP and SCNP ($p<0.01$) (Figure 37, Table 27).

**Confocal microscopic imaging studies of $GABA_B$ receptors in the cerebral cortex of experimental groups**

Confocal microscopic image of $GABA_B$ receptors in the cerebral cortex of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) in the mean pixel intensity when compared to C. There was also a significant decrease ($p<0.001$) in mean pixel intensity of GCNP and GSCNP when compared to PHNT (figure 38, Table 28).

**Scatchard analysis of [$^3$H] ketanserin binding against ketanserin to 5-HT$_{2A}$ receptors in the cerebral cortex of experimental rats**

5-HT$_{2A}$ receptors $B_{max}$ and $K_d$ was significantly decreased ($p<0.001$) in PHNT, GCNP and SCNP when compared to C. The receptor $B_{max}$ ($p<0.001$) and $K_d$ ($p<0.01$) were decreased significantly in GSCNP when compared to both C and PHNT. There was no significant change in the receptor number of GSCNP when compared to GCNP and SCNP. The $K_d$ value of 5-HT$_{2A}$ receptors in the cerebral cortex of GSCNP was significantly decreased ($p<0.001$) when compared to both GCNP and SCNP (Figure 39, Table 29).

**Real Time PCR amplification of 5-HT$_{2A}$ receptor subunit mRNA in the cerebral cortex of experimental rats**

The 5-HT$_{2A}$ receptor gene expression in all partially hepatectomised groups were decreased significantly ($p<0.001$) when compared to C. The gene expression was significantly decreased ($p<0.01$) in both GCNP and SCNP and decreased ($p<0.001$) further in GSCNP (Figure 40, Table 30).

**Confocal microscopic imaging studies of 5-HT$_{2A}$ receptors in the cerebral cortex of experimental groups**

Confocal microscopic image of GABA$_B$ receptors in the cerebral cortex of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) in the mean pixel intensity when compared to C. There was also a significant decrease ($p<0.001$) in mean pixel intensity of GCNP and GSCNP when compared to PHNT. The mean pixel intensity obtained from GSCNP was significantly decreased ($p<0.001$) compared to GCNP and SCNP (figure 41, Table 31).

**cAMP content in the cerebral cortex of experimental rats**

cAMP is a second messenger involved in the G-protein coupled receptor mediated cell signalling. cAMP content in the cerebral cortex of PHNT, GCNP, SCNP and GSCNP were significantly decreased ($p<0.001$) when compared to

both C and PHNT. The cAMP content in PHNT was significantly decreased ($p<0.001$) when compared to C. There was no significant change in the cAMP contents of the treatment groups (Figure 42).

**Real Time PCR amplification of CREB mRNA in the cerebral cortex of experimental rats**

There was a significant decrease in the expression of CREB in GCNP and SCNP ($p<0.01$) and GSCNP ($p<0.001$) when compared to PHNT. All partially hepatectomised rats showed significant decrease ($p<0.001$) in the gene expression when compared to C. In GSCNP, the CREB expression was decreased significantly ($p<0.01$) when compared to both GCNP and SCNP (Figure 43, Table 32).

**$IP_3$ content in the cerebral cortex of experimental rats**

The $IP_3$ content was significantly reduced ($p<0.001$) in GCNP, SCNP, GSCNP when compared to C and PHNT. The $IP_3$ content was significantly decreased ($p<0.001$) in PHNT when compared to C (Figure 44).

**Real Time PCR amplification of phospholipase C mRNA in the cerebral cortex of experimental rats**

Phospholipase C gene expression was significantly down regulated ($p<0.001$) in all partially hepatectomised rats when compared to C. All rats treated with GABA and 5-HT chitosan nanoparticles, individually and in combination, showed a significant decrease ($p<0.001$) in the gene expression when compared to that with no treatment. The gene expression in GSCNP was significantly decreased ($p<0.01$) when compared to GCNP (Figure 45, Table 33).

**cGMP content in the cerebral cortex of experimental rats**

cGMP is a second messenger involved in the survival mechanisms of neurons. cGMP content was significantly decreased ($p<0.001$) in GCNP, SCNP

and GSCNP when compared to PHNT and C. cGMP content in PHNT was significantly decreased ($p<0.001$) when compared to C (Figure 46).

**Real Time PCR amplification of NF-κB mRNA in the cerebral cortex of experimental rats**

NF-κB is a neuronal survival factor and activated form transcribes the genes involved in neuronal maintenance. The gene expression of NF-κB showed a significant increase ($p<0.001$) in GCNP, SCNP and GSCNP when compared to PHNT and C. The gene expression of NF-κB was increased significantly ($p<0.001$) in GSCNP compared to GCNP and SCNP (Figure 47, Table 34).

**Real Time PCR amplification of TNF-α mRNA in the cerebral cortex of experimental rats**

NF-κB activation was achieved by an important cytokine TNF-α. The TNF-α expression was significantly increased ($p<0.001$) in all partially hepatectomised rats with and without treatment when compared to control. The gene expression was also observed increased significantly ($p<0.001$) in GCNP, SCNP and GSCNP when compared to PHNT (Figure 48, Table 35).

**Real Time PCR amplification of Akt-1 mRNA in the cerebral cortex of experimental rats**

TNF-α induced NF-κB activation require Akt. The gene expression of Akt-1 showed a significant increase ($p<0.001$) in GCNP, SCNP and GSCNP when compared to PHNT. The gene expression of Akt-1 was increased significantly ($p<0.001$) in PHNT when compared to C. The gene expression was significantly increased ($p<0.01$) in GSCNP compared to GCNP and SCNP (Figure 49, Table 36).

**Real Time PCR amplification of Insulin like growth factor-1 mRNA in the cerebral cortex of experimental rats**

IGF-1 is an important growth factor present in brain which was involved in neuronal survival. IGF-1 expression was significantly increased ($p<0.001$) in all partially hepatectomised rats compared to control. The gene expression was also increased significantly ($p<0.001$) in PHNT when compared to C. The expression of IGF-1 was more prominent in GSCNP ($p<0.001$) when compared with GCNP and SCNP (Figure 50, Table 37).

**Real Time PCR amplification of SOD mRNA in the cerebral cortex of experimental rats**

The level of antioxidant enzyme SOD was elevated when the ROS content in the cell was increased. The free radical generation was a symbol of apoptosis and increased expression of SOD directly indicated the reduction in ROS. The gene expression was significantly increased ($p<0.001$) in PHNT when compared to C. A significant increase ($p<0.001$) in SOD gene expression was observed in GCNP, SCNP and GSCNP when compared to PHNT and C (Figure 51, Table 38).

**Real Time PCR amplification of Bax mRNA in the cerebral cortex of experimental rats**

The apoptotic protein, Bax was significantly down regulated ($p<0.001$) in all partially hepatectomised rats compared to control. There was significant decrease in Bax gene expression in GCNP ($p<0.01$) and GSCNP ($p<0.001$) when compared to PHNT. There was no change in the gene expression of Bax in rats treated with individual and in combination of GABA and 5-HT chitosan nanoparticles (Figure 52, Table 39).

**Real Time PCR amplification of caspase-8 mRNA in the cerebral cortex of experimental rats**

Caspase-8 is involved in the apoptotic signalling in neurons. The gene expression was significantly decreased ($p<0.001$) in all partially hepatectomised

rats compared to control. The gene expression of caspase-8 was significantly down regulated in GCNP ($p<0.01$), SCNP and GSCNP ($p<0.001$) compared to PHNT. The present study revealed a prominent decrease in the gene expression of rats treated with a combination of GABA and 5-HT chitosan nanopaticle when compared to rats treated with individual nanoparticles (Figure 53, Table 40).

**Real Time PCR amplification of BDNF mRNA in the cerebral cortex of experimental rats**

BDNF is important in differentiation, survival and plasticity of the CNS. The neurotrophic factor, BDNF, expression in PHNT, GCNP, SCNP and GSCNP were significantly up regulated ($p<0.001$) when compared to C. All the treatment groups also showed an increased ($p<0.001$) expression of BDNF. A combination of GABA and 5-HT coupled with chitosan nanoparticles treated group showed an increased ($p<0.01$) expression of BDNF when compared to individually treated groups (Figure 54, Table 41).

**Real Time PCR amplification of GDNF mRNA in the cerebral cortex of experimental rats**

GDNF is a small protein that potentially promotes the survival of many types of neurons. The GDNF expression in PHNT, GCNP, SCNP and GSCNP was significantly up regulated ($p<0.001$) when compared to C. All the treatment groups also showed an increased expression of GDNF. A combination of GABA and 5-HT coupled with chitosan nanoparticles treated group showed an increased ($p<0.001$) expression of GDNF when compared to individually treated groups (Figure 55, Table 42).

**Brain stem**

**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the brain stem of experimental rats**

$B_{max}$ of $GABA_B$ receptor binding study showed a significant decrease ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The $B_{max}$ of $GABA_B$ receptor assay of GCNP showed a significant decrease ($p<0.01$) when compared to PHNT. SCNP and GSCNP also showed a significant decrease ($p<0.001$) when compared to PHNT. $B_{max}$ was significantly decreased ($p<0.001$) in GSCNP when compared to both GCNP and SCNP. There was no significant change in the $K_d$ values of all groups (Figure 56, Table 43).

**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the brain stem of experimental rats**

Gene expression studies of $GABA_B$ receptor subunit mRNA in the brain stem of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) when compared to C. There was also a significant decrease ($p<0.001$) in receptor gene expression of GCNP, SCNP and GSCNP when compared to PHNT. The gene expression was reduced prominently ($p<0.001$) in GSCNP when compared to both GCNP and SCNP (Figure 57, Table 44).

**Confocal microscopic imaging studies of $GABA_B$ receptors in the brain stem of experimental groups**

Confocal microscopic image of $GABA_B$ receptors in the brain stem of GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) in the mean pixel intensity when compared to C. The mean pixel intensity was significantly reduced ($p<0.05$) in PHNT compared to C. There was also a significant decrease ($p<0.001$) in mean pixel intensity of GCNP, SCNP and GSCNP when compared to PHNT. The mean pixel intensity obtained from GSCNP was significantly decreased ($p<0.001$) compared to both GCNP and SCNP (figure 58, Table 45).

**Scatchard analysis of [$^3$H] ketanserin binding against ketanserin to 5-HT$_{2A}$ receptors in the brain stem of experimental rats**

$B_{max}$ of 5-HT$_{2A}$ receptor binding study showed a significant decrease ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The $B_{max}$ of 5-HT$_{2A}$ receptor binding study showed a significant decrease ($p<0.001$) in GCNP, SCNP and GSCNP when compared to PHNT. $B_{max}$ was significantly decreased ($p<0.001$) in GSCNP when compared to both GCNP and SCNP. There was no significant change in the $K_d$ values of all groups in receptor studies (figure 59, Table 46).

**Real Time PCR amplification of 5-HT$_{2A}$ receptor subunit mRNA in the brain stem of experimental rats**

Gene expression studies of 5-HT$_{2A}$ receptor mRNA in the brain stem of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) when compared to C. There was also a significant decrease ($p<0.001$) in receptor gene expression of GCNP, SCNP and GSCNP when compared to PHNT. The gene expression was reduced prominently ($p<0.01$) in GSCNP when compared to both GCNP and SCNP (Figure 60, Table 47).

**Confocal microscopic imaging studies of 5-HT$_{2A}$ receptors in the brain stem of experimental groups**

Confocal microscopic image of 5-HT$_{2A}$ receptors in the brain stem of GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) in the mean pixel intensity when compared to C. The mean pixel intensity was significantly reduced ($p<0.001$) in PHNT compared to C. There was also a significant decrease in mean pixel intensity of GCNP ($p<0.05$), SCNP ($p<0.01$) and GSCNP ($p<0.001$) when compared to PHNT. The mean pixel intensity obtained from GSCNP was significantly decreased ($p<0.01$) compared to both GCNP and SCNP (figure 61, Table 48).

**cAMP content in the brain stem of experimental rats**

The cAMP content in the brain stem of all partially hepatectomised rats showed a significant decrease ($p<0.001$) when compared to control. The cAMP content observed was decreased significantly ($p<0.001$) in all nanoparticle treated rats compared to PHNT. There was no significant change in the cAMP content among GCNP, SCNP and GSCNP (Figure 62).

**Real Time PCR amplification of CREB mRNA in the brain stem of experimental rats**

The CREB gene expression in all nanoparticle treated rats showed a significant decrease ($p<0.001$) compared to C and PHNT. The gene expression in PHNT also showed a significant decrease ($p<0.001$) compared to C. The CREB expression was decreased significantly ($p<0.01$) in GSCNP when compared to GCNP and SCNP (Figure 63, Table 49).

**$IP_3$ content in the brain stem of experimental rats**

$IP_3$ content was significantly down regulated ($p<0.001$) in all nanoparticle treated groups when compared to C and PHNT. The $IP_3$ content was significantly decreased ($p<0.001$) in PHNT compared to C. $IP_3$ content was decreased significantly ($p<0.001$) in GSCNP when compared to GCNP and SCNP (Figure 64).

**Real Time PCR amplification of phospholipase C mRNA in the brain stem of experimental rats**

The expression of phospholipase C gene was significantly down regulated ($p<0.001$) in all partially hepatectomised rats with and without nanoparticle treatment, when compared to C. Phospholipase C gene expression was significantly down regulated in both GCNP, SCNP ($p<0.01$) and GSCNP ($p<0.001$) when compared to PHNT (Figure 65, Table 50).

**cGMP content in the brain stem of experimental rats**

The cGMP content in the brain stem of all partially hepatectomised rats showed a significant decrease ($p<0.001$) when compared to control. The cGMP content observed was decreased significantly ($p<0.001$) in all nanoparticle treated rats compared to PHNT. There was no significant change in the cGMP content among GCNP, SCNP and GSCNP (Figure 66).

**Real Time PCR amplification of NF-κB mRNA in the brain stem of experimental rats**

The gene expressions of neuronal survival factor, NF-κB showed a significant increase ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The gene expression in all treatment groups showed a significant increase ($p<0.001$) when compared to PHNT. In GSCNP, a prominent increase ($p<0.001$) in NF-κB gene expression was observed when compared to individual nanoparticle treated rats (Figure 67, Table 51).

**Real Time PCR amplification of TNF-α mRNA in the brain stem of experimental rats**

The gene expression of TNF-α mRNA showed a significant increase ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The gene expression in all groups treated with respective nanoparticles showed a significant increase ($p<0.001$) when compared to PHNT. There was no significant change in TNF-α gene expression in GSCNP when compared to GCNP and SCNP (Figure 68, Table 52).

**Real Time PCR amplification of Akt-1 mRNA in the brain stem of experimental rats**

The gene expressions of Akt-1 showed a significant increase ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The Akt-1 expression in all treatment groups showed a significant increase ($p<0.001$) when compared to

PHNT. The gene expression in GSCNP showed a prominent increase ($p<0.001$) compared to both GCNP and SCNP (Figure 69, Table 53).

**Real Time PCR amplification of Insulin like growth factor-1 mRNA in the brain stem of experimental rats**

IGF-1 expression in the brain stem showed a significant increase ($p<0.001$) in GCNP, SCNP and GSCNP when compared to both C and PHNT. The gene expression in PHNT also showed a significant increase ($p<0.001$) compared to C. The IGF-1 gene expression was also increased significantly ($p<0.001$) in GSCNP when compared to both GCNP and SCNP (Figure 70, Table 54).

**Real Time PCR amplification of SOD mRNA in the brain stem of experimental rats**

SOD expression in the brain stem showed a significant increase ($p<0.001$) in GCNP, SCNP and GSCNP when compared to both C and PHNT. The gene expression in PHNT also showed a significant increase ($p<0.001$) compared to C. There was no significant change in the gene expression of SOD in GCNP, SCNP and GSCNP (Figure 71, Table 55).

**Real Time PCR amplification of Bax mRNA in the brain stem of experimental rats**

Bax gene expression in all the treatment groups were significantly decreased ($p<0.001$) when compared to PHNT and C. The gene expression in PHNT was also reduced ($p<0.001$) compared to C. The Bax gene expression was prominently reduced ($p<0.001$) in GSCNP compared to both GCNP and SCNP (Figure 72, Table 56).

**Real Time PCR amplification of caspase-8 mRNA in the brain stem of experimental rats**

Caspase-8 gene expression in all the treatment groups were significantly decreased ($p<0.001$) when compared to PHNT and C. The gene expression in PHNT was also reduced ($p<0.001$) compared to C. Caspase-8 gene expression was prominently decreased ($p<0.001$) in GSCNP compared to both GCNP and SCNP (Figure 73, Table 57).

**Real Time PCR amplification of BDNF mRNA in the brain stem of experimental rats**

The expression of neurotrophic factor, BDNF showed a significant increase ($p<0.001$) in GCNP, SCNP and GSCNP when compared to both C and PHNT. The gene expression was also significantly increased ($p<0.001$) in PHNT compared to C. There was also a prominent increase ($p<0.001$) in BDNF expression in GSCNP compared to both GCNP and SCNP. (Figure 74, Table 58).

**Real Time PCR amplification of GDNF mRNA in the brain stem of experimental rats**

The expression of neurotrophic factor, GDNF showed a significant increase ($p<0.001$) in GCNP, SCNP and GSCNP when compared to both C and PHNT. The gene expression was also significantly increased ($p<0.001$) in PHNT compared to C. There was also a prominent increase ($p<0.001$) in GDNF expression in GSCNP compared to both GCNP and SCNP (Figure 75, Table 59).

**Corpus striatum**

**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the corpus striatum of experimental rats**

$B_{max}$ of $GABA_B$ receptor binding study showed a significant decrease ($p<0.001$) in GCNP, SCNP and GSCNP when compared to C and PHNT. The $B_{max}$ of PHNT was also decreased significantly ($p<0.001$) compared to C. Both SCNP and GSCNP showed a significant decrease ($p<0.001$) when compared to GCNP. $B_{max}$ was significantly decreased ($p<0.001$) in GSCNP when compared to SCNP. There was a significant decrease ($p<0.001$) in the $K_d$ value of GSCNP compared to C (Figure 76, Table 60).

**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the corpus striatum of experimental rats**

Gene expression studies of $GABA_B$ receptor subunit mRNA in the corpus striatum of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) when compared to C. There was also a significant decrease ($p<0.001$) in receptor gene expression of GCNP, SCNP and GSCNP when compared to PHNT. The gene expression was reduced prominently ($p<0.001$) in GSCNP when compared to both GCNP and SCNP (Figure 77, Table 61).

**Confocal microscopic imaging studies of $GABA_B$ receptors in the corpus striatum of experimental groups**

Confocal microscopic image of $GABA_B$ receptors in corpus striatum of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) in the mean pixel intensity when compared to C. The mean pixel intensity was significantly reduced ($p<0.001$) in PHNT compared to C. The mean pixel intensity obtained from GSCNP was significantly decreased ($p<0.01$) compared to both GCNP and SCNP (figure 78, Table 62).

**Scatchard analysis of [$^3$H] ketanserin binding against ketanserin to 5-HT$_{2A}$ receptors in the corpus striatum of experimental rats**

$B_{max}$ of 5-HT$_{2A}$ receptor binding study showed a significant decrease ($p<0.001$) in GCNP, SCNP and GSCNP when compared to both C and PHNT. $B_{max}$ of PHNT was also decreased significantly ($p<0.001$) compared to C. $B_{max}$ was significantly decreased ($p<0.001$) in GSCNP and SCNP when compared to GCNP. $K_d$ values of PHNT ($p<0.05$), GCNP ($p<0.01$), SCNP ($p<0.001$) and GSCNP ($p<0.001$) showed a significant decrease compared to C. $K_d$ values of SCNP and GSCNP were down regulated ($p<0.01$) compared to PHNT. There was also a significant decrease ($p<0.01$) in the $K_d$ value of GSCNP compared to GCNP (Figure 79, Table 63).

**Real Time PCR amplification of 5-HT$_{2A}$ receptor subunit mRNA in the corpus striatum of experimental rats**

Gene expression studies of 5-HT$_{2A}$ receptor mRNA in the corpus striatum of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) when compared to C. There was also a significant decrease ($p<0.001$) in receptor gene expression of GCNP, SCNP and GSCNP when compared to PHNT. The gene expression was reduced prominently ($p<0.01$) in GSCNP when compared to both GCNP and SCNP (Figure 80, Table 64).

**Confocal microscopic imaging studies of 5-HT$_{2A}$ receptors in the corpus striatum of experimental groups**

Confocal microscopic image of 5-HT$_{2A}$ receptors in corpus striatum of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($p<0.001$) in the mean pixel intensity when compared to C. The mean pixel intensity was significantly reduced ($p<0.001$) in PHNT compared to C. The mean pixel intensity obtained from the sections of GSCNP was significantly decreased ($p<0.01$) compared to both GCNP and SCNP (figure 81, Table 65).

**cAMP content in the corpus striatum of experimental rats**

cAMP is a second messenger in protein kinase A (PKA) mediated G-protein signalling cascade. The cAMP content in the corpus striatum was significantly decreased ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. All the treatment groups also showed a significant decrease ($p<0.001$) when compared to the group without treatment. The cAMP content in GABA and 5-HT chitosan nanoparticle treated group was significantly reduced ($p<0.01$) when compared to GABA chitosan and 5-HT chitosan nanoparticles treated groups (Figure 82).

**Real Time PCR amplification of CREB mRNA in the corpus striatum of experimental rats**

CREB is involved in many functions in the nervous system, including neurogenesis and neuronal survival, development, differentiation, neuroprotection, axonal outgrowth and regeneration, synaptic plasticity. Gene expression of CREB mRNA was significantly decreased ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The CREB gene expression in all groups treated with respective nanoparticles showed a significant decrease ($p<0.001$) when compared to PHNT. There was also a significant decrease in the CREB expression in GSCNP when compared to both GCNP and SCNP (Figure 83, Table 66).

**$IP_3$ content in the corpus striatum of experimental rats**

$IP_3$ content was significantly down regulated ($p<0.001$) in all nanoparticle treated groups when compared to both C and PHNT. The $IP_3$ content was significantly decreased ($p<0.001$) in PHNT compared to C. $IP_3$ content was decreased significantly ($p<0.001$) in GSCNP when compared to GCNP and SCNP (Figure 84).

**Real Time PCR amplification of phospholipase C mRNA in the corpus striatum of experimental rats**

The expression of phospholipase C gene was significantly down regulated ($p<0.001$) in all partially hepatectomised rats with and without nanoparticle

treatments, when compared to C. Phospholipase C gene expression was significantly down regulated in both GCNP ($p<0.01$), SCNP ($p<0.01$) and GSCNP ($p<0.001$) when compared to PHNT (Figure 85, Table 67).

**cGMP content in the corpus striatum of experimental rats**

The cGMP content in the corpus striatum of all partially hepatectomised rats showed a significant decrease ($p<0.001$) when compared to control. The cGMP content observed was decreased significantly ($p<0.001$) in all nanoparticle treated rats compared to PHNT. There was no significant change in the cGMP content among GCNP, SCNP and GSCNP (Figure 86).

**Real Time PCR amplification of NF-κB mRNA in the corpus striatum of experimental rats**

The gene expressions of neuronal survival factor, NF-κB showed a significant increase ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The gene expression in all treatment groups like GCNP ($p<0.05$), SCNP ($p<0.05$) and GSCNP ($p<0.001$) showed a significant increase when compared to PHNT. In GSCNP, a prominent increase ($p<0.001$) in NF-κB gene expression was observed when compared to rats treated with either GABA chitosan or 5-HT chitosan nanoparticles (Figure 87, Table 68).

**Real Time PCR amplification of TNF-α mRNA in the corpus striatum of experimental rats**

The gene expression of TNF-α mRNA showed a significant increase ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The gene expression in all groups treated with respective nanoparticles showed a significant increase ($p<0.001$) when compared to PHNT. There was also a significant increase ($p<0.001$) in TNF-α gene expression in GSCNP when compared to both GCNP and SCNP (Figure 88, Table 69).

**Real Time PCR amplification of Akt-1 mRNA in the corpus striatum of experimental rats**

The gene expression of Akt-1 showed a significant increase ($p<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The Akt-1 expression in all treatment groups showed a significant increase ($p<0.001$) when compared to PHNT. The gene expression in GSCNP showed a prominent increase ($p<0.001$) compared to both GCNP and SCNP (Figure 89, Table 70).

**Real Time PCR amplification of Insulin like growth factor-1 mRNA in the corpus striatum of experimental rats**

IGF-1 expression in the corpus striatum showed a significant increase ($p<0.001$) in GCNP, SCNP and GSCNP when compared to both C and PHNT. The gene expression in PHNT also showed a significant increase ($p<0.001$) compared to C. The IGF-1 gene expression was also increased significantly ($p<0.001$) in GSCNP when compared to both GCNP and SCNP (Figure 90, Table 71).

**Real Time PCR amplification of SOD mRNA in the corpus striatum of experimental rats**

SOD expression in the corpus striatum showed a significant increase ($p<0.001$) in GCNP, SCNP and GSCNP when compared to both C and PHNT. The gene expression in PHNT also showed a significant increase ($p<0.001$) compared to C. There was no significant change in the gene expression of SOD in GCNP, SCNP and GSCNP (Figure 91, Table 72).

**Real Time PCR amplification of Bax mRNA in the corpus striatum of experimental rats**

Bax gene expression in all treatment groups was significantly decreased ($p<0.001$) when compared to both PHNT and C. The gene expression in PHNT was also reduced ($p<0.001$) compared to C. There was no significant change in the

gene expression of Bax in all groups treated with nanoparticles (Figure 92, Table 73).

**Real Time PCR amplification of caspase-8 mRNA in the corpus striatum of experimental rats**

Caspase-8 gene expression in all treatment groups was significantly decreased ($p<0.001$) when compared to both PHNT and C. The gene expression in PHNT was also reduced ($p<0.001$) compared to C. Caspase-8 gene expression was prominently decreased ($p<0.001$) in GSCNP compared to both GCNP and SCNP (Figure 93, Table 74).

**Real Time PCR amplification of BDNF mRNA in the corpus striatum of experimental rats**

The expression of neurotrophic factor, BDNF showed a significant increase ($p<0.001$) in GCNP, SCNP and GSCNP when compared to both C and PHNT. The gene expression was also significantly increased ($p<0.001$) in PHNT compared to C. There was also a prominent increase ($p<0.01$) in BDNF expression was observed in GSCNP compared to both GCNP and SCNP (Figure 94, Table 75).

**Real Time PCR amplification of GDNF mRNA in the corpus striatum of experimental rats**

The expression of neurotrophic factor, GDNF showed a significant increase ($p<0.001$) in GCNP, SCNP and GSCNP when compared to both C and PHNT. The gene expression was also significantly increased ($p<0.001$) in PHNT compared to C. There was also a prominent increase ($p<0.001$) in GDNF expression in GSCNP compared to both GCNP and SCNP (Figure 95, Table 76).

**Behavioural studies**

**Time spent by experimental rats on metallic rod in rotarod experiment**

The observations from rotarod test revealed the extent of motor deficits resulted from partial hepatectomy and regain of motor coordination due to present treatment. In rotarod test, all partially hepatectomised rats showed a significant decrease ($p<0.001$) in capacity to retain on the rotating rod when compared to C at 10, 15 and 25 rpm. Retention time on the rod for all the treatment groups also showed a significant increase ($p<0.001$) when compared to PHNT at different rpm. At 10 rpm, the experimental rats of GSCNP were retained for more time on the rod ($p<0.05$) when compared to GCNP and no significant change with respect to SCNP. At 15 and 25 rpm, rats in GSCNP group were able to retain more efficiently ($p<0.001$) on the rod when compared to other treatment groups and the retention time reversed to control (Table 77).

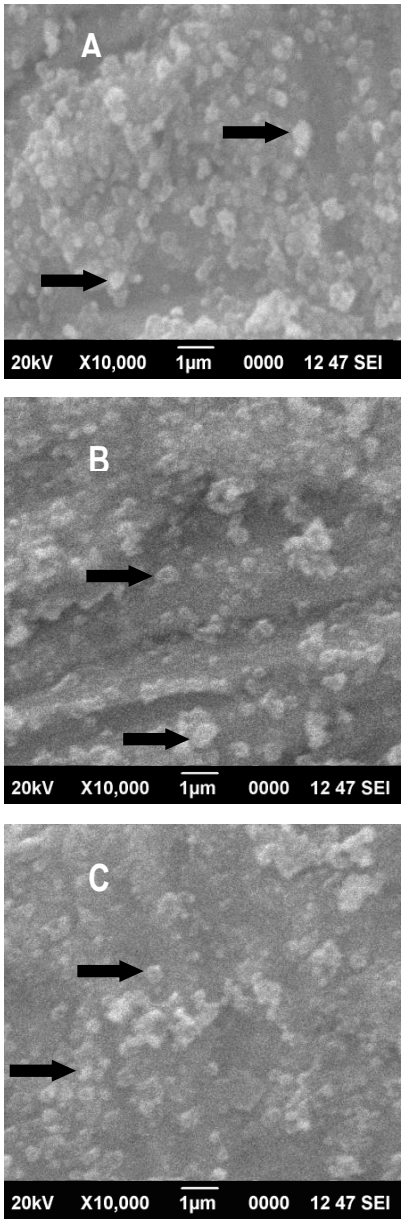
**Behavioural response of experimental rats on grid walk test**

In the Grid walk study, all experimental rats showed a significant increase ($p<0.001$) in number of foot slips on the grid due to uncontrolled motor coordination when compared to C and a significant decrease ($p<0.001$) when compared to PHNT. Among the treatment groups, GSCNP showed a prominent control to avoid foot slips ($p<0.01$) when compared to GCNP and SCNP and was reversed to control (Table 78).

**Time taken by experimental animals in narrow beam walk test**

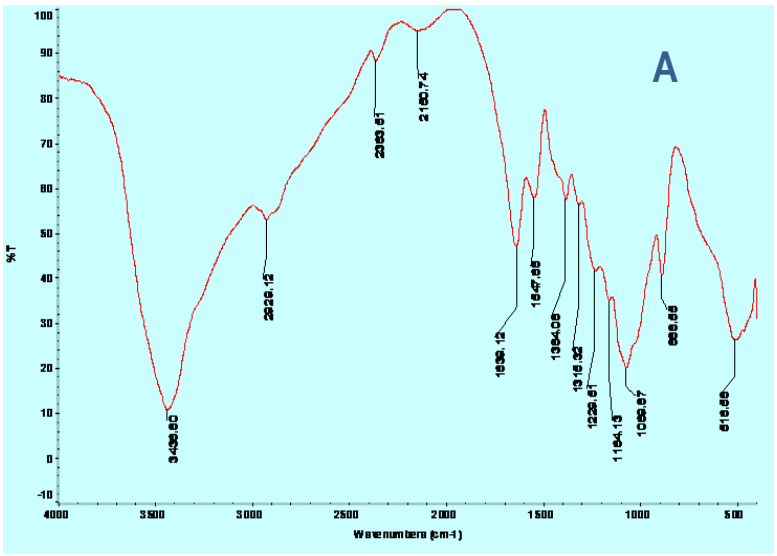
Liver injury leads to changes in motor activity of the animal. The time taken for the rats to cover the entire beam was significantly increased ($p<0.001$) in PHNT, GCNP and SCNP when compared to C and significantly decreased ($p<0.001$) in GCNP, SCNP and GSCNP when compared to PHNT. The time taken by rats in GSCNP group was significantly increased ($p<0.01$) compared to C (Figure 96).

**Figure - 1**
**Scanning electron micrograph of 'GABA chitosan nanoparticles', '5-HT chitosan nanoparticles' and 'GABA and 5-HT chitosan nanoparticles'**



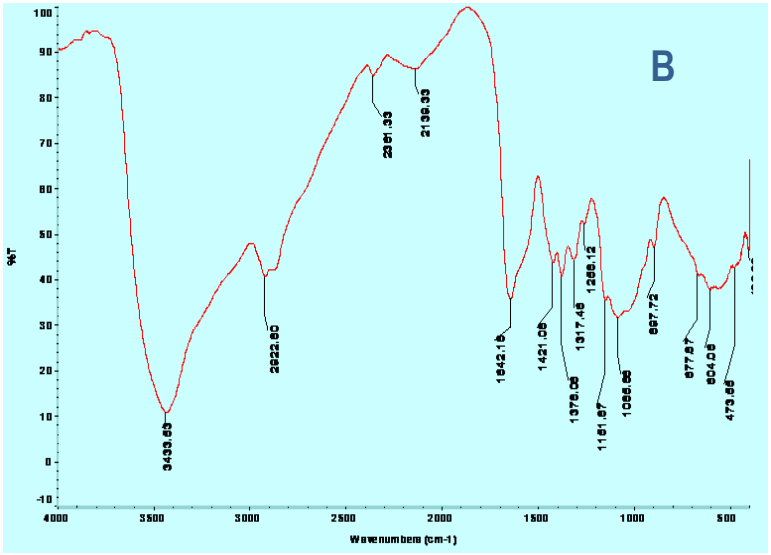

A) GABA chitosan nanoparticles B) 5-HT chitosan nanoparticles c) GABA and 5-HT chitosan nanoparticles. Scale bar represents 1µm

**Figure - 2 A**
**FT-IR spectra of chitosan flakes**



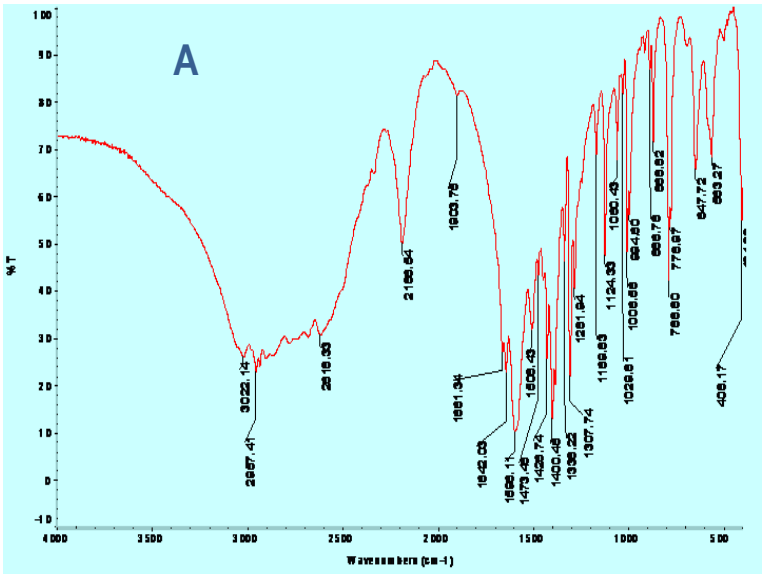

–NH bending was observed at 1642.18 $cm^{-1}$

**Figure - 2 B**
**FT-IR spectra of chitosan nanoparticles**



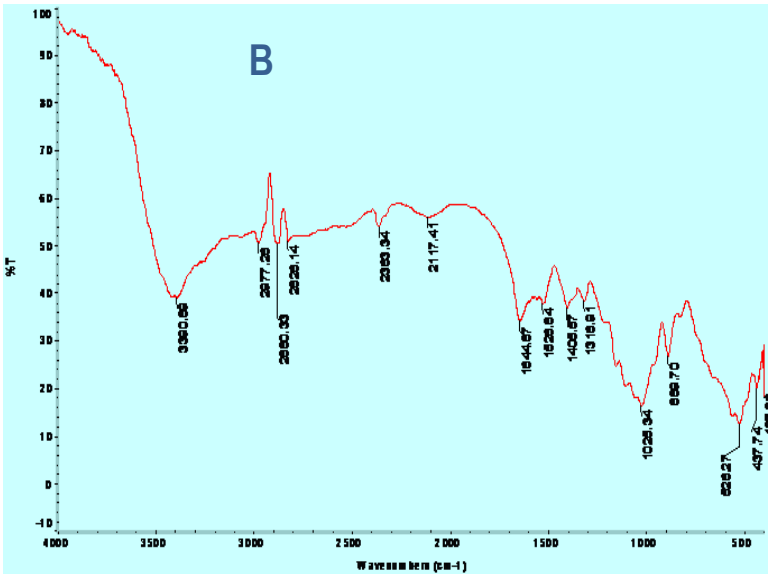

–NH bending at 1547.68 cm-1 and a new peak at 1639.12 cm-1 were observed

**Figure – 3 A**
**FT-IR spectra of GABA**



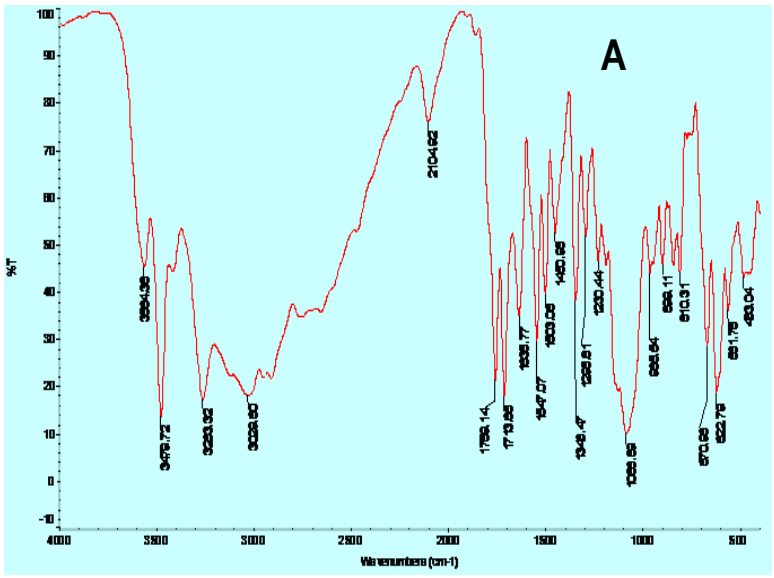

–NH stretching vibration at 3022.14 -2618.33 $cm^{-1}$, C-H aliphatic stretching at 2957 $cm^{-1}$ and 2920 $cm^{-1}$, –NH bending at 1642 $cm^{-1}$ and Strong -$COO^-$ peak at 1596.11 $cm^{-1}$ were observed

**Figure – 3 B**
**FT-IR spectra of GABA chitosan nanoparticles**



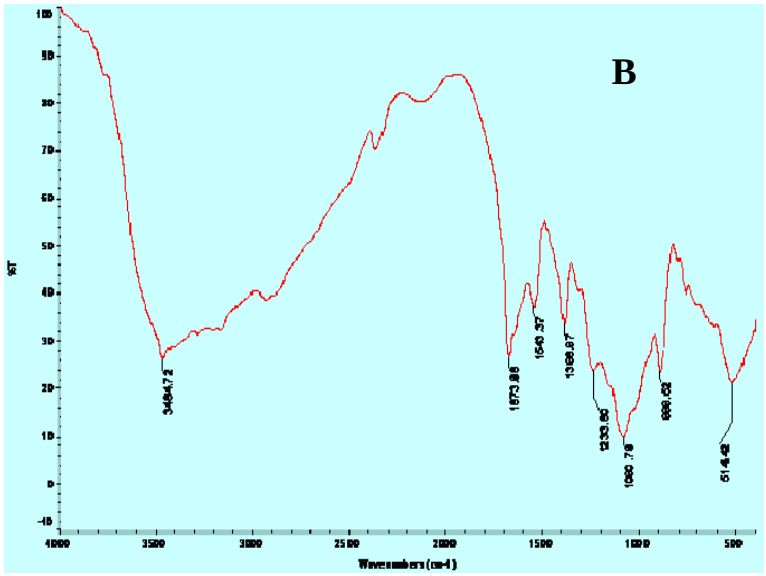

–NH stretching vibration at 3390.89 -2970 $cm^{-1}$, C-H aliphatic stretching at 2977.26 and 2880.33 $cm^{-1}$, –NH bending at 1500 $cm^{-1}$And $-COO^-$ peak 1405.57 $cm^{-1}$ (new shoulder peak) were observed

**Figure – 4 A**
**FT-IR spectra of serotonin creatinine sulphate (5-HT)**



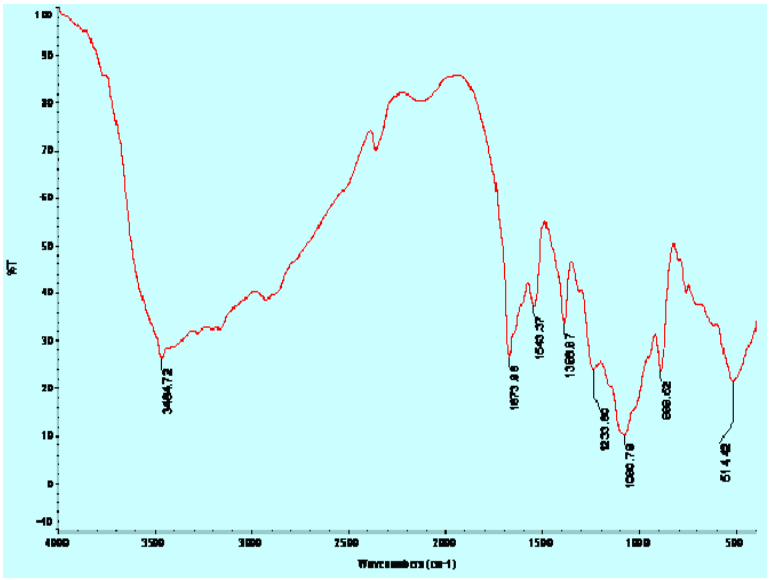

-S-O bond was observed as broad and short peak at 1230.44 $cm^{-1}$

**Figure – 4 B**
**FT-IR spectra of 5-HT chitosan nanoparticles**




–NH stretch at 3436 $cm^{-1}$ in CN shifted to broadened peaks from 3464.72 $cm^{-1}$ to 3280 $cm^{-1}$, -S-O bond at 1080.79 $cm^{-1}$ and a new peak (S-N interaction) 1386.87 $cm^{-1}$ were observed

**Figure – 5**
**FT-IR spectra of GABA and 5-HT chitosan nanoparticles**



–NH bending at 1500 $cm^{-1}$ in pure nanoparticle shifted to 1543.63 $cm^{-1}$ shows the interaction of 5-HT, C-H aliphatic stretching at 2924.8 and 2810 $cm^{-1}$ when compared to GABA chitosan nanoparticles, -S-O bond at 1072.44 $cm^{-1}$ and weak assymetric –N-H bending at 1642 $cm^{-1}$ in GABA became broader in GABA and 5-HT chitosan nanoparticles were observed

**Figure - 6**
**Encapsulation efficiency of GABA in chitosan nanoparticles**

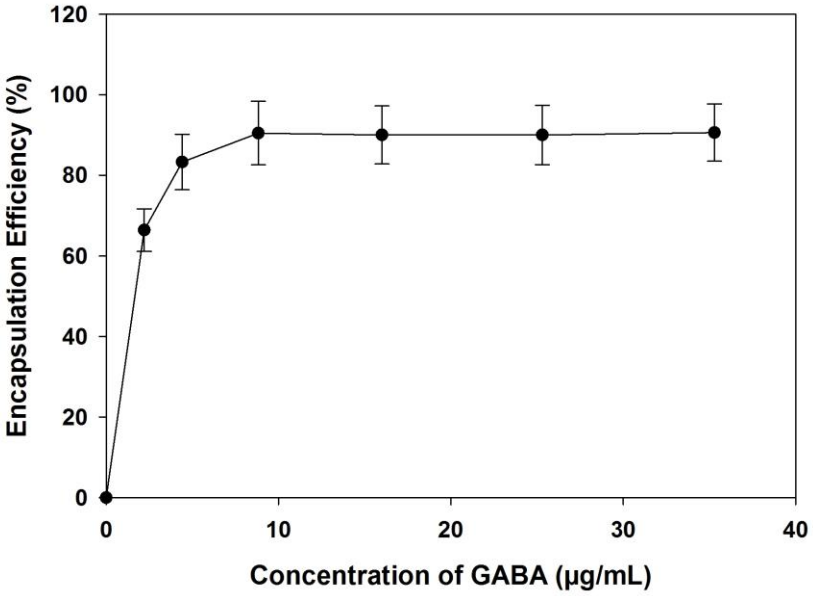



**Figure - 7**
**Encapsulation efficiency of 5-HT in chitosan nanoparticles**

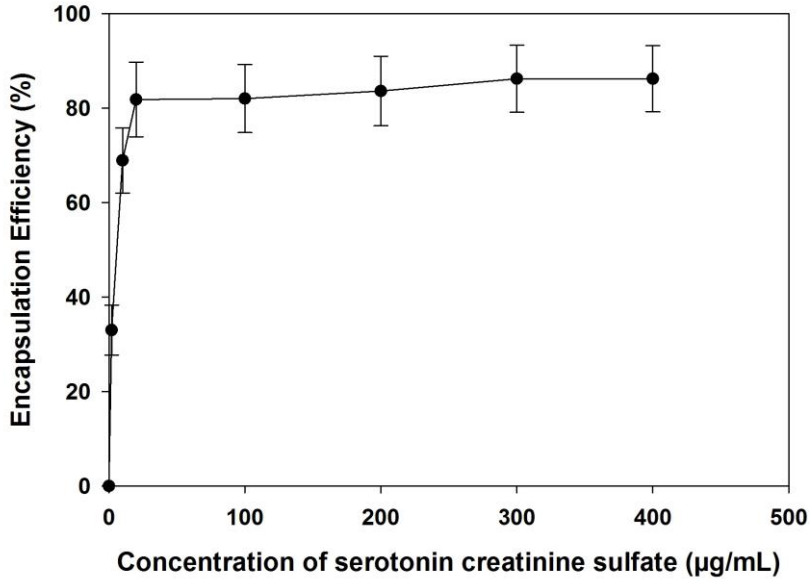


**Figure - 8**
**GABA encapsulation in GABA and 5-HT chitosan nanoparticles**

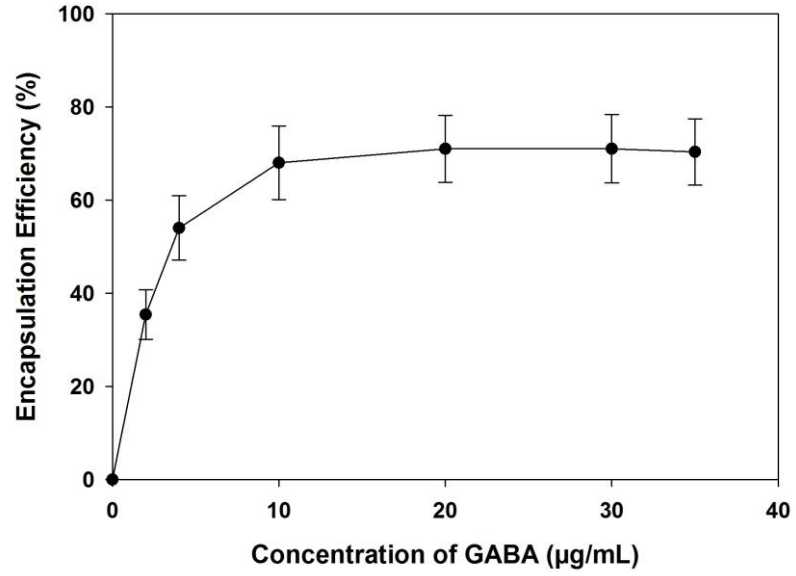



**Figure - 9**
**5-HT encapsulation in GABA and 5-HT chitosan nanoparticles**

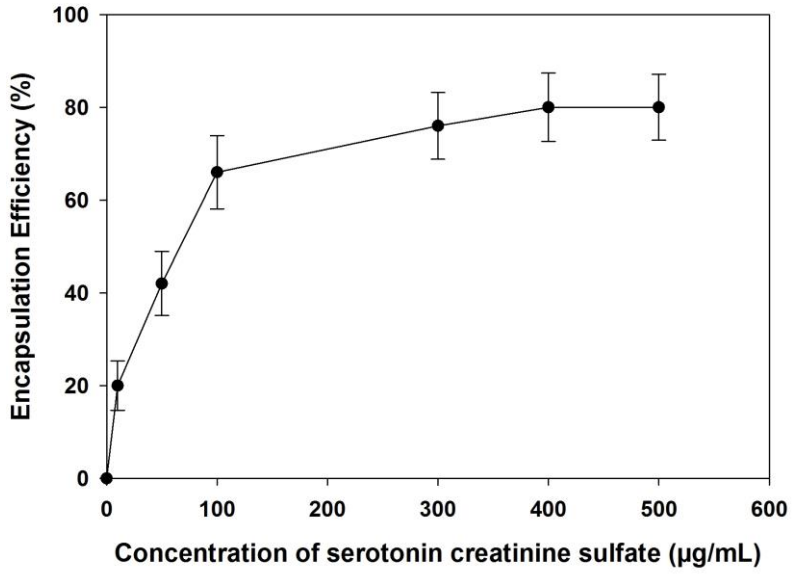


**Figure - 10**
***In vitro* release of GABA from GABA chitosan nanoparticles**

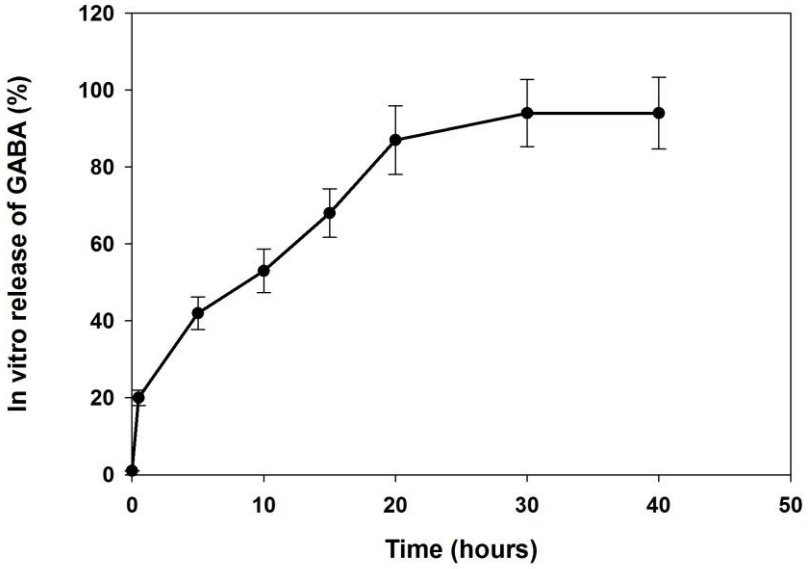



**Figure – 11**
***In vitro* release of 5-HT from 5-HT chitosan nanoparticles**

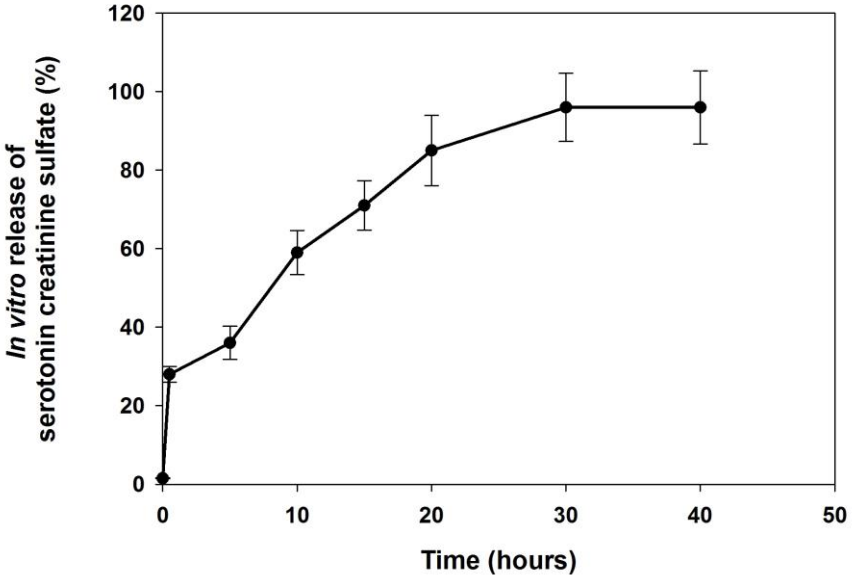


**Figure-12**
***In vitro* release of GABA and 5-HT from GABA and 5-HT chitosan nanoparticles**

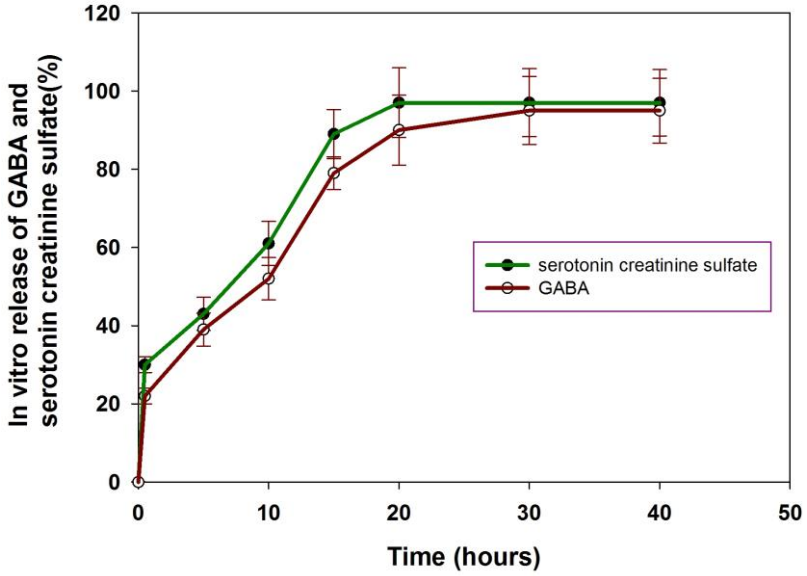
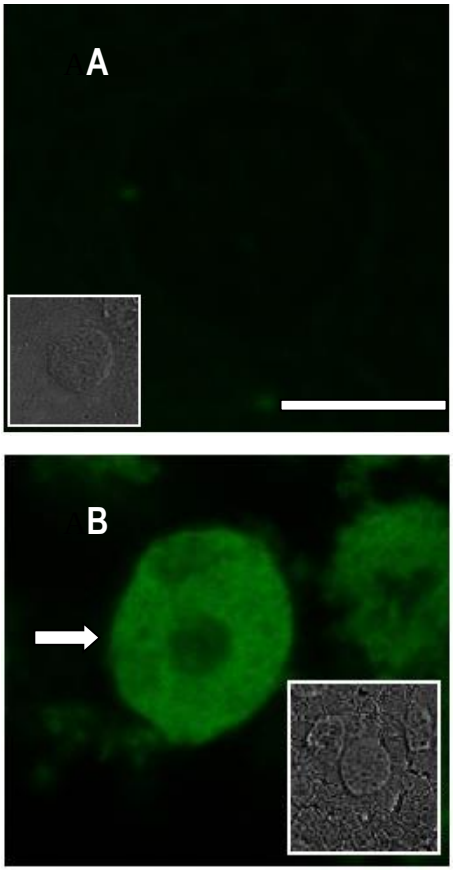


**Figure - 13**
**Confocal imaging of FITC tagged chitosan nanoparticles interacting with hepatocyte**




A) Negative control B) FITC tagged chitosan nanoparticles interacting with hepatocyte
Scale bar = 25µm

**Table - 1**
**[$^3$H] Thymidine uptake by hepatocytes of experimental rats**

| Groups | [$^3$H] Thymidine uptake DPM / mg protein |
|---|---|
| C | 15897 ± 189 |
| CN | 15800 ± 173 |
| PHNT | 18005 ± 543 [b] |
| GCNP | 23358 ± 400 [a,e] |
| SCNP | 20876 ± 302 [a,f,g] |
| GSCNP | 25969 ± 300 [a,d,h,k] |

**Table - 2**
**[$^3$H] Leucine uptake by hepatocytes of experimental rats**

| Groups | [$^3$H] Leucine uptake DPM / mg protein |
|---|---|
| C | 11218 ± 388 |
| CN | 11235 ± 395 |
| PHNT | 12658 ± 392[c] |
| GCNP | 14416 ± 823 [b,e] |
| SCNP | 14534 ± 421 [b,e] |
| GSCNP | 15984 ± 835 [a,d,h,k] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to C. [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ when compared to PHNT. [g] $p<0.001$, [h] $p<0.01$when compared to GCNP. [k] $p<0.01$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Table - 3**
**Thymidine kinase activity in hepatocytes of experimental rats**

| Groups | Vmax (nmoles/mg/min) | Km (nM) |
|---|---|---|
| **C** | 17.30 ± 8.80 | 0.92 ± 0.03 |
| **CN** | 16.98 ± 8.5 | 0.91 ± 0.03 |
| **PHNT** | 414.42 ± 50.20[a] | 0.80 ± 0.02 [c] |
| **GCNP** | 425.19 ± 69.90[a,d] | 0.80 ± 0.03 [c] |
| **SCNP** | 450.84 ± 75.45 [a,d,g] | 0.80 ± 0.03 [c] |
| **GSCNP** | 490.76 ± 83.45 [a,d,g,j] | 0.80 ± 0.05 [c] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$, [c] $p<0.05$ when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure 14**
**BrdU imaging in the liver of experimental rats**

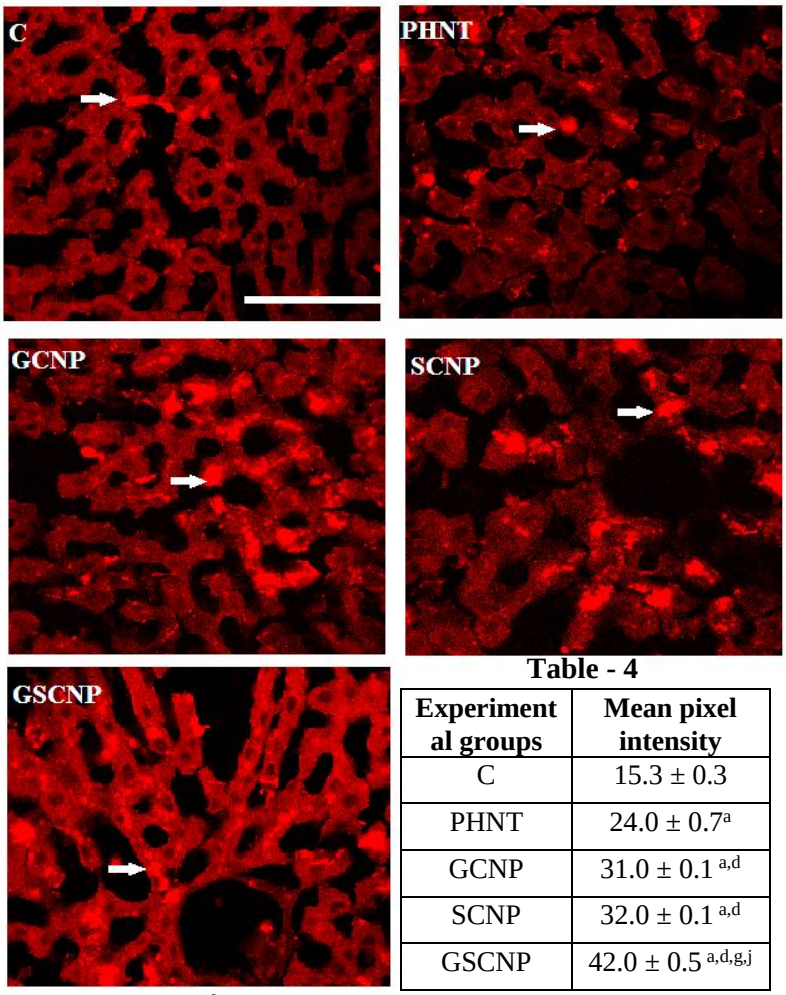



**Table - 4**

| Experimental groups | Mean pixel intensity |
|---|---|
| C | 15.3 ± 0.3 |
| PHNT | 24.0 ± 0.7[a] |
| GCNP | 31.0 ± 0.1 [a,d] |
| SCNP | 32.0 ± 0.1 [a,d] |
| GSCNP | 42.0 ± 0.5 [a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$, [c] $p<0.05$ when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment. Scale bar = 50µm.

**Figure - 15**
**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the liver of experimental rats**

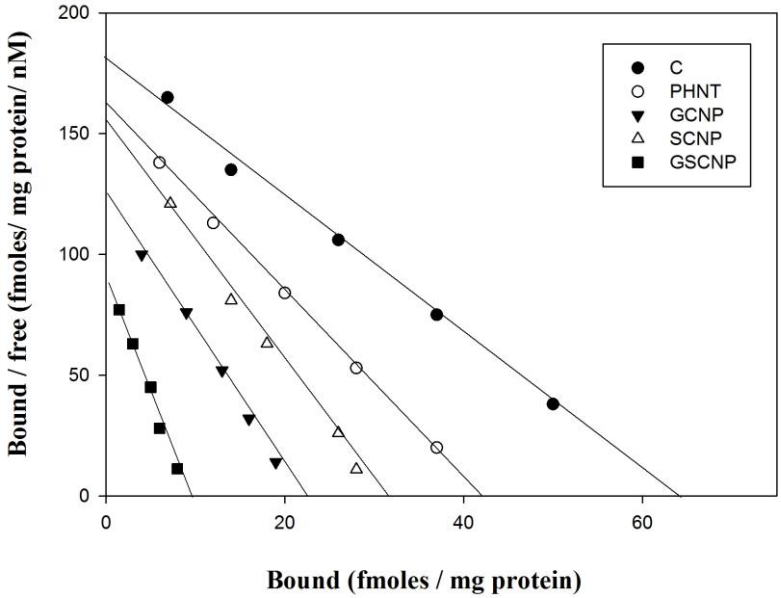



**Table - 5**
**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the liver of experimental rats**

| Experimental Groups | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
|---|---|---|
| C | 63.2 ± 1.7 | 2.8 ±0.11 |
| PHNT | 41.3 ± 2.0[a] | 3.9 ±0.05[a] |
| GCNP | 22.3 ± 2.0[a,d] | 5.6 ±0.11[a,d] |
| SCNP | 31.3 ± 2.0[a,e,h] | 5.0 ±0.08[a,d] |
| GSCNP | 9.5 ± 0.3[a,d,g,k] | 9.4 ±0.29[a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
$B_{max}$ – Maximal binding; $K_d$ – Dissociation constant.
[a] $p<0.001$when compared to C. [d] $p<0.001$, [e] $p<0.01$ when compared to PHNT. [g] $p<0.001$, [h] $p<0.01$when compared to GCNP. [j] $p<0.001$, [k] $p<0.01$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 16**
**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the liver**

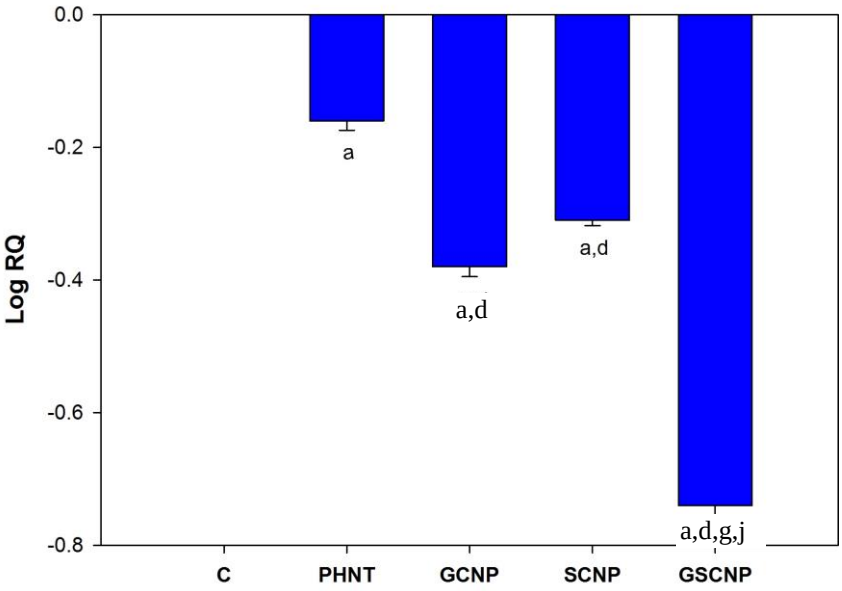



**Table- 6**
**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-0.16 \pm 0.01^{a}$ |
| GCNP | $-0.38 \pm 0.01^{a,d}$ |
| SCNP | $-0.31 \pm 0.01^{a,d}$ |
| GSCNP | $-0.74 \pm 0.02^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure 17**
**Confocal imaging of $GABA_B$ receptors in the liver of experimental rats**

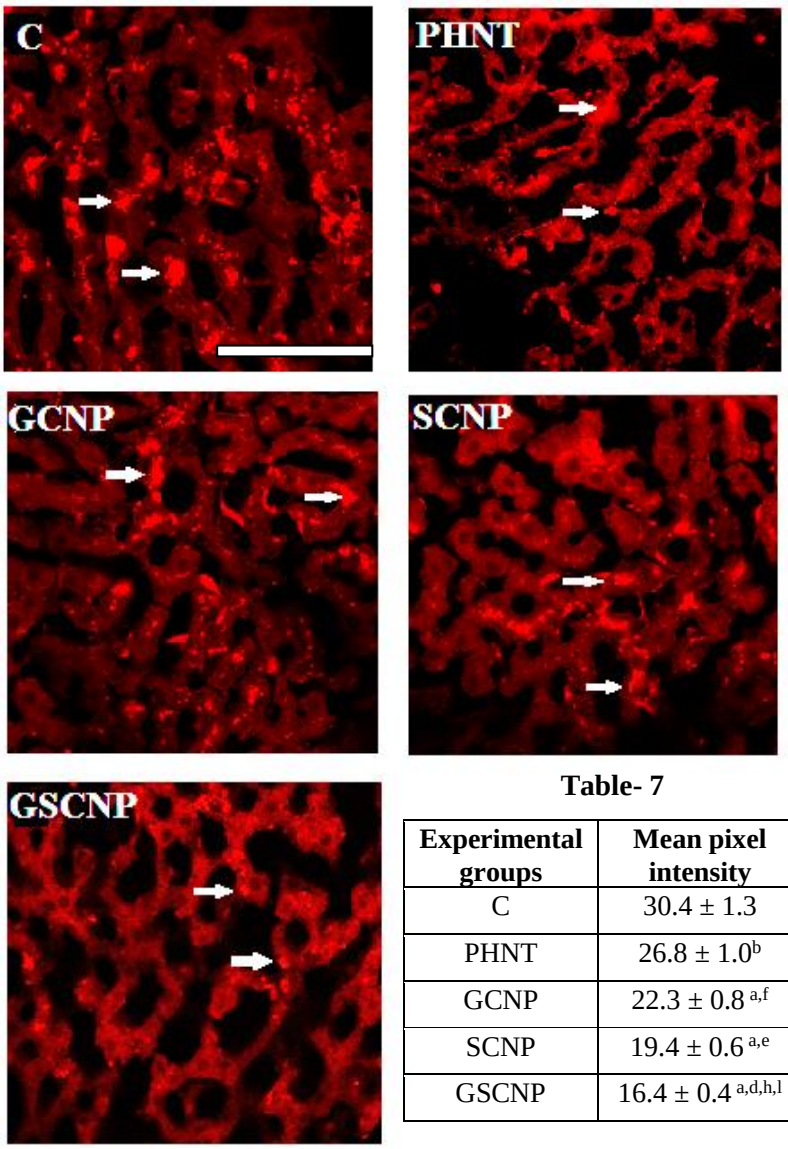



**Table- 7**

| Experimental groups | Mean pixel intensity |
|---|---|
| C | 30.4 ± 1.3 |
| PHNT | 26.8 ± 1.0[b] |
| GCNP | 22.3 ± 0.8 [a,f] |
| SCNP | 19.4 ± 0.6 [a,e] |
| GSCNP | 16.4 ± 0.4 [a,d,h,l] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [b] $p<0.01$when compared to C. [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ when compared to PHNT. [h] $p<0.01$when compared to GCNP. [l] $p<0.05$when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment. Scale bar represents 50µm.

**Figure - 18**
**Scatchard analysis of [$^3$H] ketanserin binding against ketanserin to 5-HT$_{2A}$ receptors in the liver of experimental rats**

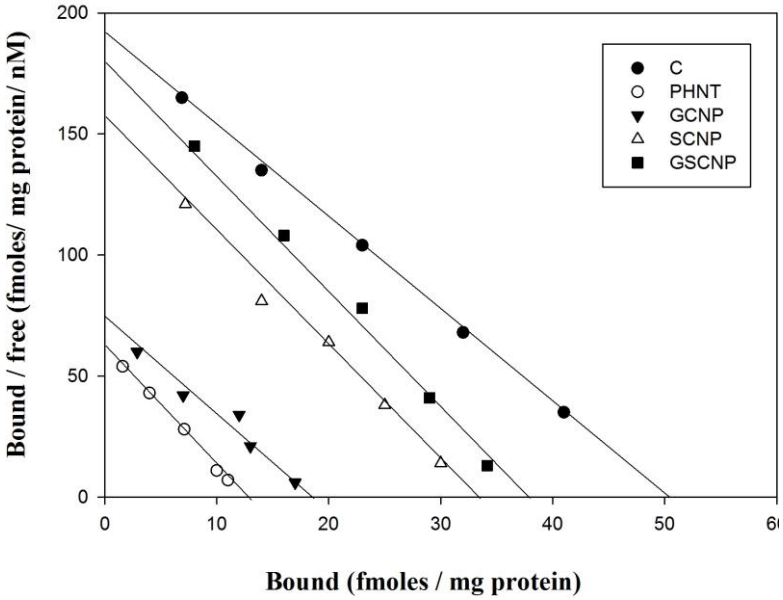



**Table - 8**
**Scatchard analysis of [$^3$H] ketanserin binding against ketanserin to 5-HT$_{2A}$ receptors in the liver of experimental rats**

| Experimental Groups | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
|---|---|---|
| C | 51.5 ± 2.0 | 3.7 ± 0.80 |
| PHNT | 12.5 ± 0.6[a] | 4.9 ± 0.64[a] |
| GCNP | 18.3 ± 0.8[a,f] | 4.7 ± 0.70[a] |
| SCNP | 33.5 ± 1.0[a,d,g] | 2.5 ± 0.52[a,d,g] |
| GSCNP | 39.0 ± 1.2[a,d,g,k] | 4.9 ± 1.2[a,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
$B_{max}$ – Maximal binding; $K_d$ – Dissociation constant
[a] $p<0.001$when compared to C. [d] $p<0.001$, [f] $p<0.05$ when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$, [k] $p<0.01$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 19**
**Real Time PCR amplification of 5-HT$_{2A}$ receptor subunit mRNA in the liver of experimental rats**

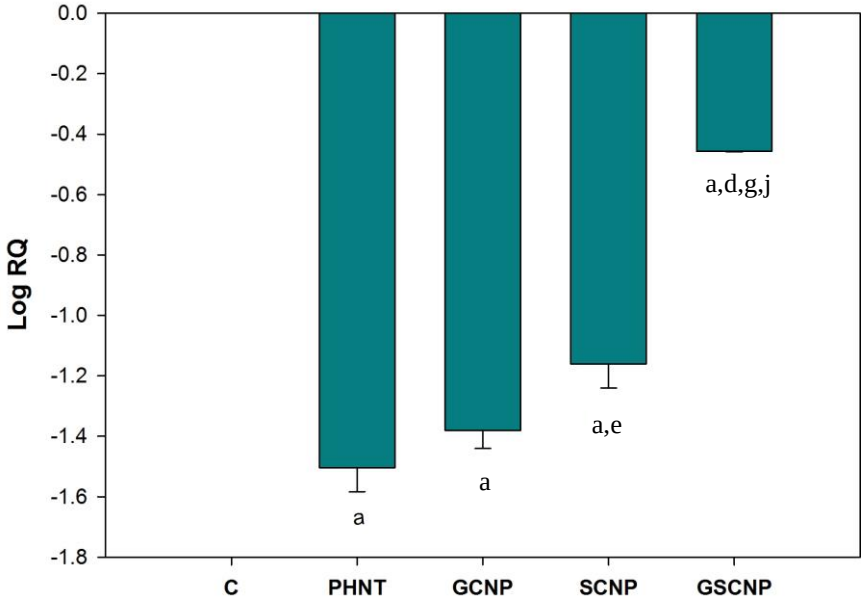



**Table - 9**
**Real Time PCR amplification of 5-HT$_{2A}$ receptor subunit mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | -1.5 ± 0.08[a] |
| GCNP | -1.3 ± 0.06[a] |
| SCNP | -1.1 ± 0.08[a,e] |
| GSCNP | -0.4± 0.01[a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$, [e] $p<0.01$ when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure 20**
**Confocal imaging of 5-$HT_{2A}$ receptors in the liver of experimental rats**

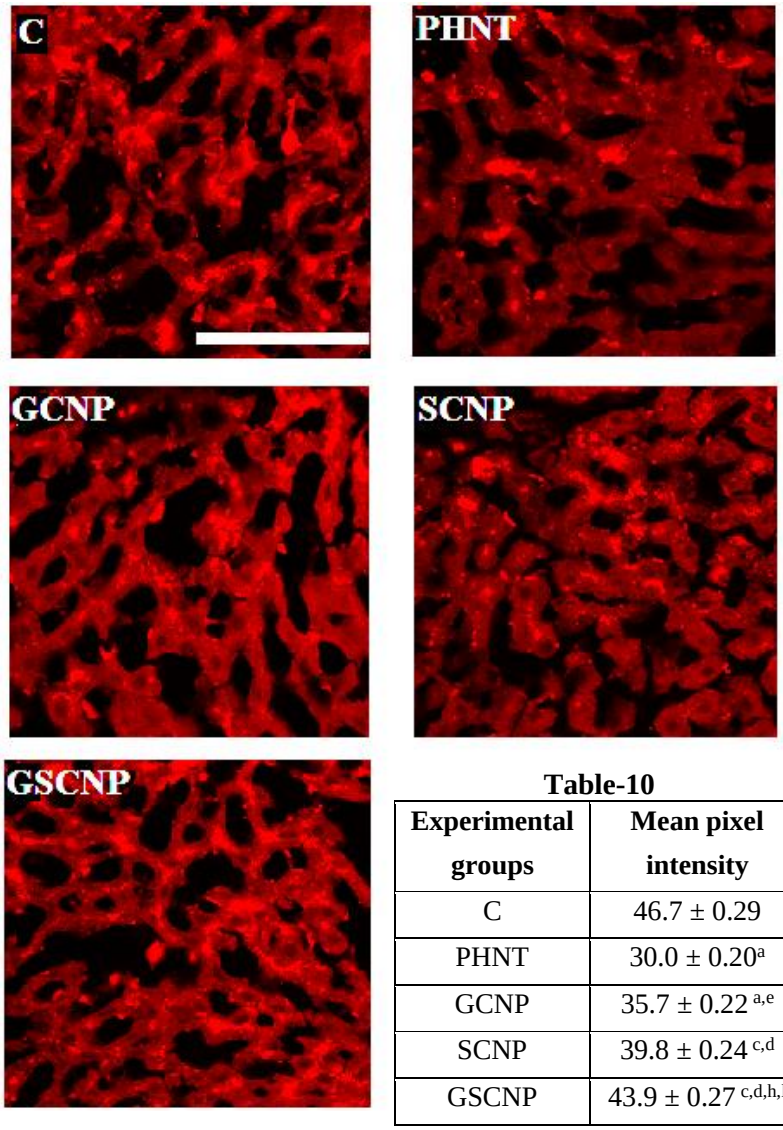
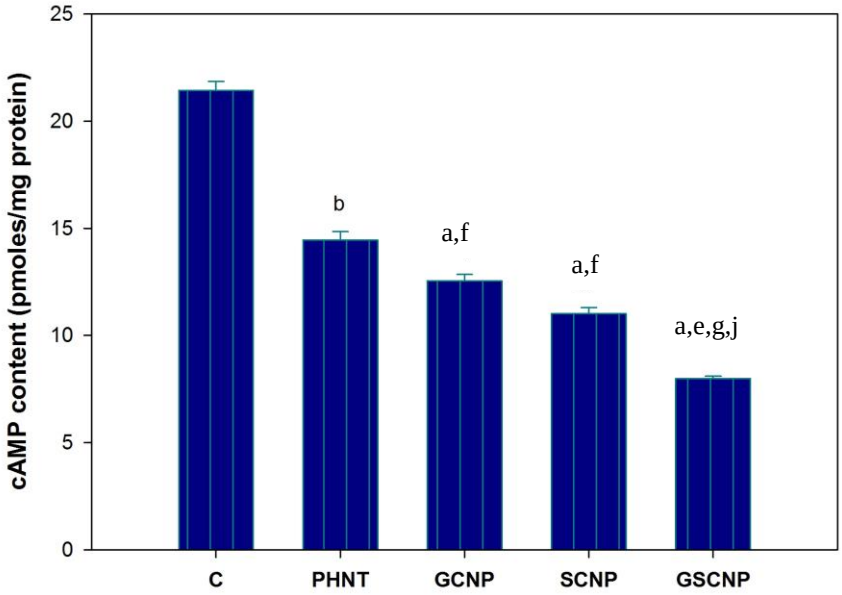



**Table-10**

| Experimental groups | Mean pixel intensity |
|---|---|
| C | 46.7 ± 0.29 |
| PHNT | 30.0 ± 0.20[a] |
| GCNP | 35.7 ± 0.22 [a,e] |
| SCNP | 39.8 ± 0.24 [c,d] |
| GSCNP | 43.9 ± 0.27 [c,d,h,l] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$, [c]$p<0.05$ when compared to C. [d] $p<0.001$, [e]$p<0.01$when compared to PHNT. [h] $p<0.01$when compared to GCNP. [l]$p<0.05$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment. Scale bar represents 50µm

**Figure - 21**
**cAMP content in the liver of experimental rats**




Values are Mean ± S.E.M. of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [b] $p<0.01$ with respect to C. [e] $p<0.01$ and [f] $p<0.05$ with respect to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP – Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP - Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment

**Figure – 22**

**Real Time PCR amplification of CREB mRNA in the liver of experimental rats**

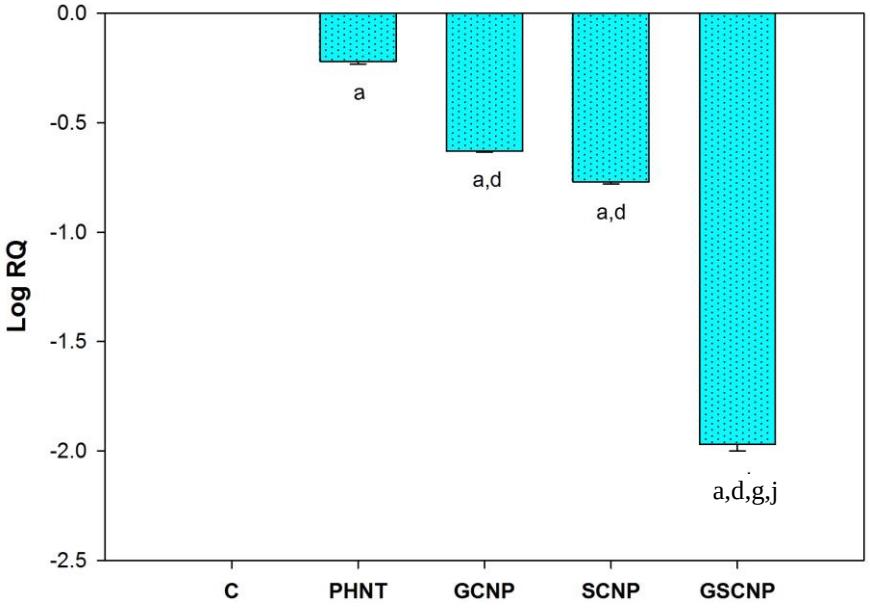


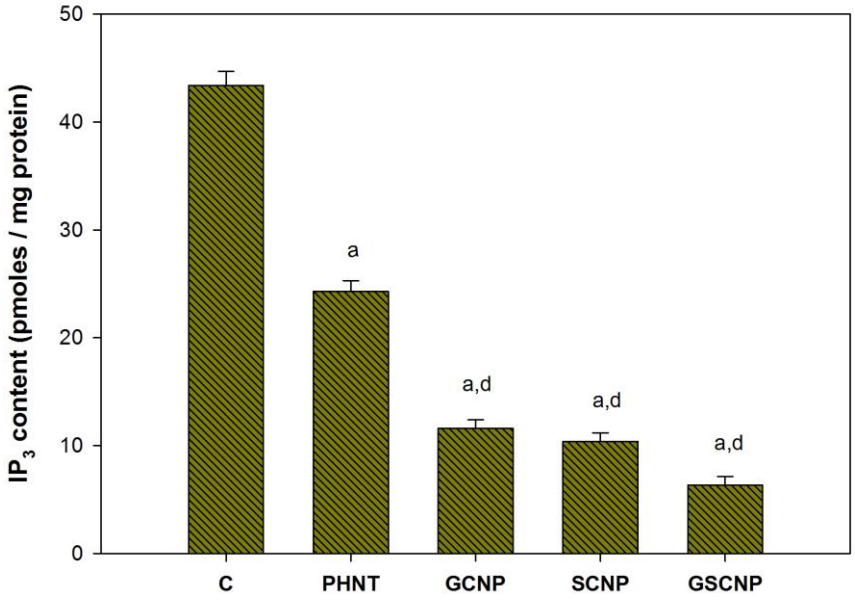

**Table - 11**
**Real Time PCR amplification of CREB mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | -0.2 ± 0.01[a] |
| GCNP | -0.6 ± 0.01[a,d] |
| SCNP | -0.7 ± 0.01[a,d] |
| GSCNP | -1.9± 0.03[a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 23**
**$IP_3$ content in the liver of experimental rats**




Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$ when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 24**
**Real Time PCR amplification of phospholipase C mRNA in the liver of experimental rats**

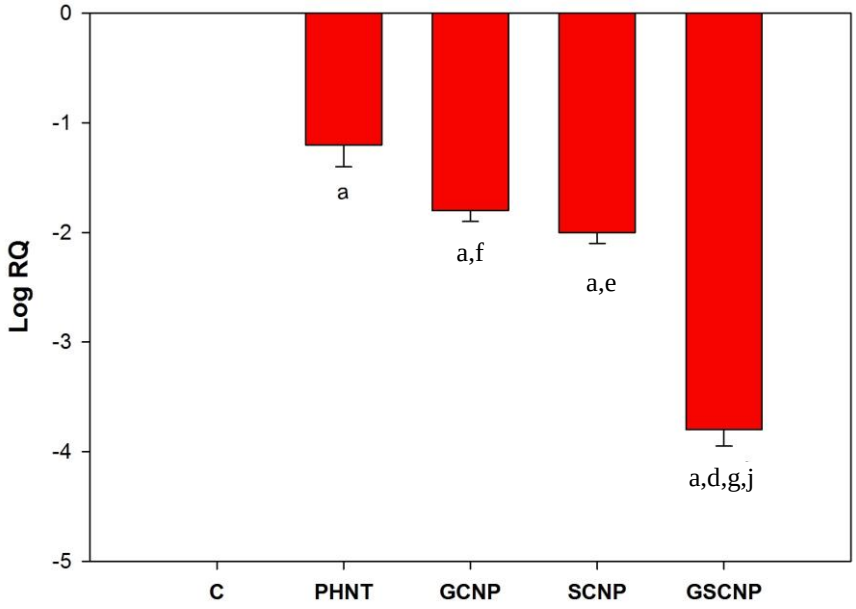



**Table – 12**

**Real Time PCR amplification of phospholipase C mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-1.2 \pm 0.2^{a}$ |
| GCNP | $-1.8 \pm 0.1^{a,f}$ |
| SCNP | $-2.0 \pm 0.1^{a,e}$ |
| GSCNP | $-3.8 \pm 0.1^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared to C. [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 25**
**Real Time PCR amplification of NF-κB mRNA in the liver of experimental rats**

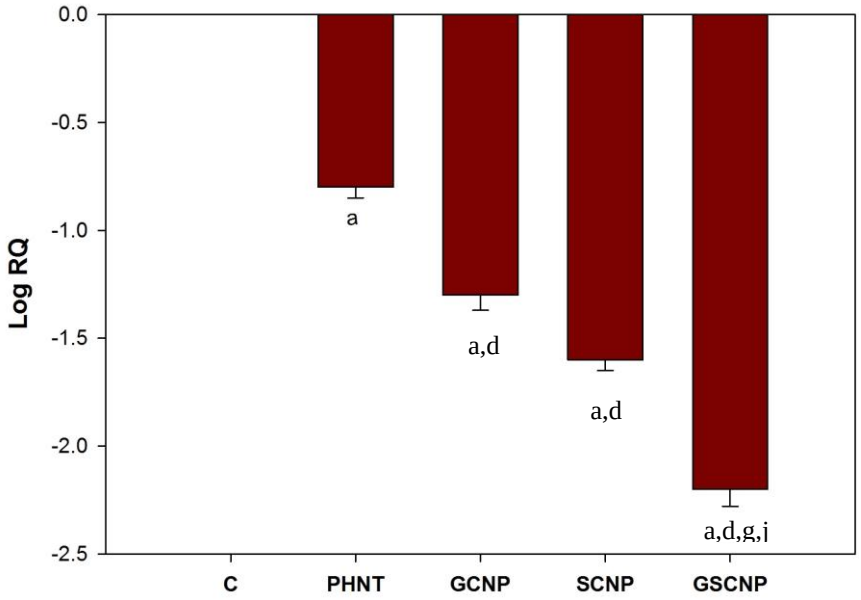



**Table - 13**
**Real Time PCR amplification of NF-κB mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-0.8 \pm 0.05^{a}$ |
| GCNP | $-1.3 \pm 0.07^{a,d}$ |
| SCNP | $-1.6 \pm 0.05^{a,d}$ |
| GSCNP | $-2.2 \pm 0.08^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 26**
**Real Time PCR amplification of TNF-α mRNA in the liver of experimental rats**

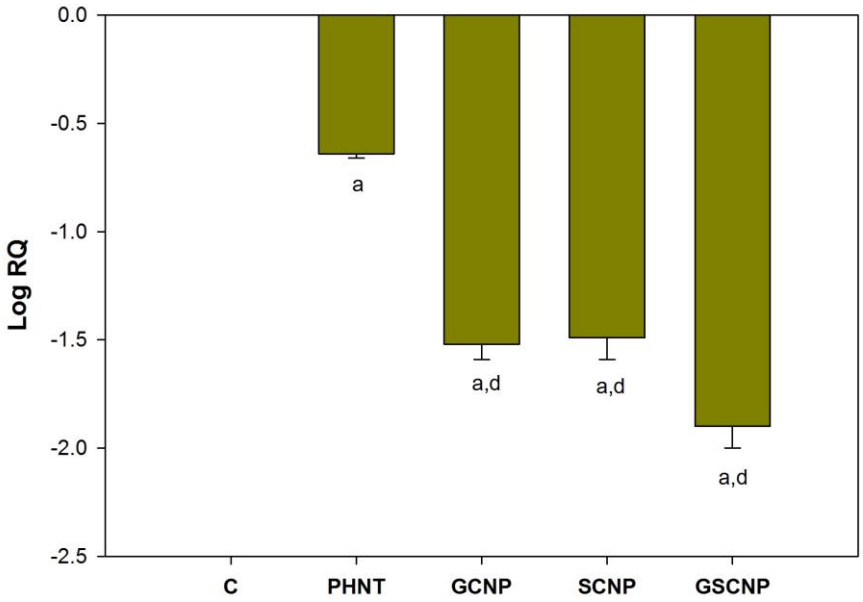



**Table - 14**
**Real Time PCR amplification of TNF-α mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | -0.64 ± 0.02[a] |
| GCNP | -1.52 ± 0.07[a,d] |
| SCNP | -1.49 ± 0.10[a,d] |
| GSCNP | -1.90± 0.10[a,d] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 27**
**Real Time PCR amplification of Akt-1 mRNA in the liver of experimental Rats**

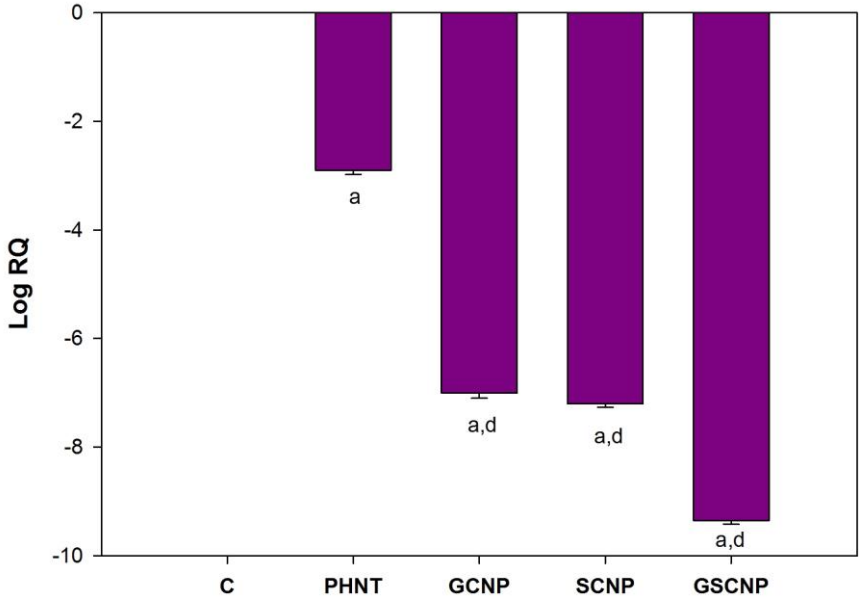



**Table - 15**
**Real Time PCR amplification of Akt-1 mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-2.9 \pm 0.08^{a}$ |
| GCNP | $-7.0 \pm 0.10^{a,d}$ |
| SCNP | $-7.2 \pm 0.07^{a,d}$ |
| GSCNP | $-9.3 \pm 0.07^{a,d}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 28**
**Real Time PCR amplification of SOD mRNA in the liver of experimental rats**

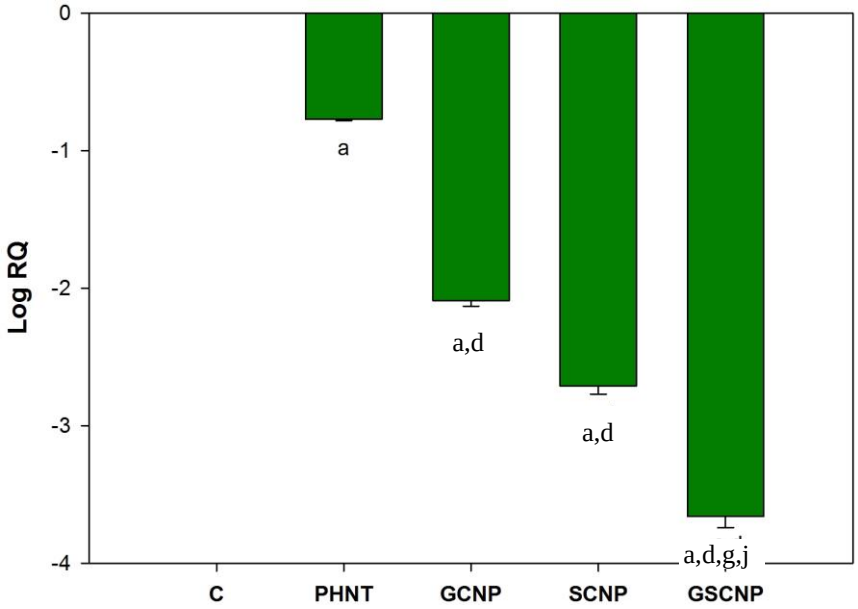



**Table – 16**
**Real Time PCR amplification of SOD mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-0.7 \pm 0.01^{a}$ |
| GCNP | $-2.0 \pm 0.04^{a,d}$ |
| SCNP | $-2.7 \pm 0.06^{a,d}$ |
| GSCNP | $-3.6 \pm 0.08^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Table- 17**
**Activity of SOD in the liver of experimental rats**

| GROUPS | SOD Activity (Units/mg protein) |
|---|---|
| **C** | 90.7 ± 3.20 |
| **PHNT** | 58.3 ± 1.59 [a] |
| **GCNP** | 29.5 ± 0.89 [a,d] |
| **SCNP** | 27.0 ± 0.73 [a,d] |
| **GSCNP** | 19.0 ± 0.54 [a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 29**
**Real Time PCR amplification of Bax mRNA in the liver of experimental rats**

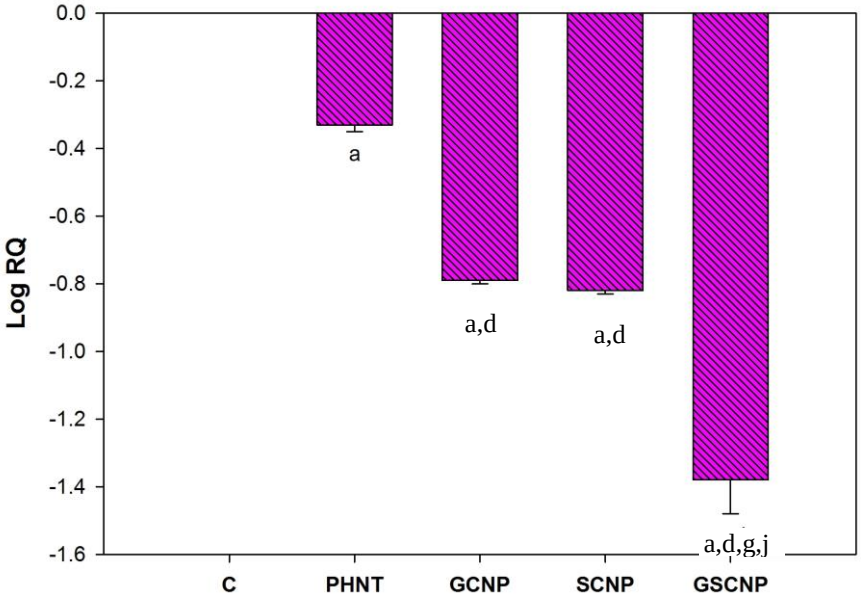



**Table - 18**
**Real Time PCR amplification of Bax mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-0.3 \pm 0.02^{a}$ |
| GCNP | $-0.7 \pm 0.01^{a,d}$ |
| SCNP | $-0.8 \pm 0.01^{a,d}$ |
| GSCNP | $-1.3 \pm 0.10^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 30**
**Real Time PCR amplification of caspase-8 mRNA in the liver of experimental rats**

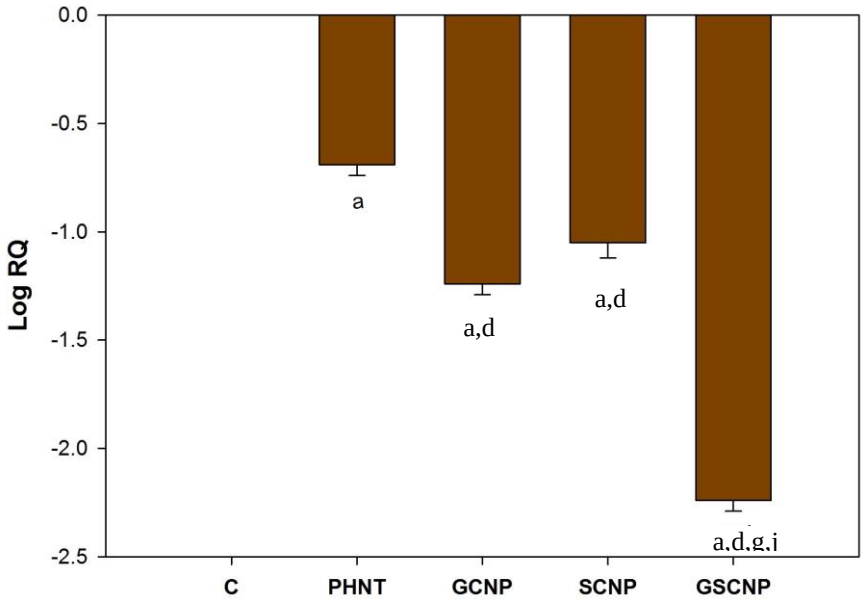



**Table - 19**
**Real Time PCR amplification of caspase-8 mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | -0.69 ± 0.05[a] |
| GCNP | -1.24 ± 0.05[a,d] |
| SCNP | -1.05 ± 0.07[a,d] |
| GSCNP | -2.24 ± 0.05[a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 31**
**Real Time PCR amplification of MAT2A mRNA in the liver of experimental rats**

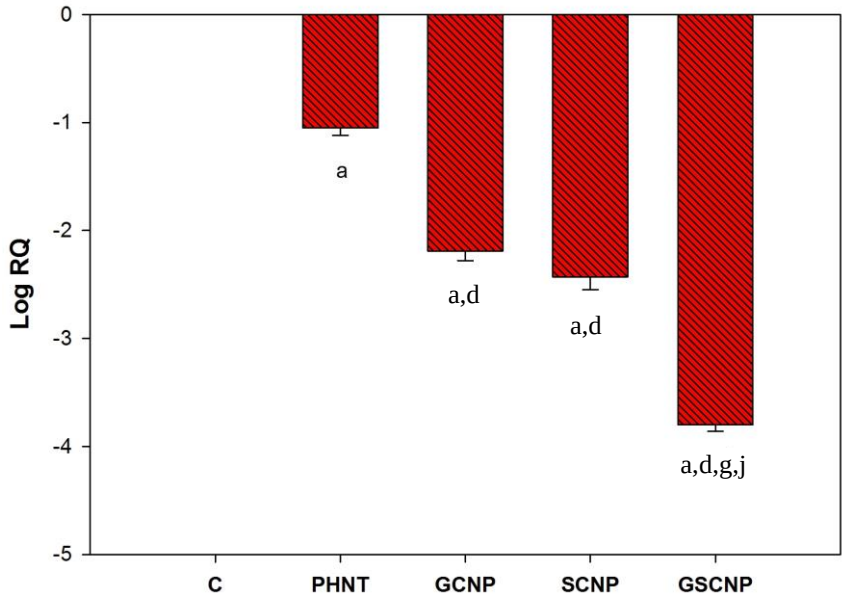



**Table - 20**
**Real Time PCR amplification of MAT2A mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-1.05 \pm 0.07^{a}$ |
| GCNP | $-2.19 \pm 0.09^{a,d}$ |
| SCNP | $-2.4 \pm 0.12^{a,d}$ |
| GSCNP | $-3.8 \pm 0.06^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 32**
**[$^3$H] methyl group incorporation on the liver DNA of experimental rats.**

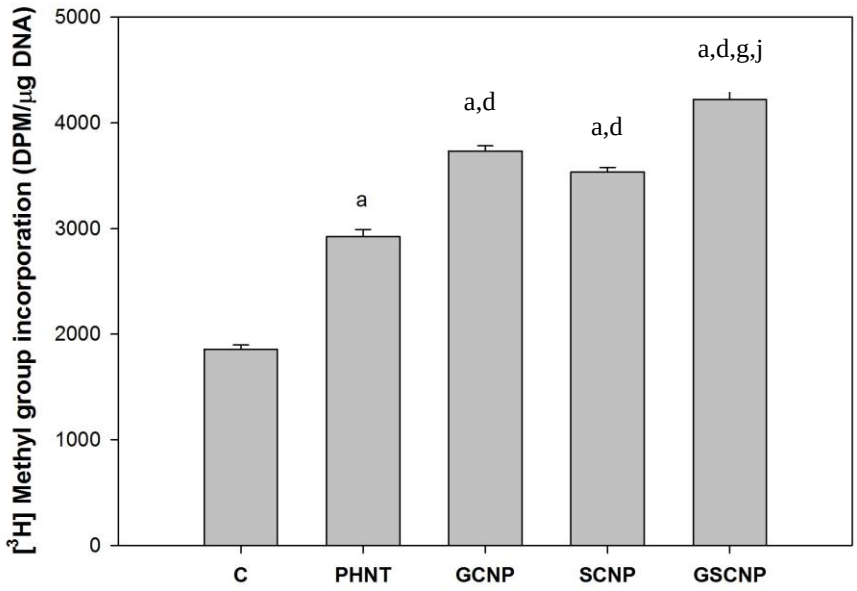



**Table - 21**
**[$^3$H] methyl group incorporation on the liver DNA of experimental rats.**

| Experimental Groups | Log RQ |
|---|---|
| C | 1857 ± 40 |
| PHNT | 2922 ± 68[a] |
| GCNP | 3536 ± 48[a,d] |
| SCNP | 3536 ± 40[a,d] |
| GSCNP | 4222 ± 82[a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Table - 22**
**DNA content in the liver of experimental rats**

| Groups | DNA content (ng/mg liver weight) |
|---|---|
| C | 0.64 ± 0.020 |
| PHNT | 0.92 ± 0 .010[a] |
| GCNP | 1.06 ± 0.010[a,d] |
| SCNP | 1.08 ± 0.005[a,d] |
| GSCNP | 1.15 ± 0.008[a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 33**
**Real Time PCR amplification of hepatocyte growth factor mRNA in the liver of experimental rats**

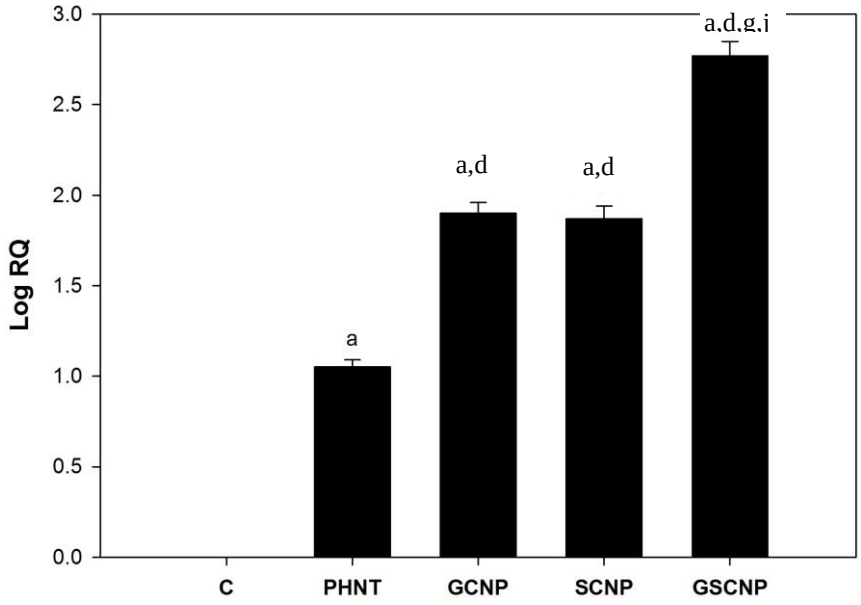



**Table - 23**
**Real Time PCR amplification of hepatocyte growth factor mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $1.05 \pm 0.04^{a}$ |
| GCNP | $1.90 \pm 0.06^{a,d}$ |
| SCNP | $1.80 \pm 0.07^{a,d}$ |
| GSCNP | $2.70 \pm 0.08^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 34**
**Real Time PCR amplification of Insulin like growth factor-1 mRNA in the liver of experimental rats**

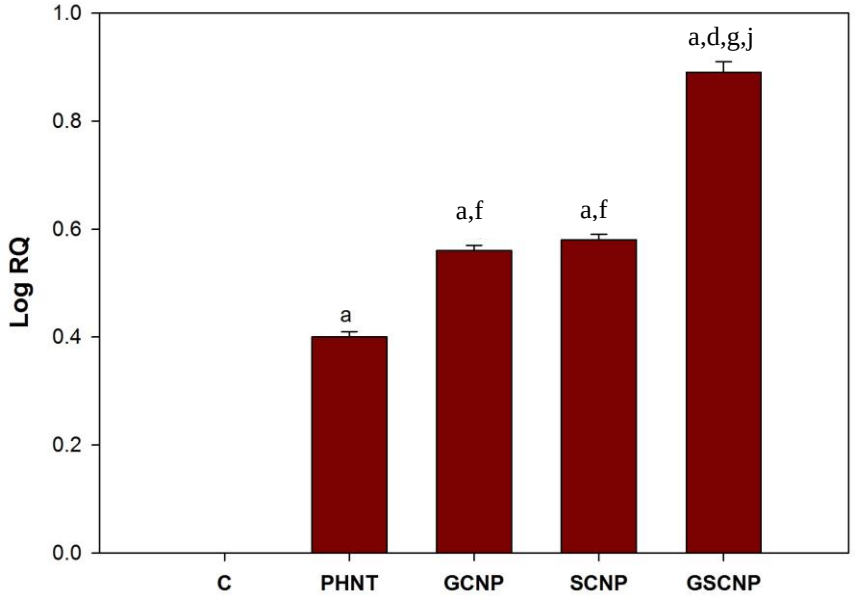



**Table - 24**
**Real Time PCR amplification of hepatocyte growth factor-1 mRNA in the liver of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $0.40 \pm 0.01^{a}$ |
| GCNP | $0.56 \pm 0.01^{a,f}$ |
| SCNP | $0.58 \pm 0.01^{a,f}$ |
| GSCNP | $0.89 \pm 0.02^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared to C. [d] $p<0.001$, [f] $p<0.05$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 35**
**Real Time PCR amplification of hepatocyte growth factor mRNA in the liver of experimental rat on seventh day of post hepatectomy**

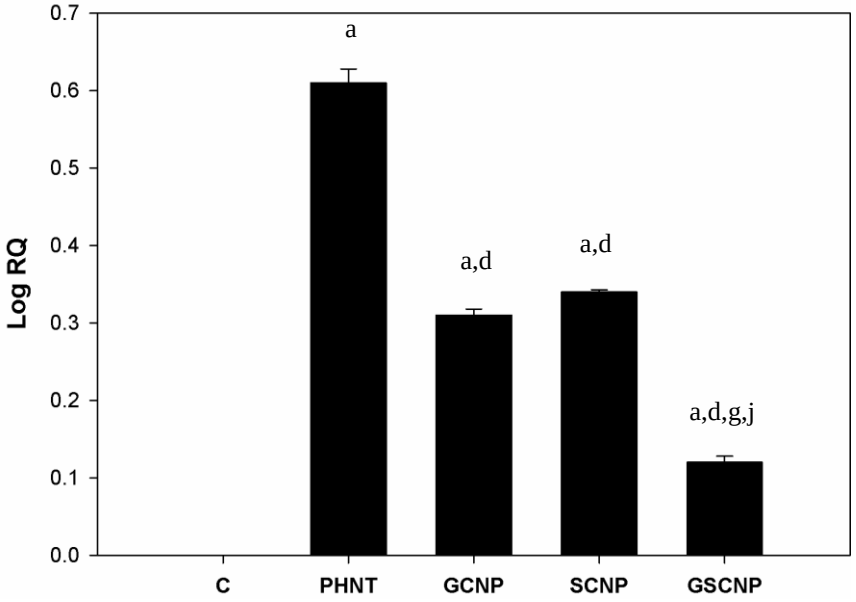



**Table 25**
**Real Time PCR amplification of hepatocyte growth factor mRNA in the liver of experimental rat on seventh day of post hepatectomy**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $0.61 \pm 0.018^{a}$ |
| GCNP | $0.31 \pm 0.008^{a,d}$ |
| SCNP | $0.34 \pm 0.003^{a,d}$ |
| GSCNP | $0.12 \pm 0.008^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$ when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 36**
**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the cerebral cortex of experimental rats**

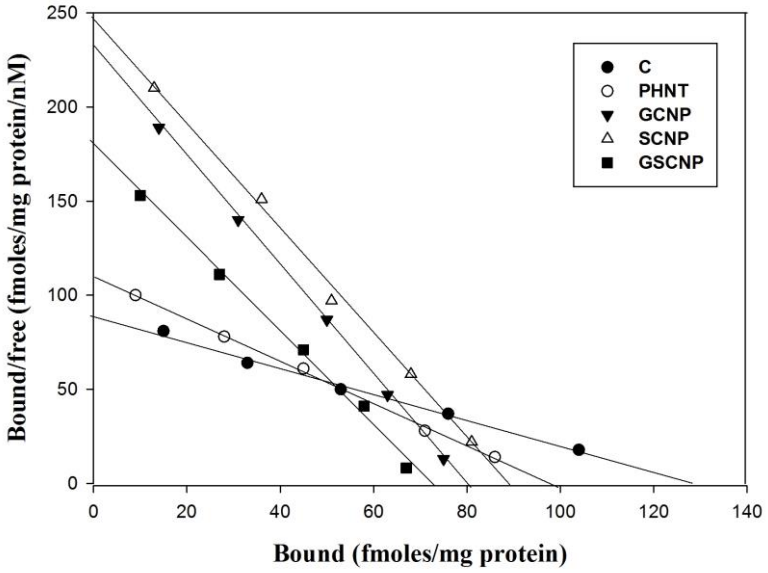



**Table - 26**
**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the cerebral cortex of experimental rats**

| Experimental Groups | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
|---|---|---|
| C | 128.0 ± 9.73 | 1.75 ± 0.14 |
| PHNT | 98.0 ± 3.732[a] | 0.87 ± 0.07[a] |
| GCNP | 81.2 ± 1.88[a,d] | 1.07 ± 0.08 [a,d] |
| SCNP | 88.2 ± 2.37[a,d] | 1.22 ± 0.14 [a,d] |
| GSCNP | 75.0 ± 0.57[a,d,h,j] | 0.86 ± 0.07 [a,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
$B_{max}$ – Maximal binding; $K_d$ – Dissociation constant
[a] $p<0.001$when compared to C .[d] $p<0.001$ when compared to PHNT. [g] $p<0.001$, [h] $p<0.01$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 37**
**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the cerebral cortex of experimental rats**

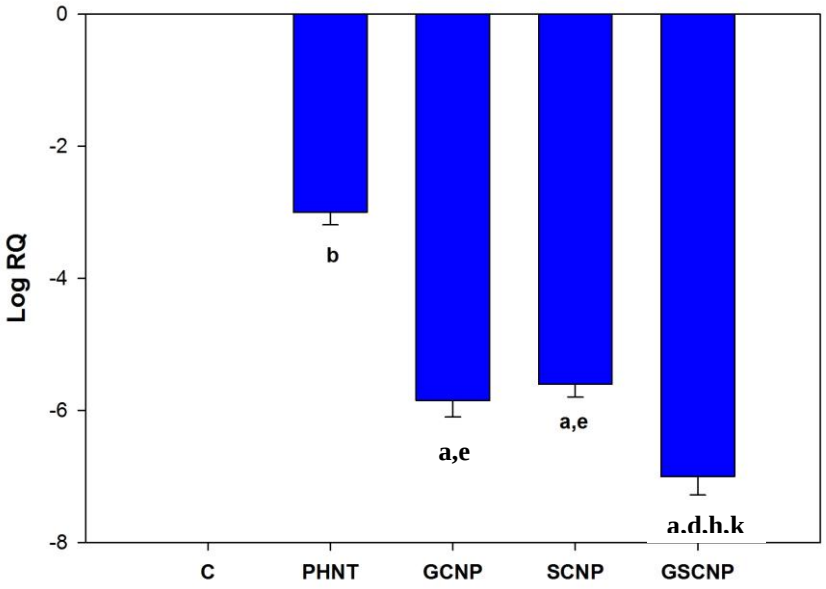



**Table - 27**
**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-3.0 \pm 0.19^{b}$ |
| GCNP | $-5.6 \pm 0.25^{a,e}$ |
| SCNP | $-5.8 \pm 0.20^{a,e}$ |
| GSCNP | $-7.0 \pm 0.28^{a,d,h,k}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$, [b] $p<0.01$ when compared to C. [d] $p<0.001$, [e] $p<0.01$ when compared to PHNT. [h] $p<0.01$ when compared to GCNP. [k] $p<0.01$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure 38**
**Confocal imaging of $GABA_B$ receptors in the cerebral cortex of experimental rats**

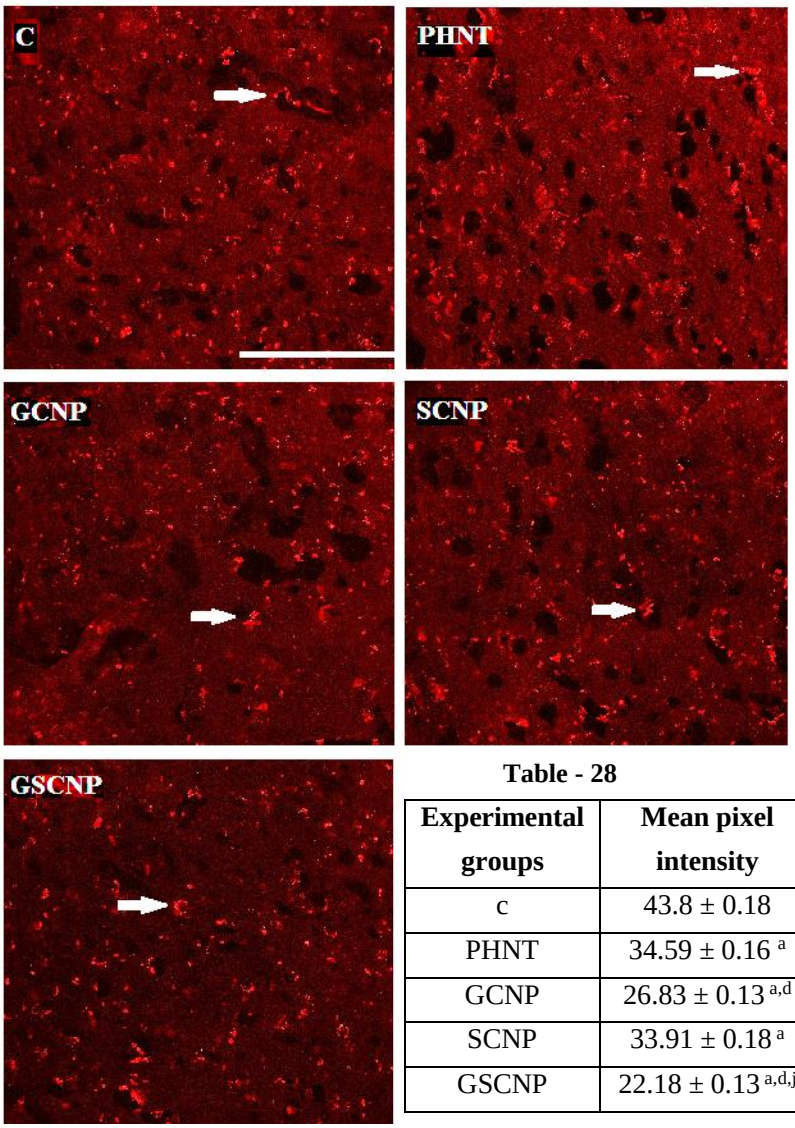



**Table - 28**

| Experimental groups | Mean pixel intensity |
|---|---|
| c | 43.8 ± 0.18 |
| PHNT | 34.59 ± 0.16 [a] |
| GCNP | 26.83 ± 0.13 [a,d] |
| SCNP | 33.91 ± 0.18 [a] |
| GSCNP | 22.18 ± 0.13 [a,d,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [j] $p<0.001$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment. Scale bar represents 50µm

**Figure - 39**
**Scatchard analysis of [$^3$H] ketanserin binding against ketanserin to 5-HT$_{2A}$ receptors in the cerebral cortex of experimental rats**

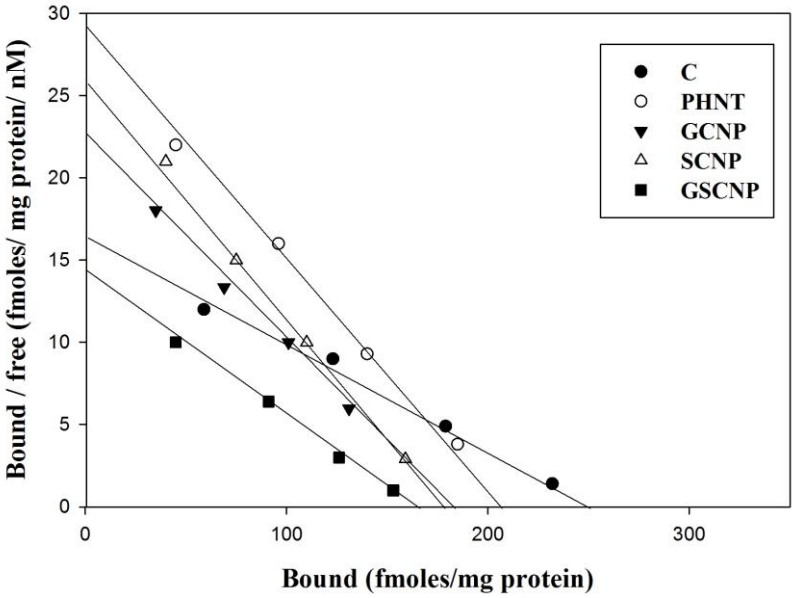



**Table - 29**
**Scatchard analysis of [$^3$H]ketanserin binding against ketanserin to 5-HT$_{2A}$ receptors in the cerebral cortex of experimental rats**

| Experimental Groups | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
|---|---|---|
| C | 251.50 ± 26.69 | 15.42 ± 0.24 |
| PHNT | 210.72 ± 16.20[a] | 7.21 ± 0.20 [a] |
| GCNP | 176.81 ± 10.91[a,d] | 7.80 ± 0.23 [a] |
| SCNP | 174.75 ± 10.69[a,d] | 6.77 ± 0.19 [a] |
| GSCNP | 166.90 ± 10.32[a,d] | 11.67 ± 0.4 [b,e,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
$B_{max}$ – Maximal binding; $K_d$ – Dissociation constant
[a] $p<0.001$, [b] $p<0.01$ when compared to C. [d] $p<0.001$, [e] $p<0.01$ when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure -40**
**Real Time PCR amplification of 5-$HT_{2A}$ receptor subunit mRNA in the cerebral cortex of experimental rats**

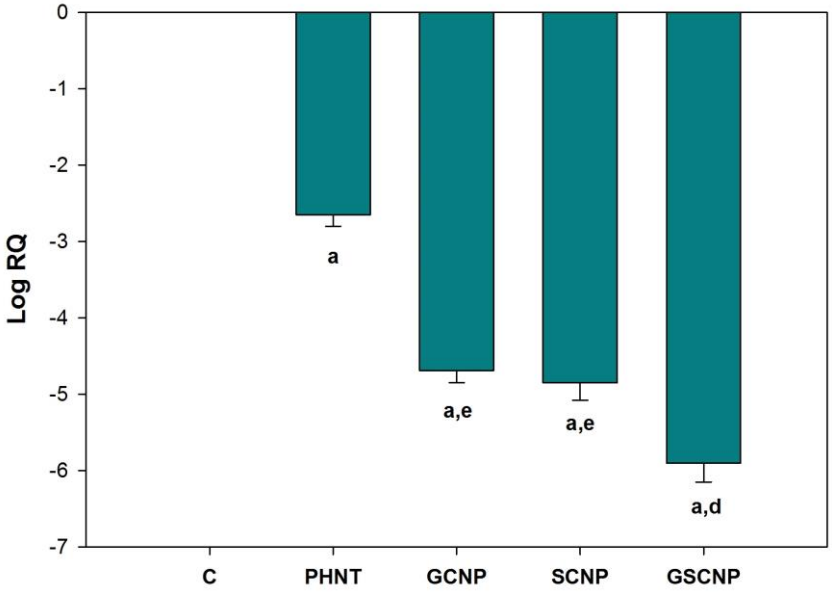



**Table - 30**
**Real Time PCR amplification of 5-$HT_{2A}$ receptor subunit mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-2.6 \pm 0.15^{a}$ |
| GCNP | $-4.6 \pm 0.16^{a,e}$ |
| SCNP | $-4.8 \pm 0.23^{a,e}$ |
| GSCNP | $-5.9 \pm 0.29^{a,d}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$, [e] $p<0.01$ when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure 41**
**Confocal imaging of 5-HT$_{2A}$ receptors in the cerebral cortex of experimental rats**

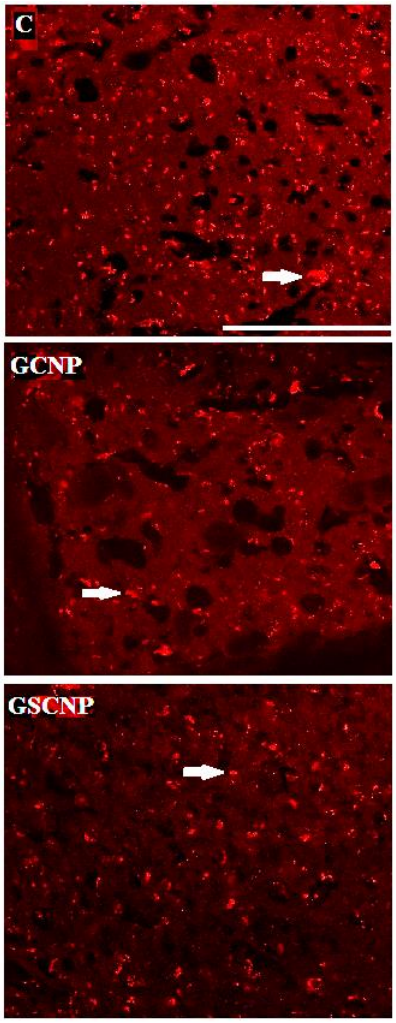
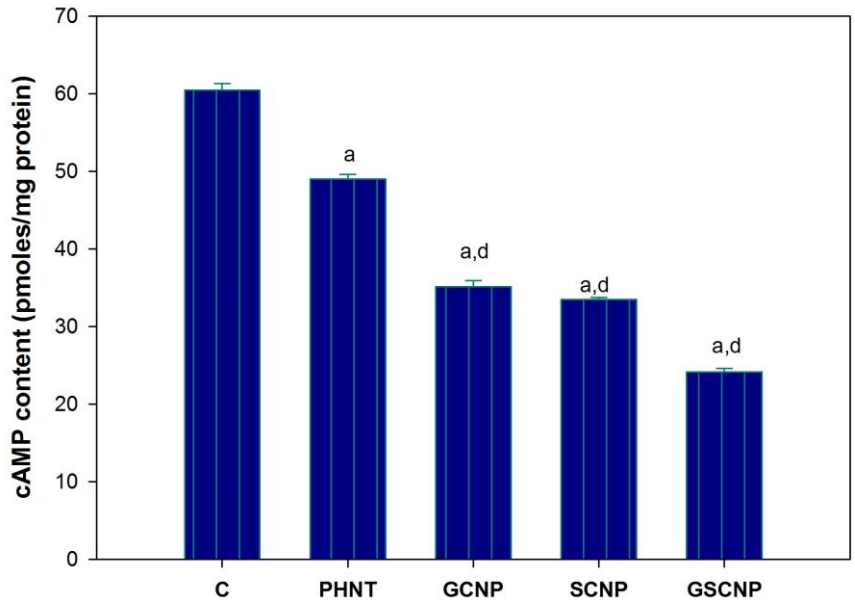



**Table- 31**

| Experimental groups | Mean pixel intensity |
|---|---|
| c | 48.9 ± 0.23 |
| PHNT | 39.5 ± 0.19[a] |
| GCNP | 37.8 ± 0.18 [a] |
| SCNP | 28.9 ± 0.14 [a,d] |
| GSCNP | 21.2 ± 0.12[a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] p<0.001when compared to C. [d] p<0.001when compared to PHNT. [g] p<0.01 when compared to GCNP. [j] p<0.001 when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment. Scale bar represents 50µm

**Figure - 42**
**cAMP content in the cerebral cortex of experimental rats**




Values are Mean ± S.E.M. of 4-6 separate experiments. Each group consists of *4-6* rats.
[a] $p<0.001$ with respect to C. [d] $p<0.001$ with respect to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP – Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP - Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment

**Figure - 43**
**Real Time PCR amplification of CREB mRNA in the cerebral cortex of experimental rats**

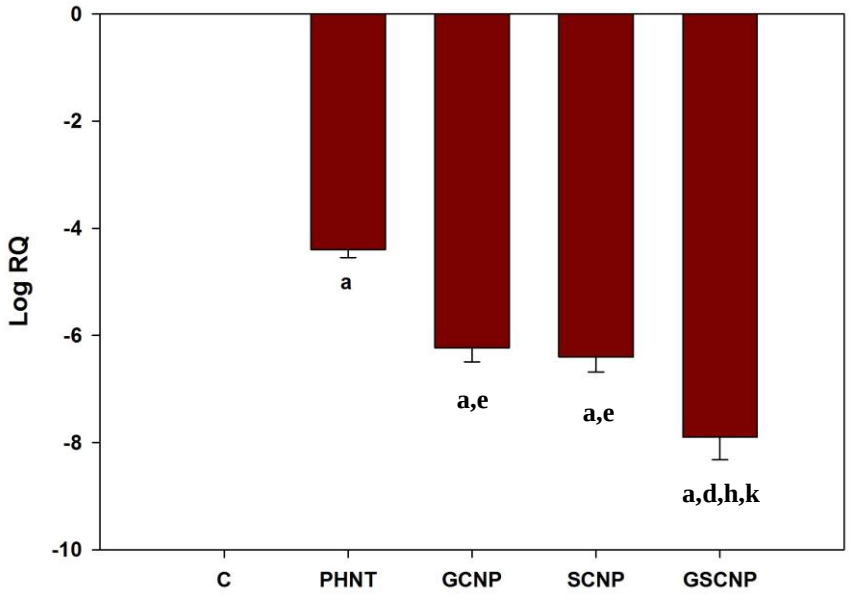
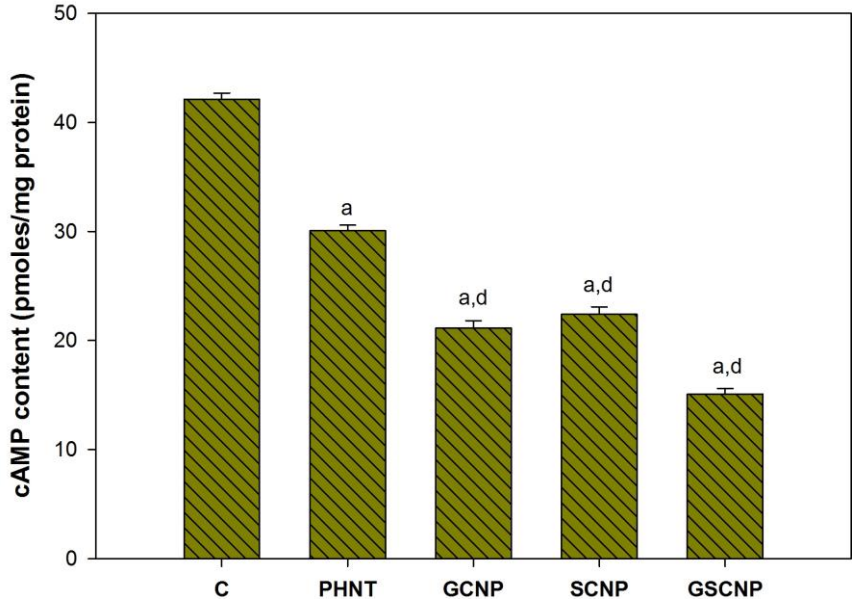



**Table - 32**
**Real Time PCR amplification of CREB mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-4.3 \pm 0.15^{a}$ |
| GCNP | $-6.2 \pm 0.26^{a,e}$ |
| SCNP | $-6.4 \pm 0.28^{a,e}$ |
| GSCNP | $-7.9 \pm 0.42^{a,d,h,k}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$, [e] $p<0.01$ when compared to PHNT. [h] $p<0.01$ when compared to GCNP. [k] $p<0.01$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure –44**
**$IP_3$ content in the cerebral cortex of experimental rats**




Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 45**
**Real Time PCR amplification of phospholipase C mRNA in the cerebral cortex of experimental rats**

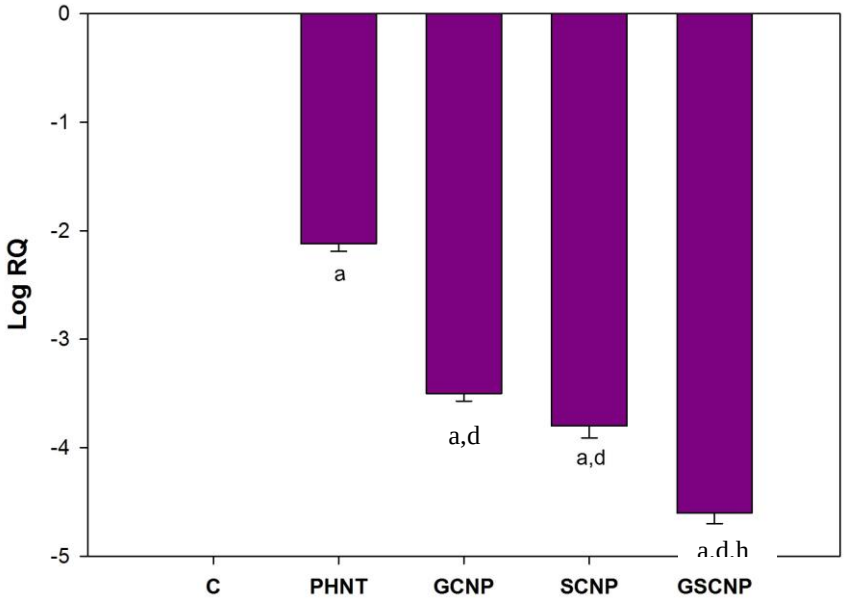


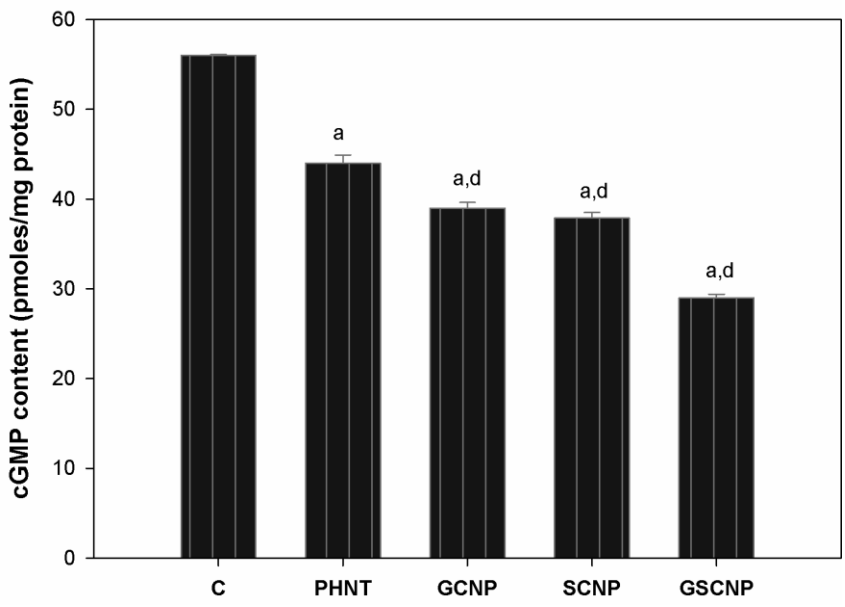

**Table - 33**
**Real Time PCR amplification of phospholipase C mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-2.1 \pm 0.07^{a}$ |
| GCNP | $-3.5 \pm 0.07^{a,d}$ |
| SCNP | $-3.8 \pm 0.11^{a,d}$ |
| GSCNP | $-4.6 \pm 0.10^{a,d,h}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [h] $p<0.01$ when compared to GCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure –46**
**cGMP content in the cerebral cortex of experimental rats**




Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 47**
**Real Time PCR amplification of NF-κB mRNA in the cerebral cortex of experimental rats**

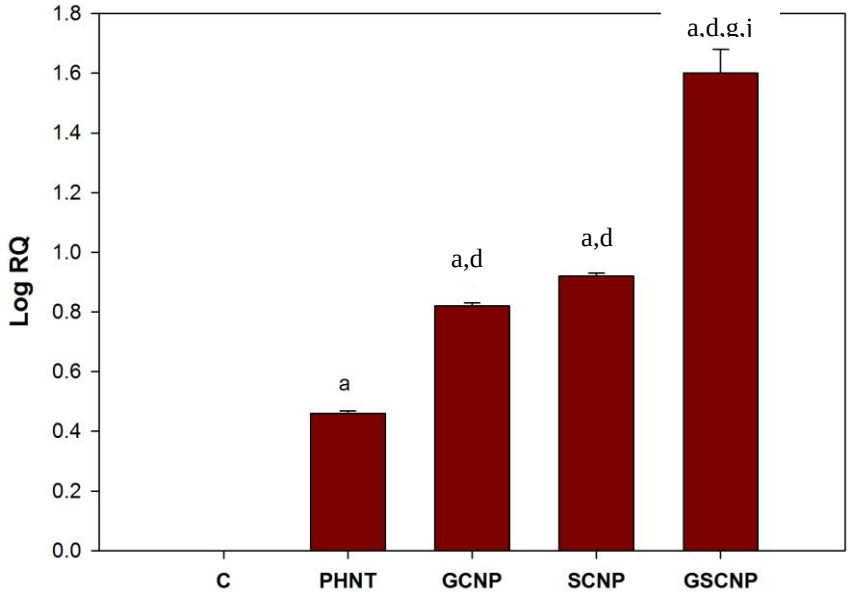



**Table - 34**
**Real Time PCR amplification of NF-κB mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $4.3 \pm 0.08^{a}$ |
| GCNP | $5.9 \pm 0.08^{a,d}$ |
| SCNP | $6.3 \pm 0.09^{a,d}$ |
| GSCNP | $7.3 \pm 0.05^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 48**
**Real Time PCR amplification of TNF-α mRNA in the cerebral cortex of experimental rats**

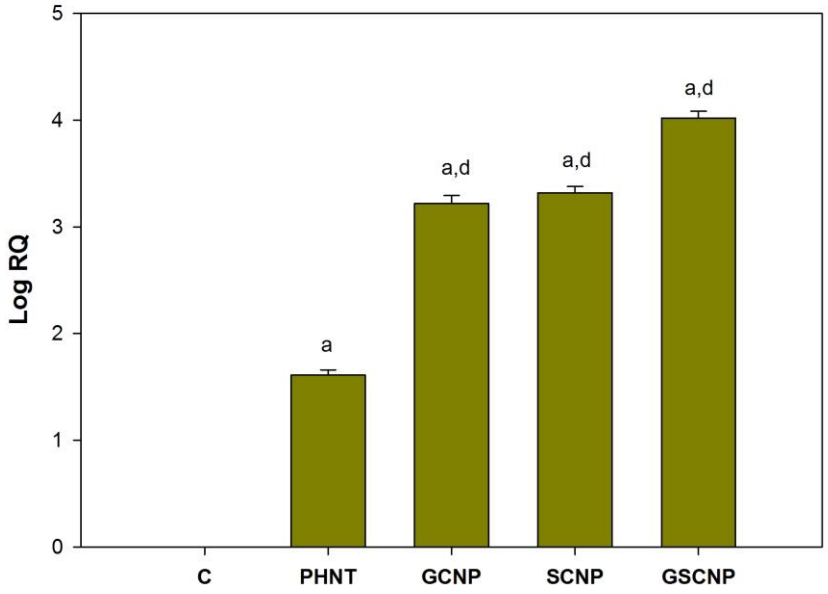



**Table -35**
**Real Time PCR amplification of TNF-α mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $1.6 \pm 0.05^{a}$ |
| GCNP | $3.2 \pm 0.07^{a,d}$ |
| SCNP | $3.3 \pm 0.05^{a,d}$ |
| GSCNP | $4.0 \pm 0.06^{a,d}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$, when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 49**
**Real Time PCR amplification of Akt-1 mRNA in the cerebral cortex of experimental rats**

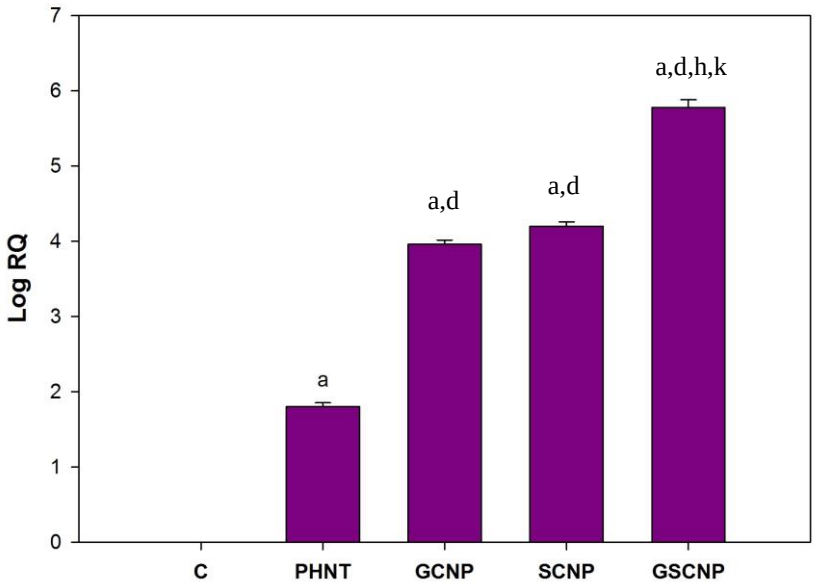



**Table -36**
**Real Time PCR amplification of Akt-1 mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $1.8 \pm 0.05^{a}$ |
| GCNP | $3.9 \pm 0.05^{a,d}$ |
| SCNP | $4.2 \pm 0.05^{a,d}$ |
| GSCNP | $5.7 \pm 0.1^{a,d,h,k}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$, when compared to PHNT. [h] $p<0.01$ when compared to GCNP. [k] $p<0.01$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure- 50**
**Real Time PCR amplification of Insulin like growth factor-1 mRNA in the cerebral cortex of experimental rats**

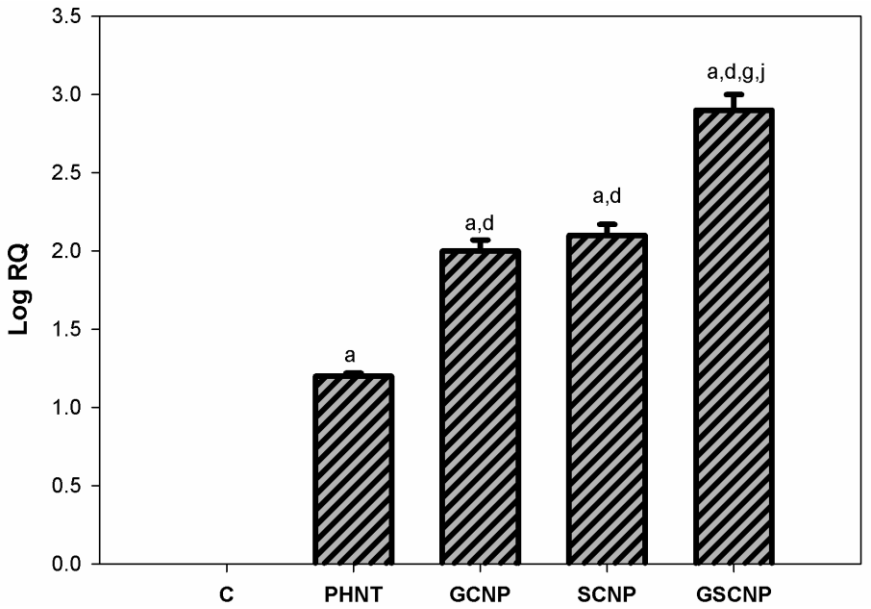



**Table-37**
**Real Time PCR amplification of Insulin like growth factor-1 mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $1.2 \pm 0.02^{a}$ |
| GCNP | $2.0 \pm 0.07^{a,d}$ |
| SCNP | $2.1 \pm 0.07^{a,d}$ |
| GSCNP | $2.9 \pm 0.1^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 51**
**Real Time PCR amplification of SOD mRNA in the cerebral cortex of experimental rats**

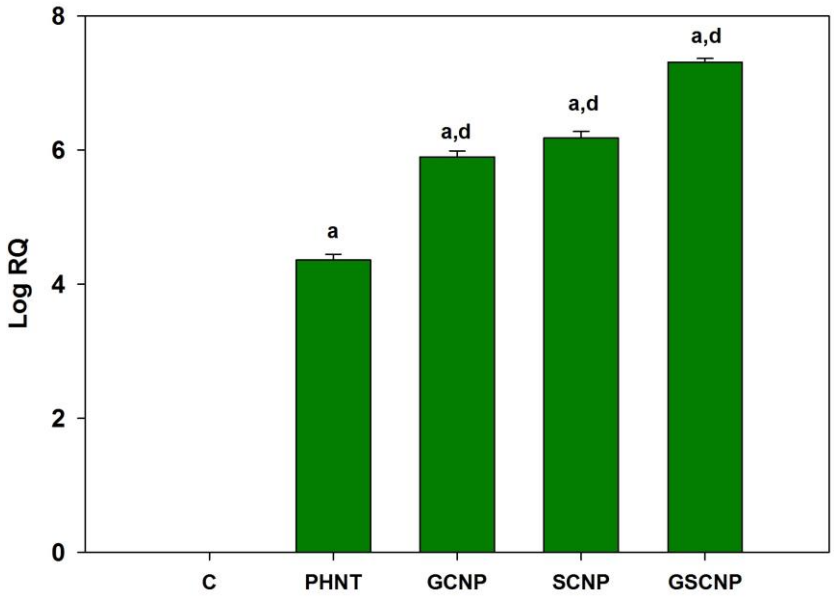



**Table - 38**
**Real Time PCR amplification of SOD mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | -4.3 ± 0.08[a] |
| GCNP | -5.9 ± 0.08[a,d] |
| SCNP | -6.3 ± 0.09[a,d] |
| GSCNP | -7.3 ± 0.05[a,d] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 52**
**Real Time PCR amplification of Bax mRNA in the cerebral cortex of experimental rats**

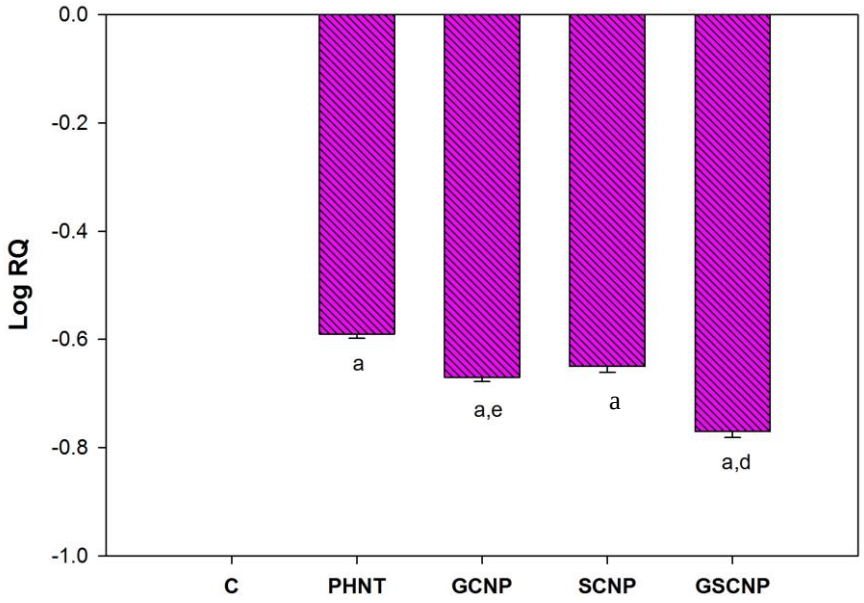



**Table- 39**
**Real Time PCR amplification of Bax mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | -0.59 ± 0.008[a] |
| GCNP | -0.67 ± 0.008[a,e] |
| SCNP | -0.65± 0.011[a] |
| GSCNP | -0.77 ± 0.011[a,d] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$, [e] $p<0.01$when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 53**
**Real Time PCR amplification of caspase-8 mRNA in the cerebral cortex of experimental rats**

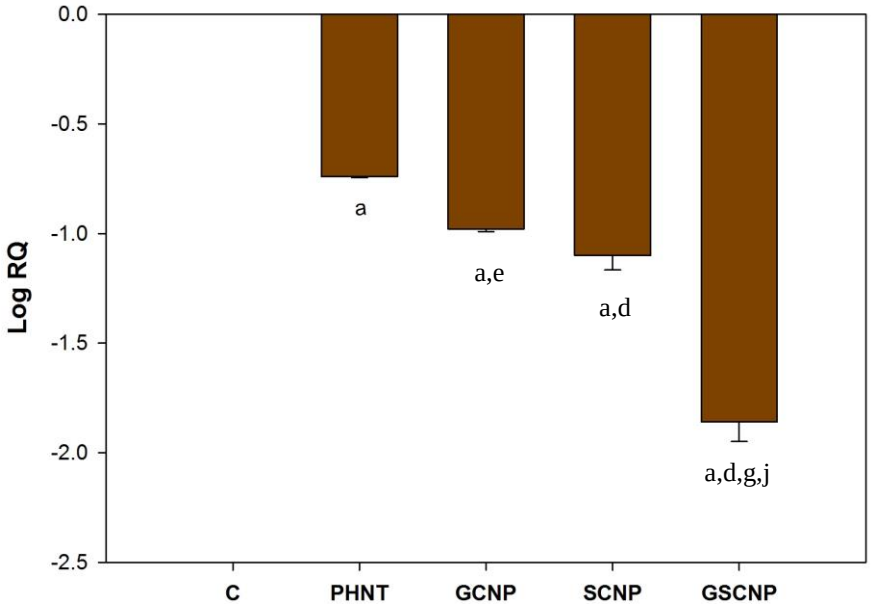



**Table- 40**
**Real Time PCR amplification of caspase-8 mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | -0.74 ± 0.005[a] |
| GCNP | -0.98 ± 0.011[a,e] |
| SCNP | -1.10± 0.066[a,d] |
| GSCNP | -1.86 ± 0.088[a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] p<0.001when compared to C. [d] p<0.001, [e] p<0.01when compared to PHNT. [g] p<0.001 when compared to GCNP. [j] p<0.001 when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 54**
**Real Time PCR amplification of BDNF mRNA in the cerebral cortex of experimental rats**

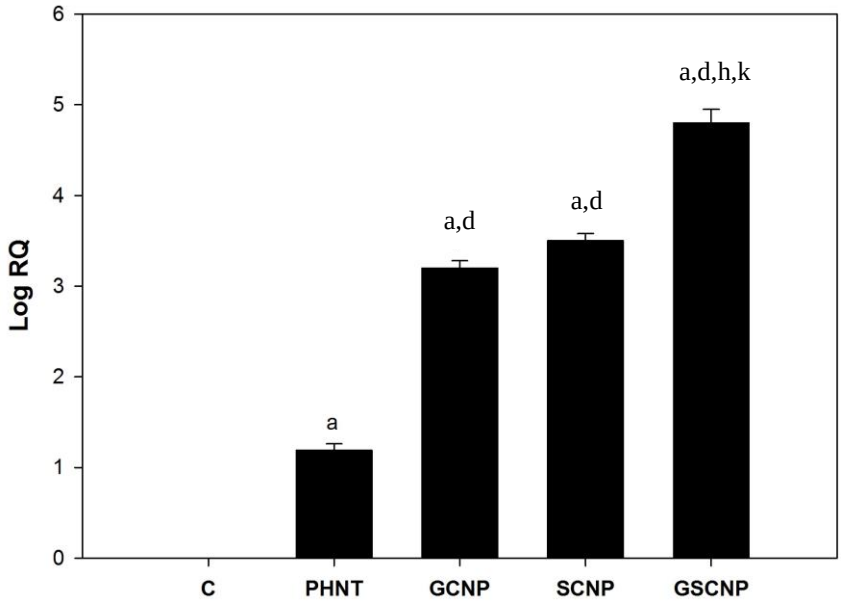



**Table- 41**
**Real Time PCR amplification of BDNF mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $1.30 \pm 0.02^{a}$ |
| GCNP | $3.20 \pm 0.08^{a,d}$ |
| SCNP | $3.50 \pm 0.08^{a,d}$ |
| GSCNP | $4.80 \pm 0.15^{a,d,h,k}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$compared to PHNT. [h] $p<0.01$ when compared to GCNP. [k] $p<0.01$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 55**
**Real Time PCR amplification of GDNF mRNA in the cerebral cortex of experimental rats**

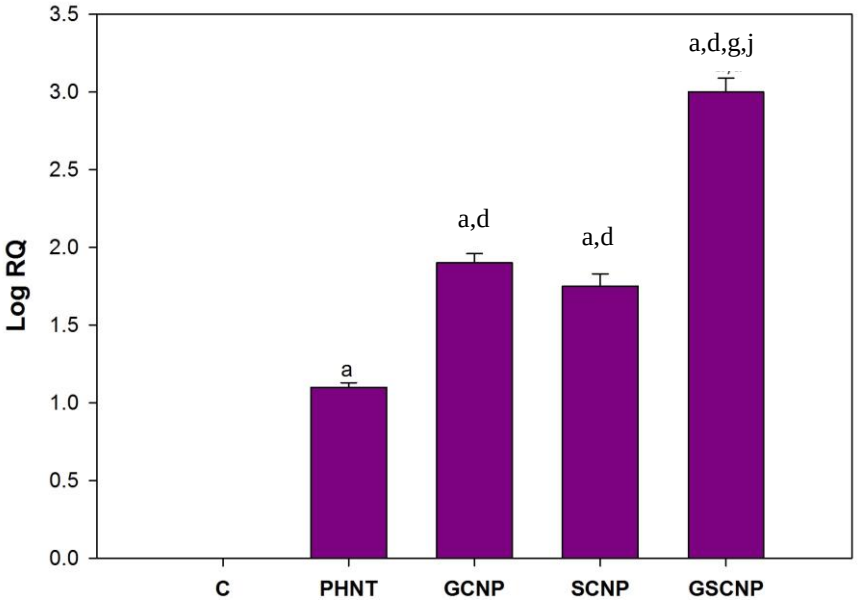



**Table- 42**
**Real Time PCR amplification of GDNF mRNA in the cerebral cortex of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $1.10 \pm 0.03^{a}$ |
| GCNP | $1.90 \pm 0.06^{a,d}$ |
| SCNP | $1.75 \pm 0.08^{a,d}$ |
| GSCNP | $3.00 \pm 0.09^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 56**
**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the brain stem of experimental rats**

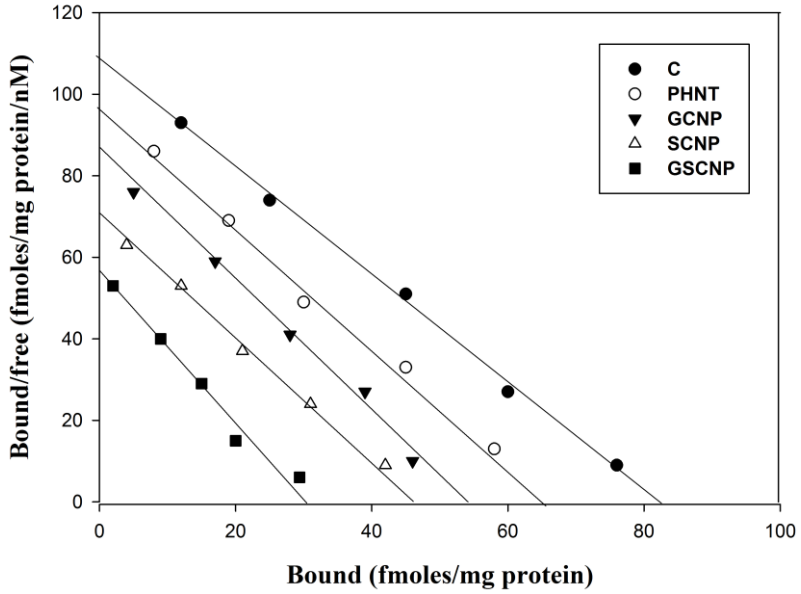



**Table- 43**
**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the brain stem of experimental rats**

| Experimental Groups | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
|---|---|---|
| C | 82.5 ± 1.4 | 1.3 ±0.11 |
| PHNT | 65.0 ± 2.8[a] | 1.4 ±0.05 |
| GCNP | 52.3 ± 2.3[a,e] | 1.6 ±0.11 |
| SCNP | 46.1 ± 2.3[a,d] | 1.5 ±0.08 |
| GSCNP | 30.3 ± 1.4[a,d,g,j] | 1.8 ±0.29 |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
$B_{max}$ – Maximal binding; $K_d$ – Dissociation constant
[a] $p<0.001$ when compared to C. [d] $p<0.001$, [e] $p<0.01$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure- 57**
**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the brain stem of experimental rats**

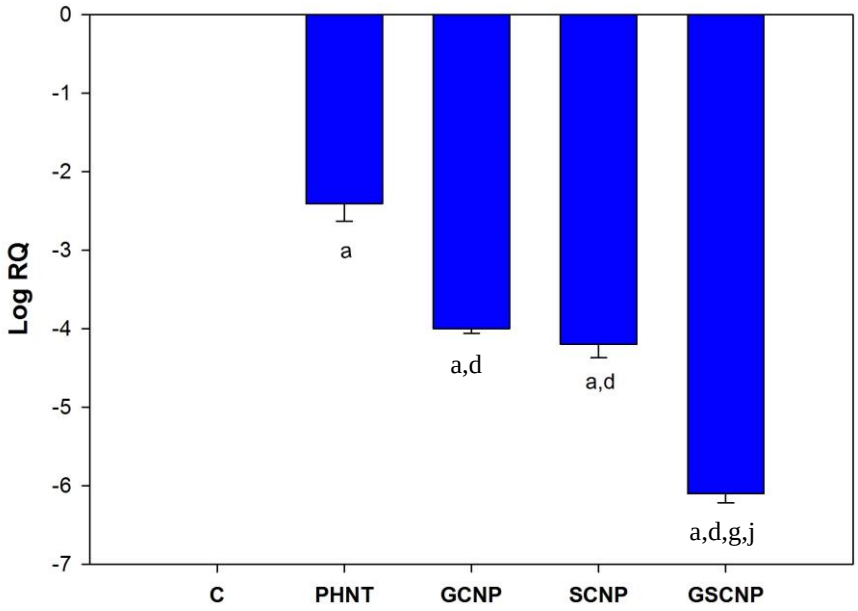


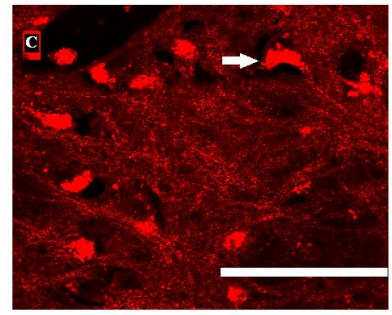

**Table- 44**
**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the brain stem of experimental rats**

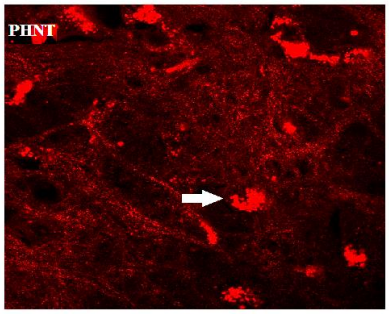
| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-2.4 \pm 0.20^{a}$ |
| GCNP | $-4.0 \pm 0.06^{a,d}$ |
| SCNP | $-4.2 \pm 0.17^{a,d}$ |
| GSCNP | $-6.1 \pm 0.12^{a,d,g,j}$ |

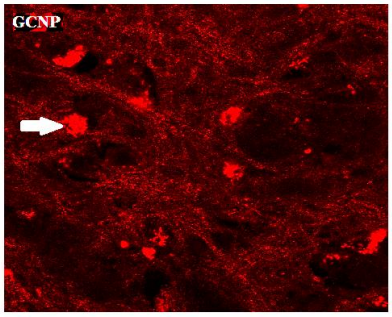
Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

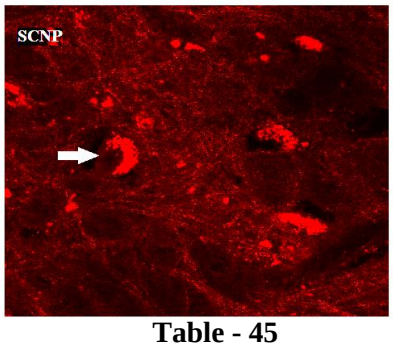
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure- 58**
**Confocal imaging of $GABA_B$ receptors in the brain stem of experimental rats**



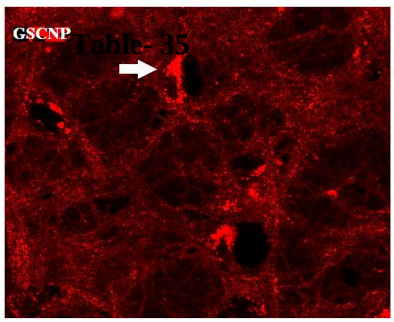




Table- 35

**Table - 45**

| Experimental groups | Mean pixel intensity |
|---|---|
| c | 52.0 ± 1.7 |
| PHNT | 45.6 ± 1.4[c] |
| GCNP | 32.6 ± 1.4 [a,d] |
| SCNP | 33.7 ± 1.4 [a,d] |
| GSCNP | 25.3 ± 1.1 [a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment. Scale bar represents 50µm

**Figure - 59**
**Scatchard analysis of [$^3$H] ketanserin binding against ketanserin to 5-HT$_{2A}$ receptors in the brain stem of experimental rats**

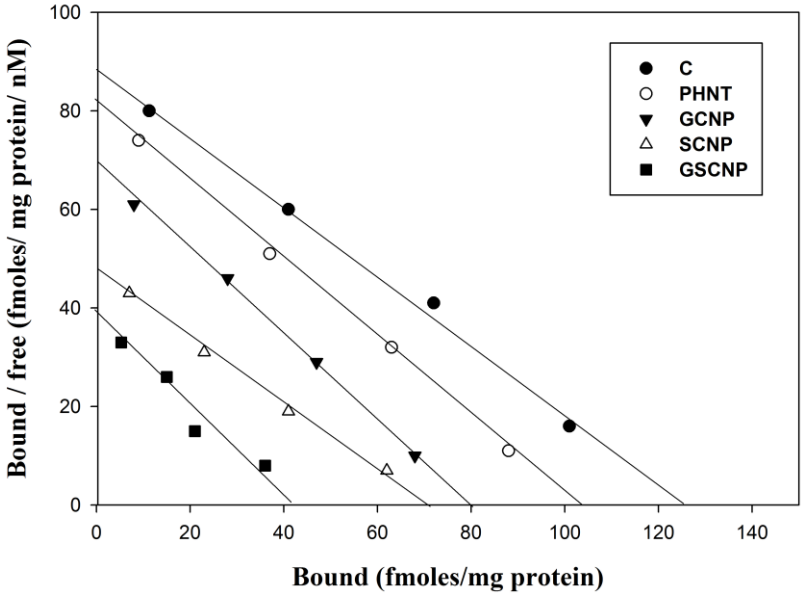



**Table- 46**
**Scatchard analysis of [$^3$H] ketanserin binding against ketanserin to 5-HT$_{2A}$ in the brain stem of experimental rats**

| Experimental Groups | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
|---|---|---|
| C | 125.3 ± 1.4 | 1.3 ± 0.10 |
| PHNT | 103.0 ± 1.1[a] | 1.2 ± 0.13 |
| GCNP | 79.0 ± 0.9[a,d] | 1.1 ± 0.11 |
| SCNP | 70.5 ± 1.0[a,d] | 1.2 ± 0.08 |
| GSCNP | 41.1± 1.1[a,d,g,j] | 1.0 ± 0.06 |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
$B_{max}$ – Maximal binding; $K_d$ – Dissociation constant
[a] $p<0.001$ when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure- 60**
**Real Time PCR amplification of 5-$HT_{2A}$ receptor subunit mRNA in the brain stem of experimental rats**

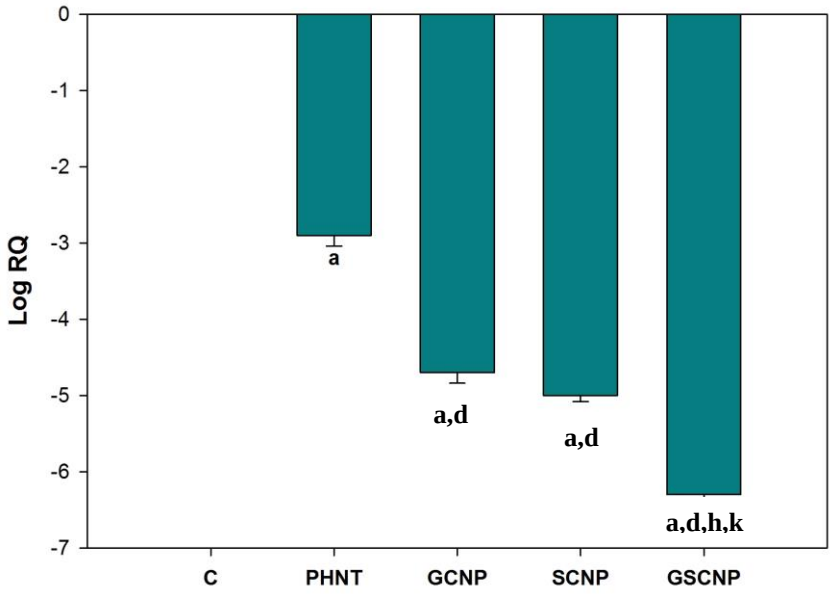



**Table- 47**
**Real Time PCR amplification of 5-$HT_{2A}$ receptor subunit mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-2.9 \pm 0.14^{a}$ |
| GCNP | $-4.7 \pm 0.14^{a,d}$ |
| SCNP | $-5.0 \pm 0.08^{a,d}$ |
| GSCNP | $-6.3 \pm 0.12^{a,d,h,k}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [h] $p<0.01$ when compared to GCNP. [k] $p<0.01$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure 61**
**Confocal imaging of 5-HT$_{2A}$ receptors in the brain stem of experimental rats**

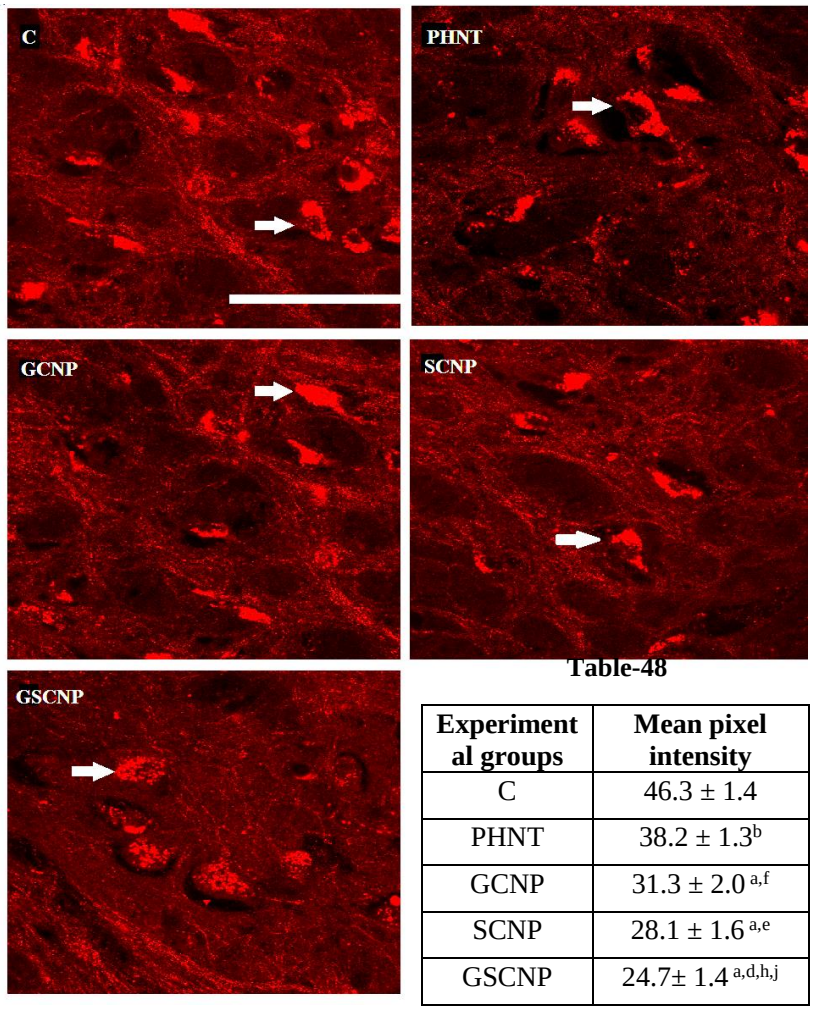
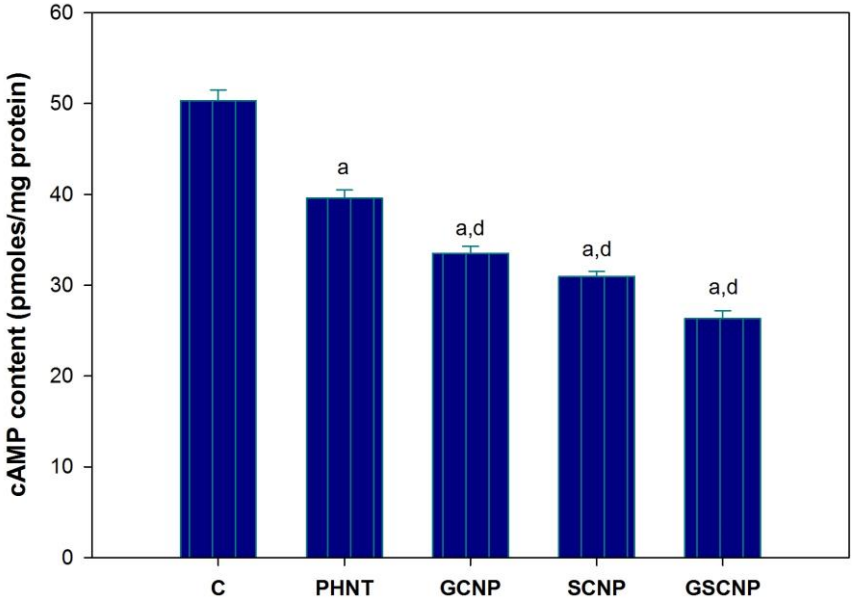



**Table-48**

| Experimental groups | Mean pixel intensity |
|---|---|
| C | 46.3 ± 1.4 |
| PHNT | 38.2 ± 1.3[b] |
| GCNP | 31.3 ± 2.0 [a,f] |
| SCNP | 28.1 ± 1.6 [a,e] |
| GSCNP | 24.7± 1.4 [a,d,h,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] p<0.001, [c] p<0.05 when compared to C. [d] p<0.001, [e] p<0.01, [f] p<0.05when compared to PHNT. [h] p<0.01 when compared to GCNP. [j] p<0.001 when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment. Scale bar represents 50µm

**Figure - 62**
**cAMP content in the brain stem of experimental rats**




Values are Mean ± S.E.M. of 4-6 separate experiments. Each group consists of *4-6* rats.
[a] $p<0.001$ with respect to C. [d] $p<0.001$ with respect to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP – Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP - Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment

**Figure- 63**
**Real Time PCR amplification of CREB mRNA in the brain stem of experimental rats**

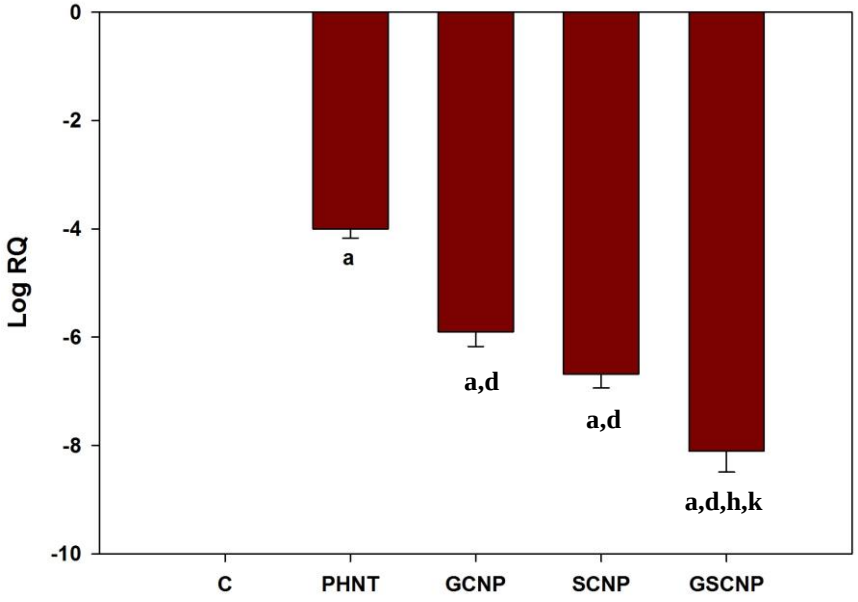


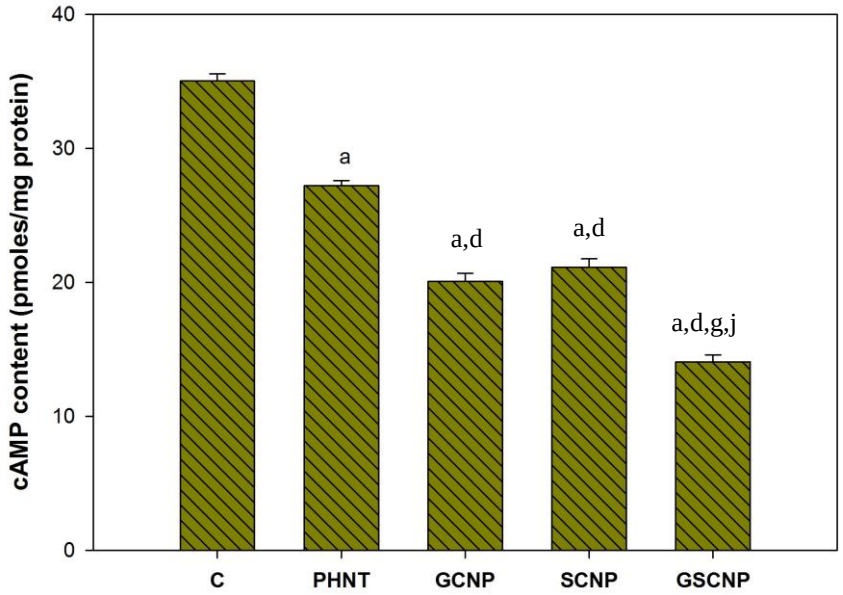

**Table- 49**
**Real Time PCR amplification of CREB mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-2.9 \pm 0.14^{a}$ |
| GCNP | $-4.7 \pm 0.14^{a,d}$ |
| SCNP | $-5.0 \pm 0.08^{a,d}$ |
| GSCNP | $-6.3 \pm 0.12^{a,d,h,k}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [h] $p<0.01$ when compared to GCNP. [k] $p<0.01$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment

**Figure- 64**
**$IP_3$ content in the brain stem of experimental rats**




Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment

**Figure- 65**
**Real Time PCR amplification of phospholipase C mRNA in the brain stem of experimental rats**

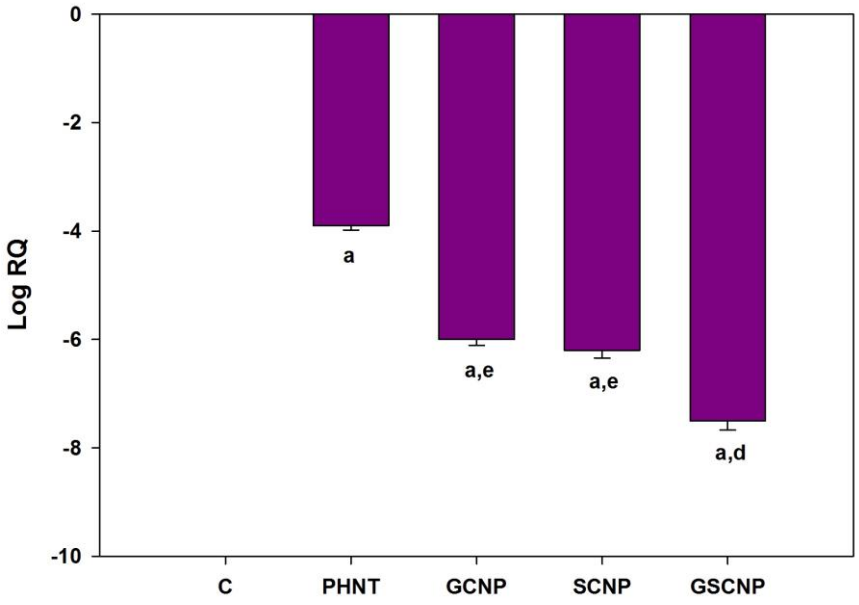


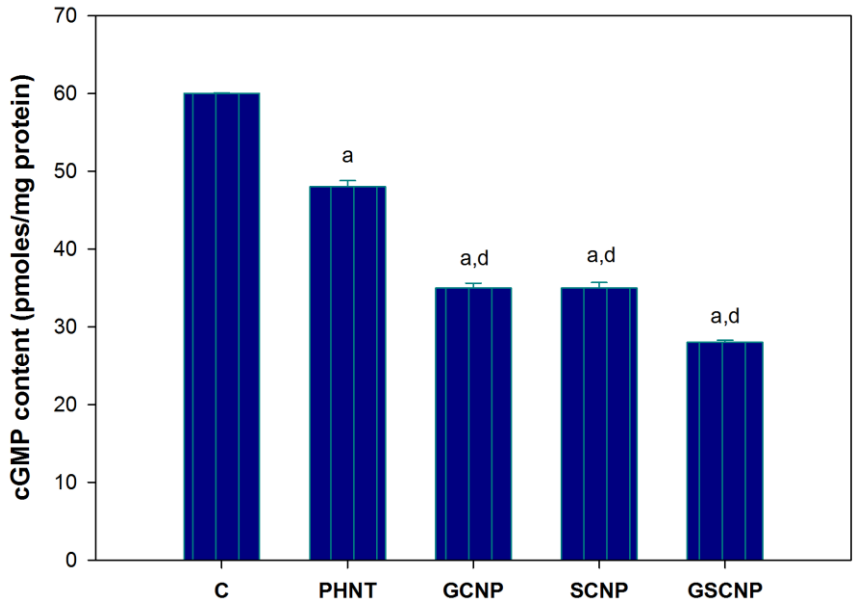

**Table- 50**
**Real Time PCR amplification of phospholipase C mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-3.9 \pm 0.08^{a}$ |
| GCNP | $-6.0 \pm 0.11^{a,e}$ |
| SCNP | $-6.2 \pm 0.14^{a,e}$ |
| GSCNP | $-7.5 \pm 0.17^{a,d}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared to C. [d] $p<0.001$, [e] $p<0.01$ when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure – 66**
**cGMP content in the brain stem of experimental rats**




Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 67**
**Real Time PCR amplification of NF-κB mRNA in the brain stem of experimental rats**

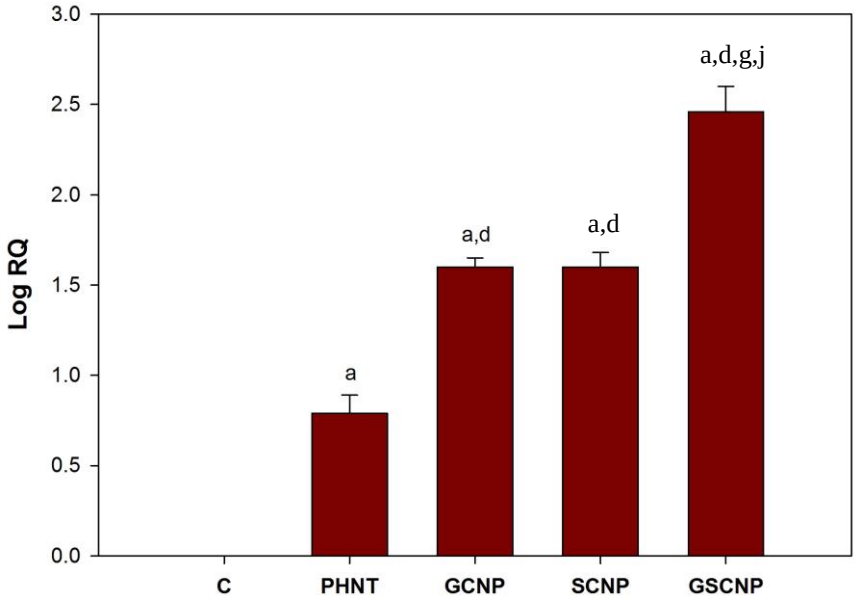



**Table -51**
**Real Time PCR amplification of NF-κB mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $0.7 \pm 0.08^{a}$ |
| GCNP | $1.6 \pm 0.08^{a,d}$ |
| SCNP | $1.6 \pm 0.09^{a,d}$ |
| GSCNP | $2.4 \pm 0.05^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 68**
**Real Time PCR amplification of TNF-α mRNA in the brain stem of experimental rats**

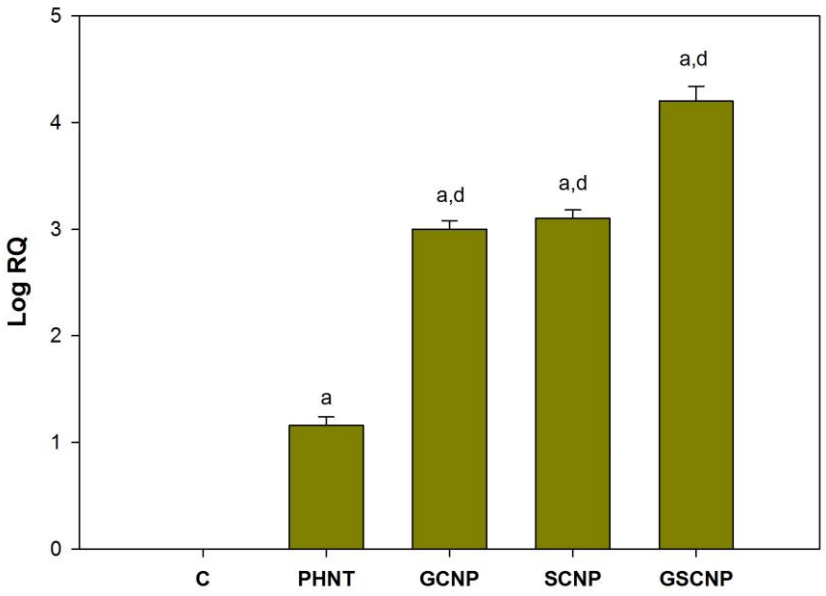



**Table - 52**
**Real Time PCR amplification of TNF-α mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $1.1 \pm 0.08^{a}$ |
| GCNP | $3.0 \pm 0.08^{a,d}$ |
| SCNP | $3.1 \pm 0.09^{a,d}$ |
| GSCNP | $4.2 \pm 0.05^{a,d}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 69**
**Real Time PCR amplification of Akt-1 mRNA in the brain stem of experimental rats**

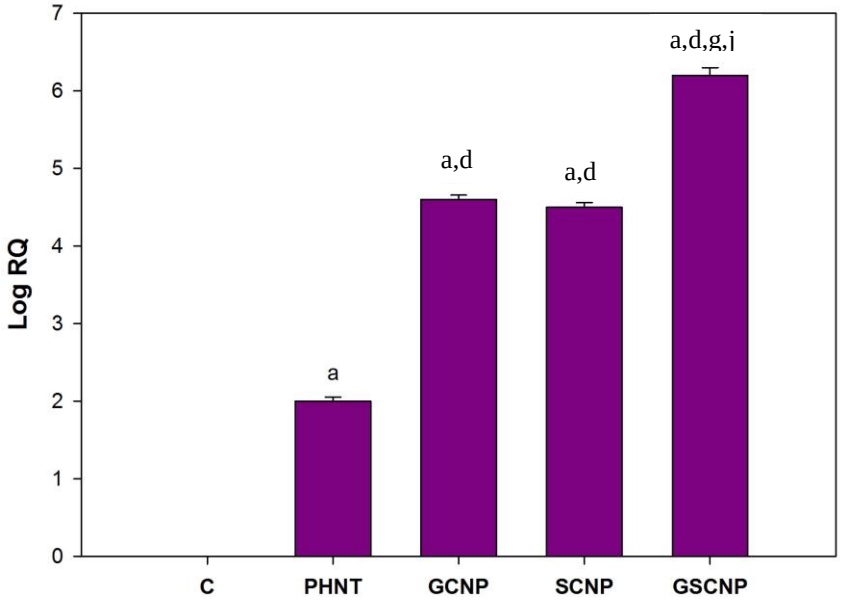



**Table - 53**
**Real Time PCR amplification of Akt-1 mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $2.0 \pm 0.05^{a}$ |
| GCNP | $4.6 \pm 0.05^{a,d}$ |
| SCNP | $4.5 \pm 0.05^{a,d}$ |
| GSCNP | $6.2 \pm 0.10^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure- 70**
**Real Time PCR amplification of Insulin like growth factor-1 mRNA in the brain stem of experimental rats**

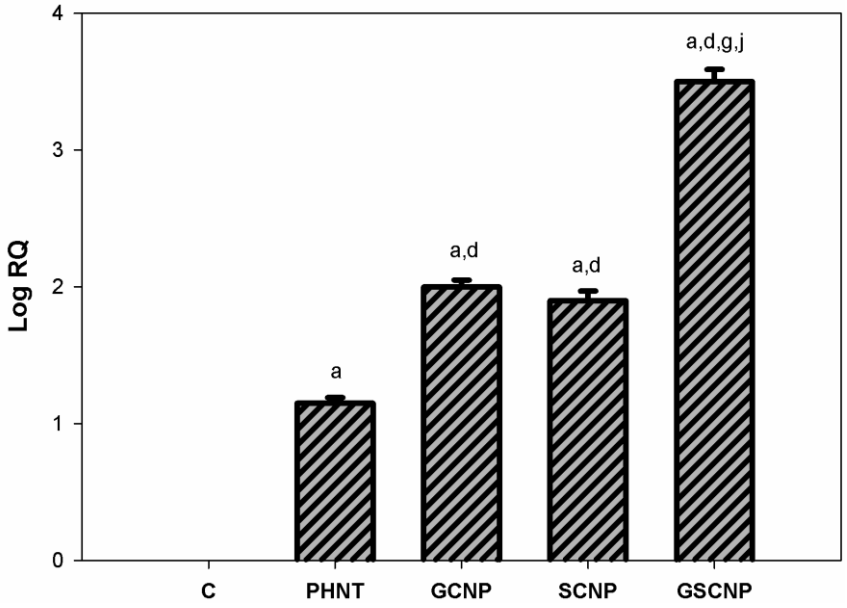



**Table- 54**
**Real Time PCR amplification of Insulin like growth factor-1 mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | 1.15 ± 0.04 |
| GCNP | 2.00 ± 0.05 |
| SCNP | 1.90 ± 0.07 |
| GSCNP | 3.50 ± 0.09 |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 71**
**Real Time PCR amplification of SOD mRNA in the brain stem of experimental rats**

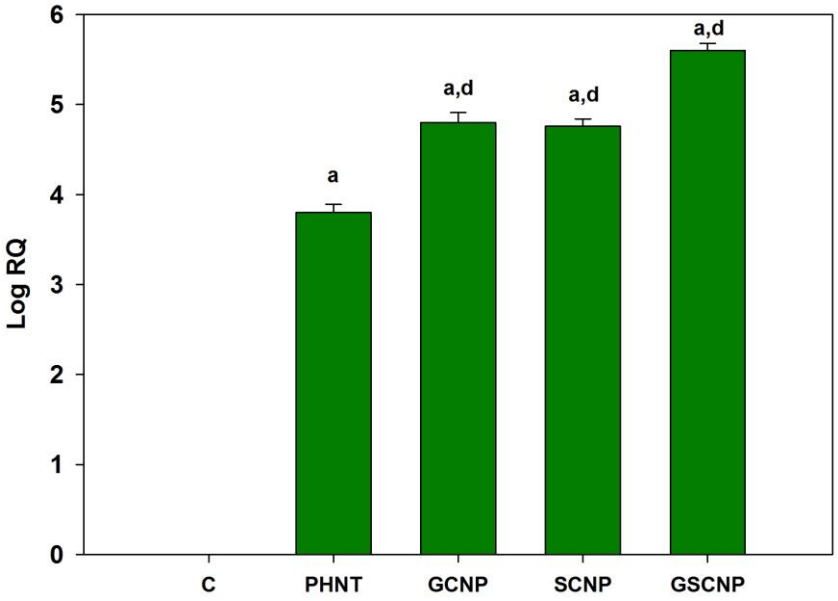



**Table - 55**
**Real Time PCR amplification of SOD mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $3.8 \pm 0.09^{a}$ |
| GCNP | $4.8 \pm 0.11^{a,d}$ |
| SCNP | $4.7 \pm 0.08^{a,d}$ |
| GSCNP | $5.6 \pm 0.08^{a,d}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 72**
**Real Time PCR amplification of Bax mRNA in the brain stem of experimental rats**

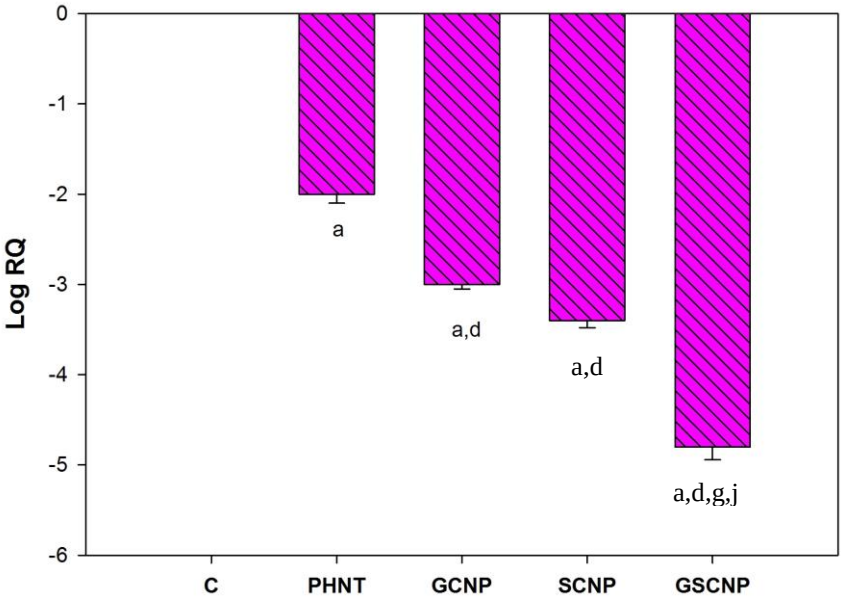



**Table - 56**
**Real Time PCR amplification of Bax mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-2.0 \pm 0.10^{a}$ |
| GCNP | $-3.0 \pm 0.05^{a,d}$ |
| SCNP | $-3.4 \pm 0.08^{a,d}$ |
| GSCNP | $-4.8 \pm 0.14^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 73**
**Real Time PCR amplification of caspase-8 mRNA in the brain stem of experimental rats**

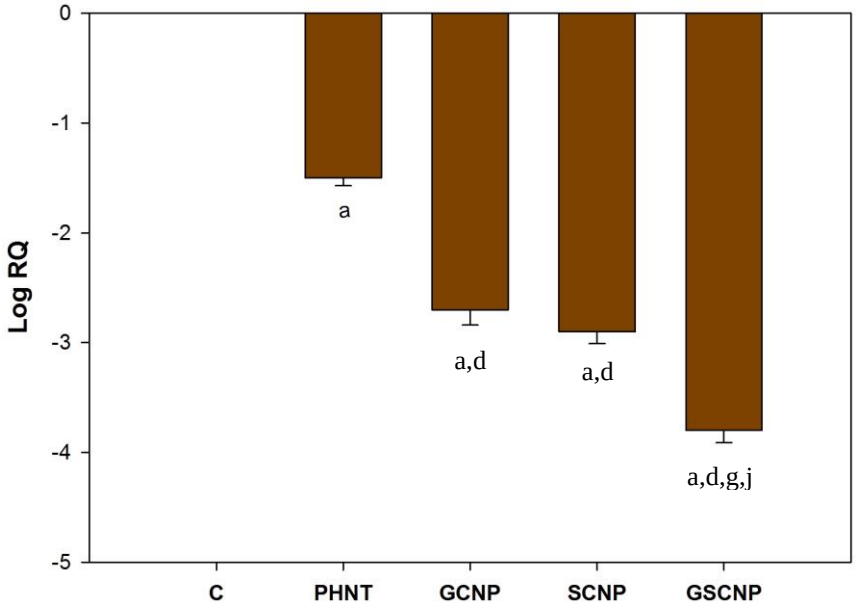



**Table- 57**
**Real Time PCR amplification of caspase-8 mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-1.5 \pm 0.07^{a}$ |
| GCNP | $-2.7 \pm 0.14^{a,d}$ |
| SCNP | $-2.9 \pm 0.11^{a,d}$ |
| GSCNP | $-3.8 \pm 0.12^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 74**
**Real Time PCR amplification of BDNF mRNA in the brain stem of experimental rats**

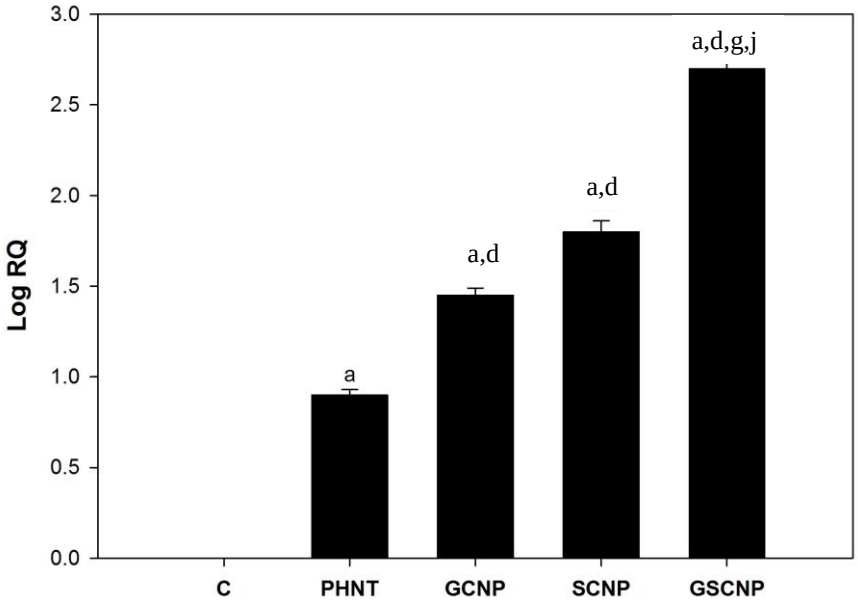



**Table- 58**
**Real Time PCR amplification of BDNF mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $0.90 \pm 0.03^{a}$ |
| GCNP | $1.45 \pm 0.04^{a,d}$ |
| SCNP | $1.80 \pm 0.06^{a,d}$ |
| GSCNP | $2.70 \pm 0.07^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 75**
**Real Time PCR amplification of GDNF mRNA in the brain stem of experimental rats**

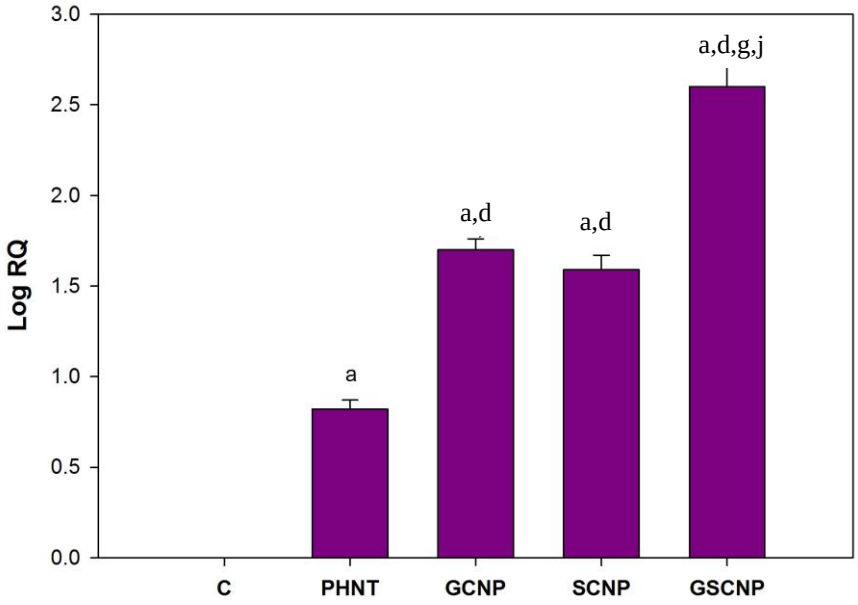



**Table- 59**

**Real Time PCR amplification of GDNF mRNA in the brain stem of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $0.82 \pm 0.05^{a}$ |
| GCNP | $1.70 \pm 0.06^{a,d}$ |
| SCNP | $1.59 \pm 0.08^{a,d}$ |
| GSCNP | $2.60 \pm 0.14^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 76**
**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the corpus striatum of experimental rats**

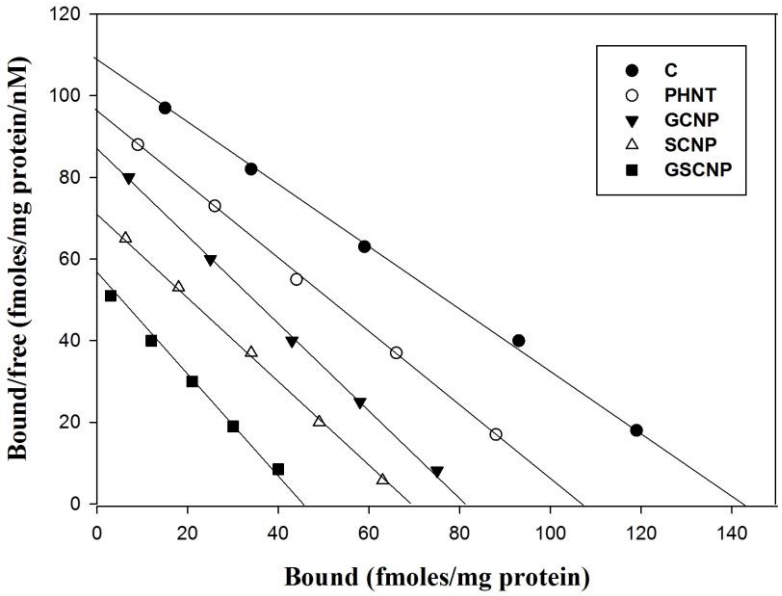



**Table- 60**
**Scatchard analysis of [$^3$H] baclofen binding against baclofen to $GABA_B$ receptors in the corpus striatum of experimental rats**

| Experimental Groups | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
|---|---|---|
| C | 141.7 ± 2.0 | 1.3 ±0.03 |
| PHNT | 106.6 ± 0.8[a] | 1.1 ±0.02 |
| GCNP | 81.8 ± 1.7[a,d] | 0.9 ±0.02 |
| SCNP | 69.6 ± 0.8[a,d,g] | 1.0 ±0.20 |
| GSCNP | 44.5 ± 1.7[a,d,g,j] | 0.7 ±0.11[c] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
$B_{max}$ – Maximal binding; $K_d$ – Dissociation constant
[a] $p<0.001$, [c] $p<0.05$ when compared to C. [d] $p<0.001$ when compared to PHNT.
[g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure- 77**
**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the corpus striatum of experimental rats**

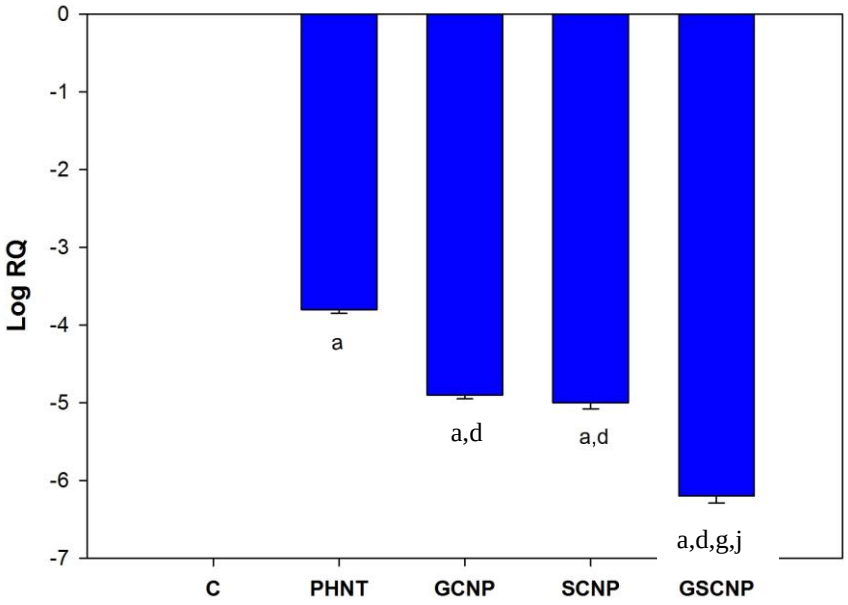



**Table- 61**
**Real Time PCR amplification of $GABA_B$ receptor subunit mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-3.8 \pm 0.05^{a}$ |
| GCNP | $-4.9 \pm 0.05^{a,d}$ |
| SCNP | $-5.0 \pm 0.08^{a,d}$ |
| GSCNP | $-6.2 \pm 0.09^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure-78**
**Confocal imaging of $GABA_B$ receptors in the corpus striatum of experimental rats**

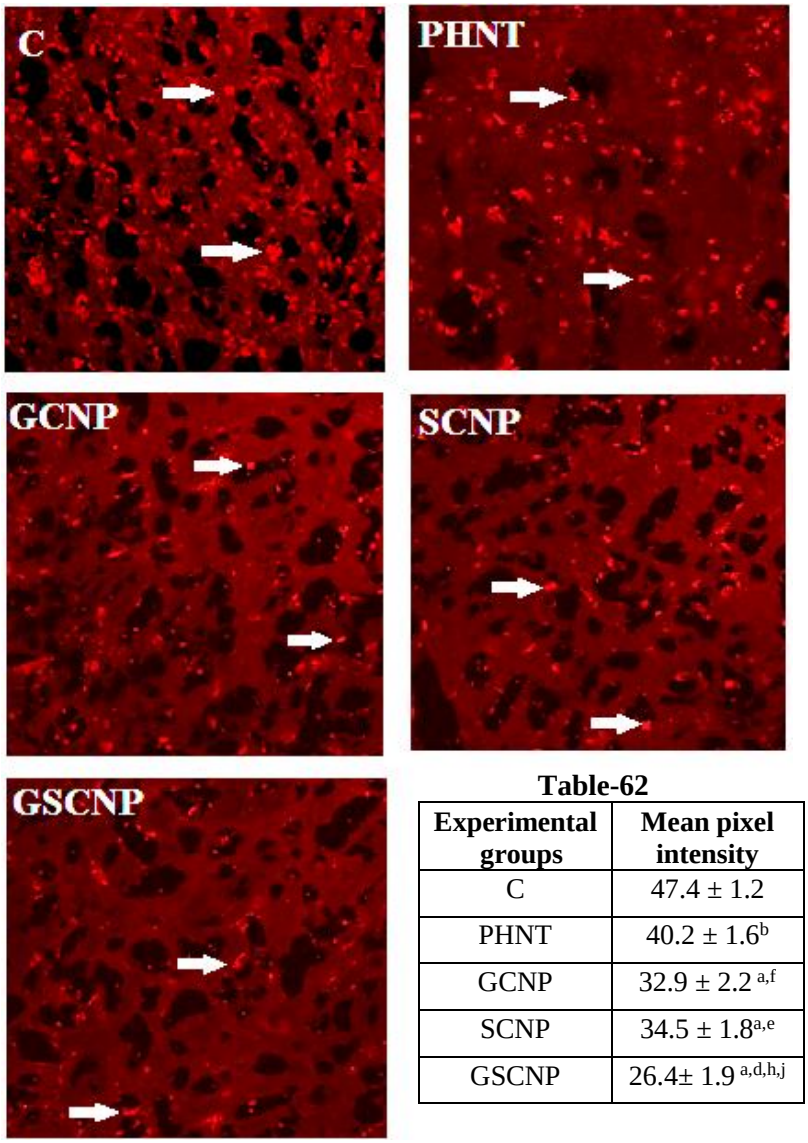



**Table-62**

| Experimental groups | Mean pixel intensity |
|---|---|
| C | 47.4 ± 1.2 |
| PHNT | 40.2 ± 1.6[b] |
| GCNP | 32.9 ± 2.2 [a,f] |
| SCNP | 34.5 ± 1.8[a,e] |
| GSCNP | 26.4± 1.9 [a,d,h,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] p<0.001when compared to C. [d] p<0.001when compared to PHNT. [h] p<0.01 when compared to GCNP. [j] p<0.001 when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment. Scale bar represents 50µm

**Figure - 79**
**Scatchard analysis of [$^3$H] ketanserin binding against ketanserin to 5-HT$_{2A}$ receptors in the corpus striatum of experimental rats**

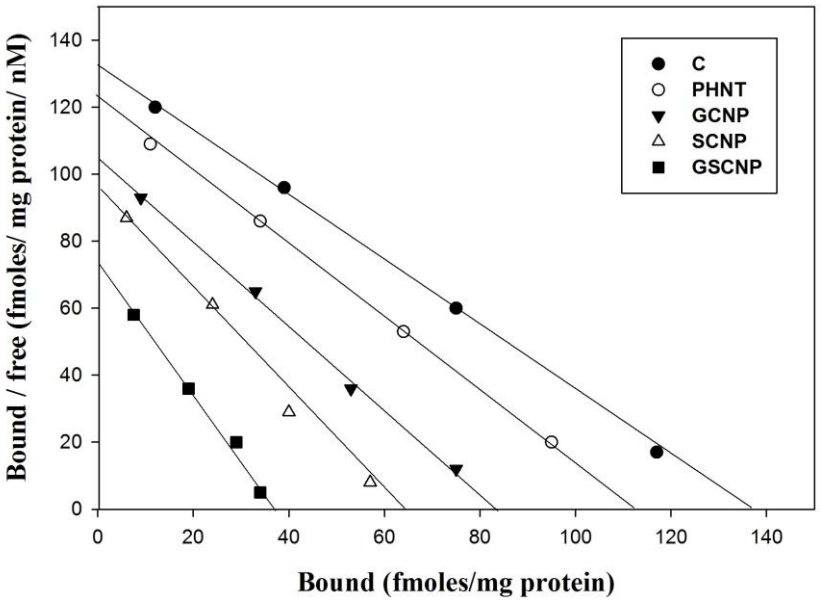



**Table- 63**
**Scatchard analysis of [$^3$H] ketanserin binding against ketanserin to 5-HT$_{2A}$ in the corpus striatum of experimental rats**

| Experimental Groups | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
|---|---|---|
| C | 136.3 ± 0.8 | 1.1 ± 0.04 |
| PHNT | 111.6 ± 0.8[a] | 0.9 ± 0.05[c] |
| GCNP | 83.4 ± 0.8[a,d] | 0.8 ± 0.05[b] |
| SCNP | 63.9 ± 0.6[a,d,g] | 0.6 ± 0.05[a,e] |
| GSCNP | 36.2 ± 0.6[a,d,g,j] | 0.5 ± 0.05[a,e,h] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
$B_{max}$ – Maximal binding; $K_d$ – Dissociation constant
[a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to C. [d] $p<0.001$, [e] $p<0.01$ when compared to PHNT. [g] $p<0.001$, [h] $p<0.01$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and SCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure- 80**
**Real Time PCR amplification of 5-$HT_{2A}$ receptor subunit mRNA in the corpus striatum of experimental rats**

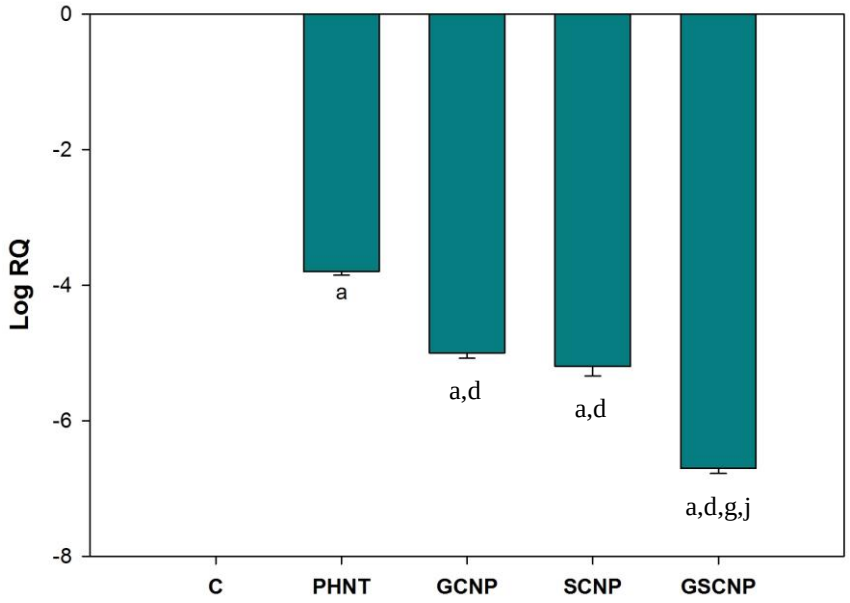



**Table- 64**
**Real Time PCR amplification of 5-$HT_{2A}$ receptor subunit mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-3.6 \pm 0.05^{a}$ |
| GCNP | $-5.0 \pm 0.08^{a,d}$ |
| SCNP | $-5.2 \pm 0.14^{a,d}$ |
| GSCNP | $-6.7 \pm 0.08^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure-81**
**Confocal imaging of 5-$HT_{2A}$ receptors in the corpus striatumof experimental rats**

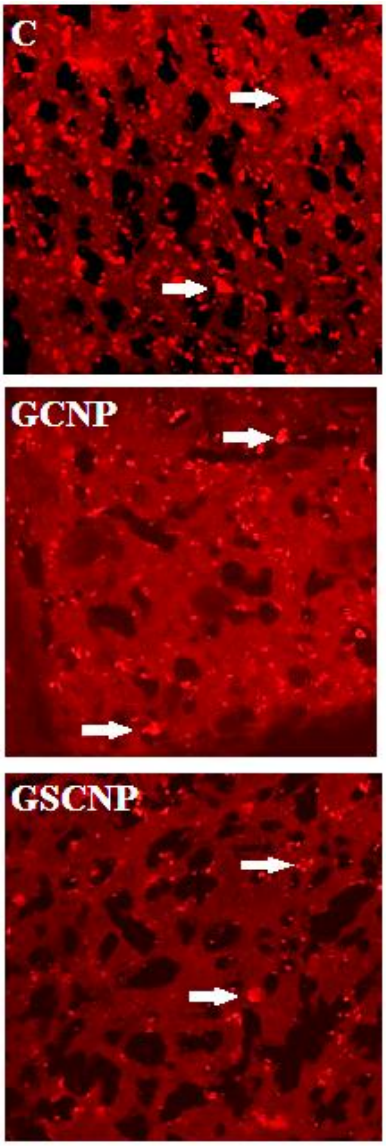
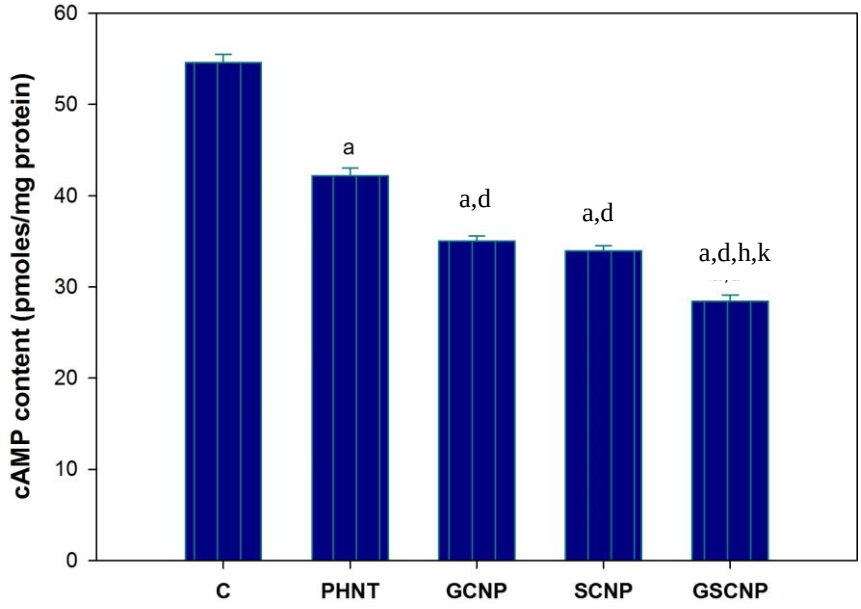



**Table-65**

| Experimental groups | Mean pixel intensity |
|---|---|
| C | 47.4 ± 1.2 |
| PHNT | 40.2 ± 1.6[b] |
| GCNP | 32.9 ± 2.2 [a,f] |
| SCNP | 34.5 ± 1.8[a,e] |
| GSCNP | 26.4± 1.9 [a,d,h,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] p<0.001when compared to C. [d] p<0.001when compared to PHNT. [h] p<0.01 when compared to GCNP. [j] p<0.001 when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment. Scale bar represents 50µm

**Figure - 82**
**cAMP content in the corpus striatum of experimental rats**




Values are Mean ± S.E.M. of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ with respect to C. [d] $p<0.001$ with respect to PHNT. [h] $p<0.01$when compared to GCNP. [k] $p<0.01$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP – Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP - Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment

**Figure- 83**
**Real Time PCR amplification of CREB mRNA in the corpus striatum of experimental rats**

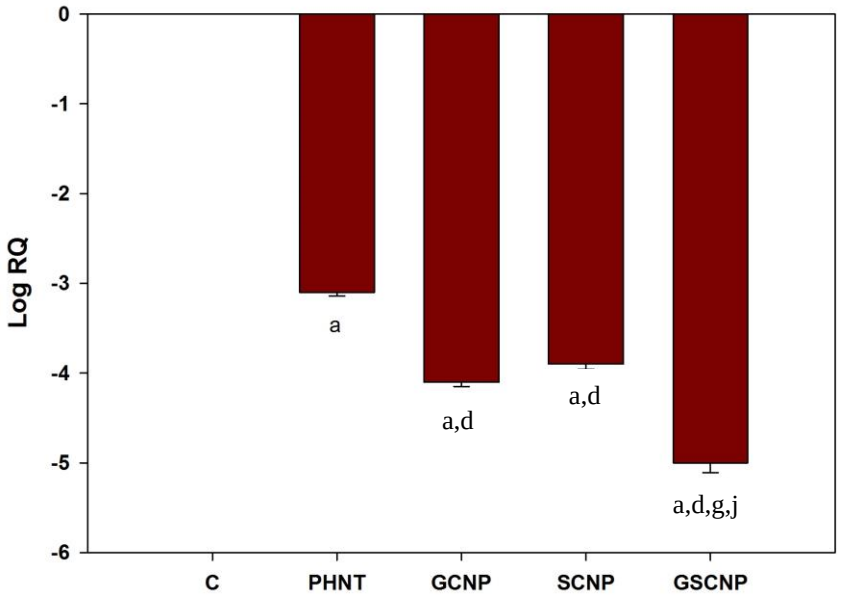


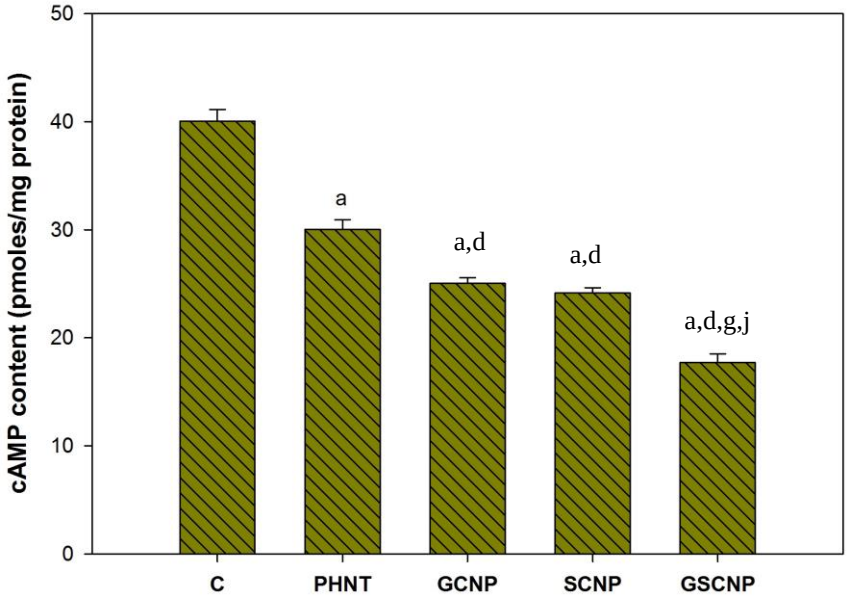

**Table- 66**
**Real Time PCR amplification of CREB mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-3.1 \pm 0.04^{a}$ |
| GCNP | $-4.1 \pm 0.05^{a,d}$ |
| SCNP | $-3.9 \pm 0.06^{a,d}$ |
| GSCNP | $-5.0 \pm 0.11^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 84**
**$IP_3$ content in the corpus striatum of experimental rats**




Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] p<0.001when compared to C. [d] p<0.001when compared to PHNT. [g] p<0.001when compared to GCNP. [j] p<0.001 when compared to SCNP.
C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure- 85**
**Real Time PCR amplification of phospholipase C mRNA in the corpus striatum of experimental rats**

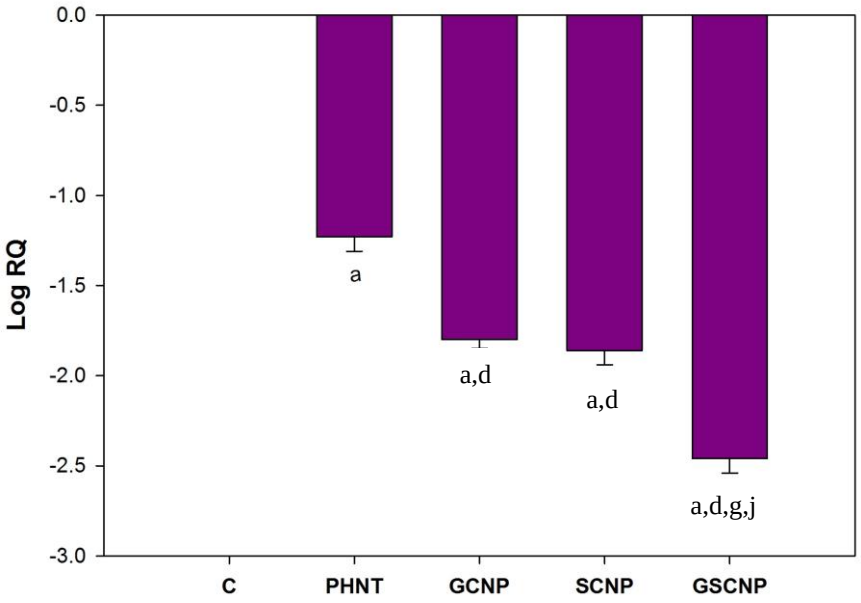


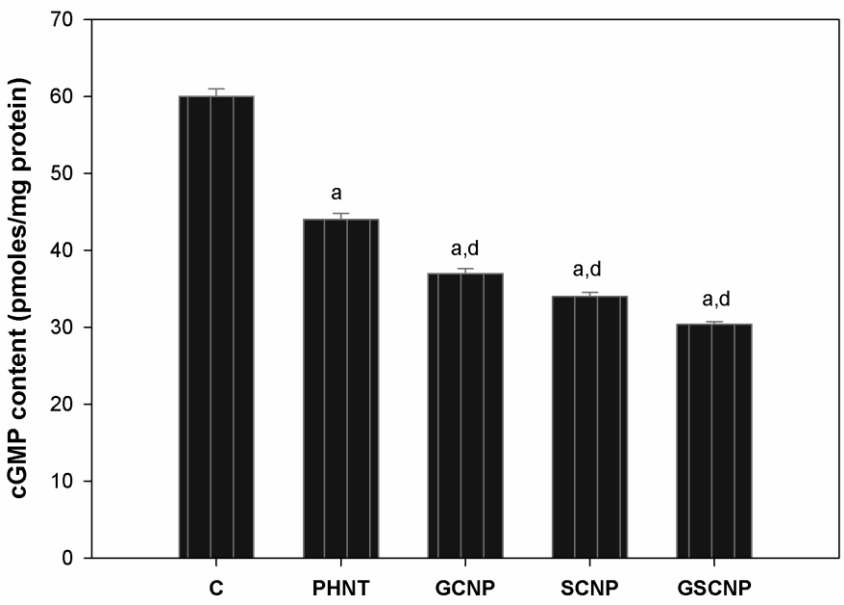

**Table- 67**
**Real Time PCR amplification of phospholipase C mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | -1.23 ± 0.08[a] |
| GCNP | -1.80 ± 0.05[a,d] |
| SCNP | -1.86 ± 0.08[a,d] |
| GSCNP | -2.46 ± 0.09[a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure – 86**
**cGMP content in the corpus striatum of experimental rats**




Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure -87**
**Real Time PCR amplification of NF-κB mRNA in the corpus striatum of experimental rats**

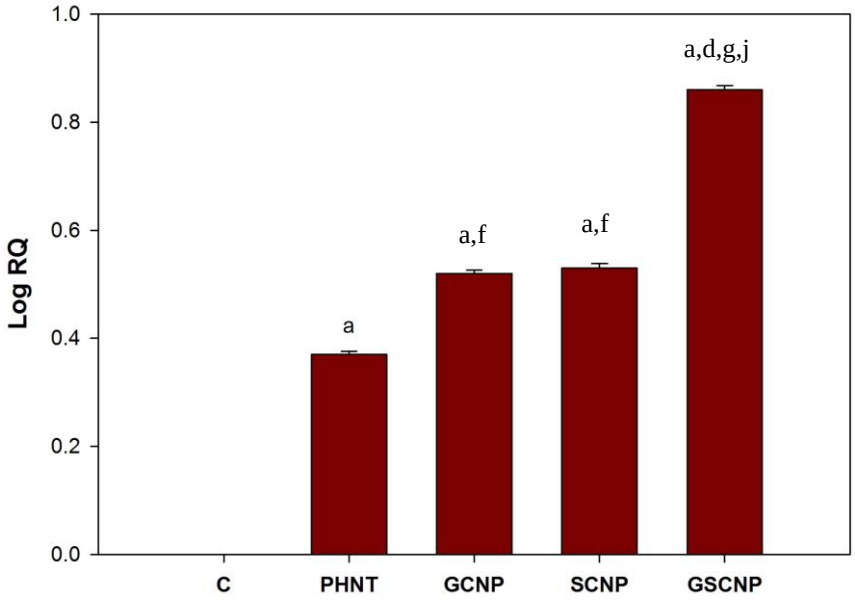



**Table- 68**
**Real Time PCR amplification of NF-κB mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $0.37 \pm 0.006^{a}$ |
| GCNP | $0.52 \pm 0.006^{a,f}$ |
| SCNP | $0.53 \pm 0.008^{a,f}$ |
| GSCNP | $0.86 \pm 0.008^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$, [f] $p<0.05$ when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 88**
**Real Time PCR amplification of TNF-α mRNA in the corpus striatum of experimental rats**

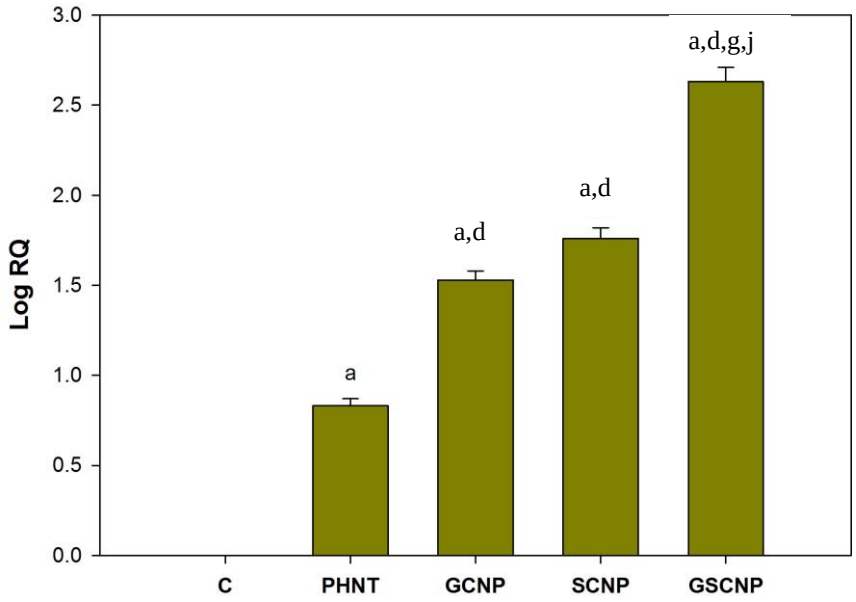



**Table- 69**
**Real Time PCR amplification of TNF-α mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $0.83 \pm 0.04^{a}$ |
| GCNP | $1.53 \pm 0.05^{a,d}$ |
| SCNP | $1.76 \pm 0.06^{a,d}$ |
| GSCNP | $2.63 \pm 0.08^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 89**
**Real Time PCR amplification of Akt-1 mRNA in the corpus striatum of experimental rats**

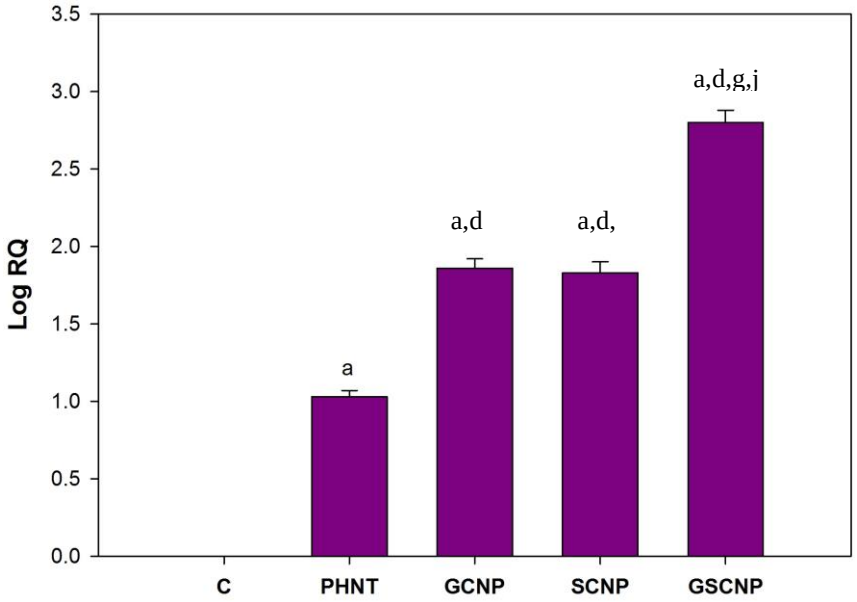



**Table- 70**
**Real Time PCR amplification of Akt-1 mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $1.03 \pm 0.04^{a}$ |
| GCNP | $1.86 \pm 0.06^{a,d}$ |
| SCNP | $1.83 \pm 0.07^{a,d}$ |
| GSCNP | $2.33 \pm 0.08^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure- 90**
**Real Time PCR amplification of Insulin like growth factor-1 mRNA in the corpus striatum of experimental rats**

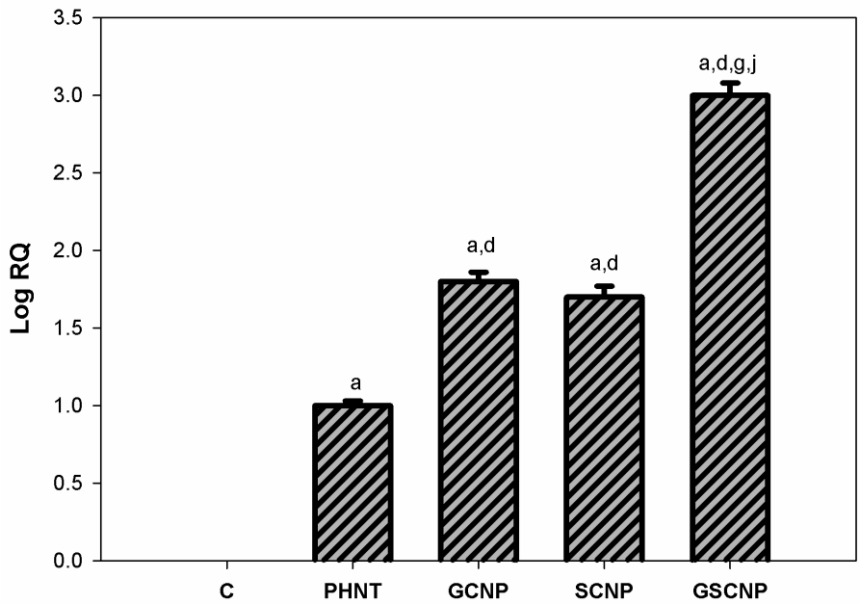



**Table- 71**
**Real Time PCR amplification of Insulin like growth factor-1 mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $1.0 \pm 0.02^{a}$ |
| GCNP | $1.8 \pm 0.07^{a,d}$ |
| SCNP | $1.7 \pm 0.07^{a,d}$ |
| GSCNP | $3.0 \pm 0.1^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT. [g] $p<0.001$ when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure- 91**
**Real Time PCR amplification of SOD mRNA in the corpus striatum of experimental rats**

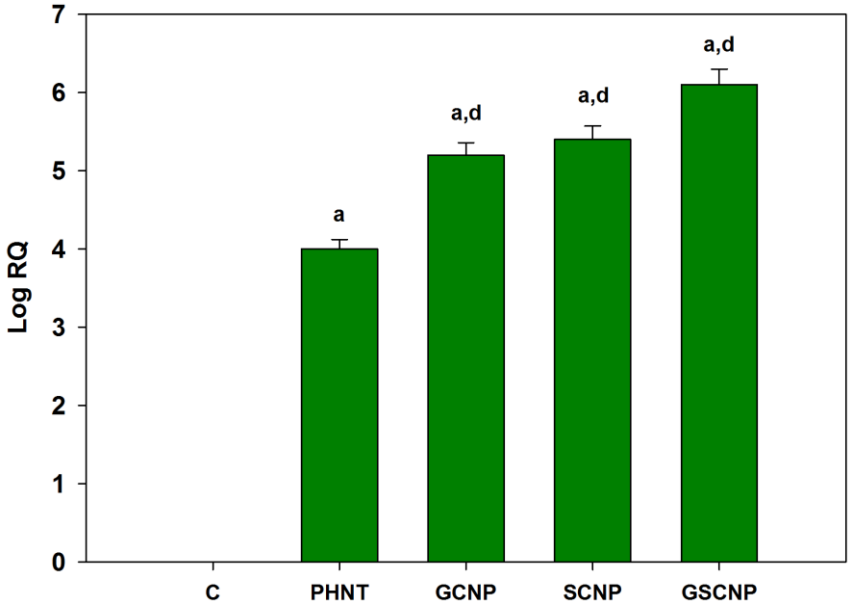



**Table- 72**
**Real Time PCR amplification of SOD mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $4.0 \pm 0.12^{a}$ |
| GCNP | $5.2 \pm 0.16^{a,d}$ |
| SCNP | $5.4 \pm 0.17^{a,d}$ |
| GSCNP | $6.1 \pm 0.20^{a,d}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$ when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 92**
**Real Time PCR amplification of Bax mRNA in the corpus striatum of experimental rats**

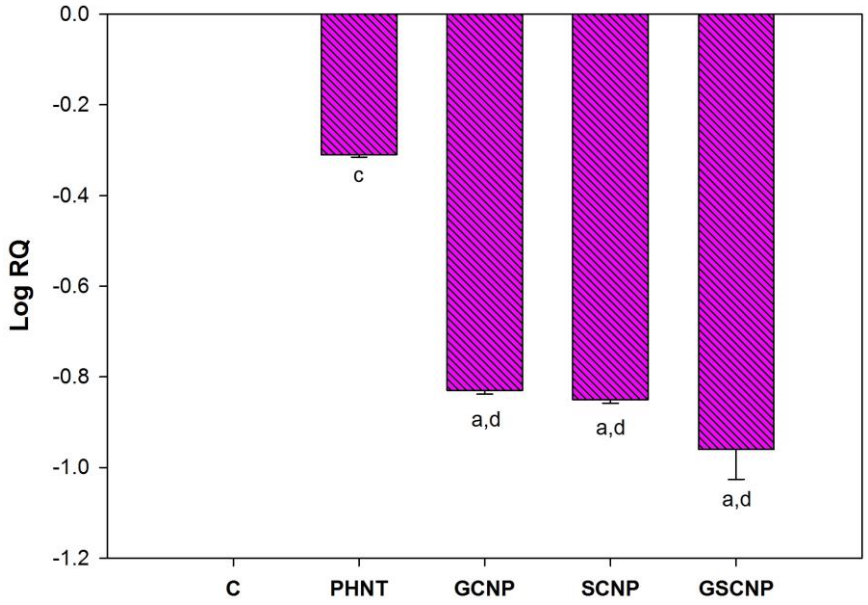



**Table- 73**
**Real Time PCR amplification of Bax mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | -0.30 ± 0.005[a] |
| GCNP | -0.83 ± 0.006[a,d] |
| SCNP | -0.85 ± 0.006[a,d] |
| GSCNP | -0.96 ± 0.009[a,d] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 93**
**Real Time PCR amplification of caspase-8 mRNA in the corpus striatum of experimental rats**

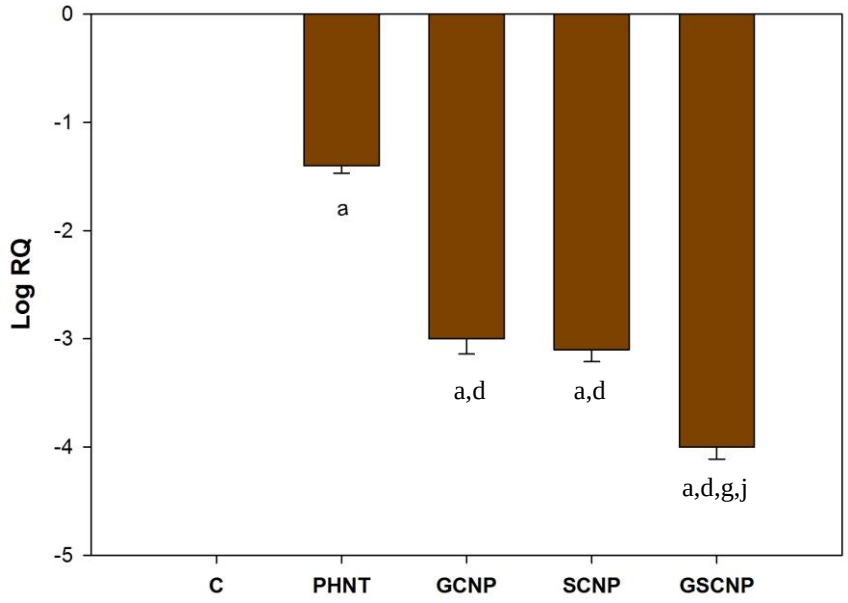



**Table - 74**
**Real Time PCR amplification of caspase-8 mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $-1.4 \pm 0.07^{a}$ |
| GCNP | $-3.0 \pm 0.14^{a,d}$ |
| SCNP | $-3.1 \pm 0.11^{a,d}$ |
| GSCNP | $-4.0 \pm 0.11^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 94**
**Real Time PCR amplification of BDNF mRNA in the corpus striatum of experimental rats**

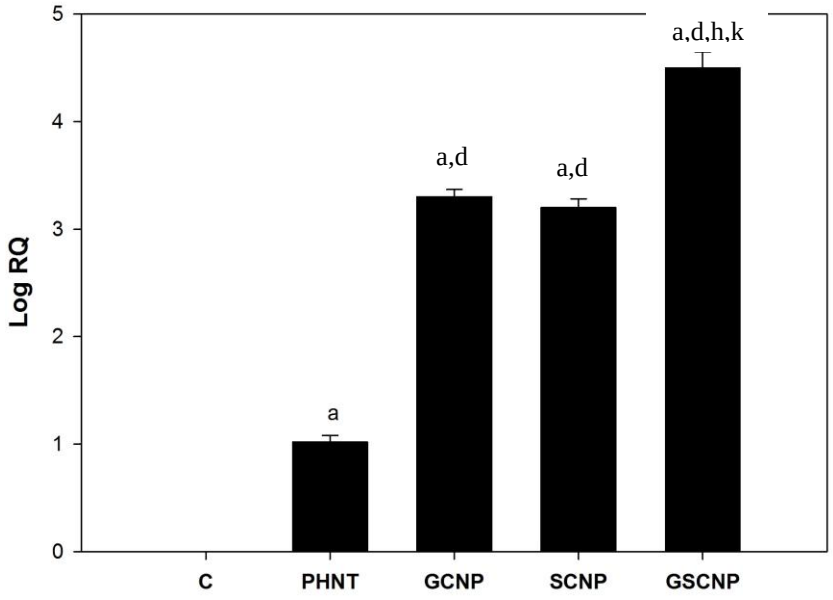



**Table- 75**
**Real Time PCR amplification of BDNF mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $1.02 \pm 0.06^{a}$ |
| GCNP | $3.30 \pm 0.07^{a,d}$ |
| SCNP | $3.20 \pm 0.08^{a,d}$ |
| GSCNP | $4.50 \pm 0.16^{a,d,h,k}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [h] $p<0.01$when compared to GCNP. [k] $p<0.01$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure - 95**
**Real Time PCR amplification of GDNF mRNA in the corpus striatum of experimental rats**

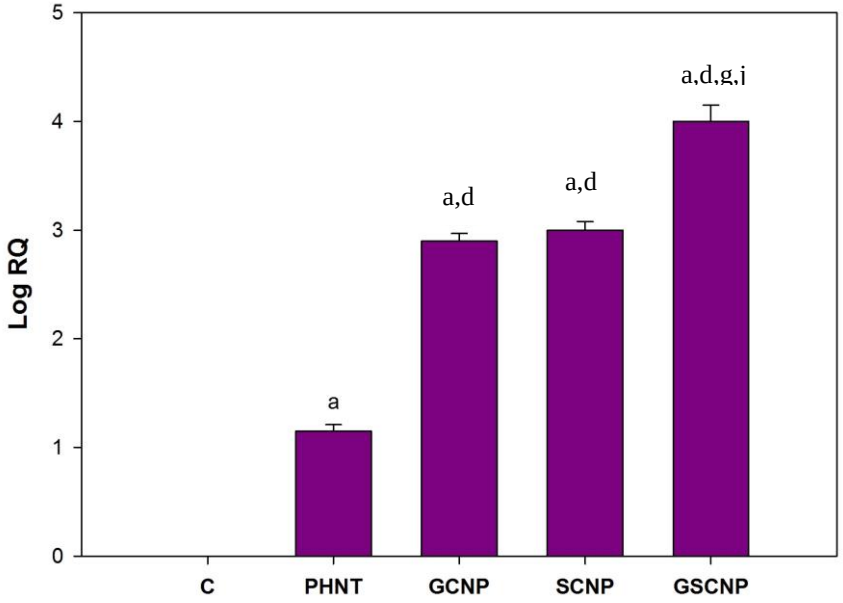



**Table- 76**
**Real Time PCR amplification of GDNF mRNA in the corpus striatum of experimental rats**

| Experimental Groups | Log RQ |
|---|---|
| C | 0 |
| PHNT | $1.15 \pm 0.06^{a}$ |
| GCNP | $2.90 \pm 0.07^{a,d}$ |
| SCNP | $3.00 \pm 0.08^{a,d}$ |
| GSCNP | $4.00 \pm 0.15^{a,d,g,j}$ |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Table- 77**
**Time spent by experimental rats on metallic rod in rotarod experiment**

| Experimental Groups | Retention time on the rod (in seconds) | | |
|---|---|---|---|
| | **10 rpm** | **15 rpm** | **25 rpm** |
| **C** | 300.0 ± 0.0 | 300.0 ± 0.0 | 182.3 ± 1.4 |
| **PHNT** | 253.6 ± 1.4[a] | 121 ± 2.0[a] | 55.6 ± 2.0[a] |
| **GCNP** | 280.6 ± 1.2[a,d] | 178.6 ± 2.0[a,d] | 91.0 ± 1.7[a,d] |
| **SCNP** | 283.3 ± 2.0[a,d] | 184.3 ± 2.6[a,d] | 87.6 ± 1.4[a,d] |
| **GSCNP** | 286.3 ± 1.4[a,d,i] | 200.3 ± 2.0[a,d,g,j] | 112.0 ± 1.5[a,d,g,j] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared to C. [d] $p<0.001$when compared to PHNT. [g] $p<0.001$, [i] $p<0.05$when compared to GCNP. [j] $p<0.001$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

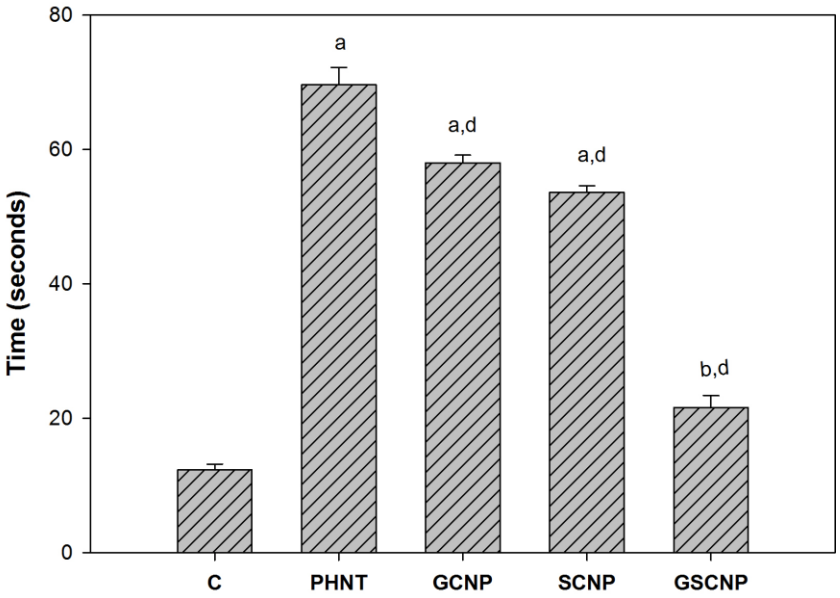
**Table- 78**
**Behavioural response of control and experimental rats on grid walk test**

| Experimental Groups | Foot slips/3minutes |
|---|---|
| **C** | 24.0 ± 1.1 |
| **PHNT** | 45.6 ± 0.8[a] |
| **GCNP** | 35.3 ± 0.8[a,d] |
| **SCNP** | 37.6 ± 1.4[a,d] |
| **GSCNP** | 29.6 ± 1.4[a,d,h,k] |

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$with respect to C. [d] $p<0.001$ with respect to PHNT. [h] $p<0.01$ when compared to GCNP. [k] $p<0.01$ when compared to SCNP.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP– Partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP – Partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP- Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

**Figure- 96**
**Time taken by experimental animals in narrow beam walk test**




Values are Mean ± S.E.M. of 5 separate experiments. Each group consists of 5 rats.

[a] $p<0.001$, [b] $p<0.01$ when compared to C. [d] $p<0.001$when compared to PHNT.

C – Sham operated control, PHNT – Partially hepatectomised group with no treatment, GCNP – Partially hepatectomised group treated with GABA chitosan nanoparticle, SCNP - Partially hepatectomised group treated with 5-HT chitosan nanoparticle and GSCNP- Partially hepatectomised group treated with GABA and 5-HT chitosan nanoparticle.

# *Discussion*

Liver is a vital organ and has a wide range of functions, which include synthesis, storage, detoxification, metabolism and redistribution of amino acids, carbohydrates, fats, vitamins and proteins. Damage to liver occurs by over consumption of alcohol, drugs with analgesic and antipyretic action, attack of parasites like *Entamoeba histolytica* and hepatotoxic chemicals. Patients often develop signs of liver dysfunction in the immediate postoperative period as a result of reduced liver mass, but normal liver function resumes once the removed liver mass is restored but not completely in advanced liver disease. The presence of various ligands in the initiation, propagation and termination of the mitotic stimulus, such as priming factors, co-mitogens, growth factors and their suppressors, is necessary for the successful and complete restoration of hepatic mass. Prolonged liver dysfunction, such as liver cirrhosis leading to hepatocyte damage, can harm the brain, leading to a serious and potentially fatal brain disorders. The liver has a remarkable capacity to regenerate after cellular damage or tissue removal. Liver regeneration is mostly the result of increased mitosis of hepatocytes.

Nanoparticulate drug delivery systems provide new hopes in solving problems in the area of drug delivery (Bosselmann & Williams, 2012). To a wide extent, biopolymer conjugated drugs coupled with nanotechnology enhances the stability and pharmacological efficiency of the active compound. Chitosan is non-toxic, biodegradable and bio compatible. Nanoparticles of chitosan coupled drugs are utilized for drug delivery in eye, brain, liver, cancer tissues, treatment of spinal cord injury and infections. Polymeric drug delivery systems can be used to deliver drugs directly to the intended site of action which results in slow release and minimized side effects elsewhere in the body and decreases the long-term use of many drugs (Sailaja *et al*., 2010).

The functional relationship between the liver and brain has been known for centuries. Neurotransmission in the brain is altered in liver diseases. A spectrum of neuropsychiatric abnormalities in patients with liver dysfunction were

observed and was characterized by intellectual impairments, personality changes and a depressed level of consciousness associated with multiple neurotransmitter systems, cerebral perfusion and astrocyte dysfunction (Avraham *et al*., 2009). 5-HT and GABA as therapeutic agents for cell proliferation and differentiation is a novel approach. 5-HT plays an important trophic role during neurogenesis, neuronal survival (Lauder *et al.*, 1981) and hepatocyte proliferation (Balasubramanian & Paulose, 1998). GABA, the main inhibitory neurotransmitter in the mature CNS, was recently implicated in playing a complex role during neurogenesis. GABA acts as a chemo attractant and is involved in the regulation of neural progenitor proliferation. GABA induces migration and motility of embryonic cortical neurons (Behar *et al.*, 1996). Report showed that a combination of GABA and 5-HT along with bone marrow cells triggered neuronal regeneration in the brain of Parkinson's disease model (Nandhu *et al*., 2011; Paul *et al*., 2011; Kuruvilla *et al.*, 2013). The foresaid combination also induced neuronal regeneration and enhanced cell survival in spinal cord injured monoplegic rats (Romeo *et al.*, 2013). Our earlier studies showed that 5-HT and GABA acting through specific receptor subtypes, $5HT_2$ (Balasubramanian & Paulose, 1998) and $GABA_B$ (Biju *et al.*, 2002) respectively, controls cell proliferation and acts as co-mitogens. In the present study, GABA and 5-HT encapsulated chitosan nanoparticles were prepared and administered for rapid hepatocyte proliferation and subsequent neuronal survival in partially hepatectomised rats.

**Encapsulation of GABA and 5-HT in chitosan nanoparticles**

Chitosan is the second-most abundant natural polysaccharide next to cellulose. Among other drug delivery strategies, a great deal of focus has been directed to chitosan nanoparticles to improve drug bioavailability, modify pharmacokinetics and/or protect the encapsulated drug (Agnihotri *et al*., 2004). Chitosan nanoparticles are prepared by the interaction of oppositely charged compounds. TPP has often been used to prepare chitosan nanoparticles because TPP is multivalent, nontoxic and able to form gels through ionic interactions. The

interaction is controlled by the charge density of TPP and chitosan, which is dependent on the pH of the solution (Zhao *et al*., 2011). Chitosan is a biopolymer that has been widely investigated as a drug delivery system. Chitosan, deacetylated chitin which is a linear nitrogenous polysaccharide, is a copolymer of β-(1, 4) linked glucosamine (deacetylated unit) and N- acetyl glucosamine (acetylated unit). Owing to the removal of acetyl moieties that are present in the amine functional groups of chitin, chitosan is readily soluble in aqueous acidic solution. The solubilisation occurs through the protonation of amino groups on the C-2 position of D-glucosamine residues whereby polysaccharide is converted into polycation in acidic media. Chitosan interacts with many active compounds due to the presence of amine group in it (Radhakumary *et al*., 2005). The presence of this reactive amine group in chitosan was exploited for the interaction with the active molecules, GABA and 5-HT, in the current study.

Spherical nanoparticles were obtained from ionic gelation of chitosan by the strong anion, TPP. The morphology was studied using SEM. In the SEM photograph, we see that there was some adhesion between the nanoparticles. This was due to high temperature in the electron beam irradiation so that the chitosan nanospheres were slowly melted. However, a bit of adhesion did not cause a significant impact during the dispersion of nanoparticles in PBS. Chitosan nanospheres could be suspended stably in buffer solution. The particle size is important for the *in vivo* distribution and cellular uptake, but also affects the release profile of the drug from the particles, as smaller particles have a larger surface area. This might result in a high burst release of the drug and a shorter period of sustained release (Singh & Lillard, 2009). The interaction of GABA and 5-HT were evaluated using FT-IR spectroscopy. GABA possesses carboxylic group which make a bond with the amino group of chitosan. Thus, a weak salt of carboxyl was formed by the interaction between GABA and chitosan. 5-HT also reacts with the active amino group of chitosan. In this study, serotonin creatinine sulphate possessing a reactive sulphate group was used. The sulphate group interacts with the amino terminal of chitosan and formed a weak interaction leading to encapsulation.

Increase in encapsulation of active molecules in chitosan matrix directly improves the pharmacological efficiency by loading maximum drug to the active site. Varying the concentration of active molecules has helped to standardize the optimum drug concentration for obtaining maximum encapsulation efficiency. A successful nanoparticulate delivery system should, apart from possessing a favorable safety profile, have a high loading capacity in order to reduce the quantity of both the drug and the excipient material to be used for therapy. Initially a burst release was observed. After that the release of GABA or 5-HT was at a slow rate. The diffusion of the drug, the erosion and swelling of polymer matrix and the degradation of polymer are the main mechanisms for the drug release. Since the degradation of chitosan is not very rapid, the release of active molecules from the nanoparticles would mainly depend on the drug diffusion and the matrix erosion. Thus, the porosity of the nanoparticles has significant effects on the release property. The observed change in release rate in the period after the burst release can be due to the distribution of GABA or 5-HT inside the nanoparticle matrix or a change in matrix degradation rate as a result of changed surface porosity and internal morphology, which have proven important parameters for the appearance of the release profile (Mao *et al*., 2007).

**Cell uptake study**

Uptake of nanoparticles by the cells was observed to verify the interaction of the particles with the receptors on hepatocytes. The interaction of nanoparticles with the hepatocytes was viewed with FITC-labelled nanoparticles under confocal microscope. A negative control showing the auto fluorescence in the cell was also obtained. Chitosan interacts with FITC because of the amino terminal of chitosan. FITC is a fluorescent dye that can bind to the surfaces with protruding amino group (Yuqing, *et al*., 2009). The only source of fluorescence in the image was due to FITC, which was tagged on the nanoparticles. In our study binding of chitosan nanoparticles with GABA and 5-HT with hepatocytes confirmed the easy interaction of these particles with the liver cells.

**Liver regeneration in partially hepatectomised rats**

The regeneration process can be viewed as a sequence of events starting from an initial signal and comprises a priming phase, followed by a progression phase, a cell cycle phase and finally a termination signal (LaBrecque, 1994; Mangnall, 2003; Michalopoulos, 2010). The increase of portal vein pressure and liver tissue perfusion and the extracellular matrix remodelling may be considered as the initial events after PH (Mangnall, 2003; Marubashi *et al*., 2004; Michalopoulos, 2007; Michalopoulos, 2010). Shortly after, a great number of genes are activated (primed) of which some encode transcriptional factors, essential for the propagation of the mitotic stimulus. Priming phase urge hepatocytes from quiescence to G1 phase of the cell cycle (Michalopoulos, 2010). Gene expression during priming phase is not capable by itself to promote liver regeneration after hepatectomy and a subsequent increase in growth factor levels is also necessary. Hepatocytes overcome the G1/S restriction point of the cell cycle only in the presence of growth factors and after this milestone they become committed to divide (Fausto, 2000; Mangnall, 2003). At least two intracellular signalling pathways, the MAPK and JAK-STAT, involved in liver regeneration have been identified.

**DNA and protein syntheses in the proliferating hepatocytes**

The nanoparticles were administered through the peritoneal cavity for achieving a better absorption of nanoparticles to partially hepatectomised liver (Harivardhan *et al*., 2005). The chitosan nanoparticles are biocompatible and are degraded by the endosomal pathways in the cell and thus the therapeutic potential of chitosan polymer conjugates are being investigated for *in vitro* and *in vivo* applications (Kavaz *et al.*, 2010). After two third hepatectomy of liver, the major metabolic pathways are disrupted. The liver starts to regenerate and the maximum DNA synthesis occurs by 24 hours post hepatectomy (Kountouras *et al*., 2001). Thymidine is the nitrogen base, which is found only in DNA and the increase in DNA synthesis leads to enhanced utilization of thymidine. In proteins the major amino acid observed is leucine and thus an increase in leucine uptake by cells *in*

*vitro* explained the enhancement in protein synthesis. Fasting animals prior to operation would probably not have diminished the variations in mitotic activity (Brues & Marble, 1937). DNA and protein syntheses were observed to be high in GCNP group compared with G and PHNT. This shows an increase in cell regeneration in the GABA- chitosan nanoparticle treatment groups. In the case of GSCNP and SCNP groups, there is less exposure of GABA and 5-HT to the internal body environment and the presence of positive charge and high cell binding affinity of chitosan (Gomez *et al*., 2007), more effective receptor activation by GABA and serotonin were achieved. The activity of liver thymidine kinase, an enzyme converts thymidine to thymidine monophospahate, is directly proportional to DNA synthesis (Ronco *et al*., 2002). We observed the highest activity of thymidine kinase in GSCNP group than PHNT, GCNP and SCNP.

Active liver cell proliferation is a value added process in medical field. Liver regeneration is a complex and multi-factorial process that is regulated by various cell signalling cascades including the interactions between growth factors, regenerative cytokines and metabolic demand of the liver following surgery (Gomez *et al*., 2007). The active compounds that mediate the cell signalling mechanism for an enhanced DNA and protein formation prior to cell division, will promote an efficient and fast proliferation of cells. Our study discussed the involvement of neurotransmitter combination such as GABA and serotonin, which were coupled to chitosan nanoparticles, in the synthesis of DNA and protein during the active cell multiplication in partially hepatectomised rat liver. The combined effect of improved stability and action of GABA and 5-HT on regenerating hepatocytes gives valuable scope for further studies in neurotransmitter involved liver regeneration.

**$GABA_B$ and 5-$HT_{2A}$ receptors expressions in the regenerating liver**

The amazing ability of the liver to regenerate following partial resection or injury is unique, especially because the highly differentiated functions of the organ are totally maintained. After two third hepatectomy of liver, the major metabolic pathways were disrupted. Liver starts to regenerate and the maximum

DNA synthesis occurs by 24 hours post hepatectomy (Kountouras *et al*., 2004). For the activation of cell division, GABA receptors functional regulation in various signalling pathways was controlled during liver regeneration (Biju *et al.*, 2002). Recently, GABAergic activity has been described in various tissues beyond the central nervous system. Usually in non- replicating tissues (group C), GABA and 5-HT receptors activation and deactivation occurs by endocytosis and exocytosis of receptor on the membrane. Previous report showed that the exposure of Gamma aminobutyric acid B agonist, baclofen, inhibited Schwann cells proliferation due to increased expression of $GABA_B$ receptor (Magnaghi *et al*, 2004). In our results $GABA_B$ receptor expression was reduced in groups with individual treatment with GABA and 5-HT chitosan nanoparticles and better in combination treatment that supported active hepatocyte proliferation. Activation of 5-$HT_{2A}$ receptors in cultured cortical neurons has been shown to inhibit GABA receptor currents via a protein kinase C mediated pathway (Feng *et al.*, 2001). 5-HT of combination treatment in GSCNP group down regulated $GABA_B$ receptor which was an indication of improved hepatocyte proliferation. Studies also described that, GABAergic activity is associated with attenuation of hepatic regeneration after partial hepatectomy, whereas decreased activity is associated with enhanced hepatic regeneration after acute and chronic liver damages (Minuk & Gauthier, 1993; Zhang *et al*., 1996; Kaita *et al*., 1998; Zhang *et al*., 1998). Thus the above reports together with our present data documenting a negative correlation between [$^3$H] thymidine and [$^3$H] leucine incorporation and $GABA_B$ receptor expression in regenerating rat livers, are consistent with the fact that GABA serves to regulate hepatic growth and development (Erdo *et al*., 1985; Gilon *et al*., 1987).

During enhanced hepatocyte proliferation, the expression of 5- $HT_{2A}$ subtype serotonin receptors in the liver was increased (James and Perkins, 2006). Our study also supported this and observed an up regulation in 5-$HT_{2A}$ receptor expression in the treatment groups exposed to 5-HT. Hepatic stellate cells (HSCs) are key cellular components of hepatic wound healing and fibrosis (Ochoa & Diehl, 2013). There is emerging evidence that the fibrogenic function of liver cells

was influenced by neurochemical and neurotrophic factors (Copple *et al*., 2010). 5-$HT_2$ receptors were strongly associated with fibrotic tissue in diseased rat liver. Treatment of HSCs with 5-$HT_2$ antagonists suppressed proliferation and elevated their rate of apoptosis. 5-HT synergized with platelet-derived growth factor to stimulate increased hepatocyte proliferation. HSCs express key regulatory components of the 5-HT system enabling them to store and release 5-HT and to respond to the neurotransmitter in a profibrogenic manner. Antagonists that selectively target the 5-HT class of receptors may be exploited as antifibrotic drugs (Richard *et al*., 2006). Researchers investigated the effect of 5-$HT_2$ blockage by ketanserin after 60–70% PH in rats and found that its administration arrests liver regeneration only when administrated close to the G1/S transition point implying that serotonin is a cofactor for DNA synthesis. In the same study, they also measured the concentration of liver serotonin which started to increase around the G1/S transition point and culminated at the time of maximal hepatic proliferative activity (Papadimas *et al*, 2006). These data were in accordance with the study by Sulaiman *et al*. (Sulaiman *et al*., 2008) which also showed that hepatic serotonin content significantly increased during hepatocyte DNA synthesis in rats after PH. Some *in vivo* and *in vitro* studies have demonstrated that serotonin exposure resulted in the desensitization and down-regulation of 5-$HT_{2A}$ receptors (Shi *et al.*, 2007). The serotonin exposure to the receptors in SCNP groups was more compared to GSCNP group. Thus, 5-$HT_{2A}$ receptor expression and activity in GSCNP group was observed to be increased due to reduced receptor desensitization, which again points the enhancement in liver cell multiplication. The $GABA_B$ and 5-$HT_{2A}$ receptor expression patterns in the experimental groups were further confirmed by confocal microscopy images. Thus, treatment with GABA and 5-HT chitosan nanoparticles increased liver cell proliferation by regulating both $GABA_B$ and 5-$HT_{2A}$ receptors functions.

**$GABA_B$ receptor expression in the brain regions**

GABA is one of the most abundant neurotransmitters in the vertebrate central nervous system and is involved in neuroendocrine processes such as

development, reproduction, feeding and stress (Martyniuk *et al*., 2005). Somatic and dendritic $GABA_B$ receptors regulate neuronal excitability (Breton & Stuart, 2012). $GABA_B$ receptors are broadly expressed in the nervous system and have been implicated in a wide variety of neurological and psychiatric disorders. For example, hypoactivity of the GABA system was linked to epilepsy, spasticity, anxiety, stress, sleep disorders, depression, addiction, and pain. On the contrary, hyperactivity of the GABAergic system was associated with schizophrenia (Benes & Berretta, 2001). $GABA_B$ receptors were repeatedly implicated in synaptic plasticity (Mott & Lewis, 1991; Vogt & Nicoll, 1999). Although still conflicting, the data linking $GABA_B$ receptors to transcription factors suggest that these receptors mediate long-term metabolic effects requiring new protein synthesis. Additionally, $GABA_B$ receptors signal through G protein- independent effector pathways that modulate neurotransmitter release (Harrison, 1990). $GABA_B$ receptor mediated signalling also affects $Ca^{2+}$ signals associated with other receptors (Chalifoux & Carter, 2010; Chalifoux & Carter, 2011)

Hepatic encephalopathy is considered to be a reversible metabolic encephalopathy, which occurs as a complication of hepatocellular failure and is associated with increased portal- systemic shunting of gut-derived nitrogenous compounds. Its manifestations are most consistent with a global depression of CNS function, which could arise as a consequence of a net increase in inhibitory neurotransmission, due to an imbalance between the functional status of inhibitory (e.g., GABA) and excitatory (e.g., glutamate) neurotransmitter systems. In liver failure, factors that contribute to increased GABAergic tone include increased synaptic levels of GABA and increased brain levels of natural central benzodiazepine (BZ) receptor agonists and a pattern of neural activity similar to that induced by drugs which activate the GABA neurotransmitter system (Schafer & Jones, 1982). Ammonia, present in modestly elevated levels, also augment GABAergic tone by direct interaction with the GABA receptor and stimulation of astrocytic synthesis and release of neurosteroid agonists of the GABA receptor. Thus, there is a rationale for therapies of HE that lower ammonia levels and

incrementally reduce increased GABAergic tone towards the physiologic norm (Jones, 2000).

$GABA_B$ receptor mediated neurotransmission is also brain region-dependent (Hensler *et al.*, 2012). Cortical neurons display synchronous fluctuations between periods of persistent activity and periods of relative quiescence in the animal (Craig *et al*., 2013). Altered cortical GABA neurotransmission contribute to disturbances in diverse functions through affecting the generation of cortical oscillations in conditions like schizophrenia. These oscillatory activities have been proposed to play critical roles in regulating the efficiency of information transfer between neurons and neuronal networks in the cortex (Hashimoto *et al*., 2010). Brain stem is involved in regulating the cardiac and respiratory functions in the body. Any stress in the brain results in decreased expression of GABA receptors. Anju *et al*., reported that, decreased $GABA_B$ receptor expression in the brain stem was observed as a response of the body to encounter hypoxic ventilatory decline (Anju *et al*., 2011). Agonists of GABA can act at the GABA receptor complex, and increased concentrations of the agonists are found in the brain stem in liver failure (Basile & Jones, 1994). Neurosteroids produced in brain during acute liver failure led to increased GABAergic tone (Ahboucha *et al*., 2012) and also, elevated intra-cerebral concentrations of GABA significantly decreased ornithine decarboxylase activity in the liver (Lapinjoki *et al*., 1983). Increased level of ammonia leads to neuronal damage and alteration in the cardiovascular and respiratory centres in brain stem. Impaired ammonia, aromatic amino acid metabolisms and the effect of neurosteroids like allopregnanolone (Ahboucha *et al*., 2006) and dehydroepiandrosterone sulphate (Ahboucha *et al*., 2012) contribute to increased GABAergic tone in brain during liver injury. The striatum is involved in mediating habit learning (Berke & Hyman, 2000) and optimizing control of motor behaviour and cognitive function (Graybiel, 2005) and is susceptible to oxygen deprivation. GABA is considered as the main transmitter released from the axons of striatal neurons projecting to the output structures of the basal ganglia. It also plays a central role in the processing of information in the striatum (Groves,

1983). Striatal neurons are known to have both $GABA_A$ and $GABA_B$ receptors (Ng & Yung, 2001), activation of which decreases potentiation evoked-glutamate and dopamine release in many brain regions including hippocampus, cortex and spinal cord (Tanaka *et al*., 2002; Matsumoto *et al*., 2003; Tanaka *et al*., 2003). GABA protects neurons not only by directly hyperpolarizing neurons but also by exerting an inhibitory influence on glutamate-mediated neuronal activity (Costa *et al*., 2004).

During liver injury ammonia metabolism is disturbed and leads to a condition called hyperammonemia. Hyperammonemia has been suggested to induce enhanced ammonia uptake by brain, subsequent glutamine synthesis and accumulation. The degree of correlation between serum and brain ammonia levels is still controversial. Ammonia levels are mildly elevated in patients with chronic cirrhosis without symptoms of hepatic encephalopathy. Severely encephalopathic patients may have normal ammonia levels. Serum ammonia has been shown to be a poor predictor as a single test for the presence of hepatic encephalopathy (Nicolao *et al*., 2003; Arora *et al*, 2006). More recent evidence suggests that there is a synergistic effect between ammonia and various other inflammatory cytokines that result in excess glutamine within astrocytes, leading to osmotic swelling of the astrocytes and the subsequent brain edema as well as other neurocytotoxic effects (Shawcross *et al*., 2007). The changes in brain glutamate and gamma aminobutyric acid could be related to altered ammonia metabolism (Dejong *et al*., 2007). The autonomic regulation of GABA was mediated through $GABA_B$ receptors (Sved *et al*., 1990) and reduction in the GABA neurotransmission in the brain regions enhanced DNA synthesis in liver by facilitating the sympathetic tone (Biju, 2000). Increased expression of $GABA_B$ Rs shifts the balance between perisomatic and dendritic inhibition (Booker *et al*., 2013). In our study, $GABA_B$ receptor expression was decreased considerably in the cerebral cortex, brain stem and corpus striatum of GABA and 5-HT chitosan nanoparticles (individually and in combination) treated groups. GABAergic tone was decreased in these rats with active liver cell proliferation by controlling the influx of metabolites enhance GABA content in brain. Thus the neurological

damage and cellular imbalance due to liver injury mediated brain damage was reduced by the current treatment.

**5-$HT_{2A}$ receptor expression in the brain regions**

Serotonin is not only a neurotransmitter but also has immunomodulatory functions (Das, 2011). The specific 5HT receptor involved appears to be of the $5HT_2$ family mainly $5HT_{2A}$ receptors (Kinkead & Mitchell, 1999; Fuller *et al*., 2001; Mitchell *et al*., 2001). Factors directly affecting brain functions in liver disease are decreased oxygen delivery, which can result from a variety of factors including gastrointestinal bleeding, sepsis, the effects of cytokines or compounds released from necrotic liver tissue (Fessel & Conn, 1972). In particular, proinflammatory cytokines have a pivotal role in impairing several brain functions (Gabduzda & Hall, 1966; Prabhakar & Bhatia, 2003). The effects of hypotension on cerebral perfusion may be magnified in liver failure because of an associated impairment in the autoregulation of cerebral blood flow (Als-Nielsen *et al*., 2004; Morgan *et al*., 1987). The serotonin transporter (5-HTT) plays a key role in central serotonergic neurotransmission by controlling its intensity and duration through the reuptake of 5-HT that has been released from serotonergic terminals, somas and/or dendrites. Since serotonin does not readily penetrate the blood brain barrier, neuronal serotonin is synthesized locally in the brain from the precursor, tryptophan (Lozeva-Thomas, 2004). These molecules are capable of affecting mental functions such as mood and appetite as well as regulating blood pressure, body temperature and other bodily processes (Medina *et al*., 2003). Serotonergic nerve fibres are part of the autonomic nervous system and nerve endings have been found on the branches of the hepatic artery, portal vein, bile ducts and connective tissue of the interlobular septa in humans (el-Salhy *et al*., 1993), as well as in portal tracts and the fibrous septa within rat hepatic lobules (Stoyanova, 2004; Frampton *et al*., 2010) .

Alterations in serotonergic neurotransmission was observed in brain regions during several critical physiological conditions like hypoxia (Anju *et al*., 2010; Anju & Paulose, 2011; Anju *et al*., 2011a; Anju *et al*., 2011b;), diabetes

(Abraham *et al*, 2010) and Parkinson's disease (Kuruvilla *et al*., 2013). In cerebral cortical development, early manipulations of the serotonergic innervation lead to altered development and plasticity in sensory areas in a variety of species (Gu & Singer, 1995; Osterheld-Haas & Hornung 1996; Janusonis *et al*., 2004). Several studies have revealed the presence of 5-$HT_{2A}$ receptors in cortical pyramidal neurons (Amargos-Bosch *et al*., 2004; Santana *et al*., 2004). 5-HT influences the descending excitatory input into limbic and motor structures, where the prefrontal cortex projects through the activation of pyramidal 5-$HT_{2A}$ receptors. During liver injury and hepatic insufficiency, the aromatic amino acid catabolism was altered. Thus the plasma levels of aromatic amino acid increases and enters the brain. The aromatic amino acid tryptophan enhanced the serotonin synthesis in brain, which lead to active serotonin mediated neurotransmission. As hepatic cell recovery progresses, the aromatic amino acid metabolism also gets reactivated. Thus, the serotonin content gets decreased in brain regions (Dejong *et al*., 2007). The neurotransmitter serotonin has a profound effect on the control of sleep and mood fluctuations, thus excess serotonin activity in the brain could be responsible for impaired consciousness during liver failure. In brain, conversion of tryptophan by pyridoxal phosphate increased the 5-HT content. Serotonin is metabolized to 5-hydyoxy indole acetic acid (5-HIAA) by the mitochondrial enzyme monoamine oxidase (MAO, primarily MAO-A). The increase in 5-HT content in brain is brought about by significant increase in 5-HT synthesis and decrease in its breakdown to 5-HIAA. There is also an interesting report suggesting that serotonin can potentially contribute to liver tissue hypoperfusion following hepatic ischemia and reperfusion (Murata *et al*., 2003). Sympathetic innervation is important for liver regeneration (Kiba *et al*., 1995). Over synthesis of 5-HT also liberates reactive oxygen species. Enzymatic oxidation of excess 5-HT at physiological pH forms tryptamine-4,5-dione (Wrona & Dryhurst, 1991). Tryptamine-4,5-dione is also formed *in vivo*, possibly by an oxidative enzyme or potent oxidizing agent, such as oxygen free radicals. If tryptamine-4, 5-dione is formed *via* a free-radical reaction in the central nervous system, it participate in the pathological process of neuronal damage induced by ischemia or traumatic

brain injury (Ikeda *et al*., 1989; Sakamoto *et al*., 1991). During active hepatocyte proliferation, the serotonin receptor number was decreased in the brain regions of partially hepatectomised rats (Sudha, 1997). The present study also showed a decreased expression of 5-$HT_{2A}$ receptors in the cerebral cortex, corpus striatum and brain stem of GABA and 5-HT (individually and in combination) chitosan nanoparticles treated groups compared to that with no treatment. Liver cell proliferation was increased in all nanoparticles treated rats, with a great emphasis on the treatment with combination of GABA and 5-HT. Thus the precursor for serotonin synthesis in brain was also metabolized rapidly in rats with active liver cell proliferation. So decrease in GABA and 5-HT receptors numbers was a homeostatic adjustment by the brain to activate the sympathetic innervation, thereby elevating DNA synthesis in the liver.

**Second messengers and transcription factors**

The regenerative capacity of the liver is well known, and the mechanisms that regulate this process have been extensively studied using experimental model systems including surgical resection and hepatotoxin exposure. The response to primary mitogens has also been used to investigate the regulation of hepatocellular proliferation. Such analyses have identified many specific cytokines and growth factors, intracellular signalling events and transcription factors that are regulated during and necessary for normal liver regeneration (Rudnick & Davidson, 2012). Second messengers are molecules that relay signals from receptors on the cell surface to target molecules inside the cell, in the cytoplasm or nucleus. They relay the signals of hormones like epinephrine (adrenaline), growth factors and others and cause some kind of change in the activity of the cell. They greatly amplify the strength of the signal. Secondary messengers are a component of signal transduction cascades. Secondary messenger systems can be synthesized and activated by enzymes, like the cyclases that synthesize cyclic nucleotides, or by opening of ion channels to allow influx of metal ions, like $Ca^{2+}$ signalling. These small molecules bind and activate protein kinases, ion channels, and other proteins, thus continuing the signalling cascade (Michalopoulos, 2010).

**Cyclic AMP and CREB**

Studies of the biological role of cAMP have indicated dual and often opposing effects on proliferation and differentiation. Elevation of the intracellular cAMP in normal and transformed cells lead to cell proliferation; in other cells, it induces changes in morphology, apoptosis and/or differentiation (Nesterova & Stratakis, 2007). Report described that cAMP is an integral downstream component of the neurotrophin signalling pathway in the adult mammalian CNS regeneration (Spencer & Filbin, 2004). CREB is a transcription factor involved in G-protein mediated cell signalling mechanism. Activation of Gαs coupled receptors leads to the stimulation of adenylyl cyclases elevating cAMP, which as a second messenger interacts with other proteins including ion channels and activates the protein kinase A (PKA). This phosphorylating enzyme also activates cAMP-responsive transcription factors like CREB modifying gene expression. CREB plays an important role in a variety of cellular processes, including proliferation, differentiation and adaptive responses. CREB phosphorylation during post-ischemic recovery is associated with neuronal survival.

**Liver**

The activation of cell division with GABA and 5-HT receptors functional regulation in various signalling pathways were controlled during liver regeneration. The expression of $Gi_2$ alpha subunit of G protein is increased by 24 hours of hepatectomy and thus the activity of adenylyl cyclase is decreased in the S phase of cell cycle (Anna *et al*., 1992). In the pre- replicative phase of liver regeneration, there is a burst in cAMP-dependent protein kinase A (PKA) activation that is partly down regulated during the phase of active regeneration (S phase). In normal regeneration of hepatocytes the G protein receptor activation occurs in the initial phase of cell division which further leads to a rise in cAMP and PKA levels. But later, during the shift from $G_1$ to S phase, the PKA mediated cell signalling gets suppressed (Ekanger *et al*., 1989) and promote protein kinase C mediated cell signalling (Arturo, 2003). The levels of cAMP were markedly

higher in quiescent liver (Liu *et al*., 2010). Activation of Akt is a critical cell survival signal during the regenerative process (Hong *et al.,* 2000). The inhibition of Akt phosphorylation leads to impaired liver regeneration, which is dependent on cAMP/PKA expression (Pang *et al*., 2009; Mei *et al*., 2011). cAMP content and CREB expression were reduced in the regenerating livers of PHNT and further reduced significantly in GCNP, SCNP and GSCNP, which promoted enhanced liver cell proliferation in the active phase of cell cycle. Three laboratories described that the COOH-terminal domain of $GABA_{B(1)}$ interacts with members of the ATF/CREB family of transcription factors, that is, ATF4/CREB2 and ATFx (Nehring *et al*., 2000; White *et al*., 2000; Vernon *et al*., 2001). The interaction between CREB2/ATF-4 and $GABA_{B(1)}$ takes place between the leucine-zipper and the coiled-coil domain, respectively. Although it is formally not ruled out that this interaction is artificial, increasing evidence points toward a physiological relevance. cAMP content and CREB expression were reduced in the regenerating livers of PHNT and further reduced in GCNP, SCNP and GSCNP, which promoted enhanced liver cell proliferation in the active phase of cell cycle.

**Brain regions**

$G_i\alpha$ and $G_o\alpha$ proteins, the predominant transducers of $GABA_B$ and $5\text{-}HT_{2A}$ receptors, inhibit most of the adenylyl cyclases (Simonds, 1999). Many studies have reported that $GABA_B$ receptors inhibit forskolin-stimulated cAMP formation, but others also observed a stimulation of cAMP production (Bowery *et al*., 2002; Calver *et al*., 2002). $G_i\alpha$ and $G_o\alpha$ proteins inhibit adenylyl cyclase. Therefore, the stimulatory action of $GABA_B$ receptors on cAMP levels is a consequence of G protein cross-talk and depends on the expression of adenylyl cyclase isoforms together with $GABA_B$ and $G_s\alpha$-coupled GPCRs. Both the inhibition and enhancement of cAMP levels by $GABA_B$ receptor activation were confirmed *in vivo* using microdialysis (Hashimoto & Kuriyama, 1997). Many ion channels are targets of the cAMP-dependent kinase (protein kinase A or PKA). Accordingly, a $GABA_B$ receptor-mediated modulation of $K^+$ channels via cAMP was reported

(Gerber & Gahwiler, 1994). Significantly, the activity of $GABA_B$ receptors on adenylyl cyclase is expected to modulate neuronal function on a longer time scale.

Until recently, it was unclear whether $GABA_B$ receptors can influence plasticity processes through the cAMP pathway. Recent experiments now demonstrate that G protein-mediated signalling through $GABA_B$ receptors retards the recruitment of synaptic vesicles during sustained activity and after short-term depression (Sakaba & Neher, 2003). This retardation occurs through a lowering of cAMP, which blocks the stimulatory effect of the increased $Ca^{2+}$ concentration on vesicle recruitment. In this signalling pathway, cAMP and $Ca^{2+}$/calmodulin cooperate to enhance vesicle priming. Our study described the down regulation of cAMP which blocked the stimulatory effect resulted from disturbed GABAergic neurotransmission in all nanoparticle treated rats.

Serotonin is one of the ubiquitous molecules acting as messengers, well known as a neurotransmitter and neuromodulator (Berger *et al*., 2009). $5\text{-}HT_2$ receptors involve an alternative signalling pathway to cAMP, where increasing $Ca^{2+}$ levels is of particular importance, relying on the crosstalk between cAMP signalling and $Ca^{2+}$ regulated adenylyl cyclases (Berumen *et al*., 2012). Disturbed serotonergic neurotransmission resulted from improper metabolism of amino acids during liver injury alters the cAMP content. Liver disease is associated with vomiting and nausea due to increase in serotonin mediated signalling and elevated cAMP (Hagbom *et al*., 2011). Serotonin influences body temperature, breathing rhythms (respiratory system), heart rate (cardiovascular function in general), eating and bowel motility (gastrointestinal system), ejaculatory latency and bladder control, muscle contraction/relaxation and locomotion, sleep, pain and sensory perception, emotions and cognition (Nichols & Nichols, 2008;Berger *et al*., 2009; Ahern, 2011; Feijo, *et al*., 2011) with a well-known signalling role in immune cells (Ahern, 2011). Thus reduced cAMP in GABA and 5-HT (individual and in combination) chitosan nanopaerticles treated rats due to decreased $5\text{-}HT_{2A}$ mediated signalling, favoured the animal to regain normal body activities.

CREB, a transcription factor, mediates responses to a number of physiological and pathological signals such as neurotransmitters, synaptic activity,

depolarization, mitogens and other stress factors (Sheng *et al*., 1991; Ginty, 1997; Vo & Goodman, 2001). CREB drives cell survival signalling (Walton & Dragunow, 2000; Ciani *et al*., 2002). GABAR mediated depolarization can also promote CREB signalling needed for cell survival (Dieni *et al*., 2012). $GABA_B$ signalling through CREB proteins was already proposed before cloning. This transcription factor promotes cell survival through the PI3K/Akt pathway to up regulate the expression of the anti-apoptotic factor Bcl-2 (Du & Montminy, 1998; Pugazhenthi *et al*., 2000). CREB is a major transcriptional target of brain derived serotonin in neurons (Oury *et al*., 2010). Serotonin function requires the expression of CREB, which regulates the expression of several genes affecting appetite (Yadav *et al*., 2011). Liver injury leads to loss of appetite and will be replenished by restoring the normal serotonin mediated neurotransmission.

In the present study the gene expression of CREB was down regulated in cerebral cortex, brain stem and corpus striatum of GABA and 5-HT chitosan nanoparticle treated rats. The cAMP level was decreased in these rats and CREB expression also declined. Acute metabolic disturbances due to liver injury triggers cell death pathways by activating pro-apoptotic genes like Bax and destabilizing jun - fos complex in brain. The activation of apoptotic pathways down regulates CREB expression thereby blocking the cAMP signalling cascade and inturn reduces over activation of apoptotic factors in rats with regenerating liver. The combined effect of GABA and 5-HT helped the animals to recover from liver injury based neuronal damage in a better way compared to those with no treatment and individual administration of chitosan nanoparticles with GABA or 5-HT.

**$IP_3$ and phopholipase C**

$IP_3$ is a second messenger in all G-protein coupled receptor mediated cell signalling cascades. Phospholipase C (PLC) is involved in transduction of neurotransmitter signals across membranes *via* hydrolysis of phosphatidylinositol-4,5-bisphosphate, leading to generation of second messengers inositol- 1,4,5-trisphosphate and diacylglycerol.. $IP_3$ functions by binding to the membrane-associated $IP_3$ receptors ($IP_3R$) (Berridge *et al*., 2003). The activation of Gαq/11

coupled receptors lead to the hydrolysis of membrane phosphoinositides resulting in the formation of diacyl glycerol (DAG) and inositol phosphates ($IP_3$). $IP_3$ can interact with the calcium reservoirs, elevating intracellular levels and activating protein kinase C (Nichols & Nichols, 2008; Werry *et al*., 2005). Binding of $IP_3$ to the receptor increases its sensitivity to $Ca^{2+}$ and only after $Ca^{2+}$ is bound the trafficking of the $Ca^{2+}$ into the cytosol take place. Notably, $Ca^{2+}$ has a biphasic action on the $IP_3R$ with a stimulatory effect at low $Ca^{2+}$ concentrations and an inhibitory effect at higher $Ca^{2+}$ concentrations (NadifKasri *et al*., 2002; Taylor & Laude, 2002). Acting as a signal transducer between two ubiquitous second messengers $IP_3$ and $Ca^{2+}$, $IP_3R$ has been implicated in a variety of cellular and physiological processes as diverse as cell division, cell proliferation, apoptosis, fertilization, development, behaviour, memory and learning. In mammals, there are three distinct types of IP3R with splice variants observed among the types (Patel *et al*., 1999). $IP_3$-receptor is dominantly expressed in neuronal cells throughout the central nervous system (Nakanishi *et al*., 1991; Furuichi *et al*., 1993). Throughout the brain, the $IP_3R1$ is the predominantly expressed member of the family and its mRNA is widely distributed.

**Liver**

The liver regeneration signalling cascades also result in the activation of transcription factors and signal transduction pathways. Among these transcription factors and corresponding signal transductions, the TNF-$\alpha$/NF-$\kappa$B and $IP_3$/Akt pathways are identified as the two major cascades during the process of liver regeneration (Nowatari *et al*., 2012). In the process of liver regeneration, the phosphoinositide (PI) cycle might be activated in the hepatocyte nucleus where the levels of phosphatidylinositol-4,5-bisphosphate ($PIP_2$) and diacylglycerol (DAG) are changed during the process (Bregoli *et al*., 2002). Indeed, it is demonstrated that the nucleus is equipped with most of the PI cycle–related enzymes, including PLC and diacylglycerol kinase (DAGK) (Goto *et al*., 2006). DAGK is an enzyme responsible for the phosphorylation of DAG, which is derived from $PIP_2$ by the action of PLC, to phosphatidic acid (PA). Both of the lipids are important

components of phospholipid biosynthesis in the PI cycle (Kanoh *et al*., 1990). A well-known functional role of DAGK is the regulation of protein kinase C (PKC), for which DAG acts as an allosteric activator and whose activity plays a central role in many different cell types (Sakane & Kanoh, 1997). The DAG-PKC pathway is implicated in widely various cellular processes such as cellular growth, differentiation, and secretion (Nishizuka, 1984; Nakano *et al*., 2012). However, heteromeric $GABA_{B(1,2)}$ receptors do activate PLC via chimeric $G_{qi}\alpha$ and $G_{qo}\alpha$ proteins, in which the five COOH-terminal residues of $G_i\alpha$ or $G_o\alpha$ replace those of $G_q\alpha$. Therefore, like other GPCRs, $GABA_B$ receptors recognize the very COOH terminus of Gα subunits (Blahos *et al*., 1998*).* Serotonin receptors coupled to Gα12/13, mediating structural changes within the cell through activation of the Rho signalling pathway (Hannon & Hoyer, 2008). *In vitro* studies showed that EGFR could activate $Ca^{2+}$ dependent pathways such as RaI and NF-κB through the phosphorylation of PLCγ. PLC is one of the possible downstream pathways activated by EGFR. It has been shown that the increased activity of nuclear PLC in regenerating rat occurs before DNA synthesis peak after PH (Albi *et al*., 2003). Moreover, Farrell's group pointed out the role of the EGFR/PLC axis in hepatocyte proliferation in a model of chronic ethanol consumption (Zhang & Farrell, 1999). An injury to liver leads to shock mediated hepatocyte apoptosis and multiplication. An improved liver regeneration depends on decreased apoptosis and increased cell proliferation. The signals evoked by $IP_3$ mediated $Ca^{2+}$ ions in mitochondria triggers apoptosis. Phospholipase C is the enzyme involved in the synthesis of $IP_3$ and thus the increased level of $IP_3$ and phospholipase C result in enhanced apoptosis (Szalai *et al*., 1999). Triggering of protein kinase C occurs through $IP_3$ mediated signalling pathway activates NF-κB and TNF-α involved cell death. Thus in our study, the $IP_3$ content and phospholipase C expression in the GABA and 5-HT chitosan nanoparticles treated rats was decreased prominently compared to other partially hepatectomised rats and the result ensures the suppression of NF-κB and TNF-α involved cell death in regenerating liver.

**Brain**

In the CNS, neurotransmitter receptor coupling to phospholipase C has been extensively documented in [$^3$H] inositol-labeled tissue slices and synaptosomes obtained from animal brains (Fisher & Agranoff, 1987; Stephens & Logan, 1989; Chandler & Crews, 1990). Previous study reports that phospholipase C-mediated signalling initiated by growth factor receptor types, are involved in long-term memory formation, a process that requires gene expression (Paul *et al*., 1999). Thus altered $IP_3$ and PLC regulation in the brain regions of partially hepatectomised rats resulted to memory deficits. In the present study, we observed, down regulation of phospholipase C gene expression in corpus striatum, cerebral cortex and brain stem of partially hepatectomised rats with and without nanoparticle treatment. Down regulation of the Phospholipase C in brain regions during liver regeneration contribute to the impaired signal transduction of G-protein coupled neurotransmitter receptors. Increased level of $IP_3$ and phospholipase C result in enhanced apoptosis (Szalai *et al*., 1999). Altered phospholipase C expression fails to modulate the activity of downstream proteins important for apoptotic signalling. Defective expression of phospholipase C results in low levels of $IP_3$ causing the impaired release of $Ca^{2+}$ and bring down the level of intracellular calcium required for apoptosis. Thus treatment with GABA and 5-HT chitosan nanoparticles favoured decreased expression of signalling molecules through phospholipase C/$IP_3$ channel that leads to apoptosis.

**DNA methylation in the regenerating liver**

DNA methylation is a modification of DNA established immediately after DNA synthesis in the 'S' phase. In our study, *in vitro* methyl group incorporation was significantly high in the treatment groups when compared to C and PHNT. This suggested the occurrence of reduced methylated DNA in the liver of each experimental group with active hepatocyte proliferation. There are studies reporting that in mammalian cells the synthesis and methylation of DNA do not occur simultaneously (Gruenbaum *et al*., 1983). The cells enter the S phase with an apparent decrease in the methylated DNA. Increased DNA content of the cells

results from DNA replication (Smith *et al*., 1983). Earlier studies pointed out that the DNA methylation was decreased in hepatocytes undergoing rapid division (Aniagu *et al*., 2009; Gearhart *et al*., 2010; Pan *et al*., 2012). Decreased methylation in DNA obtained from the regenerating liver that was treated with GABA and 5-HT chitosan nanoparticles clearly explained the increase in DNA content by DNA replication followed by cell mitosis. Earlier study reported that the methylation level of mammal fish conserved sequence 1 is low in the developing limb (Katsuyama & Paro, 2011). The *MAT2A* codes for methionine adenosyltransferase (MAT), which is an essential enzyme catalyzes the formation of *S*-adenosylmethionine, the main methyl donor for DNA methylation. In our result for MAT2A gene expression, the enzyme synthesis in the GSCNP group was significantly reduced. Thus the extent of DNA methylation in the regenerating liver of GSCNP were less compared to other experimental groups, again projected the significance of GABA and 5-HT chitosan nanoparticles treatment for active hepatocyte DNA synthesis and division.

**HGF expression in the liver of experimental rats**

Hepatocyte growth factor (HGF) is an important growth factor which is expressed and elevated during liver regeneration in partially hepatectomised rats and is a potent stimulator of hepatocyte growth and DNA synthesis identified (Su *et al*., 2002). Its ability to stimulate mitogenesis, cell motility and matrix invasion gives it a central role in angiogenesis, tumorogenesis, and tissue regeneration. HGF is recognized as one of the most important factors in the regulation of liver regeneration after surgical resection or chemical damage (Jiang *et al*., 1993). Apart from liver, HGF is also involved in the regeneration of other tissues. The pleiotrophic cytokine, HGF promotes epithelial proliferation, morphogenesis, migration, and resistance to apoptosis, as a candidate mediator of alveolar formation and regeneration (Calvi *et al*., 2013). Hepatocyte growth factor mediates MSCs stimulated functional recovery in animal models of multiple sclerosis (Bai *et al*., 2012) The required growth factors for the progression of liver regeneration through the cell cycle into the S phase are signalling through two

main tyrosine-kinase receptors: EGFR and c-Met. HGF is the main ligand of c-Met receptor. It is mainly secreted by macrophages and endothelial liver cells (Matsumoto & Nakamura, 1992). These factors are potent mitogens *in vitro* (Ito *et al*., 1994; Fausto *et al*., 1995; Block *et al*., 1996; Berasain *et al*., 2005). Over expression of HGF in the liver of transgenic mice increases hepatocyte proliferation during postnatal development and accelerated liver regeneration after PH but has minor effects at adult stage in a quiescent liver (Sakata *et al*., 1996; Shiota & H. Kawasaki, 1998;Bell *et al*., 1999). On the contrary, conditional deletion of this receptor, as well as studies using RNAi in the liver of mice, caused either a significant decrease in the peak of proliferation (Borowiak *et al*., 2004; Factor *et al*., 2010) or a delay of S-phase entry (Paranjpe *et al*., 2007). Moreover, Thorgeirsson's team indicated that c-Met is required for G2/M progression as well as entering the cell cycle *in vivo* (Factor *et al*., 2010). The increased expression of HGF gene in the early stage and decreased expression in the later stage revealed the therapeutic significance of GABA and 5-HT chitosan nanoparticle induced liver regeneration.

**Neuronal survival factors**

During any injury to the organs, a stress mediated tissue degenerative signalling gets activated in the brain. It was more prominent in the case of liver injury. Thus there are mechanisms developed by the animal's own body to alleviate the effect of stress by activating neuronal survival molecules. Ankarcrona *et al.* (1995) explained that during brain injury, glutamate accumulation leads to overstimulation of postsynaptic glutamate receptors with intracellular $Ca^{2+}$ overload and neuronal cell death. Over-stimulation of neurotransmitter glutamate was decreased by the treatment with GABA and 5-HT in Parkinson's disease rat model (Nandhu *et al*., 2011). Thus in our study, the combined effect of GABA and 5-HT treatment in GSCNP group showed an increased expression of neuronal survival factors and resulted in reduced cell death. Active liver regeneration, which is supported with increased neuronal survival helps the partially hepatectomised rats to recover easily with less brain damage.

**NF-κB, TNF-α and Akt-1**

During any injury to the organs, a stress mediated tissue degenerative signalling gets activated in the brain. It was more prominent in the case of liver injury. Thus there are mechanisms developed by the animal's own body to alleviate the effect of stress by activating neuronal survival molecules. Active liver regeneration, which is supported with increased neuronal survival, helps partially hepatectomised rats to recover easily with less brain damage. NF-κB is expressed in diverse cell types in the nervous systems (Neill & Kaltschmidt, 1997). An involvement of NF-κB in neuronal development demonstrates its activation in neurons in certain regions of the brain during neurogenesis. Inhibition of NF-κB by an inhibitor such as diethyldithiocarbamate (DDTC) was shown to increase cell death and infarct size following transient ischemic insult in rats, suggesting that NF-κB induces survival signalling in neuronal cells (Hill *et al*., 2001). NF-κB is a heterodimer transcription factor that is sequestered in the cytoplasm by an anchor protein, inhibitor of NF-κB (IκB). Phosphorylation of NF-κB on serines 32 and 36 by IκB kinase leads to its ubiquitination and degradation by proteosomal enzymes, which allows NF-κB heterodimer to translocate to the nucleus and regulate gene expression. In GCNP, SCNP and GSCNP groups the NF-κB level was increased compared to PHNT, which showed an increased neuronal survival and maintenance.

One well studied pathway that led to NF-κB activation was by the involvement of cytokine TNF-α through intracellular signalling molecules TNF receptor associated factors (TRAF2 and TRAF6) and activated NF-κB-inducing kinase (NIK), which phosphorylates the IKKs (Karin & Ben-Neriah, 2000). IKK can be phosphorylated by an alternative pathway, which involves Akt. Indeed TNF-α and platelet-derived growth factor (PDGF)-induced NF-κB activation has been reported to require Akt (Burow *et al*., 2000). Also, report showed that NF-κB appears to be a target of the anti-apoptotic Ras/PI (3)K/Akt pathway and the expression and activity of Akt was regulated by NF-κB (Meng *et al*., 2002). The

interrelated activation of NF-κB, TNF-α and Akt-1 were required for the survival of neurons in the brain.

The differential patterns of localization of TNF-α receptors in neuronal and glial cells, their state of activation and the down stream effectors, all are thought to play an important role in determining whether tumour necrosis factor (TNF-α) will exert a beneficial or harmful effect on CNS (Figiel, 2008). Even though the neurodegenerative activity of TNF-α was documented in many studies, several reports emphasise the neuroprotective role of the same. In rat ischemia model, Hurtado and colleagues (2001) demonstrated that TACE (enzyme required for the activation of TNF-α) is up regulated after ischemic brain damage and that the increase in TACE expression contributes to a rise in TNF-α and a subsequent neuroprotective effect after excitotoxic stimuli. TNF-α induces neuroprotection against excitotoxic damage in primary cortical neurons *via* sustained NF-κB activation (Dolga *et al.*, 2008). The reports of Larsen and Wendon (2002) explained clearly that the inflammatory mediators like TNF-α and neuronal survival factors like NF-κB and Akt-1 play an important role in the regulation of brain function by liver. Thus based on the evidences from the above studies, irrespective to the neurodegenerative effect, TNF-α has a neuronal protective and survival effect along with NF-κB and Akt-1 during liver cell proliferation. From our study, in group GSCNP a better neuronal survival signalling mechanism was observed when compared to PHNT.

**cGMP**

cGMP synthesis is catalyzed by guanylate cyclase (GC), which converts GTP to cGMP. Membrane-bound GC is activated by peptide hormones such as the atrial natriuretic factor, while soluble GC is typically activated by nitric oxide to stimulate cGMP synthesis. cGMP is a common regulator of ion channel conductance, glycogenolysis, and cellular apoptosis. cGMP binding activates cGMP dependant protein kinase (PKG), which phosphorylates serines and threonines on many cellular proteins, frequently resulting in changes in activity or function, subcellular localization, or regulatory features. The proteins that are so

modified by PKG commonly regulate calcium homeostasis, calcium sensitivity of cellular proteins, platelet activation and adhesion, smooth muscle contraction, cardiac function, gene expression, feedback of the NO-signalling pathway and other processes (Francis *et al*., 2010). Role of cyclic GMP in the regulation of neuronal calcium and survival by secreted forms of beta-amyloid precursor in neuro-degenerative Alzheimer's disease was already reported (Barger *et al*., 1995). cGMP directly regulates NO-signalling. Nitric oxide (NO) is a molecule that functions as a signalling agent under physiological conditions but causes nitrosative stress under pathological conditions due to its enhanced production in ROS/ RNS species activation impairing normal neurological function (Akhtar *et al*., 2012). During liver injury, non metabolized compounds enter brain will abnormally synthesise GABA and glutamate. In many systems, including primary cultures of cerebellar neurons, excessive synthesis of glutamate causes neurotoxicity by activation of NMDA receptors, leading to increased intracellular calcium which binds to calmodulin and activates neuronal nitric oxide synthase (NOS), increasing nitric oxide (NO) which in turn activates guanylate cyclase and increases cGMP (Montoliu *et al*., 1999; Montoliu *et al*., 2001). Another report also revealed that the inhibition of cGMP prevents neurotoxicity in immature cortical neurons and a hippocampal nerve cell line. It was shown here that soluble guanylyl cyclase (sGC) activity is required for nerve cell death caused by glutathione depletion. The mutual interaction between GABA and glutamate determinates the proper functioning of the CNS. Inhibitors of sGC block glutamate and GABA and a cGMP analogue potentiates cell death. These neurotransmissions also induces an elevation of cGMP that occurs late in the cell death pathway. The resultant cGMP modulates the increase in intracellular calcium that precedes cell death (Li *et al*., 1997; Sahaboglu *et al*., 2013). In our study, the cGMP content in the cerebral cortex, corpus striatum and brain stem were decreased significantly in all the nanoparticles treated rats compared to that with no treatment. This clearly suggested the treatment adopted in the present study decreased neuronal degeneration in brain regions of partially hepatectomised rats.

**BDNF**

The neurotrophins comprise a family of homologous proteins which includes NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Despite the 50-55% similarity in the amino acid composition of these molecules (Hohn *et al.*, 1990), the different neurotrophins promote the survival of distinct, yet overlapping, sets of central and peripheral neurons. BDNF is a potent trophic factor supports neurons and promotes survival and/or differentiation of neurons *in vitro*. BDNF gene contains cAMP response element. It is also a crucial neurotrophic factor and possess pro-survival and differentiation effects on several neuronal populations and synaptic plasticity. It is also a potent *in vitro* survival factor for granule neurons. Schwartz *et al*. (1997) reported that mice that are genetically deficient for the BDNF or BDNF receptor genes display an excess of apoptotic cells in the cerebellum. Disturbed homeostasis of many metabolites like ammonia, aromatic amino acids, endogenous opiates and neurotransmissions lead to neuronal death. Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) genes are the targets of $GABA_B$ and $5\text{-}HT_{2A}$ mediated transcriptional regulation. Both the production of NGF and BDNF are stimulated after treatment of rats with $GABA_B$ receptor antagonists (Heese *et al*, 2000). Several metabotropic effects of serotonin have been related to BDNF expression (Brezun & Daszuta, 1999) and BDNF itself promotes the development and function of serotonergic neurons (Martinowich & Lu, 2008). This kind of interaction between neurotrophic factors and neurotransmitters has been reported also with steroids; the regulation of HPA axis by serotonin and vice versa is well documented (Lanfumey *et al*., 2008; Haj-Dahmane & Shen, 2011) and sexual steroids have this intricate correlation as well (Banasr, 2001). The key for understanding these relationships is the existence of multiple receptors and ligand interaction for molecular signalling. Extensive research has shown that neurotrophins have profound influences upon the development, survival, regulation of function and plasticity of diverse neuronal populations in both the CNS and PNS (Lindsay *et al.*, 1994). Moreover, BDNF promotes the sprouting of mature, uninjured serotonergic axons and dramatically enhance the survival or

sprouting of 5-HT axons normally (Mamounas *et al.*, 2000). Increased BDNF mRNA expression in the brain regions of liver injured rats compared to control contributes directly to the initiation of neuronal survival as seen in many neurodegenerative disorders (Porritt *et al.*, 2005). Increased BDNF production in the treatment groups protected the neurons from the apoptotic insult derived from neurotransmitter imbalance. A prominent increase in BDNF expression in rats treated with a combination of GABA and 5-HT chitosan nanoparticles supported the above reports regarding the neuronal survival.

**GDNF**

Many studies with *in vitro* and *in vivo* models have shown that GDNF supports neuritic outgrowth or survival of mesencephalic dopaminergic neurons, cranial nerve and spinal cord motor neurons, brain stem noradrenergic neurons (Arenas *et al*., 1995), basal forebrain cholinergic neurons, Purkinje cells and certain groups of dorsal ganglion and sympathetic neurons (Lin *et al*., 1993; Kreiglestein *et al*., 1995; Siegel & Chauhan, 2000). Earlier studies described that more neuronal loss results in decreased expression of GDNF mRNA (Zhang *et al*., 2012; Wang *et al*., 2013). GDNF protects mesencephalic neurons by suppression of oxygen radical accumulation and caspase-dependent apoptosis, which are mediated by the PI3K/Akt pathway (Sawada *et al*., 2000; Ding *et al*., 2004). GDNF is up regulated following nerve regeneration and has been shown to have a role in the promotion of neuronal survival, migration of Schwann cells and to enhance myelination (Iwase *et al.*, 2005). GDNF, acts at least in part through Akt signalling and Akt activation increases GDNF expression (Cen *et al.*, 2006). Administration of 5-HT and GABA which were encapsulated in chitosan nanoparticles provided neuroprotection in the brain regions *via* Akt activation. Microdialysis studies performed on rat hippocampus demonstrated that GDNF significantly reduced free radical production and increased the activity of SOD (free radical scavengers) following kainate-induced excitotoxicity (Cheng *et al.*, 1998). Another study showed that CA3 and CA1 hippocampal regions are highly responsive to GDNF-induced neuroprotection and suggest that, upon

excitotoxicity, such neuroprotection involves a GDNF modulation of microglial cell activity (Boscia *et al*., 2009). The binding of GDNF to GFRα receptors activates a transmembrane tyrosine kinase, c-Ret and induces further downstream signalling *via* multiple pathways including the MAP kinase pathway and phospolipase Cγ pathway. Activation of the extracellular signal-regulated kinase members of the MAP kinase family (ERK or p42/p44 MAP kinase) and the PI3K/Akt signalling pathway promote cell survival. Neurotrophic factors such as NGF, BDNF, GDNF, and insulin-like growth factor I (IGF-I), activate the PI3-Akt signalling cascade through corresponding receptor tyrosine kinases such as the high affinity neurotrophin receptors (Trk's). Akt activates the CREB, an additional transcriptional regulator that promotes neuronal survival (Bonni *et al*., 1999). In addition, Akt can directly inhibit the apoptotic machinery by phosphorylation at sites both upstream (BAD) (Datta *et al*., 1997) and downstream (Caspase-9) (Cardone *et al*., 1998) of mitochondrial cytochrome C release. Therefore, the inter related expressions of CREB, BDNF and GDNF suggested an increased neuronal growth and survival in the brain regions of partially hepatectomised rats treated with GABA chitosan nanoparticles, 5-HT chitosan nanoparticles and further increased in GABA and 5-HT chitosan nanoparticles.

**Super oxide dismutase activity**

Free radical scavenging enzyme like SOD plays important role in protection against oxygen toxicity in mammalian systems. Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS, as well as to regulate redox-sensitive signalling pathways. The SOD converts superoxide radical into hydrogen peroxide and molecular oxygen (Weydert & Cullen, 2010). ROS are produced in many aerobic cellular metabolic processes. They include, but are not limited to, species such as superoxide and hydrogen peroxide which react with various intracellular targets, including lipids, proteins, and DNA (Cerutti, 1985). Although ROS are generated during normal aerobic metabolism, the biological effects of ROS on these intracellular targets are dependent on their concentration and increased levels of these species are present during oxidative

stress. Increased levels of ROS are cytotoxic, while lower levels are necessary for the regulation of several key physiological mechanisms including cell differentiation (Allen & Balon, 1989) apoptosis (Hockenbery *et al*., 1993), cell proliferation (Shibanuma *et al*., 1988) and regulation of redox-sensitive signal transduction pathways (Lo *et al*., 1996). However, increased levels also result in ROS-induced damage including cell death, mutations, chromosomal aberrations, and carcinogenesis (Cerutti, 1985).

**Liver**

Antioxidant enzyme SOD has an important role in maintaining physiological levels of free radicals by hastening the dismutation of free radicals and eliminating organic peroxides and hydro-peroxides (Pari & Latha, 2005). SOD has ability to directly neutralize a number of free radicals and reactive oxygen and nitrogen species, it stimulates several antioxidant enzymes that increase its efficiency as an antioxidant. Growth factor stimulation by platelet-derived, epidermal and insulin-like growth factors results in an increase in intracellular reactive oxygen species (Sauer *et al*., 2001). This reactive oxygen species production can inactivate phosphatases at the cell membrane (Meng *et al*., 2002), activate kinases and transcription factors (Sauer *et al*., 2001) leading to cell cycle progression. An increase in reactive oxygen species concentration leads to a decrease in SOD activity (Gajewska & Sklodowska, 2007). Previous study proved that over expression of copper/zinc-superoxide dismutase in transgenic mice markedly impairs regeneration and increases development of neuropathic pain after sciatic nerve injury (Kotulska *et al*., 2006). Another report says that induction of DNA synthesis and cell proliferation was attained by decreased SOD expression (Shibanuma *et al*., 1988). Our results also explained a decrease in SOD gene expression and activity in all partially hepatectomised rats which emphasized an increase in cell proliferation. Our finding was supported by earlier reports (Havens *et al*., 2006; Love *et al*., 2013; Park *et al*., 2012).

**Brain**

Free radicals represent a class of biologically generated species that pose a potential threat to neuronal survival. Superoxide dismutase (SOD) is the key cellular antioxidant enzymes by which neurons and other cells detoxify free radicals and protect themselves from damage. Increase in SOD activity could be due to its induction by increased production of superoxide ($O_2^-$) which has been implicated in cell dysfunction (Wiseman & Halliwell, 1996). Oxidative stress has been a common pathogenic mechanism underlying many major psychiatric disorders, such as anxiety, due to the intrinsic oxidative vulnerability of the brain (Ng *et al*., 2008). Growing evidences have suggested correlation between the imbalance of antioxidant defense mechanism and anxiety-like behaviour (Souza, *et al*., 2007; Rammal *et al*., 2008; Bouayed *et al*., 2009; Salim *et al*., 2010a; Salim *et al*., 2010b). Therefore, the role and the beneficial effects of antioxidants against various disorders and diseases induced by oxidative stress have received much attention (Fortes *et al*., 2013). Earlier study reports animals treated with SOD demonstrated greater survival than those with saline control and later regained most vital neurological functions in patients affected with stroke (Reddy & Labhasetwar, 2009). Another research explained that specific activity of antioxidant enzyme and mRNA expression of SOD was decreased in brain regions of polychlorinated biphenyls exposed animals that led to neuronal damages in all the brain regions (Venkataraman *et al*., 2010) and altered major neurotransmissions (Pratheepakumari *et al*., 2011). Oxygen radicals are also involved in cell death/survival signalling pathways (Liu *et al*., 1998; Lewen *et al*., 2000; Chan, 2001; Sugawara *et al*., 2002). In the present work, SOD expression in the cerebral cortex, corpus striatum and brain stem were increased considerably in rats treated with a combination of GABA and 5-HT chitosan nanoparticles compared to those with individual treatment of GABA and 5-HT chitosan nanoparticles and the one with no treatment. This emphasized oxidative stress occurred in brain regions due to unbalanced entry of metabolites during liver

injury and regaining of cellular free radical homeostasis through active liver cell proliferation by current treatment.

**IGF-1 expression in liver and brain regions of experimental rats**

IGF-1 is a hormone similar in molecular structure to insulin. It plays an important role in childhood growth and continues to have anabolic effects in adults. A synthetic analog of IGF-1, mecasermin is used for the treatment of growth failure. IGF-1 is produced primarily by the liver as an endocrine hormone as well as in target tissues in a paracrine/ autocrine fashion. Production is stimulated by growth hormone (GH) and can be retarded by under nutrition, growth hormone insensitivity, lack of growth hormone receptors, or failures of the downstream signalling pathway post GH receptor (Arnaldez *et al*., 2012). Its primary action is mediated by binding to its specific receptor, the insulin- like growth factor 1 receptor, present on many cell types in many tissues. Binding to the IGF-1R, a receptor tyrosine kinase, initiates intracellular signalling. IGF-1 is one of the most potent natural activators of the Akt signalling pathway, a stimulator of cell growth and proliferation, and a potent inhibitor of programmed cell death.

**Liver**

Hepatocyte proliferation is regulated by different mitogens, including hepatocyte growth factor (HGF) and ligands of the epidermal growth factor (EGF) and fibroblast growth factor receptors. In addition, recent studies revealed a role of insulin-like growth factor- 1 (IGF-1) and its receptor in hepatocyte proliferation after hepatectomy (Pennisi *et al.*, 2004; Desbois-Mouthon *et al.*, 2006). Apart from liver, insulin-like growth factor-I is also involved in the regeneration of tissues in other parts of the body like intestine (Donovan *et al*., 2004). Hepatocytes exhibit a mitogenic response to various growth factors and cytokines. IGF-1 is an important growth factor required for liver regeneration. IGF-1 is a genetically related polypeptide similar to insulin with similar three-dimensional and primary structures. IGF-1 is synthesized primarily in the liver and also in the brain. Its

synthesis is regulated by growth hormone, insulin and nutritional intake (Mathews *et al*., 1988). The growth-promoting effects of growth hormone can be direct in selected target tissues, such as liver, or indirect, via its endocrine mediator IGF-1. Growth hormone is the primary regulator of IGF-1 synthesis and secretion in hepatocytes. In turn, IGF-1 regulates growth hormone secretion through a classical negative feedback loop (Daughaday and Rotwein, 1989). Desbois-Mouthon *et al*., (2006) reported that a delayed liver regeneration was observed in liver-specific IGF type 1 receptor knockout partially hepatectomised mice. In GABA and 5-HT chitosan nanoparticle treatment the IGF-1 expression was increased compared to other single neurotransmitter treatments. Our observations also supported the earlier reports regarding the importance of IGF-1 in liver cell proliferation. Researchers proposed that the reduction in insulin/IGF-1 signalling is at least partially responsible for the regeneration defect (Beyer *et al*., 2008). This assumption is consistent with the delayed liver regeneration seen in IGF-1R-deficient animals (Desbois-Mouthon *et al*., 2006). The impaired activation of the IR and the reduced tyrosine phosphorylation of IRS-1 are likely to be responsible for the reduced phosphorylation of Akt and p38, since Tyr608 phosphorylation of IRS-1 is crucial for the activation of both signalling pathways (Cheng and Feldman, 1998; Bloch-Damti *et al.*, 2006).

**Brain regions**

Insulin-like growth factor-1 (IGF-1) protects neurons from apoptosis. Signalling through the insulin/IGF-1 pro-survival pathway is widely recognized to be neuroprotective as well as important for neuronal growth and physiology. The insulin-like growth factor 1 receptor is essential for axonal regeneration in adult central nervous system neurons (Dupraz *et al*., 2013). Therapeutic administration with neurotrophic proteins (IGF-I) is associated with potential reversal of degeneration of spinal cord motor neuron axons in certain peripheral neuropathies (Lewis *et al*., 1993). It offers neuroprotective support to hippocampal CA1 pyramidal neurons following ischemia or seizure (Wine *et al*., 2009). Another study reported that Akt is activated by trophic factors, IGF-1, through the

generation of the lipidic second messenger phosphatidylinositol 3-phosphate by phosphatidylinositol 3-kinase (PI3K). The protein kinase Akt acts as a node, playing a critical role in controlling cell survival and cell cycle progression (Aburto *et al*., 2012). In our study also IGF-1 and Akt-1 expressions were increased prominently in partially hepatectomised rats treated with GABA and 5-HT (in combination) chitosan nanoparticles. This explained the increased neuronal survival achieved with the help of present treatment. Another report supported the above fact that insulin-like growth factor-1 (IGF-I) specifically enhances the extent and rate of murine corticospinal motor neurons axonal outgrowth, mediated via the IGF-I receptor and down stream signalling pathways (Ozdinler & Macklis, 2006). Thus increased expression of IGF-1 in cerebral cortex, corpus striatum and brain stem promoted neuronal survival in rats with actively regenerating liver.

**Apoptotic factors in liver and brain regions**

Apoptosis of cells was clarified by studying the gene expression of Caspase-8 and Bax. Apoptosis is achieved by two major apoptotic pathways (extrinsic and intrinsic). The extrinsic pathway involves the binding of cytokines to death receptors, activation of caspase-8 and cleavage and activation of effector caspase-3 (Ashkenazi & Dixit, 1998). Caspase-8 is a key factor uniquely associated with this pathway. The intrinsic pathway involves translocation of Bax protein from the cytosol to the outer mitochondrial membrane, where it increases membrane permeability and promotes release of cytochrome c, which binds with apoptotic protease activating factor-1 (Apaf-1) and procaspase-9, resulting in its cleavage to form activated caspase-9 (Green & Reed, 1998).

**Bax expression in liver and brain regions**

Bax is a pro-apoptotic protein allowing apoptosis to occur through the intrinsic, damage-induced pathway and amplifying that one occurring *via* the extrinsic, receptor mediated pathway. Bax is present in viable cells and is activated by pro-apoptotic stimuli. Bax has multiple functions: it releases different mitochondrial factors such as cytochrome c, SMAC/diablo; it regulates

mitochondrial fission, the mitochondrial permeability transition pore; it promotes $Ca^{2+}$ leakage through ER membrane (Ghibelli & Diederich, 2010). Cytosolic Bax translocates to mitochondria upon death stimulus, promoting cytochrome c release (Gross *et al*., 1998). Bax mediated cell death is related to mitochondrial permeability transition (Jin & El-Deiry, 2005). The expression of proapototic protein Bax can be taken as an index of cell death. The present study observed a significant down regulation of Bax expression in the liver, cerebral cortex, brain stem and corpus striatum of partially hepatectomised rats. Bax is one of the key proteins that turn on the apoptotic cascade. The Bcl-2 family of proteins is an important determinant of apoptotic cell death. It consists of pro-apoptotic (Bax, Bcl- Xs, Bak and Bad) and anti-apoptotic (Bcl-2, Bcl-XL and Bcl-w) proteins (Adams & Cory, 1998). Bcl-2 family members determine cell death and survival by controlling mitochondrial membrane ion permeability, cytochrome *c* release and the subsequent activation of caspase (caspase 3, caspase 9) executor functions (Allen *et al.*, 1998; Banasiak *et al.*, 2000; Glasgow & Perez-Polo, 2000). Bax homodimers facilitate mitochondrial release of cytochrome c via a process requiring Bax translocation to mitochondria (Crompton, 2000). Thus, in GABA and 5-HT chitosan nanoparticles treated group, a prominent and significant decrease in Bax expression was observed in liver and brain regions, which denoted increased cell proliferation in liver and neuronal maintenance in partially hepatectomised rats.

There are evidences explaining that increase in GABA and 5-HT contents activates apoptotic signalling. Anju *et al*., (2011) reported that a trigger in the altered transcription of $GABA_B$ receptors can be related to the activation of apoptotic pathways by activating Bax expression. Serotonin also activates the mitogen- activated protein kinase that can influence the cell apoptosis (Watts, 1996). Active liver cell proliferation regains the capacity to metabolise aromatic amino acids and ammonia. Thus, decreased expression of GABA and 5-HT in the brain regions suppresses the MAPK phosphorylation and increases neuronal survival in partially hepatectomised rats. In our study, Bax expression was

decreased in all the treatment groups when compared to that with no treatment group.

**Caspase-8 expression in liver and brain regions of experimental rats**

JNK-family of Mitogen-activated protein kinase (MAPK) pathway has been observed to play a central role in both of these apoptotic pathways (Dhanasekaran & Reddy, 2008). MAPK can be phosphorylated by mitogens binding to G-protein-coupled receptors (Yagle *et al*., 2001). Due to the disturbed ammonia and aromatic amino acid metabolisms in partially hepatectomised rats, GABA and 5-HT contents in the brain gets increased, which in turn elevates their respective receptors mediated signalling and MAPK phosphorylation. JNKs activates apoptotic signalling either through the up regulation of pro-apoptotic genes via the transactivation of specific transcription factors including c-Jun or by directly modulating the activities of mitochondrial pro- and anti-apoptotic proteins through phosphorylation events. There is also an evidence for crosstalk between the Bax-regulated intrinsic mitochondrial pathway and the extrinsic death receptor pathway, in which activated caspase-8 is a key enzyme (Li *et al*., 1998). Active Caspase-8 further activates other Caspases like Caspase-3 and result in DNA fragmentation (Kuwana *et al*., 1998). Thus, reduction in caspase-8 expression showed decreased cell death in GABA and 5-HT chitosan nanoparticles treated partially hepatectomised rats.

Apoptosis and necrosis are the most widely recognized forms of hepatocyte cell death. The hepatocyte displays many unique features regarding cell death by apoptosis. Caspase-8 small interfering RNA prevents acute liver failure in mice was also reported (Zender *et al*., 2003). It is quite susceptible to death receptor-mediated injury and its death receptor signalling pathways involve the mitochondrial pathway for efficient cell killing. Also, death receptors can trigger lysosomal disruption in hepatocytes which further promotes cell and tissue injury. The hepatocyte apoptosis induced by TNF-α is correlated with the activation of caspases (Wang *et al*., 2003). From our observation, the TNF-α and caspase-8 expressions were down regulated considerably in nanoparticles treated

group compared to the one with no treatment. This emphasizes the reduction in apoptosis and enhanced liver cell proliferation.

**Behavioural studies**

Metabolic cease results in the provocation of several pathogenic factors that affects the motor control centre of brain. Elevated metabolite influx to brain during liver injury leads astrocytic swelling, tissue swelling, and neuronal toxicity in cerebral tissues (Back *et al*., 2011). These potential pathogenic factors include a direct neurotoxic effect of ammonia, oxidative stress caused by generation of reactive oxygen species, endogenous benzodiazepine-like ligands, subclinical intracellular astrocytic edema, GABA like molecules that act as GABA agonists, abnormal histamine and serotonin neurotransmission, endogenous opiates, neurosteroids, inflammatory cytokines, and potential manganese toxicity. Earlier study reported that metabolic alterations due to physiological disorders will affect the motor control seat of the brain, corpus striatum lead to motor deficits (Sherin *et al*., 2012). Neuronal death and altered neurotransmissions lead to motor control deficits in animals, which is a serious issue. The condition is marked by both altered intellectual function and emotion, as well as disturbed psychomotor and behavioural regulation (Jones & Gammal, 1988). The spectrum of hepatic encephalopathy varies from mild intellectual impairment to deep coma and includes manifestations of motor dysfunction, especially extrapyramidal signs and asterixis (Jones & Weissenborn, 1997). Striatal neurons in association with cerebellar innervations execute planning and coordination of motor action as reported by Laforce and Doyon (2001). Striatal neuronal degeneration also leads to motor deficits, which is similar to that in Huntington's disease (Mattson, 2000). Over activation of glutamate receptors due to disturbances in GABA content damage the neurons leading to impairment in the motor function and co-ordination in hyperglycaemic rats (Anu *et al.,* 2010). In our study, the animals' ability to retain on the rolling platform of rotarod, cross the narrow beam and walk along the grid with minimum foot slips were observed increasing in the nanoparticle treated groups compared to the partially hepatectomised rats with no treatment. This

inferred the fact that motor control in liver injured rats were collapsed due to neuronal damage in corpus striatum and was minimised with active liver cell proliferation followed by maintenance of normal levels of potential pathogenic factors.

Earlier studies, from our laboratory have proved the functional regulation of the central neurotransmitter receptor subtypes during hepatocyte proliferation and pancreatic regeneration (Balasubrahmanian & Paulose, 1998; Biju *et al*., 2002; Sulaiman, *et al*., 2008; Mohanan *et al.*, 2005; Kaimal *et al.*, 2007; Anu *et al*., 2010; Anitha *et al*., 2012; Sherin *et al*., 2012, Jayanarayanan *et al*., 2013; Kumar *et al*., 2013). The neurotransmitter receptor alterations by GABA and 5-HT treatment in spinal cord injury (Romeo *et al*., 2013) and Parkinson's disease (Nandhu *et al*., 2011; Paul *et al*., 2011; Kuruvilla *et al.*, 2013) rat models were also reported from our laboratory. The present study discussed the $GABA_B$ and 5-$HT_{2A}$ receptors functional regulation in brain and liver of partially hepatectomised rats treated with GABA and 5-HT chitosan nanoparticles.

Brain and liver functions are interrelated. Liver is the only organ in the adult that regenerate after a considerable loss of cells. Thus metabolism of the body with injured liver gets disturbed and leads to motor deficits, neuropsychiatristic and mood alterations. So a therapeutic system that favouring hepatocyte proliferation in damaged liver along with the neuronal protective effect gains immense importance. The present work revealed the potential of GABA and 5-HT chitosan nanoparticle treatment in enhancing liver cell proliferation and improving neuronal survival by reducing neuronal apoptosis in the brain after partial hepatectomy. We proposed that the foresaid supplementation in combination will have better implication against the neuronal loss in brain regions than individual treatment during liver injury. It was evident that GABA and 5-HT chitosan nanoparticles treatment in partially hepatectomised rats renders neuronal protection which will have therapeutic significance in the management of liver based diseases.

# *Summary*


1. GABA and 5-HT, both individually and in combination, were encapsulated in chitosan nanoparticles by ionic gelation method. Spherical particles with 80 nm size were obtained. The interaction between the amino group of chitosan with the carboxyl group of GABA and sulphate group of 5-HT were studied by FT-IR spectroscopy.
2. Encapsulation efficiency in coupling GABA and 5-HT with chitosan nanoparticles were examined to obtain maximum entrapment of neurotransmitters with the nanoparticles.
3. Uptake of fluorescently labelled GABA and 5-HT chitosan nanoparticles by hepatocytes were confirmed by confocal microscope.
4. Partially hepatectomised rats were used as a model to study the liver regeneration by GABA and 5-HT chitosan nanoparticles.
5. Liver cell proliferation in partially hepatectomised rats treated with and without nanoparticles was assessed by quantifying DNA and protein syntheses using [$^3$H] thymidine and [$^3$H] leucine uptake studies *in vitro*. Thymidine kinase activity in the cells of regenerating liver was also studied. DNA and protein syntheses were increased by individual treatment with GABA or 5-HT chitosan nanoparticles and a prominent increase was observed with a combination of GABA and 5-HT chitosan nanoparticles.
6. The incorporation of thymidine analogue BrdU was increased in the partially hepatectomised rats treated with a combination of GABA and 5-HT chitosan nanoparticles compared to the rats with single treatment of GABA or 5-HT chitosan nanoparticle and the one with no treatment. This confirmed that combination of GABA and 5-HT chitosan nanoparticles increased DNA synthesis, which is a part of rapid cell proliferation in the regenerating liver.

7. $GABA_B$ receptor functional status was analysed by Scatchard analysis using [$^3$H] baclofen. $GABA_B$ receptor number was decreased in regenerating liver of partially hepatectomised rats treated with a combination of GABA and 5-HT chitosan nanoparticles compared to the rats with single treatment of GABA or 5-HT chitosan nanoparticle and the one with no treatment. This favoured an increased cellular signalling for active liver cell proliferation.
8. $GABA_B$ receptor binding parameters were confirmed by studying the mRNA expression of the corresponding receptor subtype using Real Time PCR. The receptor expression was decreased in partially hepatectomised rats treated with a combination of GABA and 5-HT chitosan nanoparticles compared to the rats with single treatment of GABA or 5-HT chitosan nanoparticle and the one with no treatment.
9. Confocal microscopic imaging of $GABA_B$ receptors in the liver sections showed decreased mean pixel intensity for those obtained from the groups treated with both GABA and 5-HT chitosan nanoparticles compared to GABA or 5-HT chitosan nanoparticles treatment individually and the one with no treatment.
10. $5\text{-}HT_{2A}$ receptor functional status was analysed by Scatchard analysis using [$^3$H] ketanserin. $5\text{-}HT_{2A}$ receptor number was increased in rats treated with GABA and 5-HT chitosan nanoparticles individually compared to that with no treatment. A prominent increase was observed in the rats treated with a combination of GABA and 5-HT chitosan nanoparticles.
11. $5\text{-}HT_{2A}$ receptor binding parameters were confirmed by studying the mRNA expression of the corresponding receptor subtype using Real Time PCR. The receptor expression was increased by individual treatment of GABA or 5-HT chitosan nanoparticles and a prominent increase was observed in the groups with both GABA and 5-HT chitosan nanoparticles treatment.

12. Confocal microscopic imaging of 5-$HT_{2A}$ receptors in the liver sections showed increased mean pixel intensity for partially hepatectomised rats treated with a combination of GABA and 5-HT chitosan nanoparticles compared to the rats with single treatment of GABA or 5-HT chitosan nanoparticle and the one with no treatment.
13. cAMP, $IP_3$ contents, CREB and phospholipase C expressions were decreased in the actively regenerating liver of partially hepatectomised rats treated with a combination of GABA and 5-HT chitosan nanoparticles compared to the rats with single treatment of GABA or 5-HT chitosan nanoparticle and the one with no treatment. This confirmed a delayed signalling through protein kinase A and C mediated signalling cascade in S phase of cell cycle.
14. NF-κB, TNF-α, and Akt-1 were involved in the subsequent activation of downstream transcription cascades, which effect the transition of the quiescent hepatocytes to the active cell multiplication phase. The gene expressions were decreased in the rats with individual treatment of GABA and 5-HT chitosan nanoparticles and a prominent decrease was observed with a combination of GABA and 5-HT chitosan nanoparticles.
15. SOD activity was assessed by gene expression and enzyme activity studies. The SOD expression was decreased in the regenerating liver of partially hepatectomised rats treated with a combination of GABA and 5-HT chitosan nanoparticles compared to the rats with single treatment of GABA or 5-HT chitosan nanoparticle and the one with no treatment. The active cell proliferation was assisted with increased ROS content and decreased SOD.
16. Apoptotic factors like Bax and caspase-8 were decreased prominently in partially hepatectomised rats treated with a combination of GABA and 5-HT chitosan nanoparticles compared to the rats with single treatment of GABA or 5-HT chitosan nanoparticle and the one with no treatment. This decrease was due to trigger in cell proliferation rather than death.

17. Rapid DNA synthesis prior to mitosis resulted in decreased DNA methylation. [$^{3}$H] Methyl group incorporation, *in vitro* was increased in the liver DNA of rats treated with GABA and 5-HT, indiviually and in combination compared to that with no treatment. MAT2A, the enzyme required for the synthesis of methyl donor, was also decreased and supported active regeneration by GABA and 5-HT chitosan nanoparticles.
18. Hepatocyte growth factor (HGF) is the most potent stimulator of hepatocyte growth and DNA synthesis identified. The HGF gene expression was increased 24 hours post hepatectomy in GABA and 5-HT chitosan nanoparticles treatment compared to the other groups. The gene expression was decreased seventh day post hepatectomy in the rats treated with nanoparticles compared to the one with no treatment. When the liver gains complete mass after regeneration, hepatocyte growth factor expression was decreased considerably.
19. Liver cell proliferation is initiated and progressed by the combined effect of growth factors. Real Time PCR amplification of Insulin like growth factor-1 mRNA in the liver of experimental rats were increased in the rats treated with a combination of GABA and 5-HT compared to the individual treatment of GABA or 5-HT chitosan nanoparticles.
20. $GABA_B$ and $5\text{-}HT_{2A}$ receptors binding in the cerebral cortex, corpus striatum and brain stem were decreased by combination of GABA and 5-HT chitosan nanoparticles compared to individual GABA or 5-HT chitosan nanoparticles. The active liver cell proliferation by nanoparticles restored GABAergic and serotonergic neurotransmission compared to the regeneration without treatment.
21. $GABA_B$ and $5\text{-}HT_{2A}$ receptors binding parameters in the cerebral cortex, corpus striatum and brain stem were confirmed by studying the mRNA expression of the corresponding receptor subtype using Real Time PCR. Both receptors in the brain regions of rats treated with a combination of GABA and 5-HT chitosan nanoparticles showed a decreased expression

compared to individual treatment with GABA or 5-HT chitosan nanoparticles. The active liver cell proliferation by nanoparticles restored GABAergic and serotonergic neurotransmission in brain regions compared to the regeneration without treatment.

22. The confocal microscopic imaging of $GABA_B$ and $5\text{-}HT_{2A}$ receptors in the brain sections showed decrease in mean pixel intensity for partially hepatectomised rats treated with a combination of GABA and 5-HT chitosan nanoparticles compared to the rats with single treatment of GABA or 5-HT chitosan nanoparticle and the one with no treatment.
23. cAMP, $IP_3$ contents, CREB and phospholipase C gene expressions were decreased in the brain regions like cerebral cortex, brain stem and corpus striatum of partially hepatectomised rats treated with a combination of GABA and 5-HT chitosan nanoparticles compared to the rats with single treatment of GABA or 5-HT chitosan nanoparticle and the one with no treatment. Disturbed serotonergic and GABAergic neurotransmissions resulted from the over influx of improper metabolism of compounds during liver injury altered these parameters. G-protein mediated signalling was decreased in the GABA and 5-HT chitosan nanoparticles treated rats compared to the rats with no treatment, which restored the normal neuronal activity in liver injured rats.
24. NF-κB, TNF-α and Akt-1 expressions showed a significant increase in the corpus striatum, cerebral cortex and brain stem of all partially hepatectomised rats. Partial hepatectomy induces activations of factors responsible for neuronal survival. GABA and 5-HT chitosan nanoparticles, individually and in combination, activated these genes prominently compared to PHNT.
25. Decreased gene expression of apoptotic factors like Caspase-8 and Bax in cerebral cortex, corpus striatum and brain stem was observed in all partially hepatectomised rat groups due to reduction in neurodegeneration by apoptosis. Treatment with 5-HT and GABA chitosan nanoparticles in

combination increased gene expressions of caspase-8 and Bax compared with either GABA chitosan or 5-HT chitosan nanoparticle treatment. This confirmed a decreased apoptosis of neurons in the brain regions due to improper influx of metabolites and altered neurotransmission.

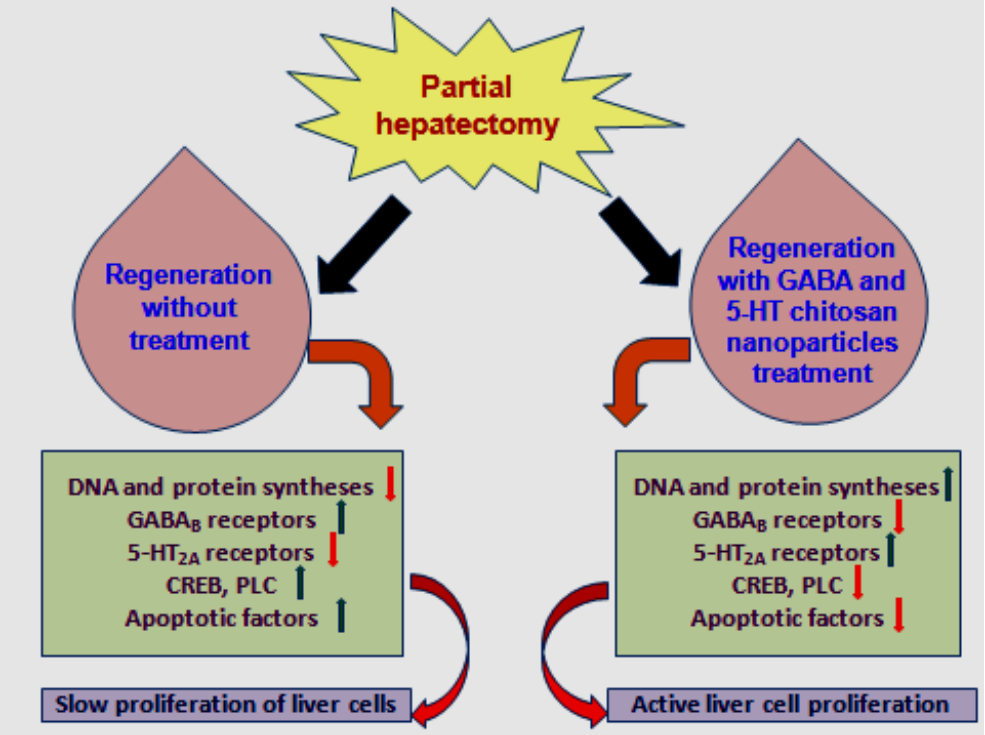
26. Neurotrophic factors BDNF and GDNF showed a significant up regulation in the corpus striatum, cerebral cortex and brain stem of all partially hepatectomised rats. Combined treatment with 5-HT and GABA encapsulated chitosan nanoparticles increased the expression of these neurotrophic factors considerably compared to individual treatment with GABA or 5-HT chitosan nanoparticles. This suggested an increase in neuronal survival achieved through GABA and 5-HT chitosan nanoparticles.
27. cGMP content and IGF-1 expression were related to neuronal survival. cGMP content was decreased and IGF-1 expression was up regulated in corpus striatum, cerebral cortex and brain stem of all partially hepatectomised rats. The neuronal survival conditions favoured by cGMP and IGF-1was high in the rats treated with a combination of GABA and 5-HT chitosan nanoparticles compared to that with individual treatment of GABA or 5-HT chitosan nanoparticles treatment.
28. Behavioural studies: Partial hepatectomy induced behavioural deficits in rats due to disturbed neurotransmission and neuronal death in brain. The rats treated with a combination of GABA and 5-HT chitosan nanoparticles showed a better capability to retain on the rotating rod, walk along the grid and cross narrow beam compared to the rats with individual treatment of GABA or 5-HT chitosan nanoparticle.

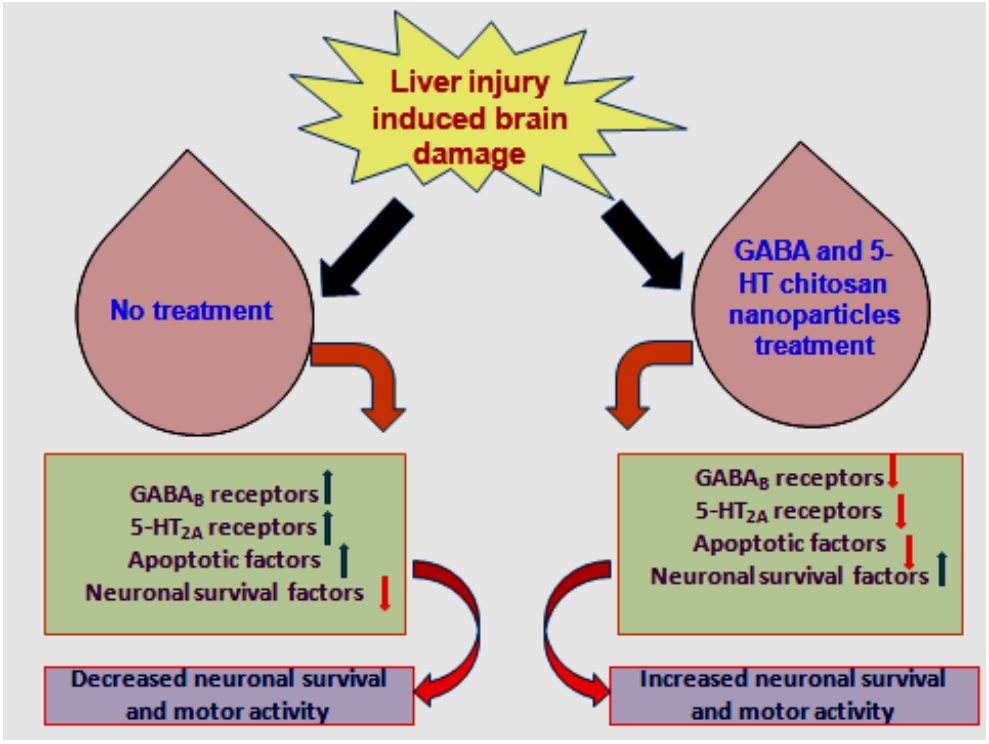
Our results thus showed that $GABA_B$ and $5\text{-}HT_{2A}$ receptors functional regulation and modulation of apoptosis and growth factors plays a critical role for enhancing liver cell proliferation that achieved through GABA and 5-HT chitosan nanoparticles. Gene expression studies of $5\text{-}HT_{2A}$ and $GABA_B$ receptors subunits

showed a prominent GABAergic and serotonergic dysregulation in neurotransmission in brain regions of partially hepatectomised rats. Partially hepatectomised rats slowly tried to restore the neuronal activity without any treatment, whereas GABA and 5-HT chitosan nanoparticles triggered the neuronal survival mechanisms and restored motor co-ordination rapidly. The findings from this study gives insight on understanding the molecular mechanisms underlying liver cell proliferation and maintaining routine neuronal functions in rats with actively regenerating liver. A combination of GABA and 5-HT encapsulated in chitosan nanoparticles showed functional recovery from liver injury mediated tissue loss and neuronal death that is of immense therapeutic significance in the management of liver diseases.

# *Conclusion*

Liver regeneration is a compensatory hyperplasia and hypertrophy that occurs as a response to viral or toxic liver injury or hepatic resection. The ability of hepatocytes to undergo cellular growth and proliferation during regeneration, while continuing to carry out their metabolic tasks, makes possible a relatively gradual restoration of the delicate homeostatic equilibrium even after serious insult to the liver. Disturbances in metabolism result in neurological dysfunctions and structural changes in the CNS. Thus achievement of enhanced liver cell proliferation with simultaneous neuronal maintenance by GABA and 5-HT chitosan nanoparticles is the present focus. The proliferative signalling through $GABA_B$ and $5\text{-}HT_{2A}$ receptors associated second messengers and transcription factors activated liver cell division. Our study showed a decreased expression of apoptotic factors - Bax and caspase-8 in both liver and brain regions, supported active liver cell multiplication and neuronal maintenance. Increased expression of growth factors - HGF and IGF-1 confirmed enhanced cell proliferation in injured liver. Restoration of GABAergic and serotonergic neurotransmissions in cerebral cortex, corpus striatum and brain stem was observed in the rats treated with GABA and 5-HT chitosan nanoparticles. Neuronal survival mechanisms in brain regions were activated with progressive liver cell proliferation associated restoration of metabolic functions. This was studied by examining the expression patterns of NF-κB, TNF-α, Akt-1, IGF-1, BDNF and GDNF. Functional disturbances in neurons of corpus striatum affected motor coordination in partially hepatectomised rats. The rotarod, grid walk and narrow beam tests gave a clear idea of regaining motor control in rats treated with GABA and 5-HT chitosan nanoparticles. Thus our results conclude the regenerating ability of GABA and 5-HT chitosan nanoparticles and successive increase in neuronal survival which has a novel therapeutic role in the management of liver diseases.


Partial hepatectomy
Regeneration without treatment
Regeneration with GABA and 5-HT chitosan nanoparticles treatment
DNA and protein syntheses ↓
GABA$_B$ receptors ↑
5-HT$_{2A}$ receptors ↓
CREB, PLC ↑
Apoptotic factors ↑
Slow proliferation of liver cells
DNA and protein syntheses ↑
GABA$_B$ receptors ↓
5-HT$_{2A}$ receptors ↑
CREB, PLC ↓
Apoptotic factors ↓
Active liver cell proliferation


Liver injury induced brain damage
No treatment
GABA and 5-HT chitosan nanoparticles treatment
GABA$_B$ receptors ↑
5-HT$_{2A}$ receptors ↑
Apoptotic factors ↑
Neuronal survival factors ↓
GABA$_B$ receptors ↓
5-HT$_{2A}$ receptors ↓
Apoptotic factors ↓
Neuronal survival factors ↑
Decreased neuronal survival and motor activity
Increased neuronal survival and motor activity

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2. **Shilpa J**, Roshni BT, Chinthu R, Paulose CS. (2012). Role of GABA and serotonin coupled chitosan nanoparticles in enhanced hepatocyte proliferation. J Mater Sci Mater Med. 23(12):2913-21.

3. **Shilpa J**, Anitha M, Paulose CS. (2013). Increased neuronal survival in the brain stem during liver injury : Role of GABA and 5-HT chitosan nanoparticles. J. Neorosci. Res., DOI: 10.1002/jnr.23233.

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5. **Shilpa J,** Anju TR, Ajayan MS, Paulose CS. (2013). Increased cortical neuronal survival during liver injury: Effect of GABA and 5-HT chitosan nanoparticles. Journal of Biomedical Nanotechnology (Accepted for publication).

6. Abraham PM, Kuruvilla KP, Mathew J, **Joy S**, Paulose CS. (2010). Alterations in hippocampal serotonergic and INSR function in streptozotocin induced diabetic rats exposed to stress: neuroprotctive role of pyridoxine and Aegle marmelose. J Biomed Sci. 17:78 1-15.

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**Abstracts Presented**

1. **Shilpa Joy**, Nandhu M. S. & C. S. Paulose. GABA - Chitosan nanoparticles induced hepatocyte proliferation in partially hepatectomised

rats. Innovations in Biotechnology, SRM University, Chennai, October, 2010.

2. Malat Anitha, Abraham Pretty Mary, **Joy Shilpa** and C. S. Paulose. Down regulation of serotonin receptors and its transporter expression in the pancreas of streptozotocin induced diabetic rats: Effect of pyridoxine and *Aegle marmelose.* Indian Ageing congress held at Banaras Hindu University (BHU), Varanasi, India 12-14 November, 2010.
3. **Shilpa Joy**, Anitha. M, C. S. Paulose. $GABA_{B,}$ cAMP AND CREB functional regulation in partially hepatectomised rats. GABA-Chitosan nanoparticles induced hepatocyte proliferation. Kerala science congress, January, 2011.
4. **Shilpa Joy**, Roshni Baby Thomas, C. S. Paulose . SOD, Bax and MAT 2A functional regulation in partially hepatectomised rats : gaba - chitosan nanoparticles induced hepatocyte proliferation. Cochin Nano, August, 2011.
5. Roshni Baby Thomas, **Shilpa Joy**, Naijil George, C.S. Paulose. "Dopamine D1 Receptor Down Regulation And Enhanced Bax Expression In Neonatal Hypoglycemic Rat Cerebral Cortex: Glucose, Bacoside A , B*acopa Monnieri* – Resusitation and Functional Recovery" . National seminar on emerging trends in Biotechnology and Annual meeting of Society for Biotechnologists, India , Acharya Nagarjuna University, Guntur, september 24 – 26, 2011.

**6.** Roshni Baby Thomas,T.R.Anju, **Shilpa Joy** and C.S.paulose. Role of Curcumin as a Nutritional Supplement to Enhance Cell Proliferation and survival in hypoxia Induced Hepatocyte Injury-*in vitro* model. (2012), $I^{st}$ Edition, Bloomsbury Publishing India Pvt.Ltd.ISBN:978-93-82563-27-3.

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# Evaluation of GABA-Chitosan Nanoparticle Induced Cell Signaling Activation During Liver Regeneration After Partial Hepatectomy

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Liver damage due to infection, cirrhosis, accidents and diseases lead to destruction of hepatocytes and their regeneration to its original form is important for the proper functioning of the body. Gamma aminobutyric acid (GABA), a neurotransmitter, was coupled with a biopolymer chitosan and the nanosized complexes were made. The morphology was studied by scanning electron microscope and the interaction of GABA with chitosan was analysed by FT-IR spectroscopy. The interaction of GABA-chitosan nanoparticles with hepatocytes were observed by FITC labeled nanoparticles. After partial hepatectomy in male Wistar rats, DNA synthesis was estimated by tritiated thymidine uptake and the activity of thymidine kinase and protein synthesis by tritiated leucine uptake in hepatocytes. There was an increase in tritiated thymidine uptake in partially hepatectomised groups with nanoparticle treatment (GCNP) when compared to partially hepatectomised groups without nanoparticle treatment (PHNT) and with pure GABA treatment (G). Inositol 1,4,5 trisphosphate ($IP_3$) content and gene expression of phospholipase C mRNA and nuclear factor kappa-light-chain-enhancer of activated B (NF-$\kappa$B) mRNA was decreased for groups G and GCNP with respect to PHNT. Thus our results showed increased hepatocyte regeneration with decreased cell death in group G and more better with GCNP when compared to PHNT.

**Keywords:** Biocompatible Material, Chitosan, GABA, Hepatocyte Proliferation, Nanoparticles, Cell Division.

RESEARCH ARTICLE

## 1. INTRODUCTION

Nanoparticulate drug delivery systems provide wide opportunities for solving problems associated with drug stability or disease states and create great expectations in the area of drug delivery.[1] Nanotechnology, in a simple way, explains the technology that deals with one billionth of a meter scale.[2] Fewer side effects, poor bioavailability, absorption at intestine, solubility, specific delivery to site of action with good pharmacological efficiency, slow release, degradation of drug and effective therapeutic outcome, are the major challenges faced by most of the drug delivery systems. To a great extend, biopolymer coated drug delivery systems coupled with nanotechnology alleviate the major drawbacks of the common delivery methods. Chitosan, deacetylated chitin, is a copolymer of $\beta$-(1,4) linked glucosamine (deacetylated unit) and *N*-acetyl glucosamine (acetylated unit).[3] Chitosan is biodegradable, non-toxic and bio compatible. Nanoparticles of chitosan coupled drugs are utilized for drug delivery in eye, brain, liver, cancer tissues, treatment of spinal cord injury and infections.[4–8] To deliver drugs directly to the intended site of action and to improve pharmacological efficiency by minimizing undesired side effects elsewhere in the body and decrease the long-term use of many drugs, polymeric drug delivery systems can be used.[9]

Gamma amino butyric acid (GABA) is a non proteinacious amino acid and is an important inhibitory neurotransmitter in the vertebrate central nervous system. Apart from the inhibitory role, it is reported that GABA involves in the cell proliferation in different regions of the body. The proliferative role of GABA was observed in the development of outer retina in rabbits,[10] TM3 Leydig cell multiplication in testis[11] and promotes neurite growth, cell proliferation and migration.[12] Baclofen, a GABA agonist, induced EGF mediated DNA synthesis in hepatocyte *in vitro*. There is an increase in hepatocyte proliferation through the activation of $GABA_B$ receptor. Also, it significantly reduced the TGF $\beta$1 suppression of EGF induced DNA synthesis. Thus the activation of GABA receptors, trigger DNA synthesis,

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mediated through the G protein, in primary cultures of rat hepatocytes.[13] The expression of the stimulatory and inhibitory $\alpha$-subunit of G proteins coupled receptors to the effector targets like adenylate cyclase cause biphasic increase in hepatic cAMP. The cell proliferation is initialized by the activation of cAMP regulated transcription factors and the phosphorylation of cAMP regulatory element binding protein which influence the induction of cAMP inducible genes in the regenerating liver.[14] GABA is synthesized from glutamate, which is decarboxylated by glutamate decarboxylase (GAD). The major catabolic route for GABA is transamination with alpha—ketoglutarate catalyzed by GABA transaminase. Succinic semialdehyde formed in this reaction is rapidly oxidized to succinate by succinic semialdehyde dehydrogenase. This is the major pathway of GABA metabolism and is termed the GABA shunt.[15] The succinate so formed is utilized in citrate cycle and promote the energy production.

The reduction in apoptosis enhances the regeneration of cells. The signals evoked by $IP_3$ mediated $Ca^{2+}$ ions in mitochondria triggers apoptosis. Phospholipase C (PLC) is the enzyme involved in the synthesis of $IP_3$ and thus the increased level of $IP_3$ and Phospholipase C result in enhanced apoptosis.[16] The triggering of protein kinase C occurs through $IP_3$ mediated signaling pathway, which further leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$\kappa$B). NF-$\kappa$B activates TNF-$\alpha$ mediated cell death.[17] Thus the suppression of NF-$\kappa$B promotes liver regeneration. Apoptosis was further studied by focusing the expression of Caspase-8 gene.

Nanoparticles of GABA entrapped in chitosan matrix were prepared for the delivery of GABA to partially hepatectomized liver. Normally the uptake of GABA from the serum by hepatocytes are occurred by a sodium dependent transporter system and the metabolism of GABA by GABA transaminase (GABA-T),[18] which is primarily associated with both plasma and mitochondrial membranes.[19] Therefore, the present study was undertaken to investigate the fastened tissue regeneration efficiency of GABA in a pharmacologically efficient way in partially hepatectomised rat model which certainly explain the therapeutic possibilities of GABA—chitosan nanoparticles for hepatocyte regeneration associated biochemical alterations in liver.

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## 2. EXPERIMENTAL DETAILS

### 2.1. Chemicals Used and Their Sources

Biochemicals, Tri-reagent kit was purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally. [$^3$H]Gamma amino butyric acid (Sp. Activity 76.2 Ci/mmol), [$^3$H] thymidine (Sp. Activity 18.0 Ci/mmol) and [$^3$H] leucine (Sp. Activity 63.0 Ci/mmol) was purchased from Amersham Life Science, UK. ABI PRISM High Capacity cDNA Archive kit, Primers and Taqman probes for Real-Time PCR were purchased from Applied Biosystems, Foster City, CA, USA. Chitosan (MW-25KDa) was a gift from Central Institute of Fisheries Technology, Cochin, India.

### 2.2. Animals

Experiments were carried out on adult male Wistar rats of 250–300 g body weight purchased from Kerala Agricultural University, Mannuthy, India. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water *ad libitum*. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health and CPCSEA guidelines.

### 2.3. Preparation of GABA-Chitosan Nanoparticles and Morphological Characterization

The chitosan nanoparticles were prepared by ionic gelation method.[20] Chitosan solution of 50 mL volume with concentration 1 mg/mL was prepared by dissolving chitosan in 2% acetic acid. The chitosan nanoparticles were precipitated from the solution by the addition of 33 mL of 1 mg/mL penta sodium tri polyphosphate (TPP) solution with vigorous stirring. To incorporate GABA in to chitosan, a solution of concentration 8.824 $\mu$g of GABA/mL of chitosan solution was prepared and the precipitation of GABA-chitosan nanoparticles were done by the above method. The precipitated nanoparticles were centrifuged at 16,000$\times$ g for 20 minutes. The pellet was washed with distilled water and then resuspended in saline. The SEM image of the nanoparticles was taken with a magnification of 20000$\times$ by scanning electron microscope (JEOL Model JSM-6390LV).

### 2.4. FT-IR Spectroscopy

The FT-IR spectrum of the GABA, chitosan, chitosan nanoparticles and GABA incorporated chitosan nanoparticles were taken using Fourier Transform Infra Red spectrometer (Thermo Nicolet, Avatar 370) with spectral range of 4000–400 cm$^{-1}$.

### 2.5. Determination of Encapsulation Efficiency and *In Vitro* Release of GABA

The maximum encapsulation efficiency[21] of GABA with chitosan nanoparticles was obtained by giving emphasis to concentration of GABA added to the chitosan solution and the duration of reaction between GABA and chitosan. The encapsulation efficiency was calculated by incorporating [$^3$H] GABA with chitosan and the radioactivity of the GABA, which was bound on the chitosan nanoparticles, were related to its concentration.[21] Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid

scintillation counter. % Encapsulation = (Concentration of GABA bound to chitosan nanoparticles/Concentration of GABA added initially) × 100. In *in vitro* release studies [$^3$H] GABA-chitosan nanoparticles were suspended in PBS, pH 7.4 and stirred gently. At different intervals of time from 0 to 40 hours, the concentration of released radioactive GABA at each time from the nanoparticles was calculated[22] to get a release profile *in vitro*.

### 2.6. Cell Uptake of GABA-Chitosan Nanoparticles

#### *2.6.1. Preparation of FITC Labeled Chitosan Nanoparticles[23]*

Chitosan solution (1 mg/mL), of volume 50 mL was prepared and the nanoparticles were precipitated by adding TPP. The nanoparticles were centrifuged and the pellet was resuspended in 5 mL DMSO and sonicated for 1 minute. Then a solution of 10 mg/mL FITC in DMSO was added to the nanoparticle suspension. Stirred the solution gently and kept overnight at dark. After stirring, particles were washed with DMSO several times until the non conjugated FITC was eliminated completely. The liver was perfused initially with $Ca^{2+}$ buffer, pH 7.4 (142 mM NaCl, 6.7 mM KCl, 10 mM HEPES and 5.5 mM NaOH) and then with collagenase buffer, pH 7.6 (67 mM NaCl, 6.7 mM KCl, 100 mM HEPES, 4.76 mM $CaCl_2 \cdot 2H_2O$, 0.66 mM NaOH and collagenase). The perfused liver was minced in PBS, pH 7.4 and kept for collagenase digestion. The cells were filtered and washed. Resuspended the cells in William's media and 150 $\mu$L of cell suspension (cell density of $1.6 \times 10^5$ cells/cm$^2$) was added to a four well glass slide. Then the cells were incubated in 5% $CO_2$ atmosphere for 24 hours at 37 °C.

#### *2.6.2. Uptake of FITC Labeled Nanoparticles by Hepatocytes[24]*

50 $\mu$L of FITC labeled and unlabelled nanoparticles were added to the corresponding cell suspension in each well and incubated for 2 hours. After the incubation, the fluorescent images were captured using confocal microscope with an excitation at 488 nm.

#### *2.6.3. Effect of GABA Encapsulated Chitosan Nanoparticles on DNA and Protein Synthesis in Hepatocytes*

Two–thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anesthesia, following a 16 hour fast.[25] Sham operations involved median excision of the body wall followed by all manipulations except removal of the lobes. All the surgeries were done between 7 and 9 A.M to avoid diurnal variations in responses. After surgery, 1 mL of 30 $\mu$g/$\mu$L GABA-chitosan nanoparticles and 0.26 mg/mL pure GABA (same concentration of GABA in GABA-chitosan nanoparticles) suspended in saline were injected intra peritoneal to the respective rats. Sham operated control (C), partially hepatectomised group without any treatment (PHNT), partially hepatectomised group with pure GABA treatment (G) and partially hepatectomised groups with GABA-chitosan nanoparticle treatment (GCNP) were the four experimental groups. The rats were sacrificed by decapitation 24 hours post hepatectomy.

The liver from the sham operated control and remaining liver from all the other three groups were perfused and cultured for 24 hours. Before incubation [$^3$H] thymidine of specific activity 18 Ci/mmol was added to one set of culture plates for all the four experimental groups to determine the measurement of DNA synthesis and [$^3$H] leucine of specific activity 63 Ci/mmol to the next set to determine the protein synthesis. All the experiments were done in triplicates. The cells were scrapped off from the culture plates and centrifuged at 2000× g for 20 minutes. The supernatant was discarded and the pellet was resuspended in 50 $\mu$L, 1 M NaOH and kept overnight. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. DNA synthesis was further determined by analyzing the activity of thymidine kinase (TK) in all the four experimental groups. A 10% liver homogenate was prepared in 50 mM Tris HCl buffer, pH 7.5. It was centrifuged at 36000× g for 30 minutes and the supernatant was collected. TK was assayed by determining the conversion of [$^3$H] thymidine in the presence of ATP to [$^3$H] thymidine monophosphate (TMP) by the binding of latter nucleotide to DEAE cellulose discs.[26] The reaction mixture contained 5 mM [$^3$H] thymidine (0.5 $\mu$Ci), 10 mM ATP, 100 mM NaF, 10 mM $MgCl_2$, 0.1 M Tris-HCl buffer, pH 8.0 and the liver supernatant fraction. After incubation at 37 °C for 15 minutes the reaction mixture was spotted in DE 81 paper discs. The bound radioactivity of [$^3$H] thymidine monophosphate was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

#### *2.6.4. Quantification of $IP_3$*

The liver was homogenised in a polytron homogeniser in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000× g for 15 min. and the supernatant was transferred to fresh tubes for $IP_3$ assay using [$^3$H] $IP_3$ Biotrak Assay System kit. The unknown concentrations were determined from the standard curve using appropriate dilutions and calculated for pmoles/mg protein. Protein was measured according to Lowry et al.[27] using bovine serum albumin as standard. The intensity of the purple blue color formed was proportional to the amount of protein which was read in a spectrophotometer at 660 nm. A standard curve was plotted with % B/Bo on the $Y$-axis and $IP_3$

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concentration (pmoles/tube) on the *X*-axis of a semi-log graph paper. % $B/B_o$ was calculated as:

$$\frac{(\text{Standard or sample cpm—NSB cpm})}{(B_0\text{cpm—NSB cpm})} \times 100$$

cpm-counts per minute, NSB-non specific binding and $B_0$-zero binding. $IP_3$ concentrations in the samples were determined by interpolation from the plotted standard curve.

#### *2.6.5. Analysis of Gene Expression by Real-Time Polymerase Chain Reaction*

RNA was isolated using Tri reagent. Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit. Real–Time PCR assays were performed in 96-well plates in ABI 7300 Real–Time PCR instrument (Applied Biosystems). PCR analyses were conducted with gene-specific primers and fluorescently labelled Taqman probe of phospholipase C, NF-$\kappa$B and Caspase 8 (designed by Applied Biosystems). Endogenous control, $\beta$-actin, was labeled with a report dye, VIC.

RNA was isolated from the liver of experimental rats using the Tri-reagent (MRC, USA) according to the procedure of Chomczynski P. Total cDNA synthesis was performed using ABI PRISM cDNA archive kit in 0.2 mL microfuge tubes. The reaction mixture of 20 $\mu$L contained 0.2 $\mu$g total RNA, 10XRT buffer, 25× dNTP mixture, 10× random primers, MultiScribe RT (50 U/$\mu$L) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 min and 37 °C for 2 h using an Eppendorf Personal Cycler. Real-time-PCR assays were performed in 96-well plates in an ABI 7300 Real-time-PCR instrument (Applied Biosystems). The specific primers and probes were purchased from Applied Biosystems, Foster City, CA, USA. The TaqMan reaction mixture of 20 $\mu$L contained 25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer and TaqMan probe for assay on demand and endogenous control $\beta$-actin and 12.5 $\mu$L of Taqman 2× Universal PCR Master Mix (Applied Biosystems) and the volume was made up with RNAse free water. The following thermal cycling profile was used (40 cycles): 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The $^{\Delta\Delta}$CT method of relative quantification was used to determine the fold change in expression. This was done by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control $\beta$-actin in the same samples ($^{\Delta}$CT = CT Target–CT$\beta$-actin). It was further normalized with the control ($^{\Delta\Delta}$CT = $^{\Delta}$CT–CT Control). The fold change in expression was then obtained as (2–$^{\Delta\Delta}$CT) and the graph was plotted using log 2–$^{\Delta\Delta}$CT.

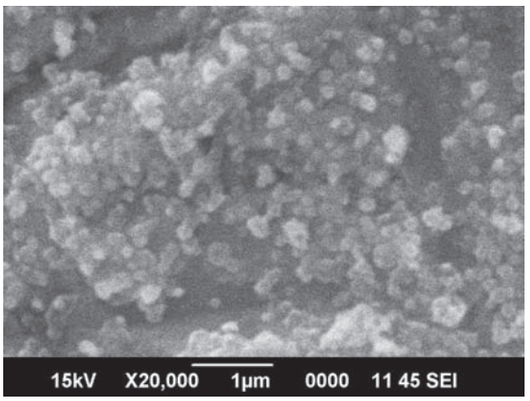


**Fig. 1.** The *SEM photograph* of magnification 20000× shows the spherical GABA coupled chitosan nanoparticles.

#### *2.6.6. Statistical Analysis*

Statistical evaluations were done with analysis of variance (ANOVA), using GraphPad Instat (version 2.04a, San Diego, USA). Student Newman-Keuls test was used to compare different groups after ANOVA. ANOVA assumes that all the data follow Gaussian distribution and the tests were done by the method of Kolmogorov and smirnov. Relative Quantification Software was used for analyzing Real-Time PCR results.

## 3. RESULTS

The SEM study for GABA-chitosan nanoparticles were prepared by ionic gelation method by using penta sodium tripolyphosphate as a strong anion for precipitating nanoparticles. Spherical particles of $80 \pm 6$ nm were observed from SEM photograph (Fig. 1).

FT-IR spectra of chitosan, GABA, chitosan-TPP nanoparticles and GABA-chitosan nanoparticles were studied. The –NH bending peak was observed at 1642.18 $cm^{-1}$in chitosan flakes. But when chitosan was precipitated as nanoparticles with the help of TPP, the –NH bending peak shifted to 1547.68 $cm^{-1}$and a new peak 1639.12 $cm^{-1}$ appeared. This showed an interaction of phosphate groups of TPP with the amino groups of chitosan. The –NH stretching vibration at 3022.14–2618.33 $cm^{-1}$ of pure GABA was shifted to 3390.89–2970 $cm^{-1}$ in GABA-chitosan nanoparticles. This was due to the interaction of GABA with chitosan. C–H aliphatic stretching of GABA at 2957 $cm^{-1}$ and 2920 $cm^{-1}$ which was overlapping with –NH stretching bands changed its positions to 2977.26 and 2880.33 $cm^{-1}$. The weak assymetrical –NH bending in GABA at 1642 $cm^{-1}$ became broad and shoulder peaks were observed in GABA-chitosan nanoparticles at 1500 $cm^{-1}$. The strong assymetrical $COO^-$ peak of GABA at 1596.11 $cm^{-1}$ disappeared due to the formation of salt by the interaction between carboxylate group

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of GABA and amino group of chitosan and thus a new shoulder peak at 1405.57 $cm^{-1}$ appeared. 1400.46 $cm^{-1}$ represented a weak $COO^-$ symmetrical peak and the influence of this was observed in GABA-chitosan nanoparticles at 1405.57 $cm^{-1}$ (salt of carboxyl) (Figs. 2(a–d)).

The efficiency of interaction of GABA with chitosan was studied by varying the time required for the interaction of GABA with chitosan and changing concentrations of GABA. A vigorous stirring of GABA with chitosan solution for 2 hours gave good encapsulation 0f $66 \pm 7.1\%$. By changing the concentration of GABA from 0 to 35 $\mu$g and considering the chitosan solution of 1mg/mL, the maximum encapsulation of $93 \pm 8.3\%$ was obtained at a concentration 8.824 $\mu$g of GABA/mL chitosan solution (Figs. 3(a, b)). From *in vitro* release studies of GABA there was an initial burst release of 20% in 0.5 hours, 87% in 20 hours and 94% in 30 hours from the nanoparticles in PBS (Fig. 3(c)).

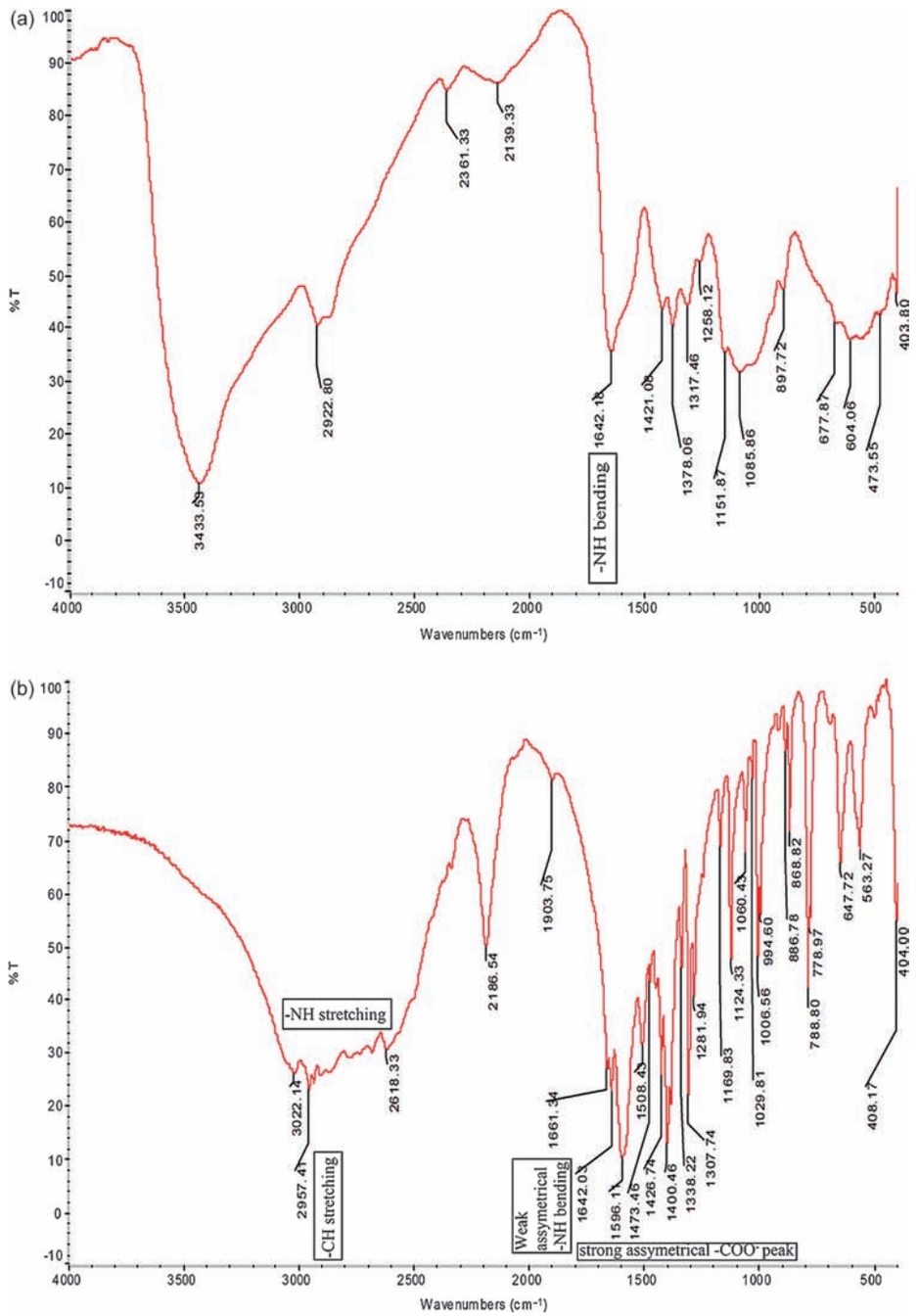


**Fig. 2.** Continued.

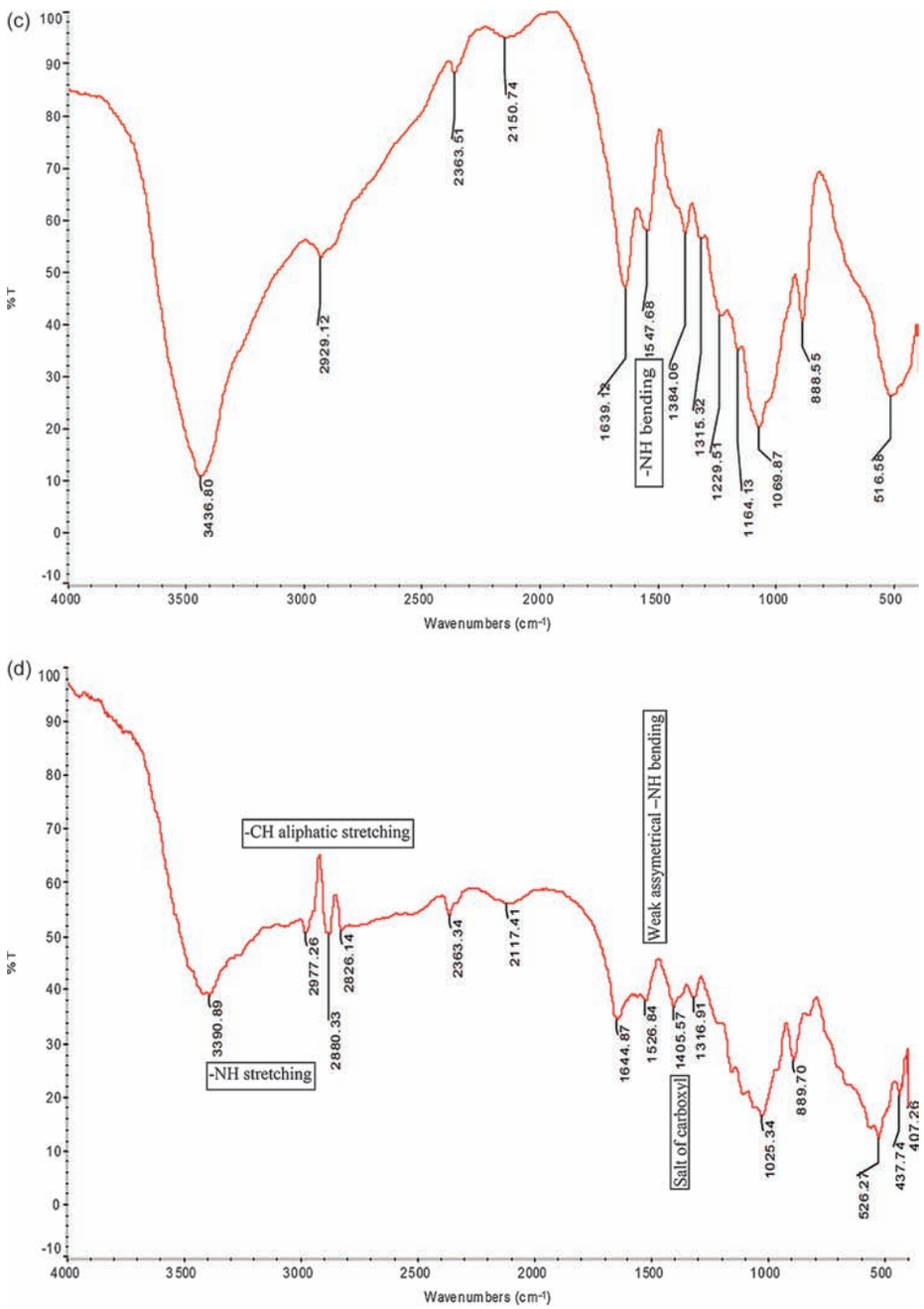


**Fig. 2.** *FT-IR spectrum* with a spectral range of 4000–400 $cm^{-1}$, using Fourier Transform Infra Red spectrometer (Thermo Nicolet, Avatar 370) of (a) chitosan flakes, (b) GABA, (c) chitosan nanoparticles and (d) GABA-chitosan nanoparticles.

The chitosan-GABA nanoparticles were labeled with FITC by incubating the nanoparticles with FITC in DMSO overnight. The cultured hepatocytes were incubated in the presence of FITC labeled GABA chitosan nanoparticles and unlabelled nanoparticles for 2 hours. A negative control showing the auto fluorescence in the cell was obtained. The fluorescent image was observed in confocal microscope with an excitation at 488 nm. The interaction of FITC labeled nanoparticles with hepatocytes was observed. The binding of fluorescent labeled GABA-chitosan nanoparticles on the surface of hepatocytes and the entry of nanoparticles in to the hepatocytes by phagocytosis led to the observation of fluorescence in the cell (Figs. 4(a, b)).

Tritiated thymidine incorporation in to replicating DNA was used as a biochemical marker for quantifying DNA synthesis. After partial hepatectomy, 1 mL of 30 $\mu g/\mu L$ concentration of GABA-chitosan nanoparticles suspended



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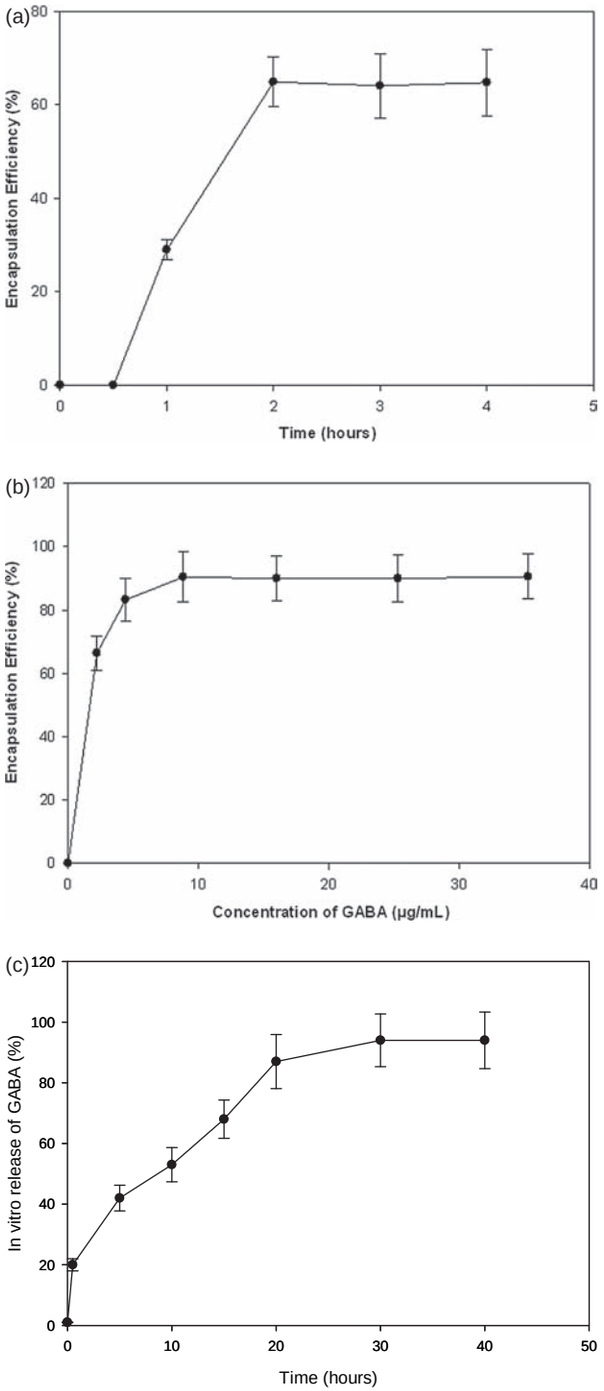


**Fig. 3.** The *maximum encapsulation efficiency* by standardizing (a) reaction time for GABA and chitosan in hours, (b) concentration of GABA in $\mu$g/mL chitosan solution, and (c) *in vitro* release of [$^3$H] GABA from nanoparticles in PBS.

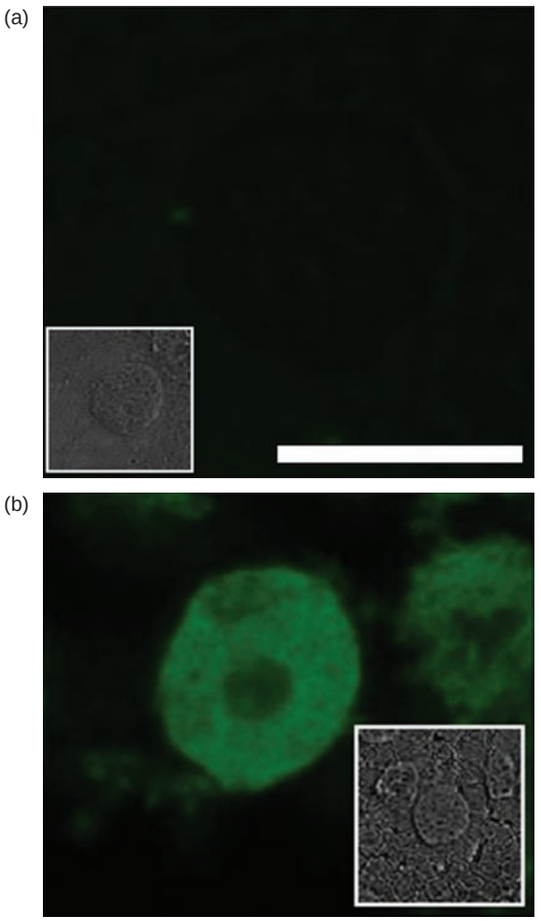


**Fig. 4.** *The study of interaction between GABA-chitosan nanoparticles and hepatocytes in vitro* using confocal microscope. (a) The bright field image (inset) and confocal image shows the interaction of non fluorescent GABA-chitosan nanoparticles with hepatocytes. (b) The bright field image (inset) and confocal image shows the interaction of FITC labeled GABA-chitosan nanoparticles with hepatocytes. The interaction of labeled nanoparticles at cell membrane and the engulfing of nanoparticles by hepatocytes lead to the fluorescence in the interior of the cell. The scale bars represent 15 $\mu$m.

in saline and 0.26 mg GABA in saline were injected intraperitonially to groups GCNP and G respectively. After 24 hours the animals were sacrificed and the remaining liver from the groups PHNT, G and GCNP which was triggered for regeneration and non triggered liver from control were perfused and cultured for 24 hours in the presence of tritiated thymidine for quantifying DNA and tritiated leucine for quantifying protein. The uptake of tritiated thymidine and leucine were observed. The tritiated thymidine uptake in PHNT was significantly increased ($p < 0.01$) with respect to sham operated control. There was also a significant increase in tritiated thymidine uptake by hepatocytes from group G when compared with control ($P < 0.01$) and PHNT ($p < 0.05$). In the GABA chitosan treated GCNP group a significant increase ($p < 0.001$) with respect to control and ($p < 0.01$) with respect to PHNT was observed. The tritiated thymidine uptake in GCNP showed a significant increase ($p < 0.01$) with respect to G. For tritiated leucine uptake there was significant increase ($p < 0.05$) in PHNT and G groups when compared with the sham operated control. There was

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**Table I.** Tritiated thymidine uptake by hepatocytes obtained in control and experimental rats.

| Groups | DPM/mg protein |
|---|---|
| C | $15897 \pm 189$ |
| PHNT | $18005 \pm 543^{b}$ |
| G | $19865 \pm 298^{b,f}$ |
| GCNP | $23358 \pm 400^{a,e,h}$ |

C—Sham operated control, PHNT—Partially hepatectomised group with no treatment, G—Partially hepatectomised group with GABA treatment and GCNP—Partially hepatectomised group with GABA-chitosan nanoparticle treatment. $^{a}p < 0.001$, $^{b}p < 0.01$ with respect to sham operated control. $^{e}p < 0.01$, $^{f}p < 0.05$ with respect to PHNT. $^{h}p < 0.01$ with respect to G. Values are mean ± S.E.M of 4–6 separate experiments.

**Table II.** Tritiated leucine uptake by hepatocytes obtained in control and experimental rats.

| Groups | DPM/mg protein |
|---|---|
| C | $11218 \pm 388$ |
| PHNT | $12658 \pm 392^{c}$ |
| G | $12986 \pm 483^{c}$ |
| GCNP | $14416 \pm 823^{b,e,i}$ |

C—Sham operated control, PHNT—Partially hepatectomised group with no treatment, G—Partially hepatectomised group with GABA treatment and GCNP—Partially hepatectomised group with GABA-chitosan nanoparticle treatment. $^{b}p < 0.01$, $^{c}p < 0.05$ with respect to sham operated control. $^{e}p < 0.01$ with respect to PHNT. $^{i}p < 0.05$ with respect to G. Values are mean ± S.E.M of 4–6 separate experiments.

**Table III.** Thymidine kinase assay in hepatocytes from control and experimental rats.

| Groups | Vmax (nmoles/mg/min) | Km (nM) |
|---|---|---|
| C | $17.30 \pm 0.88$ | $0.92 \pm 0.03$ |
| PHNT | $414.42 \pm 82.20^{a}$ | $0.80 \pm 0.02^{c}$ |
| G | $425.19 \pm 95.90^{a,d}$ | $0.80 \pm 0.03^{c}$ |
| GCNP | $479.84 \pm 23.45^{a,d,g}$ | $0.80 \pm 0.03^{c}$ |

C—Sham operated control, PHNT—Partially hepatectomised with no treatment, G—Partially hepatectomised with GABA treatment and GCNP—Partially hepatectomised with GABA- chitosan nanoparticle treatment. $^{a}p < 0.001$, $^{c}p < 0.05$ with respect to control. $^{d}p < 0.001$ with respect to PHNT. $^{g}p < 0.001$ with respect to G. Values are mean ± S.E.M of 4–6 separate experiments.

much more increase in the leucine uptake ($p < 0.01$) of GCNP compared to both control and PHNT and significant increase ($p < 0.05$) with respect to G (Tables I and II).

The enzyme thymidine kinase converts thymidine in to thymidine monophosphate, which is involved in the DNA synthesis. The thymidine kinase activity in all the four experimental groups showed a significant increase ($p < 0.001$) in the activity of thymidine kinase comparing with PHNT, G and GCNP groups with sham operated control. Also, a significant increase ($p < 0.001$) in thymidine kinase activity of liver from the groups G and GCNP with respect to PHNT was observed. A significant increase ($p < 0.001$) in the enzyme activity for group GCNP when compared to G was obtained. The $K_d$ value of the enzyme in PHNT, G and GCNP are decreased ($p < 0.05$) when compared to sham operated control (Table III).

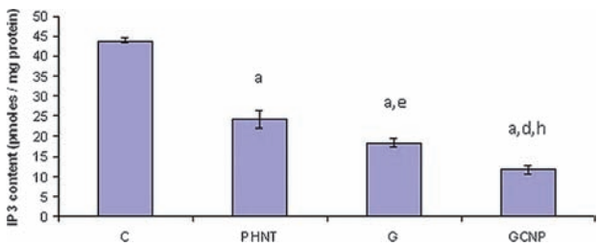


**Fig. 5.** *$IP_3$ content in the liver of experimental rats.* Values are Mean ± S.E.M. of 4-6 separate experiments. Each group consists of 6–8 rats. $^{a}p < 0.001$ with respect to C. $^{d}p < 0.001$ and $^{e}p < 0.01$ with respect to PHNT. $^{h}p < 0.01$ with respect to G. C—Sham operated control, PHNT – Partially hepatectomised group with no treatment, G—Partially hepatectomised group with GABA treatment and GCNP—Partially hepatectomised group with GABA-chitosan nanoparticle treatment.

$IP_3$ content of the regenerating liver of PHNT, G and GCNP showed a significant decrease ($p < 0.001$) compared to sham operated control. The $IP_3$ content of the liver of group G and GCNP was decreased with a $p < 0.01$ and $p < 0.001$ respectively when compared to PHNT and for group GCNP the $IP_3$ content showed a significant decrease ($p < 0.01$) when compared to G (Fig. 5).

Gene expression studies of phospholipase C (PLC) in the regenerating liver of PHNT and G showed a significant decrease ($p < 0.01$) and for GCNP with $p < 0.001$ with respect to sham operated control. There was also a significant decrease ($p < 0.05$) in PLC gene expression of the liver of G and GCNP when compared to PHNT. For group GCNP, there was a significant decrease ($p < 0.01$) in the gene expression when compared to G (Fig. 6). The NF-$\kappa$B expression in the regenerating liver of PHNT, G and GCNP showed a significant decrease ($p < 0.001$) when compared to control and $p < 0.001$ for G and GCNP with respect to PHNT and for GCNP when compared to G (Fig. 7). Caspase-8 expression in the PHNT group was significantly

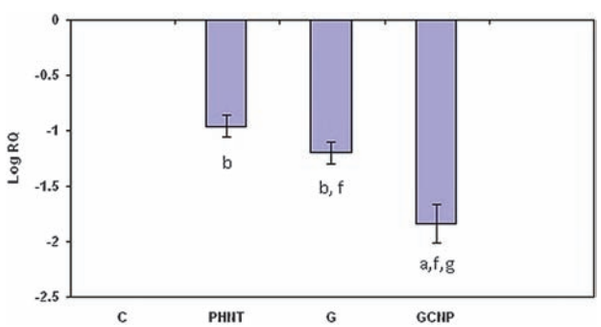


**Fig. 6.** *Real time PCR amplification of phospholipase C mRNA in the liver of experimental rats.* Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6–8 rats. $^{a}p < 0.001$ and $^{b}p < 0.01$ with respect to control. $^{f}p < 0.05$ with respect to PHNT. $^{h}p < 0.01$ with respect to G. Values are mean ± S.E.M of 4–6 separate experiments. C—Sham operated control, PHNT—Partially hepatectomised group with no treatment, G—Partially hepatectomised group with GABA treatment and GCNP—Partially hepatectomised group with GABA-chitosan nanoparticle treatment.



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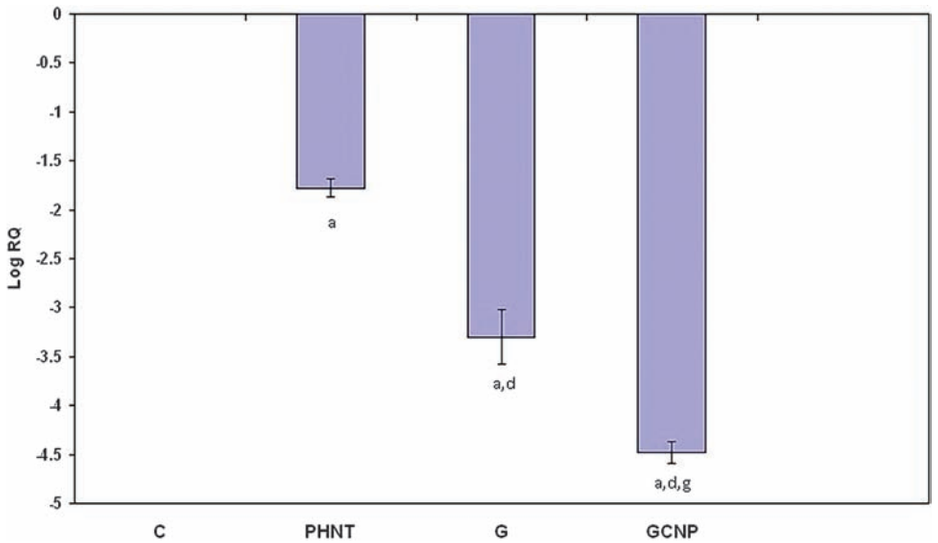


**Fig. 7.** *Real time PCR amplification of NF-κB mRNA in the liver of experimental rats*. Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6–8 rats. $^{a}p < 0.001$ with respect to control. $^{d}p < 0.001$ with respect to PHNT. $^{s}p < 0.001$ with respect to G. Values are mean ± S.E.M of 4–6 separate experiments. C—Sham operated control, PHNT—Partially hepatectomised group with no treatment, G—Partially hepatectomised group with GABA treatment and GCNP—Partially hepatectomised group with GABA-chitosan nanoparticle treatment.

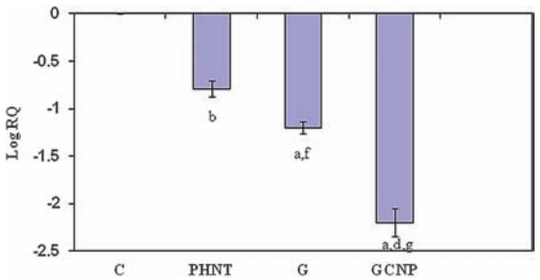


**Fig. 8.** *Real time PCR amplification of caspase-8 mRNA in the liver of experimental rats*. Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6-8 rats. $^{a}p < 0.001$ and $^{b}p < 0.01$ with respect to control. $^{d}p < 0.001$ and $^{f}p < 0.05$ with respect to PHNT. $^{s}p < 0.001$ with respect to G. Values are mean ± S.E.M of 4–6 separate experiments. C—Sham operated control, PHNT—Partially hepatectomised group with no treatment, G—Partially hepatectomised group with GABA treatment and GCNP—Partially hepatectomised group with GABA-chitosan nanoparticle treatment.

decreased ($p < 0.01$) when compared to C. There was a significant decrease ($p < 0.001$) for G and GCNP with respect to C and a significant decrease ($p < 0.05$) for G and ($p < 0.001$) for GCNP when compared to PHNT. Also a significant decrease ($p < 0.001$) in Caspase-8 expression in GCNP when compared to G was also observed (Fig. 8).

## 4. DISCUSSION

Liver cell damage occurs in many ways. Alcohol-induced cell death and inflammation result in scarring that distorts the liver's internal structure and impairs its function.[28, 29] Many drugs with analgesic and antipyretic action affect many peripheral tissues. However, an acute or cumulative over dose can cause severe liver injury with the potential to progress to liver failure. Acetaminophen is one such drug.[30] Liver cell damage is also occurred due to the attack of parasites like *Entamoeba histolytica*.[31] Apart from drugs, many hepatotoxic chemicals also cause liver cell apoptosis.[32, 33] Due to all these reasons the metabolic functions of one of the major organ in the body, liver, gets disturbed. So the proliferation of damaged hepatocytes is essential for the balanced routine body functions. Chitosan is a natural polymer produced by deacetylation of chitin. Chitin is the second abundant polymer in nature.[34] It posses positive charge and thus interact with the negatively charged cell membrane. In the last two decades, Chitosan nanoparticles have been extensively explored for pharmaceutical applications[35] like paclitaxel conjugated chitosan oligosaccharide for cancer therapy,[36] encapsulation of nitrone in chitosan nanoparticles for the treatment of stroke.[37] Gamma amino butyric acid (GABA), a neurotransmitter, enhances hepatocyte proliferation *in vitro*.[13] Apart from the inhibitory role of GABA, it can also be used for the proliferation of damaged cells.

Spherical nanoparticles of chitosan bound to GABA were prepared and the morphological study was carried out with scanning electron microscope. The interaction of GABA with chitosan was confirmed by FT-IR study. A salt formation was observed during the interaction of GABA and chitosan. The maximum encapsulation efficiency of chitosan leads to better loading of GABA in chitosan nanoparticles. The mechanism of interaction of GABA and chitosan is the bond formation between the amino group of chitosan and carboxyl group of GABA (salt formation), which is clear from the FT-IR data. We observed maximum encapsulation efficiency by standardizing the concentration

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of GABA and the reaction time for binding GABA with chitosan. The interaction of nanoparticles with the hepatocytes was observed with FITC-labelled nanoparticles under confocal microscope. The GABA-chiotsan nanoparticles were administerd through the peritoneal cavity for a better absorption of nanoparticles to partially hepatectomised liver.[38] The chitosan nanoparticles are biocompatible and are degraded by the endosomal pathways in the cell and thus the therapeutic potential of chitosan polymer conjugates are being investigated for *in vitro* and *in vivo* applications.[39, 40] Gamma amino butyric acid is a non essential amino acid synthesized in the human body and also from the earlier reports confirmed the biocompatibility of GABA.[41] After two third hepatectomy of liver, the major metabolic pathways are disrupted. The liver starts to regenerate and the maximum DNA synthesis occurs by 24 hours post hepatectomy.[42] Thymidine is the nitrogen base, which is found only in DNA and the increase in DNA synthesis leads to enhanced utilization of thymidine. In proteins the major amino acid observed is leucine and thus an increase in leucine uptake by cells *in vitro* explains the enhancement in protein synthesis. DNA and protein syntheses were observed to be high in GCNP group compared with G and PHNT. This shows an increase in cell regeneration in the GABA-chitosan nanoparticle treatment groups. In the case of GABA chitosan nanoparticles, the less exposure of GABA to the internal body environment and the presence of positive charge and high cell binding affinity of chitosan[43] the more effective receptor activation by GABA is achieved. The activity of liver thymidine kinase, an enzyme converts thymidine to thymidine monophospahate, is directly proportional to DNA synthesis.[44] We observed the highest activity of thymidine kinase in GCNP group than G, PHNT and C.

After partial hepatectomy, an increase in apoptosis is also observed due to an increase in the level of reactive oxygen species (ROS) which in turn alter protein and nucleic acid structures.[45] $IP_3$ receptor inactivation phenotypically mimics Bax deficiency by attenuating caspase-3 expression and activation.[46] The $IP_3$ content in the liver of partially hepatectomised rats are reduced due to the triggering of cell division rather than apoptosis. Our results explain a further reduction in $IP_3$ content which is achieved by GABA treatment and even decreased by the GABA-chitosan nanoparticle treatment. GABA through $GABA_B$ receptors which are G protein coupled receptors, influence many cell signaling pathways including the regulation of protein kinase C.[47] The expression of $Gi_2$ alpha subunit of G protein is increased by 24 hours of hepatectomy and thus the activity of adenylyl cyclase is also decreased.[48] It was reported that there is a synergism between Phospholipase C and Adenylyl cyclase linked growth factors.[49] Phospholipase C catalyses the hydrolysis of phosphatidylinositol (4,5)-bisphosphate results in the production of diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate ($IP_3$), which activate protein kinase C (PKC). So due to the reduction in adenylyl cyclase the expression of phospholipase C also gets reduced which in turn reduces $IP_3$. The activators of $GABA_B$ receptors, significantly inhibited the accumulation of inositol-1-phosphate and inositol-1,4,5-triphosphate in cells.[50] Thus the reduction in both $IP_3$ content and the expression of phospholipase C support the fact of reduction in apoptosis. Our results emphasize an increase in cell proliferation by the reduction of $IP_3$ and phospholipase C. Also PLC related proteins activate $GABA_A$ receptors,[51] which suppress the cell proliferation.[52] Our results also showed reduction in PLC which leads to the suppression of $GABA_A$ receptors and cause enhancement of cell division.

The gene expression of NF-$\kappa$B was significantly reduced in the GABA treated and further decreased in GABA-chitosan treated partially hepatectomised rat liver when compared to PHNT and C. In PHNT also there was a significant reduction in NF-$\kappa$B than C. PKC activates transcription factors, such as NF-$\kappa$B, which leads to the regulation of the expression of genes involved in cell proliferation. The activated, NF-$\kappa$B is dissociated from its inhibitor, I-$\kappa$B, and translocated into the nuclei, where it induces transcriptional up regulation of various proinflammatory mediators such as TNF-$\alpha$.[17] Even though TNF-$\alpha$ is an initial and mandatory cytokine for liver regeneration[53] the suppression of TNF-$\alpha$ improves liver function and facilitate liver regeneration after extended hepatectomy.[54] Apoptosis could also be induced by death domain receptor ligands such as TNF-$\alpha$ and Fas ligand.[55] So decreased NF-$\kappa$B expression leads to less activation of TNF-$\alpha$ and enhanced cell proliferation. Apoptosis after partial hepatectomy was further clarified by studying the gene expression of Caspase-8. Active Caspase-8 will further activate other Caspases like Caspase-3 and result in DNA fragmentation.[56] From our study it is clear that the gene expression of Caspase-8 is decreased significantly in GCNP when compared to PHNT and G, which shows that the apoptosis is reduced in nanoparticle treated group.

## 5. CONCLUSION

The major after effect of liver disease is the destruction of active cells that play a vital role in the metabolism of many compounds in the body. So the regeneration of hepatocytes from damaged liver for the proper functioning of body gains immense interest. Treatment with GABA-chitosan nanoparticles in partially hepatectomised rat liver improves hepatic regeneration when compared to the regeneration due to pure GABA treatment and without any treatment. This has clinical significance in liver based diseases.

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# *Role of GABA and serotonin coupled chitosan nanoparticles in enhanced hepatocyte proliferation*

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# Role of GABA and serotonin coupled chitosan nanoparticles in enhanced hepatocyte proliferation

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**Abstract** The development of nanoparticles containing active molecules having improved stability, sustained release and maximum half life helps in cell proliferation result in enhanced tissue regeneration. Our study focuses on the use of Gamma amino butyric acid (GABA) and serotonin (5-HT) coupled chitosan nanoparticles for the active liver regeneration in male Wistar rats. The nanoparticles were prepared and the morphology was studied using SEM. The FT-IR spectra of the nanoparticles and the maximum encapsulation efficiency of GABA and 5-HT binding to chitosan nanoparticles were observed. The in vitro release studies provided the percentage release of GABA and 5-HT from the nanoparticles at different time intervals. The quantification of DNA and protein syntheses was done using [$^3$H] thymidine and [$^3$H] leucine uptake studies that determined the enhancement in hepatocyte proliferation. Our results project the role of GABA and 5-HT chitosan nanoparticles in the treatment of liver based diseases.

## 1 Introduction

Nanoparticulate drug delivery systems provide wide opportunities for solving problems associated with drug stability or disease states and create great expectations in the area of drug delivery [1]. Nanotechnology is the technology that deals with one billionth of a meter scale [2]. To a great extend, biopolymer coated drugs coupled with nanotechnology reduces the major drawbacks of the common drug delivery methods. Chitosan, deacetylated chitin which is a linear nitrogenous polysaccharide, is a copolymer of $\beta$-(1,4) linked glucosamine (deacetylated unit) and *N*-acetyl glucosamine (acetylated unit) [3]. Owing to the removal of acetyl moieties that are present in the amine functional groups of chitin, chitosan is readily soluble in aqueous acidic solution. The solubilisation occurs through the protonation of amino groups on the C-2 position of D-glucosamine residues whereby polysaccharide is converted into polycation in acidic media. Chitosan interacts with many active compounds due to the presence of amine group in it. The presence of this active amine group in chitosan was exploited for the interaction with the active molecules in the current study. Chitosan is non-toxic, biodegradable and bio compatible. Nanoparticles of chitosan coupled drugs are utilized for drug delivery in eye, brain, liver, cancer tissues, treatment of spinal cord injury and infections [4–8]. Polymeric drug delivery systems can be used to deliver drugs directly to the intended site of action to improve pharmacological efficiency by minimizing undesired side effects elsewhere in the body and decrease the long-term use of many drugs [9].

A non proteinaceous amino acid Gamma amino butyric acid (GABA) is an important inhibitory neurotransmitter in the vertebrate central nervous system. Apart from the inhibitory role, GABA helps in the proliferation of cells in different parts of the body. The proliferative role of GABA was observed in the TM3 Leydig cell multiplication in testis [10], development of outer retina in rabbits [11] and promotes neurite growth, cell proliferation and migration [12]. A GABA agonist, baclofen, induced EGF mediated DNA synthesis in hepatocyte in vitro and an increase in hepatocyte proliferation was observed through the activation of $GABA_B$ receptors. Also, it significantly reduced the TGF $\beta$1 suppression of EGF induced DNA synthesis.

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Thus the activation of GABA receptors triggers DNA synthesis, which is mediated through the G protein, in primary cultures of rat hepatocytes [13]. The expression of the inhibitory and stimulatory α-subunit of G proteins coupled receptors to the targets like adenylate cyclase cause biphasic increase in hepatic cAMP and thus an enhanced signalling for cell division.

Serotonin (5-HT) has been shown to be mitogenic in many cells other than neuronal, exerting its effect by its receptor-mediated second messenger pathways. One of the factor influences the rate of dentate gyrus neurogenesis is 5-HT [14]. The $S_2$ receptor subtype of 5-HT has been shown to have mediated cell growth in fibroblasts [15]. The 5-HT $S_2$ receptor has been cloned in the human liver and has been shown to have a high degree of homology with the $S_2$ receptors of rat and mouse liver [16]. The $S_2$ receptors of 5-HT are coupled to phospho-inositide turnover and diacylglycerol formation, which activates protein kinase C (PKC), an important second messenger for cell division [17]. 5-HT S2 receptor–mediated activation of PKC has been shown to result in the phosphorylation of a 40 kDa substrate protein of PKC in human platelets [18]. Platelets are major carriers of 5-HT in the blood. It was reported that 5-HT can act as a potent hepatocyte co-mitogen and induce DNA synthesis in primary cultures of rat hepatocytes [19].

In the present study, the synthesis of nanoparticles by GABA and 5-HT with chitosan and their delivery for active liver regeneration was emphasised. The liver regeneration in partially hepatectomised rats has contributed a powerful model for evaluating the hepatocyte proliferation in vivo [20]. The liver is an important body organ responsible for various functions like bile production, storage and filtering of blood, metabolism of fats and sugars and thus making compounds which control blood volumes and clotting. Liver cell damage is occurred due to several reasons. Liver's internal structure is distorted due to the scars formed as a result of alcohol induced cell death and inflammation [21, 22]. One in twenty-five deaths Worldwide are directly related to alcohol consumption. An over dose of many drugs cause severe liver injury which leads to liver failure. Liver cell destruction is also observed due to the attack of parasites like *Entamoeba histolytica* [23]. Many hepatotoxic chemicals like dyes, preservatives and insecticides cause liver cell apoptosis [24, 25]. The regeneration of damaged liver is considered as a solution for all disturbing factors of normal hepatocytes' functions. A two-third partial hepatectomy (PH) of rats induces the remainder of the liver to undergo a synchronous first wave of DNA synthesis, followed by several rounds of mitosis [26–29]. Mitosis begins 6 h later and peaks at 30 h. The original DNA content of the regenerating liver remnant is normally restored within 96 h after PH [30].

The present work elucidates the role of GABA and 5-HT coupled chitosan nanoparticles triggering the hepatocyte proliferation in partially hepatectomised male Wistar rats, in vivo. The standardisation of nanoparticle synthesis was done to achieve a maximum binding of GABA and 5-HT with chitosan. An enhanced cell proliferation in the nanoparticle treated groups was observed by quantifying DNA and protein syntheses.

## 2 Materials and methods

### 2.1 Chemicals used and their sources

Biochemicals, including GABA (mw, 103.12) and 5-HT (mw, 387.41), were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally. [$^3$H]GABA (Sp. Activity 76.2 Ci mmol$^{-1}$), [$^3$H] thymidine (Sp. Activity 18.0 Ci mmol$^{-1}$) and [$^3$H] leucine (Sp. Activity 63.0 Ci mmol$^{-1}$) were purchased from Amersham Life Science, UK. Chitosan (mw, 25 kDa) was a gift from Central Institute of Fisheries Technology, Cochin, India.

### 2.2 Animals

Experiments were carried out on adult male Wistar rats of 250–300 g body weight purchased from Kerala Agricultural University, Mannuthy, India. They were housed in separate cages under 12 h light and 12 h dark periods and were maintained on standard food pellets and water ad libitum. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health and CPCSEA guidelines. All efforts were made to minimize the number of animals used and their suffering. Adult male Wistar rats were divided into four experimental groups: sham operated control (C), partially hepatectomised rats with no treatment (PHNT), partially hepatectomised rats with 5-HT chitosan nanoparticle treatment (SCNP) and partially hepatectomised rats with a combination of GABA and 5-HT chitosan nanoparticle treatment (GSCNP). Each group consisted of 6–8 animals.

### 2.3 Preparation of neurotransmitter-chitosan nanoparticles

The chitosan nanoparticles were prepared by ionic gelation method [31]. Chitosan was dissolved in 2 % acetic acid to get chitosan solution of concentration 1 mg mL$^{-1}$. Chitosan nanoparticles from 50 mL chitosan solution were precipitated by the addition of 33 mL of 1 mg mL$^{-1}$ penta sodium tri polyphosphate (TPP) solution with rapid stirring. To incorporate 5-HT creatinine sulphate to chitosan,

300 μg of 5-HT $mL^{-1}$ of chitosan solution was prepared and the precipitation of 5-HT chitosan nanoparticles were done by the above method. To prepare a combination of GABA and 5-HT chitosan nanoparticles, 400 μg of 5-HT and 20 μg of GABA were dissolved in chitosan solution and the nanoparticles were precipitated by the addition of TPP. The precipitated nanoparticles were centrifuged for 20 min at 16,000×*g*. The pellet was washed thoroughly with distilled water and then resuspended in saline. The SEM image of the nanoparticles was taken with a magnification of 10,000× by scanning electron microscope (JEOL Model JSM–6390LV).

### 2.4 FT-IR spectroscopy

The FT-IR spectrum of the 5-HT, 5-HT incorporated chitosan nanoparticles and GABA and 5-HT incorporated chitosan nanoparticles were taken using Fourier Transform Infra Red spectrometer (Thermo Nicolet, Avatar 370) with a spectral range of 4,000–400 $cm^{-1}$.

### 2.5 Determination of encapsulation efficiency and in vitro release studies

The maximum encapsulation efficiency [32] of 5-HT with 5-HT chitosan nanoparticles and GABA and 5-HT with GABA and 5-HT chitosan nanoparticles was obtained by giving emphasis to concentration of GABA or 5-HT added to the chitosan solution. The encapsulation efficiency of GABA in GABA and 5-HT chitosan nanoparticles was calculated by incorporating [$^3$H] GABA with chitosan and the radioactivity of the GABA, which was bound on the chitosan nanoparticles, were related to its concentration [33, 34]. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

The concentration of 5-HT, which was bound to the nanoparticles, were found by HPLC with electrochemical detector (Waters, USA) fitted with CLS-ODS reverse phase column of 5 μm particle size. After centrifugation of nanoparticle suspension, the supernatant with unbound 5-HT was filtered through 0.22 μm HPLC grade filters and injected to the column. The mobile phase consisted of 50 mM sodium phosphate dibasic, 0.03 M citric acid, 0.1 mM EDTA, 0.6 mM sodium octylsulfonate and 15 % methanol. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.22 μm (Millipore) and degassed. A Waters model 515, Milford, USA, pump was used to deliver the solvent at a rate of 1 mL $min^{-1}$. The 5-HT was identified by amperometric detection using an electrochemical detector (Waters model 2465) with a reduction potential of +0.80 V. The peaks obtained were compared with standard creatinine sulphate and quantitatively estimated using an integrator (Empower software) interfaced detector.

$$\% \text{ Encapsulation} = (\text{Concentration of GABA or 5-HT bound to chitosan nanoparticles}/\text{concentration of GABA or 5-HT added initially}) \times 100$$

In in vitro release studies, [$^3$H] GABA and 5-HT chitosan nanoparticles and 5-HT chitosan nanoparticles were suspended in PBS, pH 7.4. Both were gently stirred at different time intervals from 0 to 40 h. The concentration of released radioactive GABA [33] and 5-HT at each time from the nanoparticles was calculated to get a release profile in vitro.

### 2.6 Effect of GABA and 5-HT encapsulated chitosan nanoparticles on DNA and protein syntheses in hepatocytes

Two-thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anaesthesia, following a 16 h fast [26]. Sham operations involved median excision of the body wall followed by all manipulations except removal of lobes. All rats were undergone surgeries between 7 and 9 a.m. to avoid diurnal variations in responses. After surgery, 1 mL of 30 μg $\mu L^{-1}$ 5-HT chitosan nanoparticles and GABA and 5-HT chitosan nanoparticles suspended in saline were injected intra peritoneal to the respective rats. The DNA synthesis prior to first mitotic phase occurs between 20 and 24 h after PH [28]. So the quantification of DNA and protein syntheses performed during this period provided a significant comparison among the experimental groups. Thus the rats were sacrificed by decapitation 24 h post hepatectomy.

The liver from the group C and remaining liver from all the other three groups were perfused and cultured for 24 h [34]. Cell suspension of 150 μL (cell density of 1.6 × $10^5$ cells $cm^{-2}$) was added to a four well poly L-lysine coated glass slide. Then the cells were incubated for 24 h at 37 °C in 5 % $CO_2$ atmosphere. Before incubation, [$^3$H] leucine of specific activity 63 Ci $mmol^{-1}$ to one set of culture plates for all the four experimental groups to determine the protein synthesis and [$^3$H] thymidine of specific activity 18 Ci $mmol^{-1}$ to determine the measurement of DNA synthesis. All the experiments were done in triplicates. The cells were scrapped off from the culture plates and centrifuged at 2,000×*g* for 20 min. The supernatant was discarded and the pellet was resuspended in 50 μL, 1 M NaOH and kept overnight. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

### 2.7 Statistics

Statistical evaluations were done with analysis of variance (ANOVA), using GraphPad Instat (version 2.04a,

San Diego, USA). Student–Newman–Keuls test was used to compare different groups after ANOVA. ANOVA assumes that all the data follow Gaussian distribution and the tests were done by the method of Kolmogorov and smirnov.

## 3 Results

5-HT chitosan and GABA and 5-HT chitosan nanoparticles were prepared by the method of ionic gelation. The morphology of nanoparticles was observed by SEM (Fig. 1a, b).

FT-IR spectra of 5-HT, 5-HT chitosan nanoparticles and GABA and 5-HT in combination with chitosan nanoparticles were studied. –NH stretch at 3,436 $cm^{-1}$ in chitosan nanoparticle was changed to broadened peaks from 3,464.72 to 3,280 $cm^{-1}$ on binding with 5-HT. There was a shift in C–N stretch of amine in 5-HT when the 5-HT chitosan nanoparticles were formed. In 5-HT chitosan nanoparticles, the S–O bond at 1,080.79 $cm^{-1}$ and a short peak at 1,232.95 $cm^{-1}$ were observed. In 5-HT, the S–O bond was intense and a broad and short peak at 1,230.44 $cm^{-1}$ was seen. In the nanoparticle, a new peak at 1,386.87 $cm^{-1}$ was formed which denotes the S–N interaction due to the reaction between sulphate ion in 5-HT and $NH_3^+$ ion in the chitosan. In GABA and 5-HT combination with chitosan nanoparticles, new and modified peaks were observed due to the presence of GABA and 5-HT. In pure GABA, −CH aliphatic stretch at 2,957 and 2,920 $cm^{-1}$, which were overlapping with −NH stretching bands changed to 2,924.8 and 2,810 $cm^{-1}$ due to the interaction with chitosan. The peak at 1,384.99 in chitosan nanoparticle, is a shoulder peak in the nanoparticles with GABA and 5-HT due to the formation of salt between GABA and chitosan. A peak at 1,072.44 $cm^{-1}$, which denotes the S–O bond in the nanoparticles, shows the presence of 5-HT. The –NH bending at 1,500 $cm^{-1}$ in non encapsulated chitosan nanoparticle was shifted to 1,543.63 $cm^{-1}$ in 5-HT chitosan nanoparticles and also in GABA and 5-HT chitosan nanoparticles showed the interaction of 5-HT in chitosan. A weak asymmetric −NH binding at 1,642 $cm^{-1}$ in GABA became broad in GABA and 5-HT chitosan nanoparticles (Fig. 2a–c).

In 5-HT chitosan nanoparticles, changing the concentration of 5-HT from 0 to 400 μg $mL^{-1}$ of chitosan solution (1 mg $mL^{-1}$), a maximum encapsulation of 86 ± 7.1 % was obtained at a concentration 300 μg of 5-HT $mL^{-1}$ chitosan solution. Considering the encapsulation of 5-HT in GABA and 5-HT chitosan nanoparticles for a concentration range of 0–500 μg, a maximum encapsulation efficiency of 80 ± 7.35 % at 400 μg of 5-HT $mL^{-1}$ chitosan solution was obtained. A maximum encapsulation efficiency of 71 ± 7.2 % was observed at 20 μg GABA $mL^{-1}$ chitosan solution when the concentration range was 0–35 μg GABA $mL^{-1}$ of chitosan solution (Fig. 3a–c). From the in vitro release studies of 5-HT, there was an initial burst release of 28 ± 2 % in 0.5 h and 96 ± 8.6 % in 30 h from the 5-HT nanoparticles in PBS. An initial 5-HT release of 30 ± 2 % in 0.5 h was observed in GABA and 5-HT chitosan nanoparticles and the release continued up to 97 ± 8.9 % at 20 h. From the GABA and 5-HT chitosan nanoparticles, there was an initial burst release of 22 ± 2 % GABA by 0.5 h and 95 ± 8.6 % by 30 h (Fig. 4a, b).

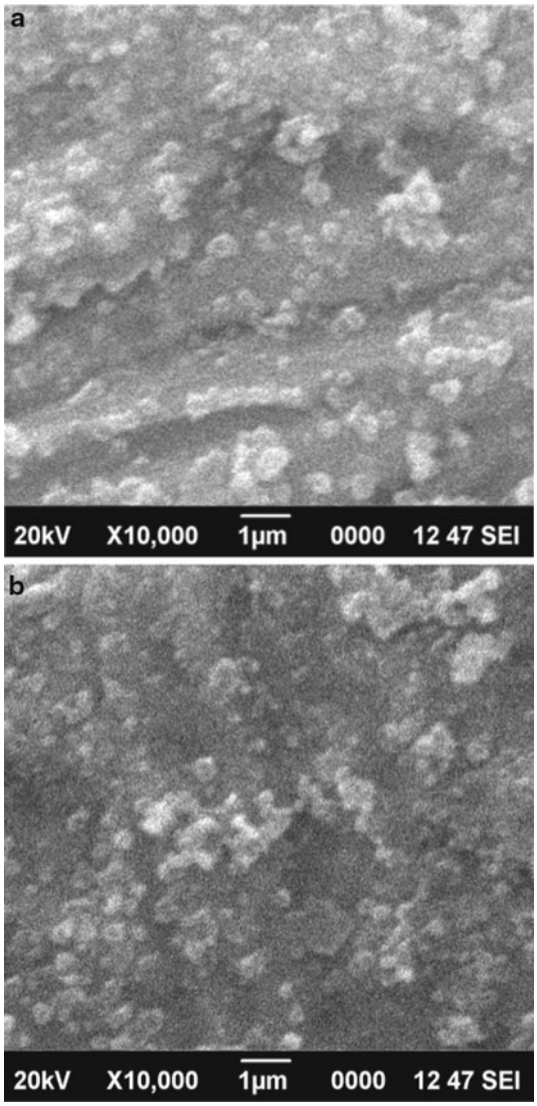


**Fig. 1** The *SEM photograph* of magnification 10,000× shows the spherical **a** 5-HT chitosan and **b** GABA and 5-HT chitosan nanoparticles

Incorporation of [$^3$H] thymidine into the replicating DNA was used as a biochemical marker to quantify DNA synthesis. The [$^3$H] thymidine uptake for PHNT was significantly increased ($p < 0.01$) when compared to C. There was a significant increase ($p < 0.001$) in [$^3$H] thymidine uptake by hepatocytes of SCNP when compared with C

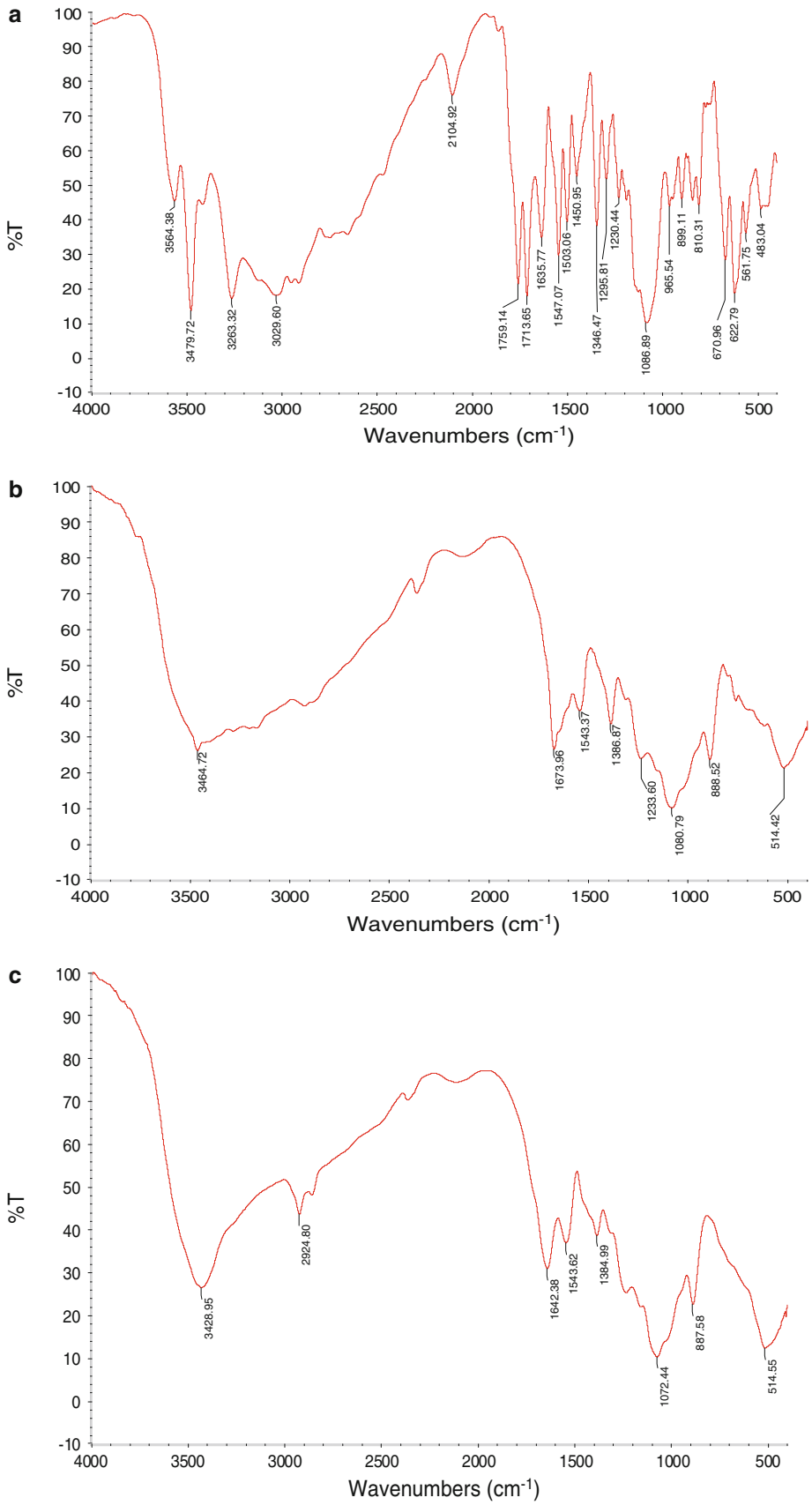


**Fig. 2** *FT-IR spectrum* with a spectral range of 4,000–400 $cm^{-1}$, using Fourier Transform Infra Red spectrometer (Thermo Nicolet, Avatar 370) of **a** serotonin creatinine sulphate (5-HT), **b** 5-HT chitosan nanoparticles, and **c** GABA and 5-HT chitosan nanoparticles

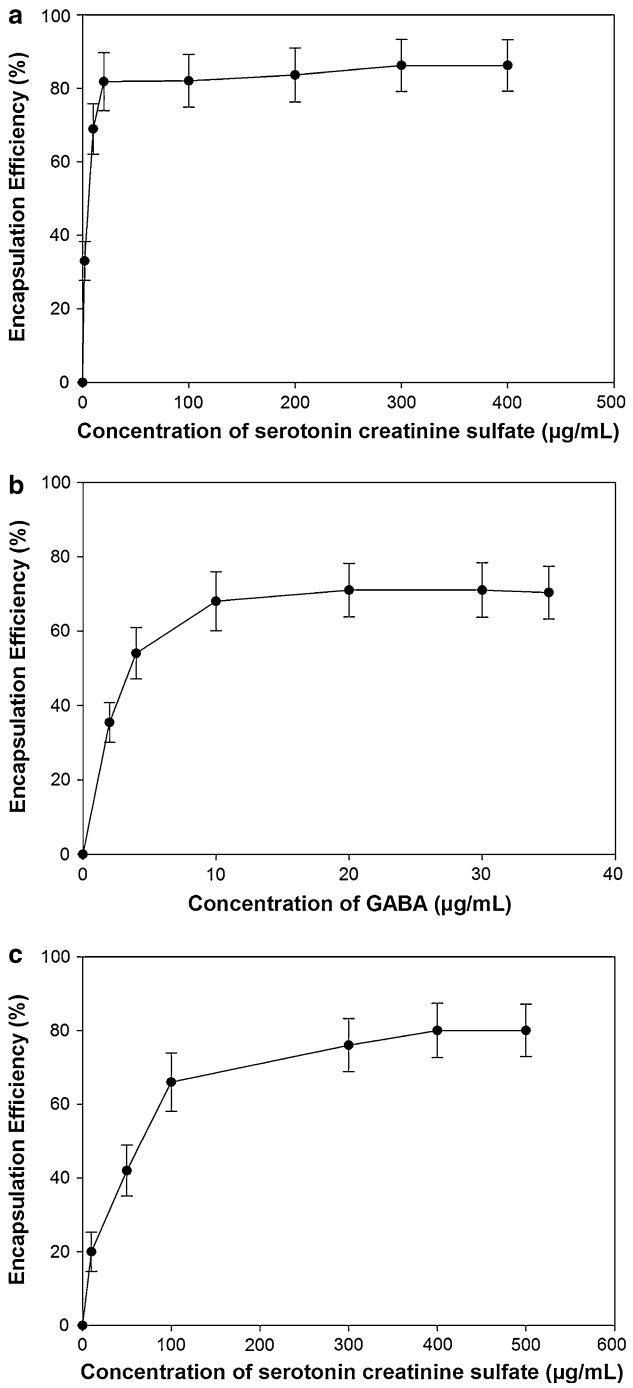


**Fig. 3** The *maximum encapsulation efficiency (%)* by standardizing the concentration of **a** 5-HT creatinine sulphate in µg $mL^{-1}$ chitosan solution for 5-HT chitosan nanoparticles, **b** GABA in µg $mL^{-1}$ chitosan solution for GABA and 5-HT chitosan nanoparticles, **c** 5-HT creatinine sulphate in µg $mL^{-1}$ chitosan solution for GABA and 5-HT chitosan nanoparticles

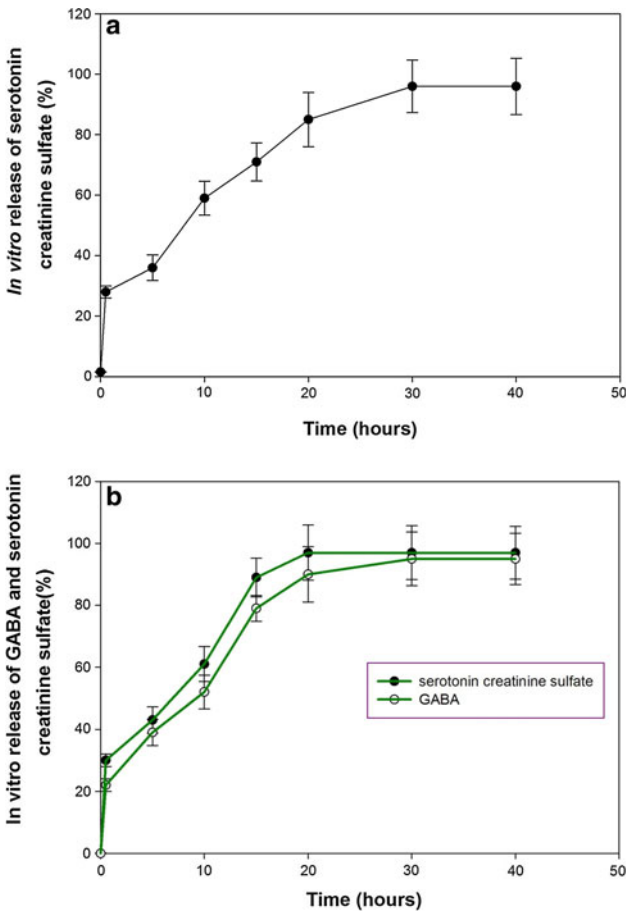


**Fig. 4** *In vitro release (%)* of **a** 5-HT creatinine sulphate from 5-HT chitosan nanoparticles, **b** GABA and 5-HT from GABA and 5-HT chitosan nanoparticles versus time of incubation

and PHNT ($p < 0.05$). In GSCNP group, a significant increase ($p < 0.001$) in [$^3$H] thymidine uptake when compared to C and PHNT was observed. For [$^3$H] leucine uptake studies, there was a significant increase ($p < 0.05$) in PHNT when compared with the C. There was a significant increase ($p < 0.01$) in the leucine uptake by hepatocytes in SCNP when compared to C and PHNT. While considering the GSCNP group, there was also a significant increase ($p < 0.05$) in [$^3$H] leucine uptake when compared to PHNT (Tables 1, 2).

## 4 Discussion

Chitosan is the second-most abundant natural polysaccharide next to cellulose. Among other drug delivery strategies, a great deal of focus has been directed to chitosan nanoparticles to improve drug bioavailability, modify pharmacokinetics and/or protect the encapsulated drug [35]. Chitosan nanoparticles are prepared by the interaction of oppositely charged compounds. TPP has often been used to prepare chitosan nanoparticles because TPP is multivalent, nontoxic and able to form gels through ionic interactions. The interaction can be controlled by the charge density of TPP and chitosan, which is dependent on the pH of the solution [36]. Fewer side effects, solubility, poor bioavailability, specific

**Table 1** [$^3$H] Thymidine uptake by hepatocytes of control and experimental rats

| Groups | DPM (mg protein) |
|---|---|
| C | 15890 ± 172 |
| PHNT | 18012 ± 540[b] |
| SCNP | 20876 ± 302[a,c] |
| GSCNP | 25969 ± 300[a,d] |

Values are mean ± SEM of 4–6 separate experiments

*C* Sham operated control, *PHNT* Partially hepatectomised group with no treatment, *SCNP* Partially hepatectomised group with 5-HT chitosan nanoparticle treatment, *GSCNP* Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment

[a] $p < 0.001$ with respect to sham operated control

[b] $p < 0.01$ with respect to sham operated control

[c] $p < 0.05$ with respect to PHNT

[d] $p < 0.001$ with respect to PHNT

**Table 2** [$^3$H] Leucine uptake by hepatocytes of control and experimental rats

| Groups | DPM (mg protein) |
|---|---|
| C | 11226 ± 380 |
| PHNT | 12663 ± 397[a] |
| SCNP | 14534 ± 421[b,c] |
| GSCNP | 15984 ± 835[d,e] |

Values are mean ± SEM of 4–6 separate experiments

*C* Sham operated control, *PHNT* Partially hepatectomised group with no treatment, *SCNP* Partially hepatectomised group with 5-HT chitosan nanoparticle treatment, *GSCNP* Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment

[a] $p < 0.05$ with respect to sham operated control

[b] $p < 0.01$ with respect to sham operated control

[c] $p < 0.01$ with respect to PHNT

[d] $p < 0.001$ with respect to sham operated control

[e] $p < 0.001$ with respect to PHNT

delivery to site of action with good pharmacological efficiency, absorption at intestine, degradation of drug, slow release and effective therapeutic outcome, are the challenges faced by the present drug delivery systems. Chitosan nanoparticles that can solve most of the above problems are widely used for drug delivery. van der Lubben et al. [37] investigated the ability of chitosan nanoparticles to enhance both systemic and local immune responses against diphtheria toxoid (DT) vaccine after the oral and nasal administration in mice. Gadopentetic acid-loaded Chitosan nanoparticles have been prepared for gadolinium neutron-capture therapy for cancer [38].

Healthy hepatocytes are necessary for the routine metabolic processes in the body. So active hepatocyte proliferation is important to reconstitute body functions after PH in rats. Earlier studies showed that GABA [13] and 5-HT [19] helped in the proliferation of hepatocytes in vitro. Our study focuses on the preparation of GABA and 5-HT combined chitosan nanoparticles for the hepatocyte proliferation in vivo. The morphology of the nanoparticles was visualized through scanning electron microscope. The interaction between GABA and 5-HT with chitosan was studied by FT-IR spectroscopy.

The maximum encapsulation efficiency of active compound to chitosan results in better loading of GABA and 5-HT in chitosan nanoparticles. From our earlier studies [34], the mechanism of interaction between GABA and chitosan is the bond formation between the amino group of chitosan and carboxyl group of GABA (salt formation). During the interaction of 5-HT and chitosan, a bond between sulphate ion in 5-HT creatinine sulphate and $NH_3^+$ ion in the chitosan is formed, which is clear from the FT-IR data. From the study, a maximum encapsulation efficiency of the active compounds with chitosan nanoparticles was observed by standardizing the concentration of GABA and 5-HT reacting with chitosan. The in vitro release studies showed the pattern of release of GABA and 5-HT from GABA and 5-HT chitosan anoparticles and also 5-HT from 5-HT chitosan nanoparticles.

The nanoparticles were administered through the peritoneal cavity for achieving a better absorption of nanoparticles to partially hepatectomised liver [39]. The chitosan nanoparticles are biocompatible and are degraded by the endosomal pathways in the cell and thus the therapeutic potential of chitosan polymer conjugates are being investigated for in vitro and in vivo applications [40]. GABA is a non essential amino acid synthesized in the human body and also earlier reports confirmed the biocompatibility of GABA [41]. Biju et al. [13] reported that GABA receptor activation occurs during liver regeneration. 5-HT receptor expression is increased after PH and it was reported that blockage of $S_2$ receptors arrested liver regeneration [42]. In thrombocytopenic mice, a 5-HT agonist reconstituted liver proliferation. The expression of 5-$HT_{2A}$ and 5-$HT_{2B}$ subtype 5-HT receptors in the liver increased after hepatectomy [43]. Thus activation of GABA and 5-HT receptor expression by the corresponding ligands achieved an enhancement in liver regeneration. Major metabolic pathways are disrupted after two-third hepatectomy of liver. During liver regeneration the maximum DNA synthesis occurs by 24 h post hepatectomy [44].

Thymidine is a nitrogen base that is found only in DNA and the increase in DNA synthesis results in enhanced utilization of thymidine. In proteins, the major amino acid observed is leucine and thus an increase in leucine uptake by cells explains the enhancement in protein synthesis. The DNA and protein synthesis quantitative studies for GABA

chitosan nanoparticle treated group were carried out in our early studies [34]. Fasting animals prior to operation would probably not have diminished the variations in mitotic activity [45]. DNA and protein syntheses were observed to be high in GSCNP when compared to other groups. This shows an increased cell proliferation in the combined GABA and 5-HT chitosan nanoparticle treated group than the individual treatment with GABA or 5-HT coupled chitosan nanoparticles. In the case of GSCNP and SCNP groups, there is less exposure of GABA and 5-HT to the internal body environment and the presence of positive charge and high cell binding affinity of chitosan [46], more effective receptor activation by GABA and 5-HT were achieved.

Active liver cell proliferation is a value added process in medical field. Liver regeneration is a complex and multifactorial process that is regulated by various cell signalling cascades including the interactions between growth factors, regenerative cytokines and metabolic demand of the liver following surgery [47]. The active compounds that mediate the cell signalling mechanism for an enhanced DNA and protein formation prior to cell division, will promote an efficient and fast proliferation of cells. Our study discussed the involvement of neurotransmitters combination such as GABA and 5-HT, which are coupled to chitosan nanoparticles, in the synthesis of DNA and protein during the active cell multiplication in partially hepatectomised rat liver. The combined effect of improved stability and action of GABA and 5-HT on regenerating hepatocytes gives valuable scope for further studies in neurotransmitter involved liver regeneration.

## 5 Conclusion

Liver is an important organ that controls major metabolic functions in the body. Damage to liver even causes mood fluctuations and behavioural alterations. The major result of liver disease is the destruction of active hepatocytes that regulate the vital metabolism of many compounds in the body. So the regeneration of hepatocytes from damaged liver for the proper functioning of body gains immense scope. Treatment with GABA and 5-HT chitosan nanoparticles in partially hepatectomised rat liver improves hepatic regeneration when compared to the natural regeneration without any treatment. This has important clinical significance in liver related diseases.

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# Increased Neuronal Survival in the Brainstem During Liver Injury: Role of γ-Aminobutyric Acid and Serotonin Chitosan Nanoparticles

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γ-Aminobutyric acid (GABA)- and serotonin (5-HT)-mediated cell signaling, neuronal survival enhancement, and reduced neuronal death in brainstem during liver injury followed by active liver regeneration have a critical role in maintaining routine bodily functions. In the present study, $GABA_B$ and $5\text{-}HT_{2A}$ receptor functional regulation, interrelated actions of neuronal survival factors, and expression of apoptotic factors in the brainstem during GABA and 5-HT chitosan nanoparticles-induced active liver regeneration in partially hepatectomized rats were evaluated. Partially hepatectomized rats were treated with the nanoparticles, and receptor assays and confocal microscopic studies of $GABA_B$ and $5\text{-}HT_{2A}$ receptors, gene expression studies of $GABA_B$ and $5\text{-}HT_{2A}$ receptors, nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), Akt-1, phospholipase C, Bax, and caspase-8 were performed with the brainstems of experimental animals. A significant decrease in $GABA_B$ and $5\text{-}HT_{2A}$ receptor numbers and gene expressions denoted a homeostatic adjustment by the brain to trigger the sympathetic innervations during elevated DNA synthesis in the liver. The neuronal apoptosis resulting from the loss of liver function after partial hepatectomy was minimized by nanoparticle treatment in rats compared with rats with no treatment during regeneration. This was confirmed from the gene expression patterns of NF-κB, TNF-α, Akt-1, phospholipase C, Bax, and caspase-8. The present study revealed the potential of GABA and 5-HT chitosan nanoparticles for increasing neuronal survival in the brainstem during liver injury following regeneration, which avoids many neuropsychiatric problems. 

**Key words:** brainstem; GABA; 5-HT; neuronal cell death; cell survival

The brain plays an important regulatory role in hepatic functions (O'Grady, 2005). The liver is a vital organ involved in routine metabolism in the body. Liver injury-related brain damage and death most frequently result from brainstem herniation resulting from increased intracranial pressure or brain edema resulting from altered ammonia and aromatic amino acid metabolism (Blei, 1991). This also affects various neurotransmitters and their receptor activation in brain. Deficiency of thiamine leads to brainstem neuronal loss (Cogan et al., 1985). Thus metabolism of the body with an injured liver is disturbed, which leads to several neuropsychiatric and mood alterations.

The regeneration of damaged liver automatically restores brain functions. Liver damage occurs due to overconsumption of alcohol, drugs with analgesic and antipyretic action, attack of parasites, and hepatotoxic chemicals. The presence of various comitogens is necessary for the successful and complete restoration of hepatic mass (Riehle et al., 2011). Prolonged liver dysfunction causes hepatocyte damage and fatal brain disorders. Several reports highlight liver injury-related brain damage (Cordoba and Blei, 1995; Butterworth, 1998; Chung et al., 2001; Larsen and Wendon, 2002), but activated liver cell proliferation coupled with increased neuronal survival has not been not well studied. The reduction in apoptosis enhances the survival of cells. The signals evoked by inositol (1,4,5)-triphosphate ($IP_3$)- and phospholipase C-mediated $Ca^{2+}$ ions in mitochondria trigger apoptosis. This was further studied from the expression patterns of caspase-8 and Bcl-2-associated X protein (Bax). Inflammatory mediators such as tumor necrosis factor-α (TNF-α) and neuronal survival factors such as nuclear factor-κB (NF-κB) and Akt-1 also play an important role in the regulation of brain function by the liver (Larsen and Wendon, 2002).

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γ-Aminobutyric acid (GABA) type B and serotonin (5-HT) type 2A receptors belong to the super family of G-protein-coupled receptors. GABA receptors are widely distributed in mammalian brain. Brain GABAergic changes are reported to regulate autonomic nerve function in rats (Martin and Haywood, 1998). In animal models of liver injury, an increase in GABAergic tone has been demonstrated (Albrecht and Jones, 1999). 5-HT is another neurotransmitter in the central and peripheral nervous systems (Lam and Heisler, 2007). The central serotonergic neurons participate in the regulation of sympathetic nerve discharge and have an inhibitory influence on central sympathetic pathways. 5-HT regulates cell proliferation, migration, and maturation in a variety of cell types. 5-HT has been implicated more in behavior, physiological mechanisms, and disease processes than any other brain neurotransmitter.

Nanoparticulate drug delivery systems provide immense hope for solving problems associated with current drug delivery methods. Chitosan, deacetylated chitin, is a copolymer of β-(1,4)-linked glucosamine (deacetylated unit) and N-acetyl glucosamine (acetylated unit; Radhakumary et al., 2005). Chitosan is a biodegradable, nontoxic, and biocompatible polymer. Chitosan nanoparticulate delivery systems are used to deliver drugs directly to the intended site of action to improve pharmacological efficiency by minimizing undesired side effects elsewhere in the body and decrease the long-term use of many drugs. Bioactive molecules are successfully encapsulated to improve bioavailability and bioactivity and to control delivery. The administration of pure neurotransmitters to the body results in degradation of the molecules and also vigorous binding to their receptors, which leads to receptor masking and cell signal arrest. Chitosan is inert to the internal body environment and thus the adverse side effects resulting from the interaction of neurotransmitters to organs other than the target organ are avoided. Chitosan encapsulation favors a controlled release than the rapid influx of active compounds.

We previously studied the role of GABA and 5-HT chitosan nanoparticle treatment individually and in combination in partially hepatectomized rats for enhanced hepatocyte proliferation (Shilpa et al., 2012a,b). Brainstem neurons are involved in the cardiovascular and respiratory control, alertness, and consciousness. Thus, brainstem damage is often a life-threatening problem. The present study assessed the potential of GABA and 5-HT chitosan nanoparticle treatment to reduce brainstem neuronal damage in partially hepatectomized rats. For achieving the aim, $GABA_B$ and $5\text{-}HT_{2A}$ receptor functional regulation, gene expression of neuronal survival, and apoptotic factors in the brainstem during GABA and 5-HT chitosan nanoparticle-induced active liver regeneration in rats were studied.

## MATERIALS AND METHODS

### Chemicals Used and Their Sources

Biochemicals and the Tri reagent kit were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade and were purchased locally. [$^3$H]baclofen (specific activity 42.9 Ci/mmol) and [$^3$H]ketanserin (specific activity 63.3 Ci/mmol) were purchased from Amersham Life Science (Amersham, United Kingdom). Chitosan (MW 25 kDa) was a gift from the Central Institute of Fisheries Technology, Cochin, India.

### Animals

Experiments were carried out on adult male Wistar rats of 250–300 g body weight purchased from Kerala Agricultural University, Mannuthy, India. They were housed in separate cages under 12-hr-light and 12-hr-dark periods and were maintained on standard food pellets and water ad libitum. All animal care and procedures were in accordance with the institutional, National Institutes of Health, and CPCSEA guidelines. All efforts were made to minimize animal suffering. Each group consisted of five animals. Sham-operated control (C), partially hepatectomized group without any treatment (PHNT), partially hepatectomized group treated with GABA chitosan nanoparticle (GCNP), partially hepatectomized group treated with 5-HT chitosan nanoparticle (SCNP), and partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle (GSCNP) were the five experimental groups.

### Preparation of GABA and 5-HT Chitosan Nanoparticles

The chitosan nanoparticles were prepared by the ionic gelation method (Calvo et al., 1997). The incorporation of GABA and 5-HT into chitosan nanoparticles individually and in combination, standardization of encapsulation efficiency, and in vitro release profile studies were performed according to Shilpa et al. (2012a,b). The nanoparticles were washed thoroughly and were dispersed in saline.

### Partial Hepatectomy and Sacrifice

Two-thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anesthesia, following a 16-hr fast (Higgins and Anderson, 1931). Sham operations involved median excision of the body wall, followed by all manipulations except removal of the lobes. All the surgeries were performed between 7 and 9 AM to avoid diurnal variations in responses. After surgery, 1 ml of 30 μg/μl GABA chitosan nanoparticles, 5-HT chitosan nanoparticles, and a combination of GABA and 5-HT chitosan nanoparticles suspended in saline were injected intraperitoneally into the rats. The rats were sacrificed by decapitation 24 hr posthepatectomy, and brain and brainstem was dissected out quickly and kept over ice according to the procedure of Glowinski and Iversen (1966). The tissues were stored at −80°C until assayed.

### $GABA_B$ and $5\text{-}HT_{2A}$ Receptor Binding Studies in the Brainstem Using [$^3$H]Baclofen and [$^3$H]Ketanserin

[$^3$H]Baclofen binding to $GABA_B$ receptor in the membrane preparations was assayed (Hills et al., 1987). Crude membrane preparation was suspended in 50 mM Tris sulfate buffer, pH 7.4, containing 2 mM $CaCl_2$ and 0.3–0.4 mg protein. In saturation binding experiments, 10–100 nM [$^3$H]baclofen was incubated with and without excess of 100 μM unlabeled baclofen. The incubations were carried out at 20°C for 20 min. The

binding reactions were terminated by centrifugation at 14,000*g* for 10 min. The dried pellet was resuspended and counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

[$^3$H]Ketanserin binding to 5-HT$_{2A}$ receptor in the crude synaptic membrane preparation was carried out according to the modified procedure of Leysen et al. (1982). Crude membrane preparation was suspended in 50 mM Tris sulfate buffer, pH 7.6, containing 0.3–0.4 mg protein. In saturation binding experiments, assays used different concentrations of 0.5–10 nM [$^3$H]ketanserin, which was incubated with and without excess of unlabeled 10 μM ketanserin. Tubes were incubated at 37°C for 15 min and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washings with 5.0 ml ice-cold 50 mM Tris sulfate buffer, pH 7.6. The bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The receptor binding parameters were determined via Scatchard analysis (Scatchard, 1949). The specific binding was determined by subtracting nonspecific binding from the total binding. The binding parameters maximal binding ($B_{max}$) and equilibrium dissociation constant ($K_d$) were derived by linear regression analysis by plotting the specific binding of the radioligand on the x-axis and bound/free on the y-axis. The maximal binding is a measure of the total number of receptors present in the tissue, and the equilibrium dissociation constant is a measure of the affinity of the receptors for the radioligand. The $K_d$ is inversely related to receptor affinity.

### Analysis of Gene Expression by Real-Time Polymerase Chain Reaction

PCR analyses were conducted with gene-specific primers and fluorescently labeled Taqman probe of GABA$_B$, 5-HT$_{2A}$, phospholipase C, NF-κB, TNF-α, Akt-1, Bax, and caspase-8, which were designed by Applied Biosystems (Foster City, CA). The endogenous control, β-actin, was labeled with a report dye, VIC. RNA was isolated from the brainstem of experimental rats using the Tri reagent according to the procedure of Chomczynski and Sacchi (1987). Total cDNA synthesis was performed with an ABI Prism cDNA archive kit in 0.2-ml microfuge tubes. The reaction mixture of 20 μl contained 0.2 μg total RNA, 10× RT buffer, 25× dNTP mixture, 10× random primers, MultiScribe RT (50 U/μl), and RNase-free water. The cDNA synthesis reactions were carried out at 25°C for 10 min and 37°C for 2 hr using an Eppendorf Personal Cycler. Real-time PCR assays were performed in 96-well plates in an ABI 7300 real-time PCR instrument (Applied Biosystems). The specific primers and probes were purchased from Applied Biosystems. The TaqMan reaction mixture of 20 μl contained 25 ng total RNA-derived cDNAs; 200 nM each of the forward primer, reverse primer, and TaqMan probe for assay on demand; endogenous control β-actin; and 12.5 μl TaqMan 2× Universal PCR Master Mix (Applied Biosystems), and the volume was made up with RNAse-free water. The following thermal cycling profile was used (40 cycles), 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min. Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCt method of relative quantification was used to determine the fold change in expression. This was done by normalizing the resulting threshold cycle (Ct) values of the target mRNAs to the Ct values of the internal control β-actin in the same samples ($\Delta Ct = Ctt\ target - Ct\beta\text{-actin}$). It was further normalized with the control ($\Delta\Delta Ct = \Delta Ct - Ct\ control$). The fold change in expression was then obtained as ($2^{-\Delta\Delta Ct}$), and the graph was plotted using log $2^{-\Delta\Delta Ct}$. Log RQ value of group C was considered to be zero, and the log RQ values for expression of the specific gene in all other experimental groups were represented by comparing with the log RQ value of C.

### Immunohistochemical Analysis by Confocal Microscopy

The experimental rats were deeply anesthetized and transcardially perfused with PBS (pH 7.4), followed by 4% paraformaldehyde in PBS (Chen et al., 2007). After perfusion, the brainstem from each experimental group was dissected out and fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in PBS (0.1 M). Ten-micrometer brainstem sections were cut with a cryostat (Leica CM1510 S). The sections were washed with PBS and then blocked for 1 hr with PBS containing 5% normal goat serum and 0.1% Triton X-100. The primary antibodies of GABA$_B$ (1:500 dilution in PBS with 5% normal goat serum and 0.1% Triton X-100) and 5-HT$_{2A}$ (1:1,000 dilution in PBS with 5% normal goat serum and 0.1% Triton X-100) were added to the various sections and incubated overnight at 4°C. After overnight incubation, the brain slices were rinsed with PBS and then incubated with fluorescence-labeled secondary antibody (Alexa Fluor 594; code A11012) prepared in PBS with 5% normal goat serum and 0.1% Triton X-100 at 1:1,000 dilution. The sections were washed with PBS thoroughly and then observed and photographed using a confocal imaging system (Leica SP 5). The specificity of the immunocytochemical procedure was validated by negative controls (data not shown) to ensure that the labeling method accurately identified the antibody bound to the specific receptors in the brainstem. Expression of GABA$_B$ and 5-HT$_{2A}$ receptors was analyzed by using the pixel intensity method. The given pixel value is the net value deducted from the negative control pixel value (Joseph et al., 2010). Quantification was in the Leica application suit advanced fluorescence (LASAF) software, considering the mean pixel intensity of the image. The fluorescence obtained depends on the number of receptors specific to the added primary antibody. The mean pixel intensity was directly related to the fluorescence emitted from the sections and calculated with the LASAF software. All the imaging parameters in the confocal imaging system, such as PMT, pinhole, and zoom factor, were kept same for imaging the sections of all experimental groups.

### Narrow Beam Walk Test

The motor deficit was studied by using a narrow beam walk test. After 3 days posthepatectomy and treatment, the animals were tested for balance and motor coordination on a narrow beam maze (Allbutt and Henderson, 2007). This has a smooth, wooden, narrow beam 105 cm long, 4 cm wide, and 3 cm thick. The beam was elevated above the ground by 1 m with additional supports. It has a start platform of 20 cm in dimension from the

start of the beam and an end platform of 20 cm dimension at the end of 105-cm-long beam. There was food on the end platform as reward for the animals. The journey time between start and end was measured. The time was recorded when the animal placed a weight-bearing step entirely over the start line. The stopwatch was then stopped when all four feet were placed entirely upon the finishing platform at the opposite end of the beam. The maximum time allowed for the task was 2 min.

### Statistical Analysis

Statistical evaluations used analysis of variance (ANOVA) in GraphPad Instat (version 2.04a; GraphPad, San Diego, CA). Student Newman-Keuls test was used to compare different groups after ANOVA. Linear regression Scatchard plots were made in Sigma Plot (version 2.03). Relative Quantification software was used for analyzing real-time PCR results.

## RESULTS

### $GABA_B$ and 5-$HT_{2A}$ Receptor Analysis in the Brainstem of Experimental Rats

$B_{max}$ represents the number of receptors, and $K_d$ denotes the affinity of receptors toward the ligand. $B_{max}$ of $GABA_B$ and 5-$HT_{2A}$ receptor binding studies showed a significant decrease ($P < 0.001$) in PHNT, GCNP, SCNP, and GSCNP compared with C. The $B_{max}$ of $GABA_B$ receptor assay of GCNP showed a significant decrease ($P < 0.01$) compared with PHNT. SCNP and GSCNP also showed a significant decrease ($P < 0.001$) compared with PHNT. The $B_{max}$ of 5-$HT_{2A}$ receptor binding study showed a significant decrease ($P < 0.001$) in GCNP, SCNP, and GSCNP compared with PHNT. There was no significant change in the $K_d$ values of any groups in either receptor study (Table I).

### Real-Time PCR Analysis of $GABA_B$, 5-$HT_{2A}$, NF-κB, TNF-α, Akt-1, Bax, Caspase-8, and Phospholipase C mRNA in the Brainstem of Experimental Rats

Gene expression studies of $GABA_B$ and 5-$HT_{2A}$ receptors mRNA in the brainstem of PHNT, GCNP, SCNP, and GSCNP showed a significant decrease ($P < 0.001$) compared with C. There was also a significant decrease ($P < 0.001$) in both receptors' gene expression of GCNP, SCNP, and GSCNP compared with PHNT (Figs. 1, 2).

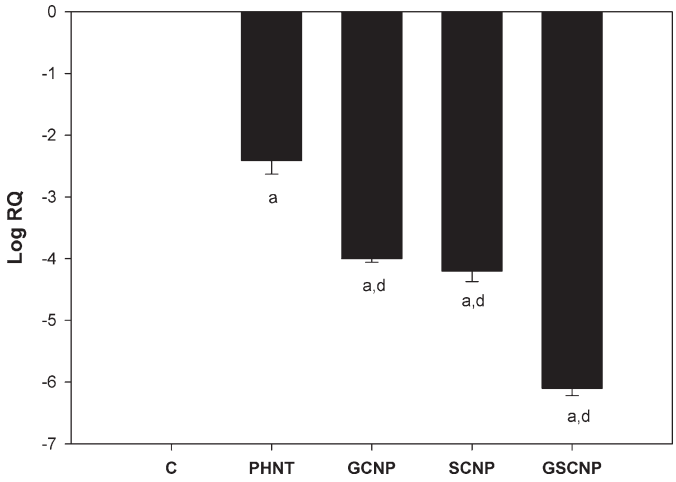


Fig. 1. Real-time PCR amplification of $GABA_B$ mRNA in the brainstem of experimental rats. Values are mean ± SEM of five separate experiments. Each group consists of five rats. [a]$P < 0.001$ compared with C. [d]$P < 0.001$ compared with PHNT. C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle.

The gene expressions of NF-κB, TNF-α, and Akt-1 mRNA showed a significant increase ($P < 0.001$) in PHNT, GCNP, SCNP, and GSCNP compared with C. The expression of these neuronal survival factors in all treatment groups showed a significant increase ($P < 0.001$) compared with PHNT (Figs. 3–5).

Neuronal apoptosis was studied by observing the gene expression patterns of Bax, caspase-8, and phospholipase C. The expressions of these three genes were significantly downregulated ($P < 0.001$) in all partially hepatectomized rats with and without nanoparticle treatment, compared

**TABLE I. $GABA_B$ and 5-$HT_{2A}$ Receptor Analysis in the Brainstem of Experimental Rats[a]**

| | $GABA_B$ | | 5-$HT_{2A}$ | |
|---|---|---|---|---|
| Group | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
| C | 82.5 ± 1.4 | 1.3 ± 0.11 | 125.3 ± 1.4 | 1.3 ± 0.10 |
| PHNT | 65.0 ± 2.8[b] | 1.4 ± 0.05 | 103.0 ± 1.1[b] | 1.2 ± 0.13 |
| GCNP | 52.3 ± 2.3[b,d] | 1.6 ± 0.11 | 79.0 ± 0.9[b,c] | 1.1 ± 0.11 |
| SCNP | 46.1 ± 2.3[b,c] | 1.5 ± 0.08 | 70.5 ± 1.0[b,c] | 1.2 ± 0.08 |
| GSCNP | 30.3 ± 1.4[b,c] | 1.8 ± 0.29 | 41.1± 1.1[b,c] | 1.0 ± 0.06 |

[a]C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle. $B_{max}$ represents the number of receptors and $K_d$ represents the affinity of receptors towards the ligand. Values are mean ± SEM of five separate experiments.
[b]$P < 0.001$ compared with C.
[c]$P < 0.001$ compared with PHNT.
[d]$P < 0.01$ compared with PHNT.

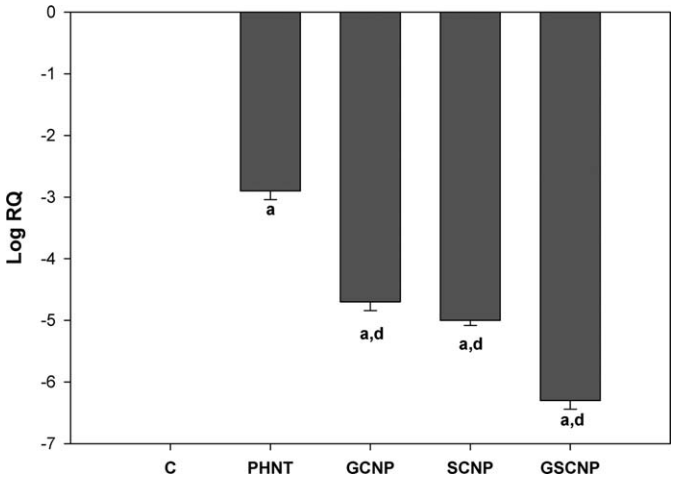


Fig. 2. Real-time PCR amplification of 5-$HT_{2A}$ mRNA in the brainstem of experimental rats. Values are mean ± SEM of five separate experiments. Each group consists of five rats. $^{a}P < 0.001$ compared with C. $^{d}P < 0.001$ compared with PHNT. C, sham operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle.

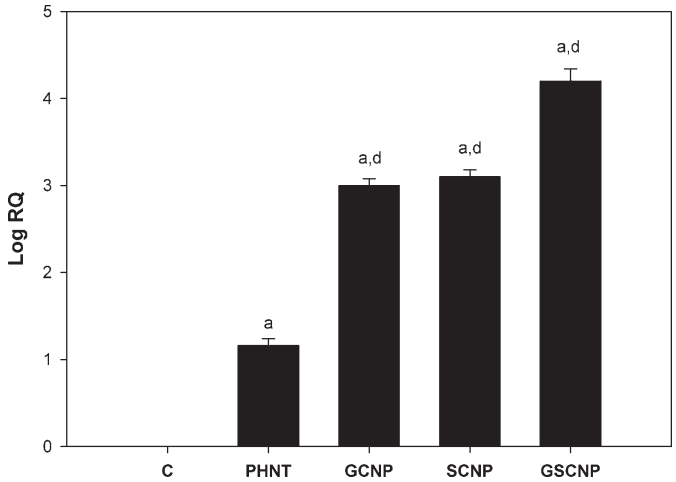


Fig. 4. Real-time PCR amplification of TNF-α mRNA in the brainstem of experimental rats. Values are mean ± SEM of five separate experiments. Each group consists of five rats. $^{a}P < 0.001$ compared with C. $^{d}P < 0.001$ compared with PHNT. C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle.

with C. Bax and caspase-8 gene expressions in all the treatment groups were significantly decreased ($P < 0.001$) compared with PHNT. Phospholipase C gene expression was significantly down regulated in GCNP and SCNP ($P < 0.01$) and also in GSCNP ($P < 0.001$) compared with

## $GABA_B$ and 5-$HT_{2A}$ Receptor Antibody Staining in the Brainstem of Experimental Rats Using Confocal Microscopy

$GABA_B$ and 5-$HT_{2A}$ receptor staining using receptor-specific primary antibody and fluorescence-labeled secondary

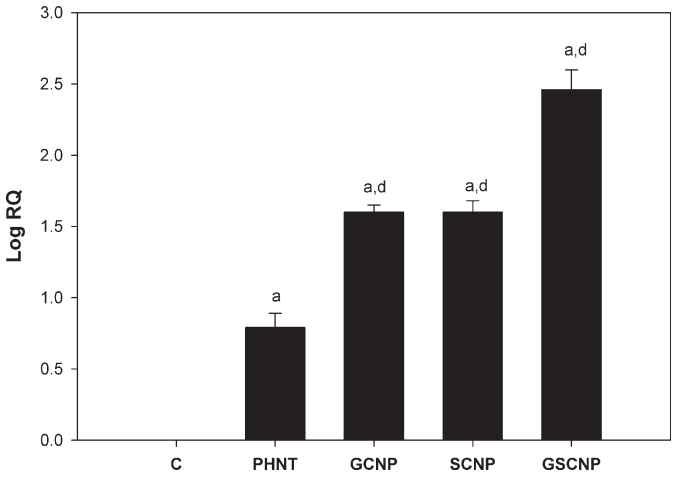


Fig. 3. Real-time PCR amplification of NF-κB mRNA in the brainstem of experimental rats. Values are mean ± SEM of five separate experiments. Each group consists of five rats. $^{a}P < 0.001$ compared with C. $^{d}P < 0.001$ compared with PHNT. C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle.

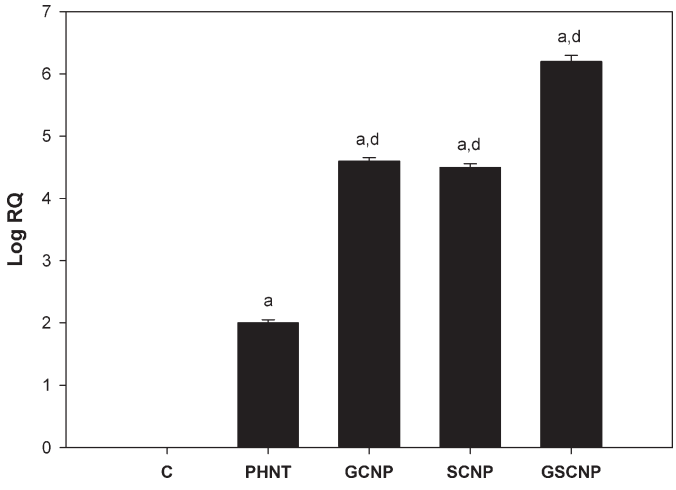


Fig. 5. Real-time PCR amplification of Akt-1 mRNA in the brainstem of experimental rats. Values are mean ± SEM of five separate experiments. Each group consists of five rats. $^{a}P < 0.001$ compared with C. $^{d}P < 0.001$ compared with PHNT. C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle.

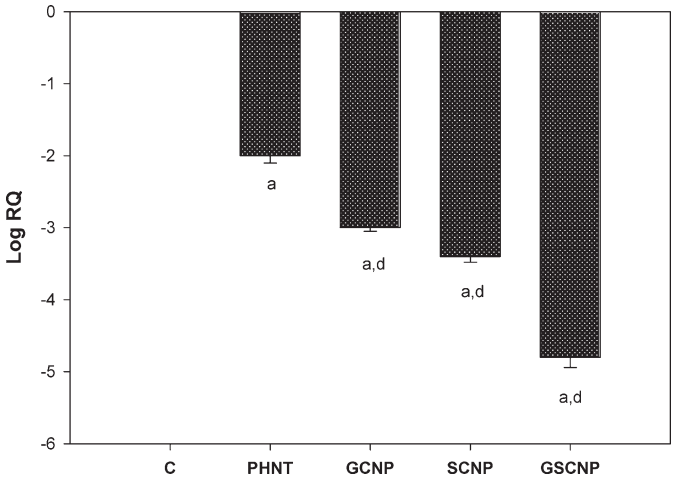


Fig. 6. Real-time PCR amplification of Bax mRNA in the brainstem of experimental rats. Values are mean ± SEM of five separate experiments. Each group consists of five rats. [a]$P < 0.001$ compared with C. [d]$P < 0.001$ compared with PHNT. C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle.

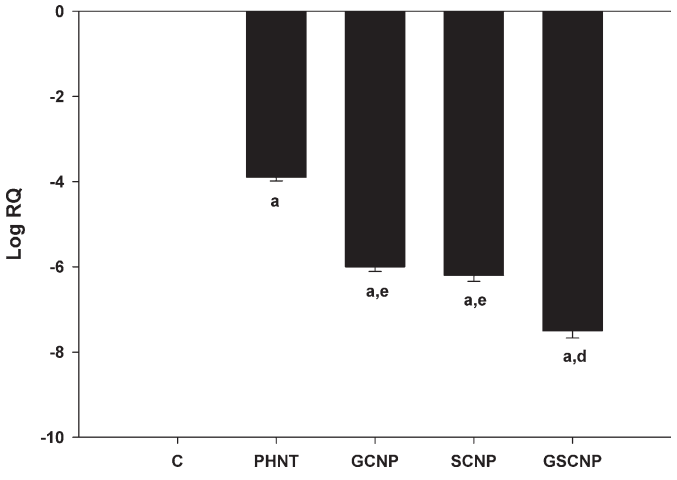


Fig. 8. Real-time PCR amplification of phospholipase C mRNA in the brainstem of experimental rats. Values are mean ± SEM of five separate experiments. Each group consists of five rats. [a]$P < 0.001$ compared with C. [d]$P < 0.001$, [e]$P < 0.01$ compared with PHNT. C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle.

antibody showed a significant change in all the groups. There was a significant decrease ($P < 0.001$) in the pixel intensity of brainstem sections with $GABA_B$- and 5-$HT_{2A}$-stained receptors in GCNP, SCNP, and GSCNP compared with C. There was a significant decrease in $GABA_B$ ($P < 0.05$) and 5-$HT_{2A}$ ($P < 0.01$) receptors in PHNT compared with C. The $GABA_B$ receptors viewed were significantly decreased ($P < 0.001$) in GCNP, SCNP, and GSCNP compared with PHNT (Table II, Fig. 9). The 5-$HT_{2A}$ receptors were decreased in GCNP ($P < 0.05$), SCNP ($P < 0.01$). and GSCNP ($P < 0.001$) compared with PHNT (Table III, Fig. 10).

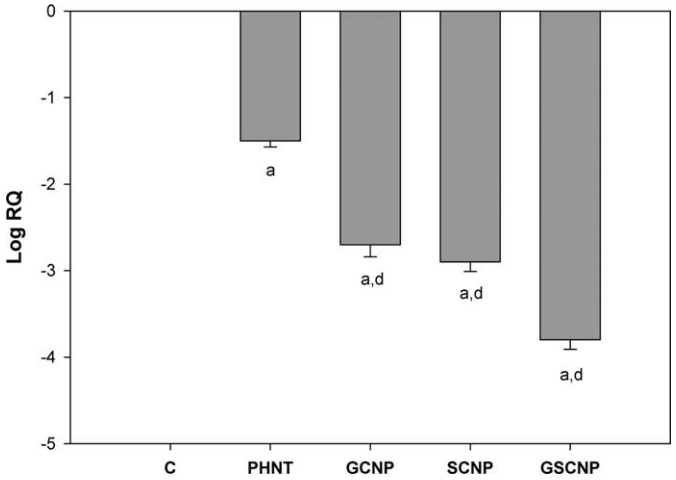


Fig. 7. Real-time PCR amplification of caspase-8 mRNA in the brainstem of experimental rats. Values are mean ± SEM of five separate experiments. Each group consists of five rats. [a]$P < 0.001$ compared with C. [d]$P < 0.001$ compared with PHNT. C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle.

## Narrow Beam Walk Test

Liver injury leads to changes in motor activity of the animal. The time taken for the rat to cover the entire beam showed a significant increase ($P < 0.001$) in PHNT, GCNP, SCNP, and GSCNP compared with C and a significant decrease ($P < 0.001$) in GCNP and SCNP compared with PHNT. The time taken for the rats in

**TABLE II. Confocal Imaging Studies of $GABA_B$ Receptors in the Brainstem of Experimental Groups[a]**

| Experimental group | Mean pixel intensity |
|---|---|
| C | 52.0 ± 1.7 |
| PHNT | 45.6 ± 1.4[c] |
| GCNP | 32.6 ± 1.4[b,d] |
| SCNP | 33.7 ± 1.4[b,d] |
| GSCNP | 25.3 ± 1.1[b,d] |

[a]C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle. Values are mean ± SEM of five separate experiments.
[b]$P < 0.001$ compared with C.
[c]$P < 0.05$ compared with C.
[d]$P < 0.001$ compared with PHNT.

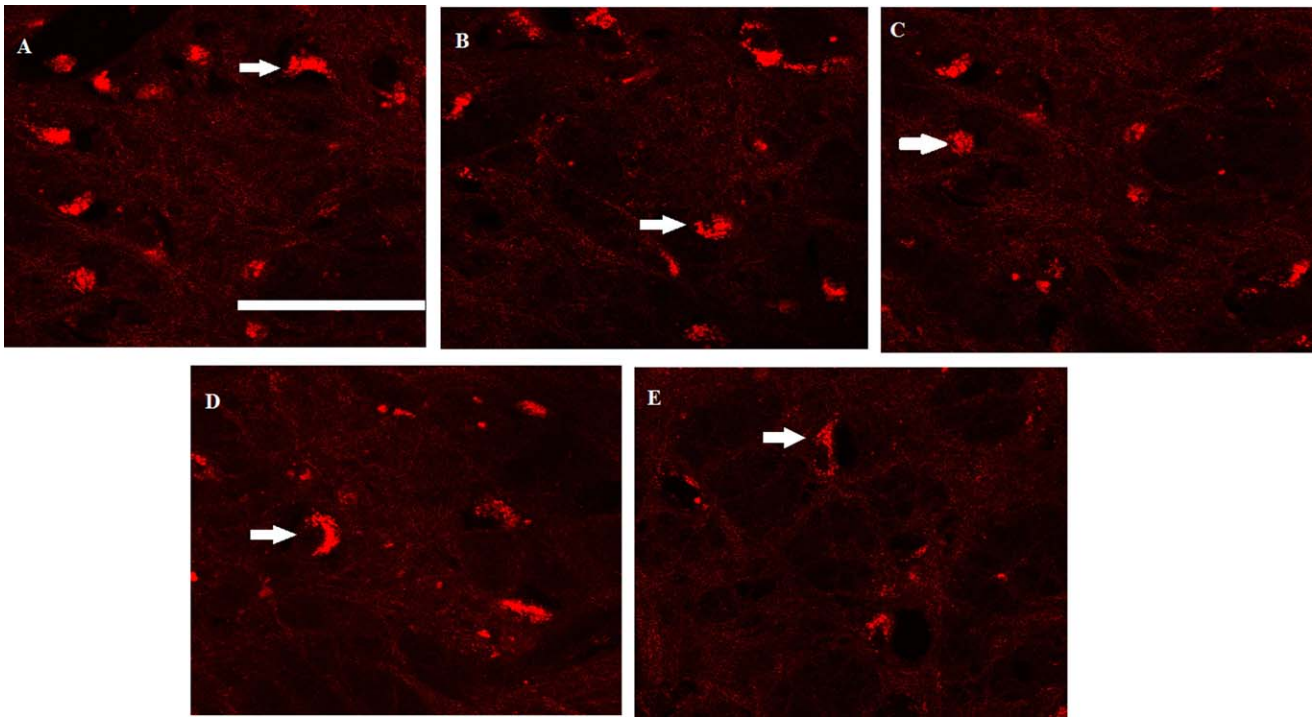


Fig. 9. Confocal image of $GABA_B$ receptors in the brainstem of control and experimental rats using immunofluorescent $GABA_B$ receptor-specific primary antibody and Alexa Fluor 594 as secondary antibody (arrows). **A:** Sham-operated control. **B:** Partially hepatectomized group with no treatment. **C:** Partially hepatectomized group treated with GABA chitosan nanoparticle. **D:** Partially hepatectomized group treated with 5-HT chitosan nanoparticle. **E:** Partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticles. Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

GSCNP showed a significant decrease ($P < 0.01$) compared with PHNT (Fig. 11).

## DISCUSSION

The functional relationship between the liver and the brain has been known for centuries. Butterworth (1995) reported that neurotransmission in the brain is altered in liver diseases. A spectrum of neuropsychiatric abnormalities in patients with liver dysfunction were observed and was characterized by intellectual impairments, personality changes, and a depressed level of consciousness associated with multiple neurotransmitter systems, cerebral perfusion, and astrocyte dysfunction (Avraham et al., 2009). Jain et al. (1991) reported an onset of mitochondrial damage in brain resulting from decreased synthesis of glutathione by damaged liver, which is the major glutathione synthesis site.

**TABLE III. Confocal Imaging Studies of 5-$HT_{2A}$ Receptors in the Brainstem of Experimental Groups[a]**

| Experimental group | Mean pixel intensity |
|---|---|
| C | 46.3 ± 1.4 |
| PHNT | 38.2 ± 1.3[c] |
| GCNP | 31.3 ± 2.0[b,f] |
| SCNP | 28.1 ± 1.6[b,e] |
| GSCNP | 24.7± 1.4[b,d] |

[a]C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle. Values are mean ± SEM of five separate experiments.
[b]$P < 0.001$ compared with C.
[c]$P < 0.01$ compared with C.
[d]$P < 0.001$ compared with PHNT.
[e]$P < 0.01$ compared with PHNT.
[f]$P < 0.05$ compared with PHNT.

The liver has a remarkable capacity to regenerate after cellular damage or tissue removal. Liver regeneration is mostly the result of increased mitosis of hepatocytes. Agonists of GABA can act at the GABA receptor complex, and increased concentrations of the agonists are found in the brainstem in liver failure (Basile and Jones, 1994). Neurosteroids produced in the brain during acute liver failure lead to increased GABAergic tone (Ahboucha et al., 2012a), and elevated intracerebral concentrations of GABA significantly decrease ornithine decarboxylase activity in the liver (Lapinjoki et al., 1983). This is an index for decreased liver cell proliferation and function. There is also an interesting report suggesting that 5-HT can potentially contribute to liver tissue hypoperfusion following hepatic ischemia and reperfusion (Murata et al., 2003).

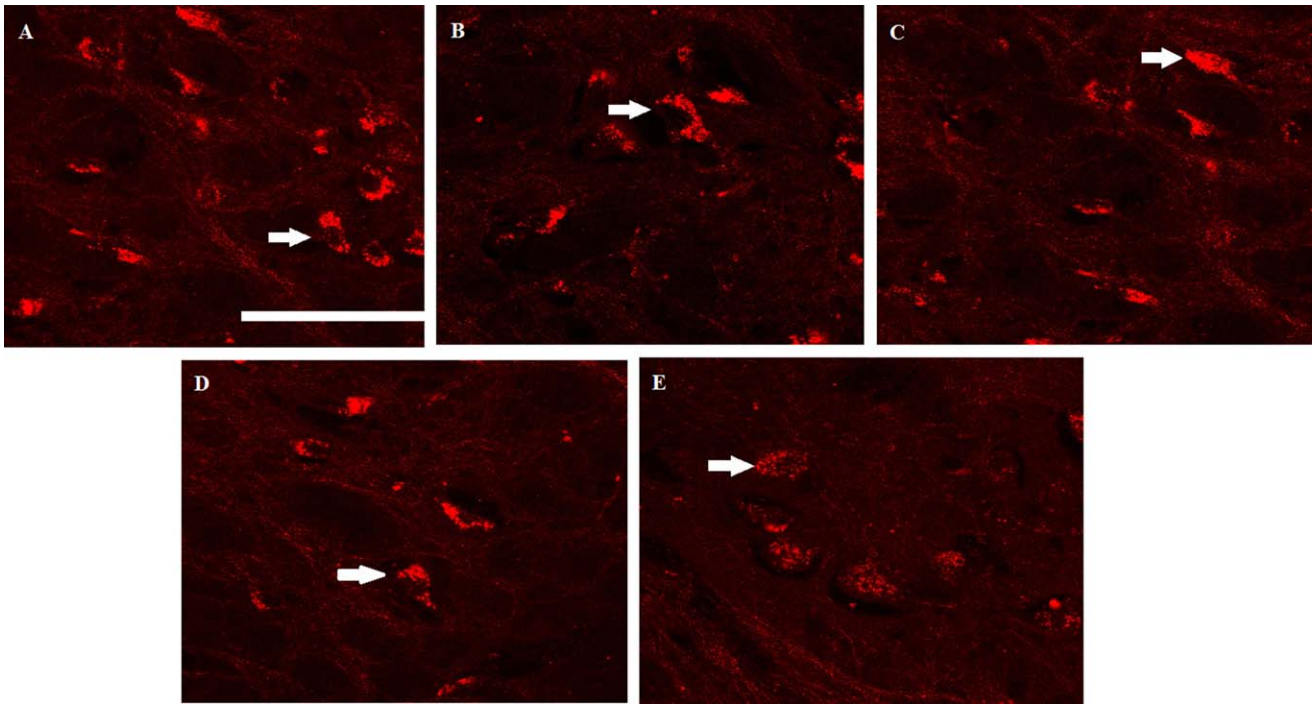


Fig. 10. Confocal image of 5-$HT_{2A}$ receptors in the brainstem of control and experimental rats using immunofluorescent 5-$HT_{2A}$ receptor specific primary antibody and Alexa Fluor 594 as secondary antibody (arrows). **A:** Sham-operated control. **B:** Partially hepatectomized group with no treatment. **C:** Partially hepatectomized group treated with GABA chitosan nanoparticle. **D:** Partially hepatectomized group treated with 5-HT chitosan nanoparticle. **E:** Partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle. Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

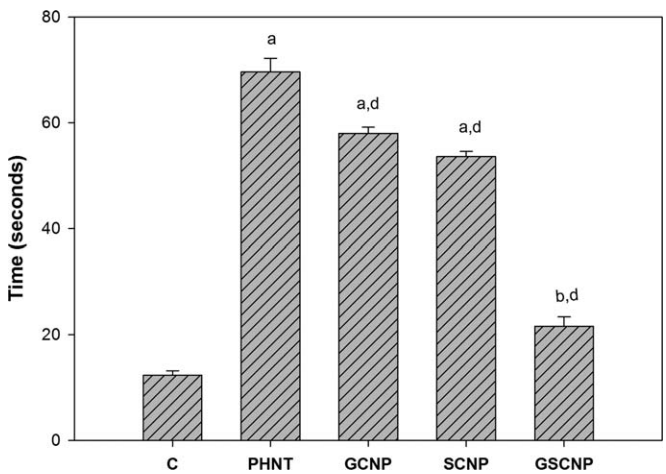


Fig. 11. Time taken by the experimental animals in narrow beam walk test. Values are mean ± SEM of five separate experiments. Each group consists of five rats. $^{a}P < 0.001$, $^{b}P < 0.01$ compared with C. $^{d}P < 0.001$compared with PHNT. C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle.

Sympathetic innervation is important for liver regeneration (Kiba et al., 1995). Our previous study showed increased DNA and protein syntheses, which are cell division markers, in the regenerating liver of GCNP, SCNP, and GSCNP compared with PHNT after 24 hr posthepatectomy (Shilpa et al., 2012a,b). During liver injury, ammonia metabolism is disturbed, leading to a condition called *hyperammonemia*. Hyperammonemia has been suggested to induce enhanced brainstem ammonia uptake and subsequent glutamine and GABA synthesis and accumulation. The changes in brain glutamate and GABA could be related to altered ammonia metabolism (Dejong et al., 1992). An increased level of ammonia leads to neuronal damage and alteration in the cardiovascular and respiratory centers in the brainstem (Saul et al., 2010). The autonomic regulation of GABA was mediated through $GABA_B$ receptors (Sved and Sved, 1990), and reduction in the GABA neurotransmission in the brain regions enhanced DNA synthesis in liver by facilitating the sympathetic tone (Biju, 2000; Biju et al., 2002). The reestablishment of ammonia metabolism in the body and GABA signaling in the brain as a result of liver cell proliferation was clearly studied by observing the reduced $GABA_B$ receptor expression in the treatment groups. GABA is an inhibitory neurotransmitter and an increased GABAergic neurotransmission is observed

during acute liver injury. Alterations of astrocytic–neuronal cross-talk affect brain function. In acute liver failure, astrocytes undergo swelling, which results in increased intracranial pressure and may lead to brain herniation (Ahboucha and Butterworth, 2007). Thus GABA and 5-HT chitosan nanoparticle treatment provided hope in rescuing the liver-injured animal. During active hepatocyte proliferation, the 5-HT receptor number was decreased in the brains of partially hepatectomized rats (Sudha, 1997). During liver injury and hepatic insufficiency, the aromatic amino acid catabolism was altered. Thus the plasma levels of aromatic amino acids increase, and they enter the brain. The aromatic amino acid tryptophan enhanced 5-HT synthesis in the brain, which leads to active 5-HT-mediated neurotransmission. As hepatic cell recovery progresses, the aromatic amino acid metabolism also was reactivated. Thus the 5-HT content is decreased in brain (Dejong et al., 1992). The neurotransmitter 5-HT has a profound effect on the control of sleep and mood fluctuations, so excess 5-HT activity in the brain could be responsible for impaired consciousness during liver failure. Our study reported the efficiency of GABA and 5-HT chitosan nanoparticle treatment in reducing the 5-HT receptor expression and signaling in brainstem, which was a good sign of recovery. In our results, the $GABA_B$ and $5\text{-}HT_{2A}$ receptor expression was downregulated in GCNP and SCNP and further decreased in GSCNP compared with PHNT, which supports the previous reports regarding the regulation of neurotransmission mediated through GABA and 5-HT receptors during liver regeneration. Thus, decrease in GABA and 5-HT receptors numbers was a homeostatic adjustment by the brainstem to activate the sympathetic innervation, thereby elevating DNA synthesis in the liver.

During any injury to the organs, a stress-mediated tissue degenerative signaling is activated in the brain. It was more prominent in the case of liver injury. Thus, mechanisms have been developed by the animal's own body to alleviate the effect of stress by activating neuronal survival molecules. Active liver regeneration, which is supported by increased neuronal survival helps the partially hepatectomized rats to recover easily, with less brain damage. NF-κB is expressed in diverse cell types in the nervous systems (Neill and Kaltschmidt, 1997). An involvement of NF-κB in neuronal development demonstrates its activation in neurons in certain regions of the brain during neurogenesis. Inhibition of NF-κB by an inhibitor such as diethyldithiocarbamate (DDTC) was shown to increase cell death and infarct size following transient ischemic insult in rats, suggesting that NF-κB induces survival signaling in neuronal cells (Hill et al., 2001). NF-κB is a heterodimer transcription factor that is sequestered in the cytoplasm by an anchor protein, inhibitor of NF-κB (IκB). Phosphorylation of NF-κB on serines 32 and 36 by IκB kinase leads to its ubiquitination and degradation by proteosomal enzymes, which allows NF-κB heterodimer to translocate to the nucleus and regulate gene expression. In GCNP, SCNP, and GSCNP, the NF-κB level was increased compared with PHNT, which showed an increased neuronal survival and maintenance. One well-studied pathway that leads to NF-κB activation is the involvement of the cytokine TNF-α through intracellular signaling molecules, TNF receptor-associated factors (TRAF2 and TRAF6), and activated NF-κB-inducing kinase (NIK), which phosphorylates the IKKs (Karin and Ben-Neriah, 2000). IKK can be phosphorylated by an alternative pathway, which involves Akt. Indeed TNF-α and platelet-derived growth factor (PDGF)-induced NF-κB activation has been reported to require Akt (Burow et al., 2000). Also, studies show that NF-κB appears to be a target of the antiapoptotic Ras/$PI_3K$/Akt pathway, and the expression and activity of Akt is regulated by NF-κB (Meng et al., 2002). The interrelated activation of NF-κB, TNF-α, and Akt-1 was required for the survival of neurons in the brainstem.

The differential patterns of localization of TNF-α receptors in neuronal and glial cells, their state of activation, and the downstream effectors all are thought to play an important role in determining whether TNF-α will exert a beneficial or harmful effect on the CNS (Figiel, 2008). Even though the neurodegenerative activity of TNF-α has been documented in many studies, several reports emphasize the neuroprotective role of the same. With a rat ischemia model, Hurtado and colleagues (2001, 2002) demonstrated that TACE (an enzyme required for the activation of TNF-α) is upregulated after ischemic brain damage and that the increase in TACE expression contributes to a rise in TNF-α and a subsequent neuroprotective effect after excitotoxic stimuli. TNF-α induces neuroprotection against excitotoxic damage in primary cortical neurons via sustained NF-κB activation (Dolga et al., 2008). The report of Larsen and Wendon (2002) explains clearly that inflammatory mediators such as TNF-α and neuronal survival factors such as NF-κB and Akt-1 play an important role in the regulation of brain function by liver. Thus, based on the evidence from these studies, irrespective of the neurodegenerative effect, TNF-α has a neuron-protective and survival effect along with NF-κB and Akt-1 during liver cell proliferation. From our study, in group GSCNP, a better neuron-survival signaling mechanism in the brainstem was observed compared with PHNT. Ankarcrona et al. (1995) explained that, during brain injury, glutamate accumulation leads to overstimulation of postsynaptic glutamate receptors with intracellular $Ca^{2+}$ overload and neuronal cell death. Overstimulation of neurotransmitter glutamate was decreased by treatment with GABA and 5-HT in a Parkinson's disease rat model (Nandhu et al., 2011). Thus, in our study, the combined effect of GABA and 5-HT treatment in GSCNP showed an increased expression of neuronal survival factors and resulted in reduced cell death.

Apoptosis of cells in the brainstem was clarified by studying the gene expression of caspase-8 and Bax. Neuronal apoptosis is achieved by two major apoptotic pathways (extrinsic and intrinsic). The extrinsic pathway involves the binding of cytokines to death receptors, activation of caspase-8 and cleavage and activation of effector caspase-3 (Ashkenazi and Dixit, 1998). Caspase-8 is a key factor uniquely associated with this pathway. The intrinsic

pathway involves translocation of Bax protein from the cytosol to the outer mitochondrial membrane, where it increases membrane permeability and promotes release of cytochrome c, which binds with apoptotic protease activating factor-1 (Apaf-1) and procaspase-9, resulting in its cleavage to form activated caspase-9 (Green and Reed, 1998). The JNK family of mitogen-activated protein kinase (MAPK) pathway has been observed to play a central role in both of these apoptotic pathways (Dhanasekaran and Reddy, 2008). MAPK can be phosphorylated by mitogens binding to G-protein-coupled receptors (Yagle et al., 2001). Because of the disturbed ammonia and aromatic amino acid metabolisms in partially hepatectomized rats, GABA and 5-HT contents in the brain are increased, which in turn elevates their receptor-mediated signaling and MAPK phosphorylation. JNKs activate apoptotic signaling either through the upregulation of proapoptotic genes via the transactivation of specific transcription factors including c-Jun or through directly modulating the activities of mitochondrial pro- and antiapoptotic proteins through phosphorylation events. There is also evidence for cross-talk between the Bax-regulated intrinsic mitochondrial pathway and the extrinsic death receptor pathway, in which activated caspase-8 is a key enzyme (Li et al., 1998).

There is evidence explaining how that increase in GABA and 5-HT contents activates apoptotic signaling. Anju et al. (2011) reported that a trigger in the altered transcription of $GABA_B$ receptors can be related to activation of the apoptotic pathways by activating Bax expression. 5-HT also activates the mitogen-activated protein kinase, which can influence cell apoptosis (Watts, 1996). Active liver cell proliferation regains the capacity to metabolize aromatic amino acids and ammonia. Thus, decreased expression of GABA and 5-HT in the brainstem suppresses the MAPK phosphorylation and increases neuronal survival in partially hepatectomized rats. In our study, caspase-8 and Bax expression were decreased in all the treatment groups compared with PHNT. Active caspase-8 further activates other caspases such as caspase-3 and results in DNA fragmentation (Kuwana et al., 1998). Thus, reduction in caspase-8 expression showed decreased cell death in nanoparticle-treated rats. Phospholipase C catalyzes the hydrolysis of phosphatidylinositol (4,5)-bisphosphate, resulting in the production of diacylglycerol (DAG) and $IP_3$, which activate protein kinase C (PKC). Phospholipase C (PLC) is the enzyme involved in the synthesis of $IP_3$, so the increased levels of $IP_3$ and PLC result in enhanced apoptosis (Szalai et al., 1999). The activators of inhibitory subunit of G-protein receptors significantly suppressed the accumulation of inositol-1-phosphate and inositol-1,4,5-triphosphate in cells (Ohmori and Kuriyama, 1989). $IP_3$ receptor inactivation phenotypically mimics Bax deficiency by attenuating caspase-3 expression and activation (Prasitsilp et al., 2000). From our study it was clear that the gene expression of caspase-8, Bax, and phospholipase C were decreased significantly in GCNP and SCNP and further decreased in GABA and 5-HT chitosan nanoparticle-treated groups compared with PHNT, which showed a reduction in neuronal apoptosis in brainstem. The apoptosis and neuronal functional instability resulting from disturbed metabolism and storage capabilities after liver injury suppress many motor activities of the animal. The narrow beam walk test gave a vivid idea about the time spent to traverse the entire length of the rod by the partially hepatectomized rats with no treatment. Motor activity was regained by the animals treated with GABA and 5-HT chitosan nanoparticles individually and in combination. Thus the time taken to traverse the entire length of the rod is decreased in the treated groups.

Hepatocyte proliferation is promoted at the expense of liver function. This was supported by Tanaka et al. (1999), who found that functional regeneration measured by $^{99m}$Tc-GSA scintigraphy was impaired, compared with the volumetric regeneration in patients who had undergone extensive liver resection. With the loss of active liver cells, the metabolism and function of all body organs are disturbed. From our previous study, uptake of [$^3$H]thymidine and [$^3$H]leucine, which are the markers for DNA and protein syntheses, was significantly increased in the treatment groups compared with the untreated group. This clearly shows an increase in hepatic mass after partial hepatectomy, which further relates to an increase in liver function (Shilpa et al., 2012a,b). Impaired ammonia and aromatic amino acid metabolisms and the effect of neurosteroids such as allopregnanolone (Ahboucha et al., 2006) and dehydroepiandrosterone sulfate (Ahboucha et al., 2012b) contribute to increased GABAergic tone in hepatic encephalopathy. Abrasive changes in GABA and 5-HT neurotransmission furthermore lead to coma, mood alterations, lack of consciousness, and disturbances in cardiovascular and respiratory centers of brainstem. As liver regeneration progresses, the brain regains normal functional capacity. The motor control deficit observed in the untreated partially hepatectomized rats was regained to a great extend in GCNP, SCNP, and GSCNP. This emphasizes the liver and brain function recovery in the present study.

Brain and liver functions are interrelated. The present work reveals the potential for GABA and 5-HT chitosan nanoparticle treatment in improving neuronal survival and reducing neuronal apoptosis in the brainstem after partial hepatectomy in rats. We propose that this supplementation in combination will have better effects against the neuronal loss in brainstem than individual treatment during liver injury. It was evident that GABA and 5-HT chitosan nanoparticle treatment in partially hepatectomized rats provides neuronal protection that will have therapeutic significance in the management of liver-based diseases.

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Molecular and cellular pharmacology

# Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors functional regulation during enhanced liver cell proliferation by GABA and 5-HT chitosan nanoparticles treatment

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ABSTRACT

Liver is one of the major organs in vertebrates and hepatocytes are damaged by many factors. The liver cell maintenance and multiplication after injury and treatment gained immense interest. The present study investigated the role of Gamma aminobutyric acid (GABA) and serotonin or 5-hydroxytryptamine (5-HT) coupled with chitosan nanoparticles in the functional regulation of Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors mediated cell signaling mechanisms, extend of DNA methylation and superoxide dismutase activity during enhanced liver cell proliferation. Liver injury was achieved by partial hepatectomy of male Wistar rats and the GABA and 5-HT chitosan nanoparticles treatments were given intraperitoneally. The experimental groups were sham operated control (C), partially hepatectomised rats with no treatment (PHNT), partially hepatectomised rats with GABA chitosan nanoparticle (GCNP), 5-HT chitosan nanoparticle (SCNP) and a combination of GABA and 5-HT chitosan nanoparticle (GSCNP) treatments. In GABA and 5-HT chitosan nanoparticle treated group there was a significant decrease ($P<0.001$) in the receptor expression of Gamma aminobutyric acid B and a significant increase ($P<0.001$) in the receptor expression of 5-hydroxy tryptamine 2A when compared to PHNT. The cyclic adenosine monophosphate content and its regulatory protein, presence of methylated DNA and superoxide dismutase activity were decreased in GCNP, SCNP and GSCNP when compared to PHNT. The Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors coupled signaling elements played an important role in GABA and 5-HT chitosan nanoparticles induced liver cell proliferation which has therapeutic significance in liver disease management.





## 1. Introduction

Liver is an important organ responsible for storage, metabolism and synthesis of major compounds in the body (Rubin and Farber, 1999). Among all organs, the liver has the ability to repair itself after suffering loss of tissue mass. Therefore faster liver regeneration with healthy hepatocytes helps the animals for effective recovery from the metabolic break (Corbin et al., 2003).

Gamma aminobutyric acid (GABA) is an important inhibitory neurotransmitter in the vertebrate central nervous system. The cell proliferative role of GABA is observed in different regions of the body including the development of outer retina in rabbits (Messersmith and Redburn, 1993), leydig cell multiplication in testis (Geigerseder et al., 2004) and promotes neuronal cell proliferation and migration (Ben-Yaakov and Golan, 2003). Biju et al. (2002) reported that baclofen, a GABA agonist, induced epidermal growth factor mediated DNA synthesis in hepatocytes *in vitro*. The cell proliferation is initialized by the activation of cyclic adenosine monophosphate (cAMP) regulated transcription factors in the regenerating liver (Diehl and Rai, 1996).

Serotonin or 5-hydroxytryptamine (5-HT) has been shown to be mitogenic in many cells, exerting its effect through receptor mediated second messenger pathways. The serotonin 2 receptor has been shown to have mediated cell growth in fibroblasts (Van Obberghen-Schilling et al., 1991). The serotonin 2 receptors are coupled to phospho-inositide turnover and diacylglycerol formation, which activates protein kinase C, an important second messenger for cell division (DeCourcelles et al., 1985). Balasubramanian and Paulose (1998) reported that serotonin can act as a potent co-mitogen and induce DNA synthesis in primary cultures of rat hepatocytes.

The use of nanotechnology in the field of medicine could revolutionize the way we detect and treat the damage to human body and disease in the future. Chitosan, deacetylated chitin, is a copolymer of

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β-(1,4) linked glucosamine (deacetylated unit) and N-acetyl glucosamine (acetylated unit) (Radhakumary et al., 2005). Chitosan nanopartcles are biodegradable, non-toxic and biocompatible.

Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radical into $H_2O_2$ and high levels of SOD decreases the reactive oxygen species concentration. Reactive oxygen species has been proposed to stimulate cell cycle progression as an intrinsic cellular signal (Burdon, 1995). Methionine adenosyltransferase (MAT) is an essential enzyme during liver regeneration because it catalyzes the formation of *S*-adenosylmethionine, the main methyl donor for DNA methylation. *MAT2A*, MAT-encoding gene, is found in mammals and expressed in the proliferating liver (Rodríguez et al., 2007). Thus extend of methylation and gene expression of MAT2A enzyme provided an idea about the variations in DNA modification in the regenerating liver.

Our previous studies explained the preparation and standardization of GABA and 5-HT chitosan nanoparticles and their advantages on pure neurotransmitter treatment for the active hepatocyte proliferation (Shilpa et al., 2012a, 2012b). The present study focused on the analysis of functional regulation of Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors by GABA and 5-HT chitosan nanoparticles in partially hepatectomised rat liver followed by their signaling through cAMP linked pathways, the extend of DNA methylation and the activity of SOD.

## 2. Materials and methods

### 2.1. Chemicals used and their sources

Biochemicals and Tri-reagent kit were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally. [$^3$H] baclofen (Sp. Activity 42.9 Ci/mmol) and [$^3$H] ketanserin (Sp. Activity 63.3 Ci/mmol) were purchased from Amersham Life Science, UK. Chitosan (MW–25KDa) was a gift from Central Institute of Fisheries Technology, Cochin, India.

### 2.2. Animals

Experiments were carried out on adult male Wistar rats of 250—300 g body weight purchased from Kerala Agricultural University, Mannuthy, India. They were housed in separate cages under 12 h light and 12 h dark periods and were maintained on standard food pellets and water *ad libitum*. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health and CPCSEA guidelines. All efforts were made to minimize animal suffering. Each group consisted of 6–8 animals. Sham operated control (C), partially hepatectomised group without any treatment (PHNT), partially hepatectomised group with GABA chitosan nanoparticle treatment (GCNP), partially hepatectomised group with 5-HT chitosan nanoparticle treatment (SCNP) and partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment (GSCNP) were the five experimental groups. Chitosan nanoparticles modified with drugs, could be recognized by their respective receptors on cells and were transferred into hepatocytes via receptor mediated endocytosis. This enhanced their ability to target to the liver, in which receptor mediated cell signaling was activated, and enabled the longevity of these nanoparticles in the liver. In contrast, chitosan nanoparticles without modification, targeted sparely to the liver, and a large part of these nanoparticles were cleared from the body in urine (Tian et al., 2010; Park et al., 2007). So giving importance to the above fact and based on our previous observations, a control group treated with chitosan nanoparticle alone was not included.

### 2.3. Experimental procedures

#### 2.3.1. Preparation of GABA and 5-HT chitosan nanoparticles

The chitosan nanoparticles were prepared by ionic gelation method (Calvo et al., 1997). The incorporation of GABA and 5-HT into chitosan nanoparticles individually and in combination, standardization of encapsulation efficiency and *in vitro* release profile studies were done according to Shilpa et al. (2012a, 2012b). The nanoparticles were washed thoroughly and were dispersed in saline.

#### 2.3.2. Partial hepatectomy and treatment

Two-thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anesthesia, following a 16 h fast (Higgins and Anderson, 1931). Sham operations involved median excision of the body wall followed by all manipulations except removal of the lobes. All the surgeries were done between 7 and 9 A.M. to avoid diurnal variations in responses. After surgery, 1 ml of 30 μg/μl GABA chitosan nanoparticles, 5-HT chitosan nanoparticles and a combination of GABA and 5-HT chitosan nanoparticles suspended in saline were injected intra peritoneal to the respective rats. The rats were killed by decapitation 24 h post hepatectomy and liver was dissected out quickly and kept over ice. The tissues were stored at −80 °C until assayed.

#### 2.3.3. Quantification of cAMP

The liver from each experimental group was homogenized in a polytron homogenizer in 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000*g* for 15 min and the supernatant was transferred to fresh tubes for cAMP assay using [$^3$H] cAMP Biotrak Assay System kit. The unknown concentrations were determined from the standard curve using appropriate dilutions and calculated for pmoles/mg protein. $C_o/C_x$ was plotted on the *y*-axis against picomoles of inactive cAMP standards on the *x*-axis of a linear graph paper, where $C_o$ is the counts per minute bound in the absence of unlabeled cAMP and $C_x$ is the counts per minute bound in the presence of standard or unknown unlabeled cAMP. From the $C_o/C_x$ value for the sample, the number of picomoles of unknown cAMP was calculated. Protein was measured according to Lowry et al. (1951) using bovine serum albumin as standard. The intensity of the purple blue color formed was proportional to the amount of protein which was read in a spectrophotometer at 660 nm.

#### 2.3.4. Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors binding studies using [$^3$H] baclofen and [$^3$H] ketanserin

[$^3$H] Baclofen binding to GABA receptor in the membrane preparations were assayed (Hills et al., 1987). Crude membrane preparation was suspended in 50 mM Tris sulfate buffer, pH 7.4 containing 2 mM $CaCl_2$ and 0.3–0.4 mg protein. In saturation binding experiments, 10–100 nM of [$^3$H] baclofen was incubated with and without excess of 100 μM unlabeled baclofen. The incubations were carried out at 20 °C for 20 min. The binding reactions were terminated by centrifugation at 14000*g* for 10 min. The dried pellet was resuspended and counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

[$^3$H] Ketanserin binding to 5-hydroxy tryptamine 2A receptor in the crude synaptic membrane preparation was done according to the modified procedure of Leysen et al. (1982). Crude membrane preparation was suspended in 50 mM Tris sulfate buffer, pH 7.6 containing 0.3–0.4 mg protein. In saturation binding experiments, assays were done using different concentrations of 0.5–10 nM of [$^3$H] ketanserin which was incubated with and without



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excess of unlabeled 10 μM ketanserin. Tubes were incubated at 37 °C for 15 min and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washings with 5.0 ml of ice cold 50 mM Tris sulfate buffer, pH 7.6. The bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The receptor binding parameters were determined using Scatchard analysis (Scatchard, 1949). The specific binding was determined by subtracting non-specific binding from the total binding. The binding parameters, maximal binding ($B_{max}$) and equilibrium dissociation constant ($K_d$), were derived by linear regression analysis by plotting the specific binding of the radioligand on *x*-axis and bound/free on *y*-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The $K_d$ is inversely related to receptor affinity.

### 2.3.5. Superoxide dismutase assay

The liver from each experimental group was homogenized in a buffer containing 10 mM EDTA, 50 mM Tris–HCl, pH 8.2 and centrifuged at 40000*g* for 15 min. The protein content of whole supernatant of the total homogenate was estimated (Lowry et al., 1951). SOD was analyzed after the inhibition by SOD of the pyrogallol autoxidation (Marklund and Marklund, 1974) at pH 8.2. Briefly, 1 ml reaction mixture contained 0.2 mM pyrogallol, 1 mM EDTA and 50 mM Tris–HCl buffer. Pyrogallol autoxidation was monitored at 420 nm for 3 min with or without 250 μg enzyme protein. The inhibition of pyrogallol was linear with the activity of the enzyme present. Fifty percent inhibition/(mg protein $min^{-1}$) was considered as one unit of enzyme activity.

### 2.3.6. Analysis of gene expression by real-time polymerase chain reaction

PCR analyses were conducted with gene-specific primers and fluorescently labeled Taqman probe of Gamma aminobutyric acid B, 5-hydroxy tryptamine 2A, cAMP regulatory element binding protein (CREB), MAT2A, SOD and hepatocyte growth factor (HGF) which were designed by Applied Biosystems. Endogenous control, β-actin, was labeled with a report dye, VIC.

RNA was isolated from the liver of experimental rats using the Tri-reagent according to the procedure of Chomczynski and Sacchi (1987). Total cDNA synthesis was performed using ABI PRISM cDNA archive kit in 0.2 ml microfuge tubes. The reaction mixture of 20 μl contained 0.2 μg total RNA, 10XRT buffer, 25X dNTP mixture, 10 X random primers, MultiScribe RT (50 U/μl) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 min and 37 °C for 2 h using an Eppendorf Personal Cycler. Real-time-PCR assays were performed in 96-well plates in an ABI 7300 Real-time-PCR instrument (Applied Biosystems). The specific primers and probes were purchased from Applied Biosystems, Foster City, CA, USA. The TaqMan reaction mixture of 20 μl contained 25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer and TaqMan probe for assay on demand and endogenous control β-actin and 12.5 μl of Taqman 2 × Universal PCR Master Mix (Applied Biosystems) and the volume was made up with RNAse free water. The following thermal cycling profile was used (40 cycles): 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The $^{\Delta\Delta}$CT method of relative quantification was used to determine the fold change in expression. This was done by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($^{\Delta}$CT=CT Target−CTβ-actin). It was further normalized with the control ($^{\Delta\Delta}$CT=$^{\Delta}$CT−CT Control). The fold change in expression was then obtained as (2−$^{\Delta\Delta}$CT) and the graph was plotted using log 2−$^{\Delta\Delta}$CT.

### 2.3.7. DNA methylation study

The DNA was isolated from the liver of experimental rats using TRI reagent according to the procedure of Chomczynski (1993). DNA concentration was determined by ultraviolet spectrophotometry (UV-1700 Pharma Spec, Shimadzu) with absorbance at 260 and 280 nm. All DNA samples had 260 to 280 absorbance ratios ≥1.7. DNA methylation was determined by using the modified method of Balaghi and Wagner (1993), in which DNA is incubated with [$^3$H] methyl *S*-adenosylmethionine in the presence of the CpG Methyl transferase. The reaction mixture contained 0.25 μg DNA, 0.015 U CpG Methyl transferase enzyme (product no. M0226S; New England Biolabs, Beverly, MA), [$^3$H] methyl *S*-adenosylmethionine (80 Ci/mmol, American radiolabeled chemicals, Inc., Saint Louis, USA), 1.5 μl NEB buffer (New England Biolabs, Beverly, MA), and sterile-filtered water to a total reaction volume of 15 μl. The mixture was incubated at 30 °C for 1 h and placed on ice for 5 min. The reaction mixture were loaded onto a 2.5 cm, round, Whatman DE81 ion-exchange paper filter. The filter was washed successively three times with 7.5 ml of 0.5 M sodium phosphate buffer (pH 8.0), then with 1 ml 70% ethanol, and finally with 1 mL 100% ethanol. The filter was dried at room temperature and the radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The ability of DNA to incorporate [$^3$H] methyl groups *in vitro* is inversely related to endogenous DNA methylation.

### 2.3.8. Immunohistochemical analysis by confocal microscope

The experimental rats were deeply anesthetized and was transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. After perfusion the liver from each experimental group was dissected out and fixed in 4% paraformaldehyde for 1 h and then equilibrated with 30% sucrose solution in PBS (0.1 M). 10 μm liver sections were cut using Cryostat (Leica, CM1510 S). The sections were washed with PBS and then blocked for 1 h with PBS containing 5% normal goat serum and 0.1% triton X-100. The primary antibodies of Gamma aminobutyric acid B (1500 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) and 5-hydroxy tryptamine 2A (1:1000 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) were added to the respective sections and incubated overnight at 4 °C. After overnight incubation, the liver slices were rinsed with PBS and then incubated with fluorescent labeled secondary antibody (Alexa Fluor 594, code-A11012) prepared in PBS with 5% normal goat serum and 0.1% triton X-100 at 1:1000 dilution. The sections were washed with PBS thoroughly and then observed and photographed using confocal imaging system (Leica SP 5). Quantification was done using Leica application suit advanced fluorescence (LASAF) software by considering the mean pixel intensity of the image. The fluorescence obtained depends on the number of receptors specific to the added primary antibody. The mean pixel intensity was directly related to the fluorescence emitted from the sections and calculated with the LASAF software. All the imaging parameters in the confocal imaging system like PMT, pinhole and zoom factor were kept same for imaging the sections of all experimental groups.

### 2.3.9. Statistical analysis

Statistical evaluations were done with analysis of variance (ANOVA), using GraphPad Instat (version 2.04a, San Diego, USA). Student Newman-Keuls test was used to compare different groups after ANOVA. Linear regression Scatchard plots were made using



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SIGMA PLOT (Ver 2.03). Relative Quantification Software was used for analyzing Real-Time PCR results.

## 3. Results

### 3.1. Analysis of Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptor studies

Gamma aminobutyric acid B receptor and 5-hydroxy tryptamine 2A receptor subtypes mediated liver cell multiplication in partially hepatectomised rats were studied. $B_{max}$ represents the number of receptors and $K_d$ represents the affinity of receptors towards the ligand. $B_{max}$ of Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptor study showed a significant decrease ($P<0.001$) for groups PHNT, GCNP, SCNP and GSCNP when compared to C. The $B_{max}$ of Gamma aminobutyric acid B receptor was significantly decreased in GCNP ($P<0.001$) and SCNP ($P<0.01$) when compared to PHNT. The 5-hydroxy tryptamine 2A receptor $B_{max}$ was significantly increased in GCNP ($P<0.05$) and SCNP ($P<0.001$) when compared to PHNT. In GSCNP group there was a significant decrease ($P<0.001$) in the receptor $B_{max}$ of Gamma aminobutyric acid B and a significant increase ($P<0.001$) in the receptor $B_{max}$ of 5-hydroxy tryptamine 2A when compared to PHNT. The $K_d$ value of Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors for PHNT, GCNP and GSCNP showed a significant increase ($P<0.001$) with respect to C. In SCNP group, $K_d$ value of Gamma aminobutyric acid B receptor showed a significant increase and 5-hydroxy tryptamine 2A receptor showed a significant decrease ($P<0.001$) with that of PHNT. While comparing with PHNT, $K_d$ value of Gamma aminobutyric acid B receptor binding in GCNP, SCNP and GSCNP and 5-hydroxy tryptamine 2A receptor binding in SCNP showed a significant decrease ($P<0.001$) (Table 1).

### 3.2. Quantification of cAMP

cAMP is involved in G protein coupled receptor mediated cell signaling during liver regeneration. cAMP content of the regenerating liver of PHNT was significantly decreased ($P<0.01$) compared to C. For groups GCNP, SCNP and GSCNP also the cAMP content was significantly reduced ($P<0.001$) when compared to C. The cAMP content of groups GCNP and SCNP showed a significant decrease ($P<0.05$) and group GSCNP also showed a significant decrease ($P<0.01$) when compared to PHNT (Fig. 1).

### 3.3. SOD assay

The concentration of antioxidant enzyme SOD depends on the formation of reactive oxygen species in the body. The reactive oxygen species levels were increased due to any kind of damage to the cells, which further led to an increase in the SOD activity also. The SOD activity was significantly decreased ($P<0.001$) for groups PHNT, G and GCNP when compared to C. There was a significant decrease ($P<0.001$) in SOD concentration in GCNP, SCNP and GSCNP when compared to PHNT (Table 2).

### 3.4. Real-time PCR analysis of Gamma aminobutyric acid B, 5-hydroxy tryptamine 2A, CREB, MAT2A, SOD and HGF mRNA

Gene expression study of Gamma aminobutyric acid B receptor mRNA in the regenerating liver of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($P<0.001$) when compared to C and also a significant decrease ($P<0.001$) in each of the nanoparticle treated groups when compared to PHNT (Fig. 2).

For 5-hydroxy tryptamine 2A receptor, when compared to C the gene expression was observed to be decreased significantly ($P<0.001$) in all the partially hepatectomised groups with or without nanoparticle treatment. There was no significant change in the expression of the receptor for GCNP when compared to PHNT. Considering the other groups, there was a significant increase in 5-hydroxy tryptamine 2A receptor gene expression in SCNP ($P<0.01$) and GSCNP ($P<0.001$) when compared to PHNT (Fig. 3).

The gene expression of transcription factor CREB, which was activated by cAMP, involved in the signaling cascade of G protein coupled receptors showed a significant decrease ($P<0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The gene expression of CREB was decreased significantly ($P<0.001$) in GCNP, SCNP and GSCNP when compared to PHNT (Fig. 4).

The methionine adenosyltransferase (MAT), which catalyzes the formation of the methyl donor *S*-adenosylmethionine, helps in DNA methylation. Its gene expression was significantly down

**Table 1**
Q5 Gamma aminobutyric acid B and 5-Hyhroxy tryptamine 2A receptors analysis in the liver of experimental rats.

| Groups | Gamma aminobutyric acid B receptor | | 5-Hydroxy tryptamine 2A receptor | |
|---|---|---|---|---|
| | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
| **C** | $63.2 \pm 1.7$ | $2.8 \pm 0.11$ | $51.5 \pm 2.0$ | $3.7 \pm 0.80$ |
| **PHNT** | $41.3 \pm 2.0^{a}$ | $3.9 \pm 0.05^{a}$ | $12.5 \pm 0.6^{a}$ | $4.9 \pm 0.64^{a}$ |
| **GCNP** | $22.3 \pm 2.0^{a,d}$ | $5.6 \pm 0.11^{a,d}$ | $18.3 \pm 0.8^{a,f}$ | $4.7 \pm 0.70^{a}$ |
| **SCNP** | $31.3 \pm 2.0^{a,e}$ | $5.0 \pm 0.08^{a,d}$ | $33.5 \pm 1.0^{a,d}$ | $2.5 \pm 0.52^{a,d}$ |
| **GSCNP** | $9.5 \pm 0.3^{a,d}$ | $9.4 \pm 0.29^{a,d}$ | $39.0 \pm 1.2^{a,d}$ | $4.9 \pm 1.2^{a}$ |

C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.
$B_{max}$ represents the number of receptors and $K_d$ represents the affinity of receptors towards the ligand.
Values are mean ± S.E.M of 4–6 separate experiments.
[a] $P<0.001$ with respect to C.
[d] $P<0.001$.
[e] $P<0.01$.
[f] $P<0.05$ with respect to PHNT.

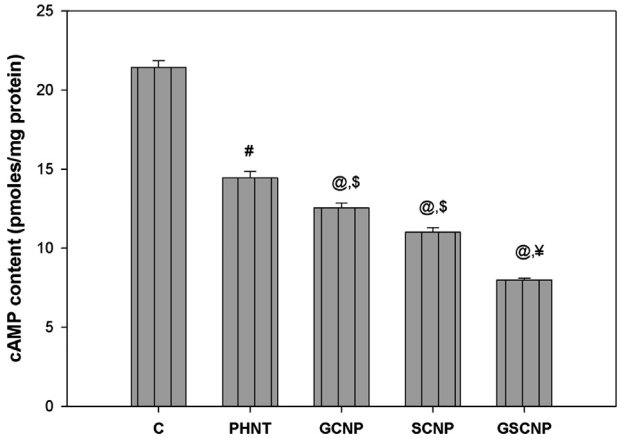


**Fig. 1. cAMP content in the liver of experimental rats.** Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6–8 rats. [@]$P<0.001$, [#]$P<0.01$ with respect to C. [¥]$P<0.01$ and [$]$P<0.05$ with respect to PHNT. C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.



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**Table 2**
Superoxide dismutase assay in the liver of experimental rats.

| Groups | SOD concentration (ng/mg protein) |
|---|---|
| C | 90.7 ± 3.20 |
| PHNT | 58.3 ± 1.59[a] |
| GCNP | 29.5 ± 0.89[a,d] |
| SCNP | 27.0 ± 0.73[a,d] |
| GSCNP | 19.0 ± 0.54[a,d] |

C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.Values are mean ± S.E.M of 4—6 separate experiments.

[a] $P < 0.001$ with respect to Sham operated control.
[d] $P < 0.001$with respect to PHNT.

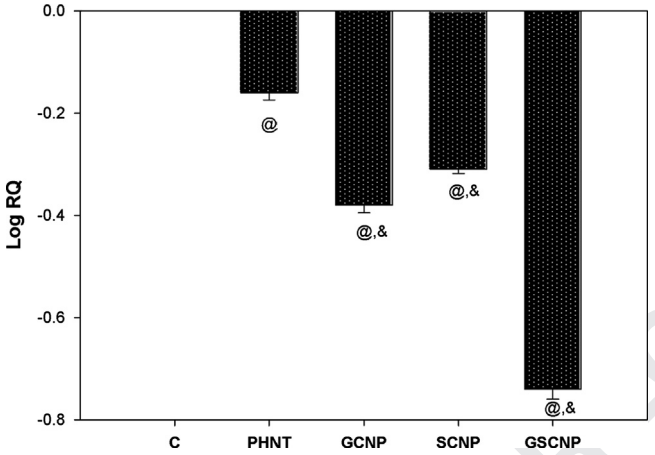


**Fig. 2. Real-time PCR amplification of $GABA_B$ mRNA in the liver of experimental rats.** Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6–8 rats. $^{@}P < 0.001$ with respect to C. $^{\&}P < 0.001$ with respect to PHNT. C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

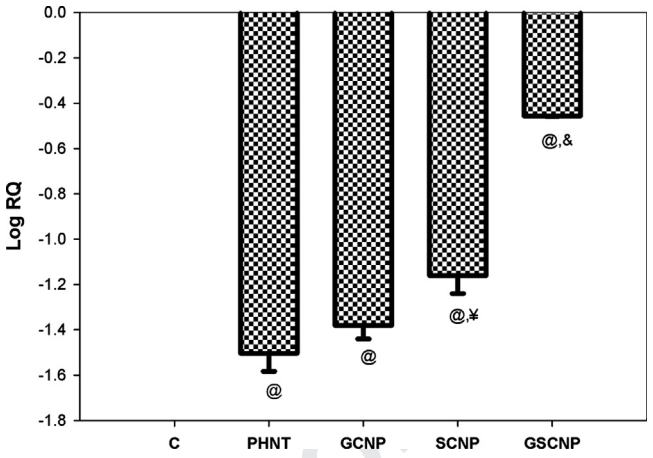


**Fig. 3. Real-time PCR amplification of 5-$HT_{2A}$ mRNA in the liver of experimental rats.** Values are Mean ± S.E.M. of 4—6 separate experiments. Each group consists of 6–8 rats. $^{@}P < 0.001$ with respect to C. $^{\&}P < 0.001$, $^{¥}P < 0.01$ with respect to PHNT. C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

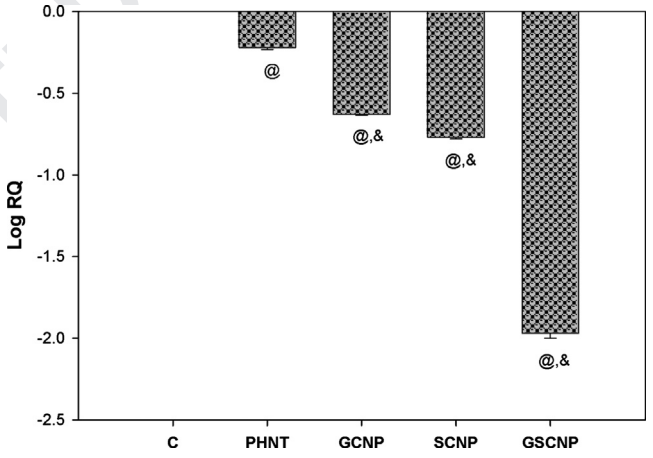


**Fig. 4. Real-time PCR amplification of CREB mRNA in the liver of experimental rats.** Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6–8 rats. $^{@}P < 0.001$ with respect to C. $^{\&}P < 0.001$ with respect to PHNT. C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

regulated ($P < 0.001$) in GCNP, SCNP and GSCNP when compared to C and PHNT. While considering PHNT, the gene expression was significantly reduced ($P < 0.001$) when compared to C (Fig. 5).

Superoxide dismutase is an antioxidant enzyme and its expression was directly dependant on the level of reactive oxygen species, which activates the apoptotic signaling. The SOD gene expression was significantly down regulated ($P < 0.001$) in GCNP, SCNP and GSCNP when compared to C and PHNT. The gene expression of SOD in PHNT was significantly reduced ($P < 0.001$) when compared to C (Fig. 6).

Hepatocyte growth factor (HGF) is the most potent stimulator of hepatocyte growth and DNA synthesis identified. Thus increase in HGF gene expression was considered as a marker for liver regeneration. HGF gene expression was significantly up regulated ($P < 0.001$) in GCNP, SCNP and GSCNP when compared to C and PHNT (Fig. 7).

### 3.5. DNA methylation study

Rapid DNA synthesis prior to mitosis resulted in decreased DNA methylation. Thus *in vitro* methyl group incorporation to the DNA, which were isolated from rapidly dividing cells, was observed to be high when compared to normal cells. The [$^3$H] methyl group incorporation in the DNA was significantly up regulated ($P < 0.001$) in GCNP, SCNP and GSCNP when compared to C and PHNT. For PHNT, the incorporation in PHNT was significantly increased ($P < 0.001$) when compared to C (Fig. 8). To support the experimental outcomes of methylation study, the DNA content was also increased significantly ($P < 0.001$) in nanoparticle treated group when compared to partially hepatectomised groups with no treatment (Table 3).

### 3.6. Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors antibody staining in the liver of control and experimental rats using confocal microscope

Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors staining using receptor specific primary antibody and

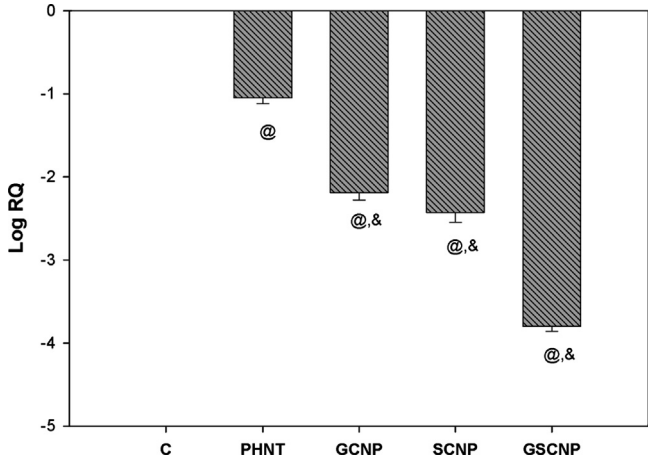


**Fig. 5.** Real-time PCR amplification of MAT2A mRNA in the liver of experimental rats. Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6–8 rats. $^{@}P < 0.001$ with respect to C. $^{\&}P < 0.001$ with respect to PHNT. C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

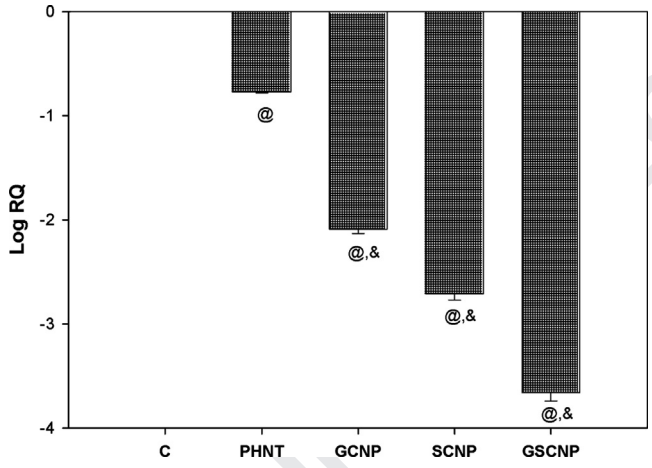


**Fig. 6. Real-time PCR amplification of superoxide dismutase mRNA in the liver of experimental rats.** Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6—8 rats. $^{@}P < 0.001$ with respect to C. $^{\&}P < 0.001$ with respect to PHNT. C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

fluorescent labeled secondary antibody showed a significant change in receptor expression. There was a significant decrease ($P < 0.001$) in Gamma aminobutyric acid B expression in GCNP, SCNP and GSCNP when compared to C. For PHNT the receptor expression was down regulated significantly ($P < 0.01$) with respect to C. The receptor expression was down regulated significantly in GCNP ($P < 0.05$), SCNP ($P < 0.01$) and GSCNP ($P < 0.01$) when compared to PHNT (Fig. 9; Table 4).

Confocal imaging of 5-hydroxy tryptamine 2A receptor expression in the liver of experimental groups showed a significant decrease in PHNT and GCNP ($P < 0.001$) and also a decrease in SCNP and GSCNP ($P < 0.05$) when compared to C. The receptor expression was up regulated significantly in GCNP ($P < 0.01$) and also in SCNP and GSCNP ($P < 0.001$) when compared to PHNT (Fig. 10; Table 5).

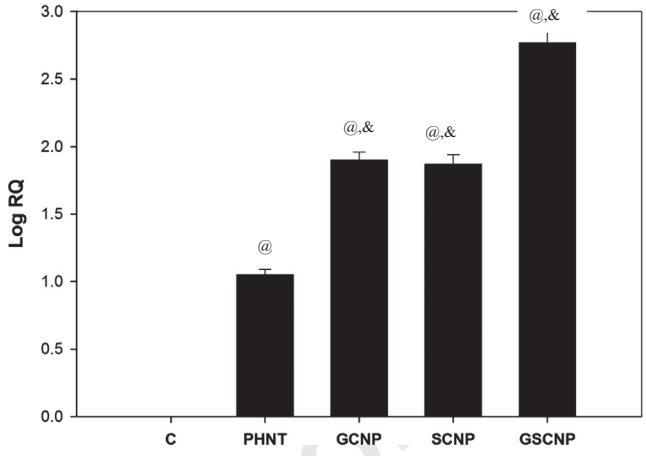


**Fig. 7. Real-time PCR amplification of hepatocyte growth factor mRNA in the liver of experimental rats.** Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6–8 rats. $^{@}P < 0.001$ with respect to C. $^{\&}P < 0.001$ with respect to PHNT. C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

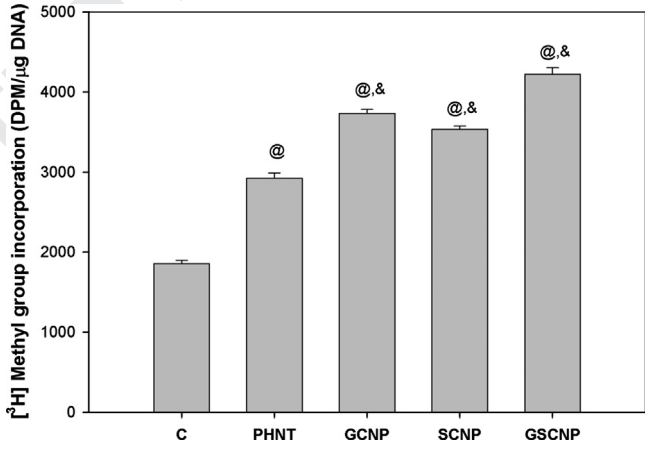


**Fig. 8. [$^{3}$H] methyl group incorporation on the liver DNA of experimental rats.** Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6–8 rats. $^{@}P < 0.001$ with respect to C. $^{\&}P < 0.001$ with respect to PHNT. C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

## 4. Discussion

Drug delivery is likely to benefit from the development of nanotechnology. Nanomedicine minimizes the damage to healthy cells in the body (Zajtchuk, 1999). In the last two decades, Chitosan nanoparticles have been extensively explored for pharmaceutical application (Sailaja et al., 2010). Chitosan is a natural and second abundant polymer produced by deacetylation of chitin (Illum, 1998). It posseses positive charge and interact with the negatively charged cell membrane. These properties render chitosan a very attractive material for drug delivery. As previously reported, several factors, such as particle size, polymer composition, molecular weight and surface characteristic of nanoparticles determine the particle distribution in the various organs of the body (Van Oss, 1978; Tabata and Ikada, 1989; Gref et al., 1994; Storm et al., 1995; Gao et al., 2004; Cho et al., 2007). The GABA and 5-HT chitosan nanoparticles composition was the main reason for their distinct body distribution over unmodified chitosan nanoparticles.

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Liver is a body organ responsible for numerous functions such as storage and metabolism. Liver cell damage occurs in many ways. Alcohol-induced cell death and inflammation result in scarring on liver (Lands, 1995; Maher and Friedman, 1995). An over dose of many drugs, parasite attack (Seydel and Stanley, 1998) and hepatotoxic chemicals (Boobis et al., 1992; Fawthrop et al., 1991) can cause severe liver injury with the potential to progress to liver failure. Due to all these reasons the metabolic functions of liver gets disturbed. So the regeneration of damaged hepatocytes is essential for the balanced routine body functions.

After two-third hepatectomy of liver, the major metabolic pathways were disrupted. Liver starts to regenerate and the maximum DNA synthesis occurs by 24 h post hepatectomy (Kountouras et al., 2001). Our earlier studies explained an enhancement in DNA and protein syntheses in hepatocytes, markers of active cell division, in GABA and 5-HT chitosan nanoparticle treated partially hepatectomised rats (Shilpa et al., 2012a, 2012b). For the activation of cell division, GABA and 5-HT receptors functional regulation in various signaling pathways were controlled during liver regeneration. Usually in non replicating tissues (group C), GABA and 5-HT receptors activation and deactivation occurs by endocytosis and exocytosis of receptor on the membrane. Previous report showed that the exposure of Gamma aminobutyric acid B agonist, baclofen, inhibited Schwann cells proliferation due to increased expression of Gamma aminobutyric acid B receptor (Magnaghi et al., 2004). In our results Gamma aminobutyric acid B receptor expression was reduced in groups with GABA and 5-HT chitosan nanoparticle treatment individually and better in combination that supported active hepatocyte proliferation. Activation of 5-hydroxyl tryptamine 2 receptors in cultured cortical neurons has been shown to inhibit GABA receptor currents via a protein kinase C mediated pathway (Feng et al., 2001). The 5-HT of combination treatment in GSCNP group down regulated Gamma aminobutyric acid B receptor which was an indication of improved hepatocyte proliferation.

During enhanced hepatocyte proliferation, the expression of 5-hydroxy tryptamine 2A subtype serotonin receptors in the liver was increased (James and Perkins, 2006). Our study also supported this and observed an up regulation in 5-hydroxy tryptamine 2A receptor expression in the treatment groups exposed to 5-HT. Some *in vivo* and *in vitro* studies have demonstrated that serotonin exposure resulted in the desensitization and down-regulation of 5-hydroxy tryptamine 2A receptors (Shi et al., 2007). The serotonin exposure to the their receptors in SCNP groups is more compared to GSCNP group. Thus 5-hydroxy tryptamine 2A receptor expression and activity in GSCNP group was observed to be increased due to reduced receptor desensitization, again pointed the enhancement in liver cell multiplication. The Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptor expression patterns in the experimental groups were further confirmed by confocal microscopy images. Thus treatment with GABA and 5-HT chitosan nanoparticles increased liver cell proliferation by regulating both Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors functions.

**Table 3**
DNA content in the liver of experimental rats.

| Groups | DNA content (ng/mg liver weight) |
|---|---|
| C | $0.64 \pm 0.020$ |
| PHNT | $0.92 \pm 0.010^{a}$ |
| GCNP | $1.06 \pm 0.010^{a,d}$ |
| SCNP | $1.08 \pm 0.005^{a,d}$ |
| GSCNP | $1.15 \pm 0.008^{a,d}$ |

C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.
Values are mean ± S.E.M of 4–6 separate experiments.
[a] $P < 0.001$ with respect to Sham operated control.
[d] $P < 0.001$ respect to PHNT.

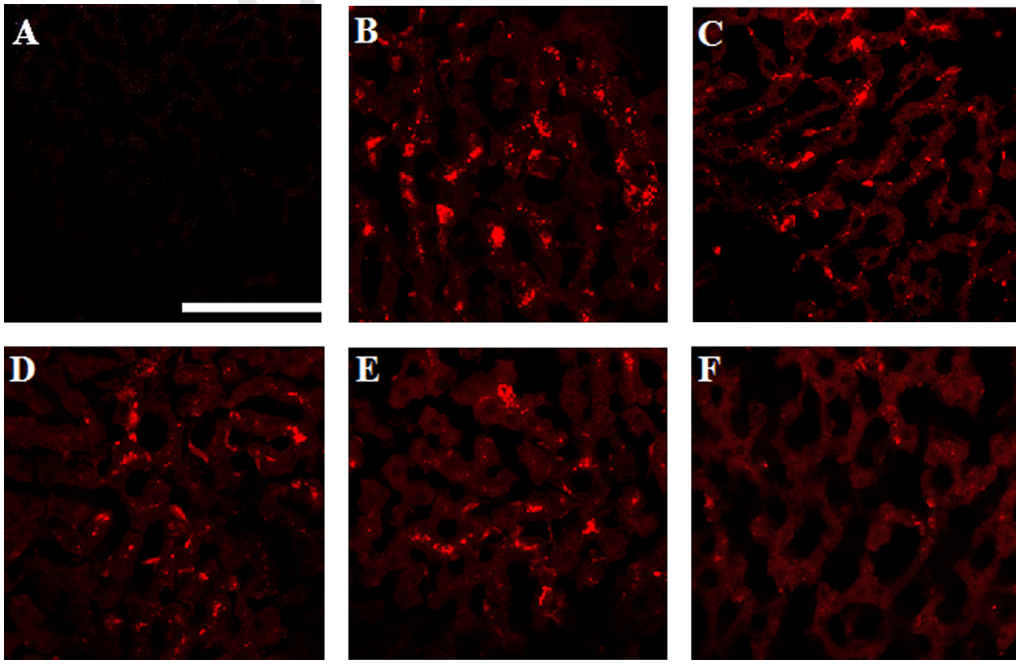


**Fig. 9. Confocal image of Gamma aminobutyric acid B receptors in the liver of control and experimental rats using immunofluorescent Gamma aminobutyric acid B receptor specific primary antibody and Alexa Fluor 594 as secondary antibody.** A–negative control, B–Sham operated control (C), C–partially hepatectomised group with no treatment (PHNT), D–partially hepatectomised group with GABA chitosan nanoparticle treatment (GCNP), E–partially hepatectomised group with 5-HT chitosan nanoparticle treatment (SCNP) and F–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment (GSCNP).

The expression of $Gi_2$ alpha subunit of G protein is increased by 24 h of hepatectomy and thus the activity of adenylyl cyclase is decreased in the S phase of cell cycle (Anna et al., 1992). In the pre-replicative phase of liver regeneration, there is a burst in cAMP-dependent protein kinase A (PKA) activation that is partly down regulated during the phase of active regeneration (S phase). In normal regeneration of hepatocytes the G protein receptor activation occurs in the initial phase of cell division which further leads to a rise in cAMP and PKA levels. But later, during the shift from $G_1$ to S phase, the PKA mediated cell signaling gets suppressed (Ekanger et al., 1989) and promote protein kinase C mediated cell signaling (Arturo, 2003). So cAMP content and CREB expression were reduced in the regenerating livers of PHNT and further reduced significantly in GCNP, SCNP and GSCNP, which promoted enhanced liver cell proliferation in the active phase of cell cycle.

**Table 4**
Confocal imaging studies of Gamma amiobutyric acid B receptors in the liver of experimental groups.

| Experimental groups | Mean pixel intensity |
|---|---|
| C | $30.4 \pm 1.3$ |
| PHNT | $26.8 \pm 1.0^{b}$ |
| GCNP | $22.3 \pm 0.8^{a,f}$ |
| SCNP | $19.4 \pm 0.6^{a,e}$ |
| GSCNP | $16.4 \pm 0.4^{a,d}$ |

C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.Values are mean ± S.E.M of 4–6 separate experiments.

[a] $P < 0.001$.
[b] $P < 0.01$ with respect to C.
[d] $P < 0.001$.
[e] $P < 0.01$.
[f] $P < 0.05$ with respect to PHNT.

Growth factor stimulation by platelet-derived, epidermal and insulin-like growth factors results in an increase in intracellular reactive oxygen species (Sauer et al., 2001). This reactive oxygen species production can inactivate phosphatases at the cell membrane (Meng et al., 2002), activate kinases and transcription factors (Sauer et al., 2001) leading to cell cycle progression. An increase in reactive oxygen species concentration leads to a decrease in SOD activity (Gajewska and Sklodowska, 2007). Our results also explained a decrease in SOD gene expression and activity in the treatment groups than PHNT, which emphasized an increase in cell proliferation. DNA methylation is a modification of DNA established immediately after DNA synthesis in the 'S' phase. In our study, the *in vitro* methyl group incorporation was significantly high in the treatment groups when compared to C and PHNT. This suggested the occurrence of reduced methylated DNA in the liver of each experimental group with active hepatocyte proliferation. There are studies reporting that in mammalian cells the synthesis and methylation of DNA do not occur simultaneously (Gruenbaum et al., 1983) and when the cells enter the S phase it was found that the apparent decrease in the methylated DNA was resulted due to increased DNA content of the cells resulting from DNA replication (Smith et al., 1980). Decreased methylation in DNA Q3 obtained from the regenerating liver that was treated with GABA and 5-HT chitosan nanoparticles clearly explained the increase in DNA content by DNA replication followed by cell mitosis. The *MAT2A* codes for methionine adenosyltransferase (MAT), which is an essential enzyme catalyzes the formation of *S*-adenosylmethionine, the main methyl donor for DNA methylation. In our result for MAT2A gene expression, the enzyme synthesis in the GSCNP group was significantly reduced. Thus the extend of DNA methylation in the regenerating liver of GSCNP were less compared to other experimental groups, again projected the significance of GABA and 5-HT chitosan nanoparticles treatment for active hepatocyte DNA synthesis and division. Hepatocyte growth factor (HGF) is an important growth factor which is expressed and elevated during liver regeneration in partially hepatectomised rats and is a potent stimulator of hepatocyte growth and DNA



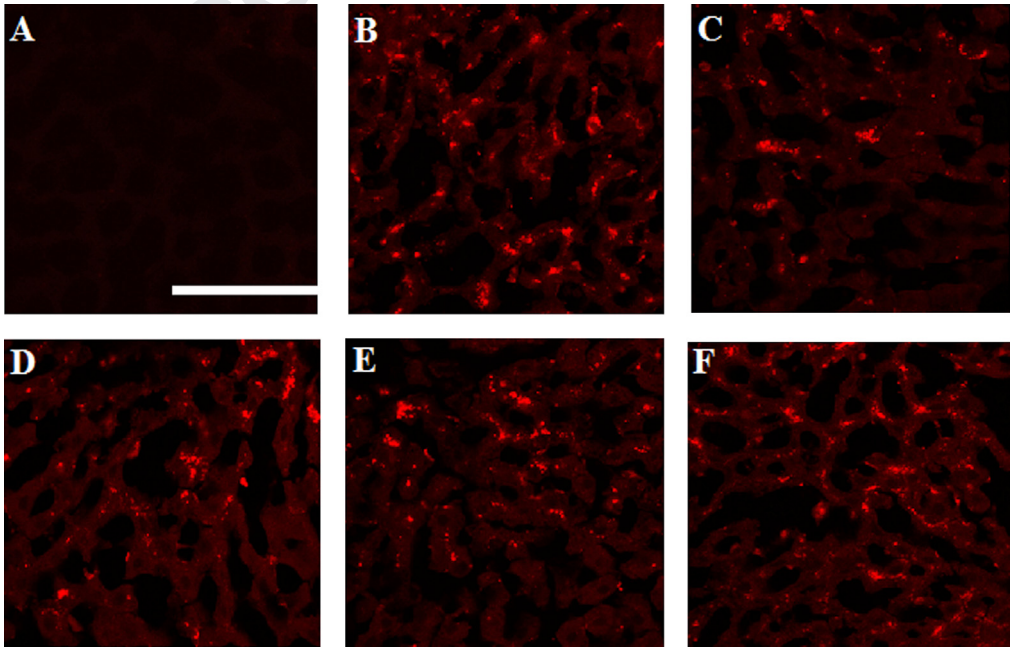


**Fig. 10. Confocal image of 5-Hydroxy tryptamine 2A receptors in the liver of control and experimental rats using immunofluorescent 5-Hydroxy tryptamine 2A receptor specific primary antibody and Alexa Fluor 594 as secondary antibody.** A–negative control, B–Sham operated control (C), C–partially hepatectomised group with no treatment (PHNT), D–partially hepatectomised group with GABA chitosan nanoparticle treatment (GCNP), E–partially hepatectomised group with 5-HT chitosan nanoparticle treatment (SCNP) and F–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment (GSCNP).

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**Table 5**
Confocal imaging studies of 5-Hyhroxy tryptamine 2A receptors in the liver of experimental groups.

| Experimental groups | Mean pixel intensity |
|---|---|
| C | $46.7 \pm 0.29$ |
| PHNT | $30.0 \pm 0.20$[a] |
| GCNP | $35.7 \pm 0.22$ [a,e] |
| SCNP | $39.8 \pm 0.24$ [c,d] |
| GSCNP | $43.9 \pm 0.27$ [c,d] |

C–Sham operated control, PHNT–partially hepatectomised group with no treatment, GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.
Values are mean ± S.E.M of 4–6 separate experiments.

[a] $P < 0.001$.
[c] $P < 0.05$ with respect to C.
[d] $P < 0.001$.
[e] $P < 0.01$ with respect to PHNT.

synthesis identified (Su et al., 2002). HGF is recognized as one of the most important factors in the regulation of liver regeneration after surgical resection or chemical damage (Jiang et al., 1993). The increased expression of HGF gene in the present study supported the concept of therapeutic significance of GABA and 5-HT chitosan nanoparticle induced liver regeneration.

## 5. Conclusion

Liver is associated with many metabolic functions in the body. Damaged hepatocytes impair the major liver functions and lead to death. So the active proliferation of hepatocytes from damaged liver for the balanced functioning of body is important. Treatment with GABA and serotonin chitosan nanoparticles to partially hepatectomised rat liver improved the cell signaling mechanisms during hepatic regeneration when compared to the regeneration without any treatment. An active antioxidant enzyme system was developed during the treatment that facilitated protection from reactive oxygen species induced hepatocyte damage. It was evident that GABA and 5-HT chitosan nanoparticles treatment in partially hepatectomised rats renders fast hepatocyte proliferation with reduced damage which will have therapeutic significance in the management of liver based diseases.

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# Increased Cortical Neuronal Survival During Liver Injury: Effect of Gamma Aminobutyric Acid and 5-HT Chitosan Nanoparticles

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Use of nanoparticulate drug delivery system for an effective therapeutic outcome against diseases gains immense hope in the study of drug delivery to partially hepatectomised rats. In the present study, partially hepatectomised rats treated with Gamma aminobutyric acid (GABA) and serotonin (5-HT) chitosan nanoparticles, individually and in combination, were evaluated to analyse their role in $GABA_B$ and $5\text{-}HT_{2A}$ receptors functional regulation, interrelated neuronal survival mechanisms by nuclear factor kappa *B* (NF-$\kappa B$), tumour necrosis factor-$\alpha$ (TNF-$\alpha$), Akt-1 and the antioxidant enzyme superoxide dismutase (SOD) in the cerebral cortex. A significant decrease in $GABA_B$ and $5\text{-}HT_{2A}$ receptor numbers and gene expressions denoted a homeostatic adjustment by the cerebral cortex to trigger the sympathetic innervations during elevated DNA synthesis in the liver. $GABA_B$ and $5\text{-}HT_{2A}$ signalling directly influenced the cyclic AMP response element binding protein (CREB) expression, neuronal survival and ROS mediated cell damage which was confirmed from the gene expression of NF-$\kappa B$, TNF-$\alpha$, Akt-1 and SOD. In addition to enhanced hepatocyte proliferation, GABA and 5-HT chitosan nanoparticle treatment protected the neurons from ROS mediated cell damage and promoted their survival in the cerebral cortex. This has application in liver based diseases and treatments with nanosized active compounds.

**KEYWORDS:** *Chitosan Nanoparticles, GABA, Serotonin, Neuronal Survival, Liver Regeneration, Antioxidant System.*

## INTRODUCTION

Nanoparticulate drug delivery systems provide new hopes in solving problems in the area of drug delivery.[1] Nanotechnology is the technology that deals with one billionth of a meter scale.[2] To a wide extend, biopolymer conjugated drugs coupled with nanotechnology enhances the stability and pharmacological efficiency of the active compound. Chitosan, deacetylated chitin, is a copolymer of $\beta$-(1, 4) linked glucosamine (deacetylated unit) and *N*-acetyl glucosamine (acetylated unit).[3] Chitosan is nontoxic, biodegradable and bio compatible. Nanoparticles of chitosan coupled drugs are utilized for drug delivery in eye, brain, liver, cancer tissues, treatment of spinal cord injury and infections. Polymeric drug delivery systems can be used to deliver drugs directly to the intended site of action which results in slow release and minimized side effects elsewhere in the body and decrease the long-term use of many drugs.

The liver is a vital organ and has a wide range of functions, which include synthesis, storage, detoxification, metabolism and redistribution of amino acids, carbohydrates, fats, vitamins and proteins. Damage to liver occurs by over consumption of alcohol death, drugs with analgesic and antipyretic action, attack of parasites like *Entamoeba histolytica* and hepatotoxic chemicals. Patients often develop signs of liver dysfunction in the immediate postoperative period as a result of reduced liver mass, but normal liver function resumes once the removed liver mass is restored[4] but not completely in advanced liver disease. The presence of various ligands in the initiation, propagation and termination of the mitotic stimulus, such as priming factors, co-mitogens, growth factors and their suppressors, is necessary for the successful and complete restoration of hepatic mass.[5] Prolonged liver dysfunction, such as liver cirrhosis leading to hepatocyte damage, can harm the brain, leading to a serious and potentially fatal brain disorders.

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Brain plays an important regulatory role in hepatic functions.[6] The cerebral cortex plays a key role in memory, attention, perceptual awareness, thought, language, and consciousness. In acute liver failure, high ammonia levels raise cerebral blood flow and increased inflammatory response have been identified as major contributors to the development of hepatic encephalopathy[7] and the related brain herniation, coma and brain swelling. This affects neurotransmitter level and its receptor activation in brain. Auto regulation of blood flow in different brain regions is impaired and is associated with anaerobic glycolysis and oxidative stress. Inflammatory mediators like tumour necrosis factor (TNF-$\alpha$) also play important role in regulation of cortical function by liver.[8] So the regeneration of damaged liver restores the functions of cerebral cortex. The liver is richly innervated and the central thyrotropin-releasing hormone, one of the important peptide transmitter substances, regulates hepatic proliferation through autonomic nervous system.[9] Extirpation of the brain cortex was shown to increase the rate of liver cell proliferation describing that the cortex exerts an inhibitory function on liver cell division, growth and also transection of the spinal cord above the area innervating the liver resulted in decreased DNA synthesis.[10] There are several reports highlighting the regulation of hepatic proliferation by the cerebral cortex, but the role of neurotransmitters and their receptors in mediating neuronal survival during neurotransmitter conjugated chitosan nanoparticle induced liver regeneration are not well studied.

Gamma aminobutyric acid (GABA), a neurotransmitter, is reported to have an inhibitory effect on sympathetic outflow. Brain GABAergic changes are reported to regulate autonomic nerve function in rats.[11] GABA and GABA receptors were widely distributed in mammalian brain and are in high concentration in cortical, hippocampal, thalamic, basal ganglia, and cerebellar structures. In animal models of liver injury, an increase in GABAergic tone has been demonstrated due to both an increase in GABA release and enhanced activation of the GABA receptor complex.[12] Serotonin (5-hydroxytryptamine [5-HT]) is also another neurotransmitter in the central nervous system and peripheral nervous systems and a hormone produced by the gut and transported in platelets.[13] The central serotonergic neurons participate in the regulation of sympathetic nerve discharge and have an inhibitory influence on central sympathetic pathways. 5-HT regulates cell proliferation, migration and maturation in a variety of cell types and alters the cytoskeleton of cells and thus influences the formation of cell contacts. 5-HT has been implicated more in behaviour, physiological mechanisms, and disease processes than any other brain neurotransmitter.

The present study explained the functional regulation of $GABA_B$ and $5\text{-}HT_{2A}$ receptors mediated signalling and their role in the activation of neuronal survival factors and antioxidant enzyme in the cerebral cortex during active liver regeneration triggered by GABA and 5-HT chitosan nanoparticles. In this work we tried to elucidate the therapeutic application of GABA and 5-HT chitosan nanoparticles in balancing the non obstructive cortical functions and enhanced liver cell proliferation after partial hepatectomy.

## MATERIALS

### Chemicals Used and Their Sources

Biochemicals and Tri-reagent kit were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally. [$^3$H] baclofen (Sp. Activity 42.9 Ci/mmol) and [$^3$H] ketanserin (Sp. Activity 63.3 Ci/mmol) was purchased from Amersham Life Science, UK. Chitosan (MW-25 KDa) was a gift from Central Institute of Fisheries Technology, Cochin, India.

### Animals

Experiments were carried out on adult male Wistar rats of 250–300 g body weight purchased from Kerala Agricultural University, Mannuthy, India. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water *ad libitum*. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health and CPCSEA guidelines. All efforts were made to minimize animal suffering. Each group consisted of 6 animals. Sham operated control treated with saline (C), partially hepatectomised rats with no treatment (PHNT), GABA chitosan nanoparticles treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle treatment (GSCNP) were the five experimental groups.

## EXPERIMENTAL PROCEDURES

### Preparation of GABA and 5-HT Chitosan nanoparticles

The chitosan nanoparticles were prepared by ionic gelation method.[14] The incorporation of GABA and 5-HT in to chitosan nanoparticles individually and in combination, standardization of encapsulation efficiency and *in vitro* release profile studies were done according to Shilpa et al.[15, 16] The nanoparticles were washed thoroughly and were dispersed in saline.

### Partial Hepatectomy and Sacrifice

Two–thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anesthesia, following a 16 hour fast.[17] Sham operations involved median excision of the body wall followed by all manipulations except removal of the lobes. All the surgeries were done between 7 and 9 A.M to avoid diurnal variations in responses. After surgery, 1 ml of

30$\mu$g/$\mu$l GABA chitosan nanoparticles, 5-HT chitosan nanoparticles and a combination of GABA and 5-HT chitosan nanoparticles suspended in saline were injected intra peritoneal to the respective rats. The rats were sacrificed by decapitation 24 hours post hepatectomy and brain cerebral cortex was dissected out quickly and kept over ice according to the procedure of Glowinski and Iversen.[18] The tissues were stored at $-80$ °C until assayed.

## $GABA_B$ and 5-$HT_{2A}$ Receptor Binding Studies in the Cerebral Cortex Using [$^3$H] Baclofen and [$^3$H] Ketanserin

[$^3$H] Baclofen binding to $GABA_B$ receptor in the membrane preparations were assayed.[19] Crude membrane preparation was suspended in 50 mM Tris sulphate buffer, pH 7.4 containing 2 mM $CaCl_2$ and 0.3–0.4 mg protein. In saturation binding experiments, 10–100 nM of [$^3$H] baclofen was incubated with and without excess of 100 $\mu$M unlabelled baclofen. The incubations were carried out at 20 °C for 20 minutes. The binding reactions were terminated by centrifugation at $14000 \times g$ for 10 minutes. The dried pellet was re-suspended and counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

[$^3$H] Ketanserin binding to 5-$HT_{2A}$ receptor in the crude synaptic membrane preparation was done according to the modified procedure of Leysen et al.[20] Crude membrane preparation was suspended in 50 mM Tris sulphate buffer, pH 7.6 containing 0.3–0.4 mg protein. In saturation binding experiments, assays were done using different concentrations of 0.5–10 nM of [$^3$H] ketanserin which was incubated with and without excess of unlabelled 10 $\mu$M ketanserin. Tubes were incubated at 37 °C for 15 minutes and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washings with 5.0 ml of ice cold 50 mM Tris sulphate buffer, pH 7.6. The bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The receptor binding parameters were determined using Scatchard analysis.[21] The specific binding was determined by subtracting non-specific binding from the total binding. The binding parameters, maximal binding ($B_{max}$) and equilibrium dissociation constant ($K_d$), were derived by linear regression analysis by plotting the specific binding of the radioligand on $x$-axis and bound/free on $y$-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The $K_d$ is inversely related to receptor affinity.

## Analysis of Gene Expression by Real-Time Polymerase Chain Reaction

PCR analyses were conducted with gene-specific primers and fluorescently labeled Taqman probe of $GABA_B$, 5-$HT_{2A}$, cAMP response element-binding protein (CREB), NF-$\kappa B$, TNF-$\alpha$, Akt-1 and superoxide dismutase (SOD) which were designed by Applied Biosystems. Endogenous control, $\beta$-actin, was labeled with a report dye, VIC. RNA was isolated from the cerebral cortex of experimental rats using the Tri-reagent according to the procedure of Chomczynski and Sacchi.[22] Total cDNA synthesis was performed using ABI PRISM cDNA archive kit in 0.2 ml microfuge tubes. The reaction mixture of 20 $\mu$l contained 0.2 $\mu$g total RNA, $10\times$ RT buffer, $25\times$ dNTP mixture, $10\times$ random primers, MultiScribe RT (50 U/$\mu$l) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 min and 37 °C for 2 h using an Eppendorf Personal Cycler. Real-time-PCR assays were performed in 96-well plates in an ABI 7300 Real-time-PCR instrument (Applied Biosystems). The specific primers and probes were purchased from Applied Biosystems, Foster City, CA, USA. The TaqMan reaction mixture of 20 $\mu$l contained 25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer and TaqMan probe for assay on demand and endogenous control $\beta$-actin and 12.5 $\mu$l of Taqman $2\times$ Universal PCR Master Mix (Applied Biosystems) and the volume was made up with RNAse free water. The following thermal cycling profile was used (40 cycles), 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The $^{\Delta\Delta}$CT method of relative quantification was used to determine the fold change in expression. This was done by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control $\beta$-actin in the same samples ($^{\Delta}$CT = CT Target–CT $\beta$-actin). It was further normalized with the control ($^{\Delta\Delta}$CT = $^{\Delta}$CT–CT Control). The fold change in expression was then obtained as ($2-^{\Delta\Delta}$CT) and the graph was plotted using log2$-^{\Delta\Delta}$CT.

## SOD Assay in the Cerebral Cortex of Experimental Rats

The cerebral cortex was homogenized in a buffer containing 10 mM EDTA, 50 mM Tris-HCl, pH 8.2 and centrifuged at $40000 \times g$ for 15 minutes. The protein content of whole supernatant of the total homogenate was estimated.[23] Cytoplasmic SOD was analyzed after the inhibition by SOD of the pyrogallol autoxidation[24] at pH 8.2. Briefly, 1 ml reaction mixture contained 0.2 mM pyrogallol, 1 mM EDTA and 50 mM Tris-HCl buffer. Pyrogallol autoxidation was monitored at 420 nm for 3 minutes with or without 250 $\mu$g enzyme protein. The inhibition of pyrogallol was linear with the activity of the enzyme present. Fifty percent inhibition/(mg protein $\cdot$ min) was considered as one unit of enzyme activity.

### Immunohistochemical Analysis by Confocal Microscope

The experimental rats were deeply anesthetized and was transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. After perfusion the brain from each experimental group was dissected out and fixed in 4% paraformaldehyde for 1 hour and then equilibrated with 30% sucrose solution in PBS (0.1 M). 10 $\mu$m cerebral cortex sections were cut using Cryostat (Leica, CM1510 S). The sections were washed with PBS and then blocked for 1 hour with PBS containing 5% normal goat serum and 0.1% triton X-100. The primary antibodies of $GABA_B$ (Chemicon, 1:500 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) and 5-$HT_{2A}$ (Chemicon, 1:1000 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) were added to the respective sections and incubated overnight at 4 °C. After overnight incubation, the brain slices were rinsed with PBS and then incubated with fluorescent labelled secondary antibody (Alexa Fluor 594, code-A11012, Invitrogen) prepared in PBS with 5% normal goat serum and 0.1% triton X-100 at 1, 1000 dilution. The sections were washed with PBS thoroughly and then observed and photographed using confocal imaging system (Leica SP 5). The specificity of the immunocytochemical procedure was validated by negative controls (data not shown) to ensure that the labelling method accurately identifies the antibody bound to the specific receptors in the cerebral cortex. Expression of $GABA_B$ and 5-$HT_{2A}$ receptors was analysed using pixel intensity method. The given pixel value is the net value which is deducted from the negative control pixel value.[25] All the imaging parameters in the confocal imaging system like PMT, pinhole and zoom factor were kept same for imaging the sections of all experimental groups.

### Statistical Analysis

Statistical evaluations were done with analysis of variance (ANOVA), using GraphPad Instat (version 2.04a, San Diego, USA). Student Newman-Keuls test was used to compare different groups after ANOVA. Linear regression Scatchard plots were made using SIGMA PLOT (Ver. 2.03). Relative Quantification Software was used for analyzing Real-Time PCR results.

## RESULTS

### $GABA_B$ and 5-$HT_{2A}$ Receptor Analysis in the Cerebral Cortex of Experimental Rats

$B_{max}$ represents the number of receptors and $K_d$ denotes the affinity of receptors towards the ligand. $B_{max}$ of $GABA_B$ receptors in GCNP and GSCNP showed a significant decrease ($p < 0.05$) and SCNP showed no significant change with respect to PHNT. In GCNP there was no significant change in 5-$HT_{2A}$ receptor $B_{max}$ when compared to PHNT. The $B_{max}$ of 5-$HT_{2A}$ receptor showed a significant decrease ($p < 0.05$) in SCNP and GSCNP when compared to PHNT. $K_d$ value of the receptors in GCNP and GSCNP showed a significant decrease ($p < 0.01$) when compared to PHNT (Tables I, II).

**Table I. $GABA_B$ receptor analysis in the cerebral cortex of experimental rats.**

| Groups | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
|---|---|---|
| C | $129.30 \pm 8.66$ | $68.9 \pm 5.80$ |
| PHNT | $98.72 \pm 6.20^{a}$ | $77.42 \pm 4.32^{c}$ |
| GCNP | $76.02 \pm 5.40^{a,f}$ | $61.24 \pm 4.10^{e}$ |
| SCNP | $95.75 \pm 5.66^{a}$ | $79.96 \pm 3.52^{c}$ |
| GSCNP | $74.68 \pm 5.32^{a,f}$ | $60.02 \pm 4.09^{e}$ |

*Notes*: Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment. $B_{max}$ represents the number of receptors and $K_d$ represents the affinity of receptors towards the ligand. $^{a}p < 0.001$, $^{c}p < 0.05$ with respect to C. $^{e}p < 0.01$ and $^{f}p < 0.05$ with respect to PHNT. Values are mean $\pm$ S.E.M of five separate experiments.

### Real Time PCR Analysis of $GABA_B$, 5-$HT_{2A}$, CREB, NF-$\kappa B$, TNF-$\alpha$, Akt-1 and SOD mRNA in the Experimental Rats

Gene expression studies of $GABA_B$ receptor in the cerebral cortex was significantly decreased in PHNT ($p < 0.01$), GCNP and SCNP ($p < 0.001$) when compared to C. While considering the receptor gene expression in SCNP and GCNP, there was a significant decrease ($p < 0.01$) when compared to PHNT. The receptor gene expression was significantly decreased in GSCNP when compared to PHNT ($p < 0.001$) and both GCNP and SCNP ($p < 0.01$). The 5-$HT_{2A}$ receptor gene expression in SCNP and GSCNP showed a significant decrease ($p < 0.01$) and GCNP showed no significant change when compared to PHNT (Fig. 1).

There was a significant decrease in the expression of CREB in GCNP and SCNP ($p < 0.01$) and GSCNP ($p < 0.001$) when compared to PHNT (Fig. 2). The gene expression of NF-$\kappa B$, TNF-$\alpha$ and Akt-1 showed a significant

**Table II. 5-$HT_{2A}$ receptor analysis in the cerebral cortex of experimental rats.**

| Groups | $B_{max}$ (fmoles/mg protein) | $K_d$ (nM) |
|---|---|---|
| C | $251.50 \pm 26.69$ | $39.32 \pm 4.90$ |
| PHNT | $210.72 \pm 16.20^{a}$ | $43.45 \pm 6.32^{c}$ |
| GCNP | $211.32 \pm 15.40^{a}$ | $36.81 \pm 4.18^{e}$ |
| SCNP | $174.75 \pm 10.69^{a,f}$ | $45.26 \pm 3.56^{c}$ |
| GSCNP | $177.68 \pm 10.32^{a,f}$ | $34.12 \pm 4.04^{e}$ |

*Notes*: Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment. $B_{max}$ represents the number of receptors and $K_d$ represents the affinity of receptors towards the ligand. $^{a}p < 0.001$, $^{c}p < 0.05$ with respect to C. $^{e}p < 0.01$ and $^{f}p < 0.05$ with respect to PHNT. Values are mean $\pm$ S.E.M of five separate experiments.

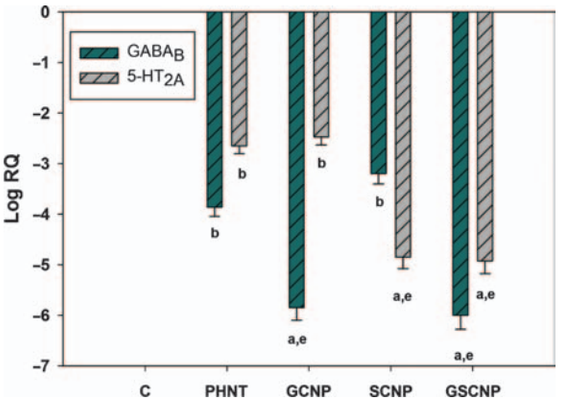


**Figure 1.** **Real time PCR amplification of GABA$_B$ and 5-HT$_{2A}$ mRNA in the cerebral cortex of experimental rats.**
***Notes*: Values are Mean ± S.E.M. of five separate experiments. Each group consists of five rats. $^{a}p < 0.001$ and $^{b}p < 0.01$ with respect to C. $^{e}p < 0.01$ with respect to PHNT. Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment.**

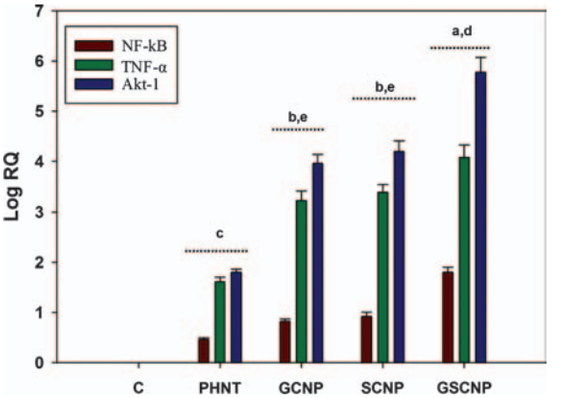


**Figure 3.** **Real time PCR amplification of NF-$\kappa B$, TNF-$\alpha$ and Akt-1 mRNA in the cerebral cortex of experimental rats.**
***Notes*: Values are Mean ± S.E.M. of five separate experiments. Each group consists of five rats. $^{a}p < 0.001$, and $^{b}p < 0.01$, $^{c}p < 0.05$ with respect to C. $^{d}p < 0.001$ and $^{e}p < 0.01$ with respect to PHNT. Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment.**

increase ($p < 0.01$) in GCNP and SCNP when compared to PHNT. The gene expression of neuronal survival factors were increased significantly ($p < 0.001$) in GSCNP when compared to PHNT (Fig. 3).

The level of antioxidant enxyme SOD and its activity were elevated when the ROS content in the cell was increased. The free radical generation was a symbol of apoptosis and increased expression of SOD directly indicated the reduction in ROS. A significant increase ($p < 0.05$) in SOD gene expression was observed in GCNP, SCNP and GSCNP when compared to PHNT (Fig. 4).

## SOD Activity in the Cerebral Cortex of Experimental Rats

The activity of cytoplasmic SOD was significantly increased in GCNP, SCNP ($p < 0.01$) and GSCNP ($p < 0.001$) when compared to PHNT (Table III).

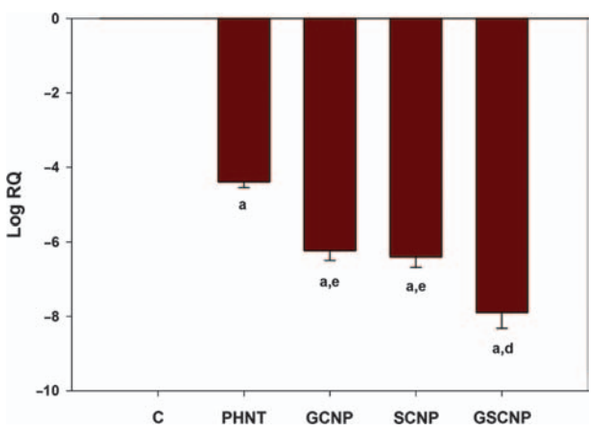


**Figure 2.** **Real time PCR amplification of CREB mRNA in the cerebral cortex of experimental rats.**
***Notes*: Values are Mean ± S.E.M. of five separate experiments. Each group consists of five rats. $^{a}p < 0.001$ with respect to C. $^{d}p < 0.001$ and $^{e}p < 0.01$ with respect to PHNT. Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment.**

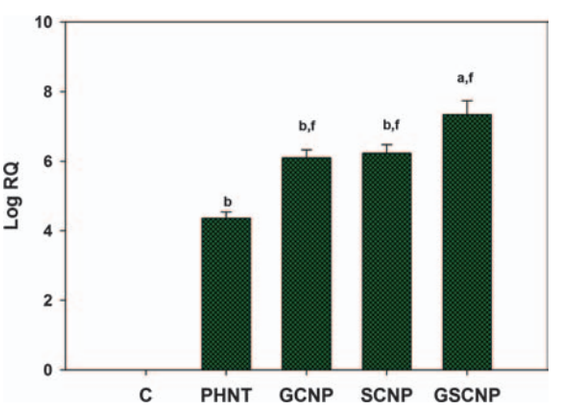


**Figure 4.** **Real time PCR amplification of SOD mRNA in the cerebral cortex of experimental rats.**
***Notes*: Values are Mean ± S.E.M. of five separate experiments. Each group consists of five rats. $^{b}p < 0.01$ with respect to C. $^{f}p < 0.05$ with respect to PHNT. Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment.**

**Table III. SOD activity in the cerebral cortex of experimental rats.**

| Groups | SOD concentration (ng/mg protein) |
|---|---|
| C | $03.76 \pm 0.20$ |
| PHNT | $23.32 \pm 1.57^{a}$ |
| GCNP | $42.50 \pm 3.23^{a,e}$ |
| SCNP | $48.98 \pm 3.66^{a,e}$ |
| GSCNP | $60.04 \pm 4.09^{a,d}$ |

*Notes*: Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment. $^{a}p < 0.001$ with respect to sham operated control. $^{d}p < 0.001$ and $^{e}p < 0.01$ with respect to PHNT. Values are mean $\pm$ S.E.M of five separate experiments.

**Table IV. Confocal imaging studies of $GABA_B$ receptors in the cerebral cortex of experimental groups.**

| Experimental groups | Mean pixel intensity |
|---|---|
| C | $43.8 \pm 0.18$ |
| PHNT | $34.59 \pm 0.16^{a}$ |
| GCNP | $26.83 \pm 0.13^{a,d}$ |
| SCNP | $33.91 \pm 0.18^{a}$ |
| GSCNP | $22.18 \pm 0.13^{a,d}$ |

*Notes*: Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment. $^{a}p < 0.001$ with respect to sham operated control. $^{d}p < 0.001$ with respect to PHNT. Values are mean $\pm$ S.E.M of five separate experiments.

**Table V. Confocal imaging studies of 5-$HT_{2A}$ receptors in the cerebral cortex of experimental groups.**

| Experimental groups | Mean pixel intensity |
|---|---|
| C | $48.9 \pm 0.23$ |
| PHNT | $39.5 \pm 0.19^{a}$ |
| GCNP | $37.8 \pm 0.18^{a,d}$ |
| SCNP | $28.9 \pm 0.14^{a}$ |
| GSCNP | $21.2 \pm 0.12^{a,d}$ |

*Notes*: Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment. $^{a}p < 0.001$ with respect to sham operated control. $^{d}p < 0.001$ with respect to PHNT. Values are mean $\pm$ S.E.M of five separate experiments.

## $GABA_B$ and 5-$HT_{2A}$ Receptors Antibody Staining in the Cerebral Cortex of Control and Experimental Rats Using Confocal Microscope

$GABA_B$ and 5-$HT_{2A}$ receptors staining using receptor specific primary antibody and fluorescent labelled secondary antibody showed a significant change in receptor expression. The mean pixel intensity of fluorescent labelling of $GABA_B$ receptors was decreased ($p < 0.001$) in GCNP and GSCNP and no change in SCNP when compared to PHNT (Table IV, Fig. 5). The mean pixel intensity of 5-$HT_{2A}$ receptor staining was decreased ($p < 0.001$) in SCNP and GSCNP and no change in GCNP when compared to PHNT (Table V, Fig. 6).

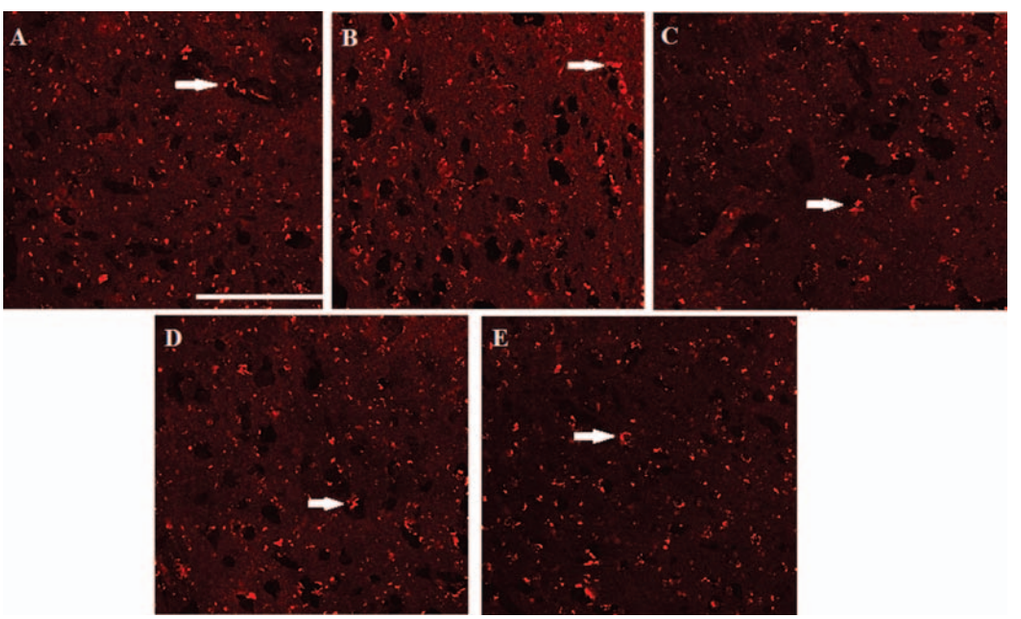


**Figure 5. Confocal image of $GABA_B$ receptors in the cerebral cortex of control and experimental rats using immunofluorescent $GABA_B$ receptor specific primary antibody and Alexa Fluor 594 as secondary antibody.**
***Notes*: The scale bar represents 40 $\mu$m. Sham operated control (A), Partially hepatectomised group with no treatment (B), GABA chitosan nanoparticle treatment (C), 5-HT chitosan nanoparticle treatment (D) and GABA and 5-HT chitosan nanoparticle treatment (E).**

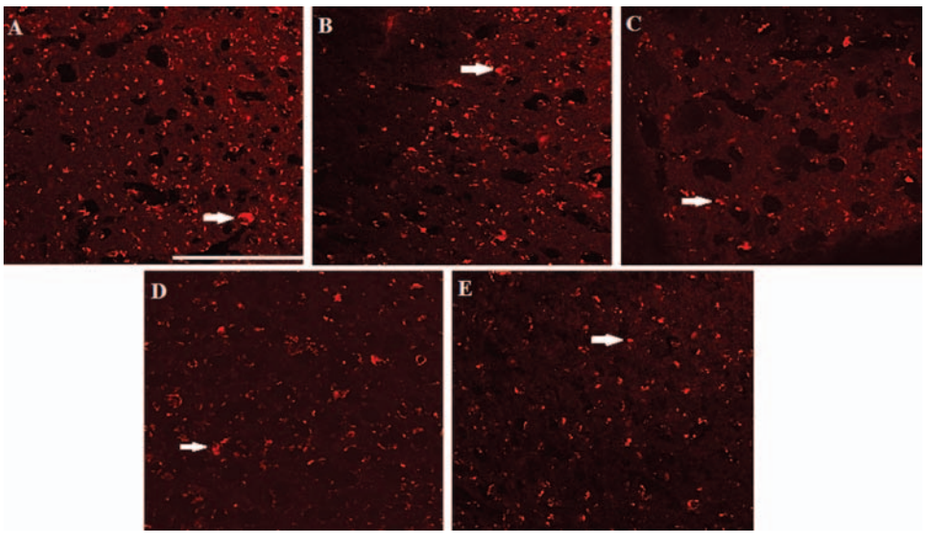


**Figure 6. Confocal image of 5-HT$_{2A}$ receptors in the cerebral cortex of control and experimental rats using immunofluorescent 5-HT$_{2A}$ receptor specific primary antibody and Alexa Fluor 594 as secondary antibody.**
***Notes*: The scale bar represents 40 $\mu$m. Sham operated control (A), Partially hepatectomised group with no treatment (B), GABA chitosan nanoparticle treatment (C), 5-HT chitosan nanoparticle treatment (D) and GABA and 5-HT chitosan nanoparticle treatment (E).**

## DISCUSSION

A great deal of focus has been directed to chitosan nanoparticles to improve drug bioavailability, modify pharmacokinetics and/or protect the encapsulated drug.[26] Chitosan is the second-most abundant natural polysaccharide next to cellulose. Fewer side effects, solubility, poor bioavailability, specific delivery to site of action with good pharmacological efficiency, absorption at intestine, degradation of drug, slow release and effective therapeutic outcome, are the challenges faced by the present drug delivery systems. Chitosan nanoparticles can very well interact with the negatively charged cell membrane by the amino group and make the polymer one of the best carrier for the active compounds to delivery site.

The relationship between the functional status of liver and that of the brain has been known for centuries. In liver diseases, neurotransmission in the brain is reported to be altered.[27] A spectrum of neuropsychiatric abnormalities in patients with liver dysfunction were observed and was characterized by personality changes, intellectual impairments and a depressed level of consciousness associated with multiple neurotransmitter systems, astrocyte dysfunction and cerebral perfusion.[28] Report also showed that there is an onset of mitochondrial damage in brain due to decreased synthesis of glutathione by damaged liver, which was the major glutathione synthesis site.[29]

The liver has a remarkable capacity to regenerate after cellular damage or tissue removal. Liver regeneration is mostly the result of increased mitosis of hepatocytes. Agonists of GABA can act at the GABA receptor complex, and increased concentrations of the agonists are found in the brain in liver failure.[30] Neurosteroids produced in brain during acute liver failure led to increased GABAergic tone[31] and also, elevated intra-cerebral concentrations of GABA significantly decreased ornithine decarboxylase activity in the liver.[32] This was an index for decreased hepatic proliferation and function. There is also an interesting report suggesting that serotonin can potentially contribute to liver tissue hypoperfusion following hepatic ischemia and reperfusion.[33] Sympathetic innervation is important for liver regeneration.[34] Our previous study showed an increased DNA and protein syntheses, which are the cell division markers, in the regenerating liver of GCNP, SCNP and GSCNP when compared to that of PHNT after 24 hours post hepatectomy.[15, 16] During liver injury ammonia metabolism is disturbed and led to a condition called hyperammonemia. Hyperammonemia has been suggested to induce enhanced cerebral cortex ammonia uptake, subsequent glutamine synthesis and accumulation. The changes in cerebral cortex glutamate and gamma-aminobutyric acid could be related to altered ammonia metabolism.[35] The autonomic regulation of GABA was mediated through GABA$_B$ receptors[36] and reduction in the GABA neurotransmission in the brain regions enhanced DNA synthesis in liver by facilitating the sympathetic tone.[37] During active hepatocyte proliferation, the serotonin receptor number was decreased in the cerebral cortex of partially hepatectomised rats.[38] During liver injury and hepatic insufficiency, the aromatic amino acid catabolism was altered. Thus the plasma levels of aromatic amino acid increases and enters the brain. The aromatic amino acid tryptophan enhanced the serotonin synthesis in cerebral cortex, which lead to active serotonin mediated neurotransmission. As hepatic cell recovery progresses, the aromatic

amino acid metabolism also reactivated. Thus the serotonin content gets decreased in cerebral cortex.[35] In our results also the 5-$HT_{2A}$ receptor expression was down regulated in GCNP, SCNP and further decreased in GSCNP when compared to PHNT that supported the regulation of neurotransmission mediated through GABA and 5-HT receptors during liver regeneration.

The transcription factor CREB is a rapidly responding intracellular effector of neurotransmitter signalling and growth factor signalling in mature neurons.[39] CREB was controlling the neuronal survival, in part, by controlling transcription of neuroprotective genes. Reports stated that neurotransmitter receptor activation is directly proportional to the CREB gene expression and its translocation to the nucleus.[40] In our study, $GABA_B$ and 5-$HT_{2A}$ receptors mediated signalling through CREB was decreased in PHNT, GCNP, SCNP and further decreased in GSCNP that implied decreased GABA and serotonin neurotransmission, which was a sign of increased cell division in regenerating liver.

NF-$\kappa B$ is expressed in diverse cell types in the nervous systems.[41] An involvement of NF-$\kappa B$ in neuronal development demonstrates its activation in neurons in certain regions of the brain during neurogenesis. Inhibition of NF-$\kappa B$ by an inhibitor increases cell death and infarct size following transient ischemic insult in rats, suggesting that NF-$\kappa B$ induces survival signalling in neuronal cells.[42] Phosphorylation of NF-$\kappa B$ on serines 32 and 36 by $I\kappa B$ kinase leads to its ubiquitination and degradation by proteosomal enzymes, which allows NF-$\kappa B$ heterodimer to translocate to the nucleus and regulate gene expression. In GCNP, SCNP and GSCNP groups the NF-$\kappa$B level was increased compared to PHNT, which showed an increased neuronal survival and maintenance. One well studied pathway that led to NF-$\kappa B$ activation was by the involvement of cytokine TNF-$\alpha$ through intracellular signalling molecules TNF receptor associated factors (TRAF2 and TRAF6) and activated NF-$\kappa B$-inducing kinase (NIK), which phosphorylates the IKKs.[43] IKK can be phosphorylated by an alternative pathway, which involves Akt. Indeed TNF-$\alpha$ and platelet-derived growth factor (PDGF)-induced NF-$\kappa$B activation has been reported to require Akt[44]. Also, report showed that NF-$\kappa$B appears to be a target of the anti-apoptotic Ras/PI (3)K/Akt pathway and the expression and activity of Akt was regulated by NF-$\kappa B$.[45] Thus it was clear that during the damage due to partial hepatectomy, the interrelated activation of NF-$\kappa$B, TNF-$\alpha$ and Akt-1 were required for the survival of neurons in the cerebral cortex. From our observation, in group GSCNP a better cortical neuronal survival signalling mechanism was observed when compared to PHNT. Neuronal death induced by glutamate accumulation[46] can be decreased by the treatment with GABA and 5-HT.[47] Thus in our study, the combined effect of GABA and 5-HT treatment in GSCNP group showed an increased expression of neuronal survival factors and resulted in reduced cell death.

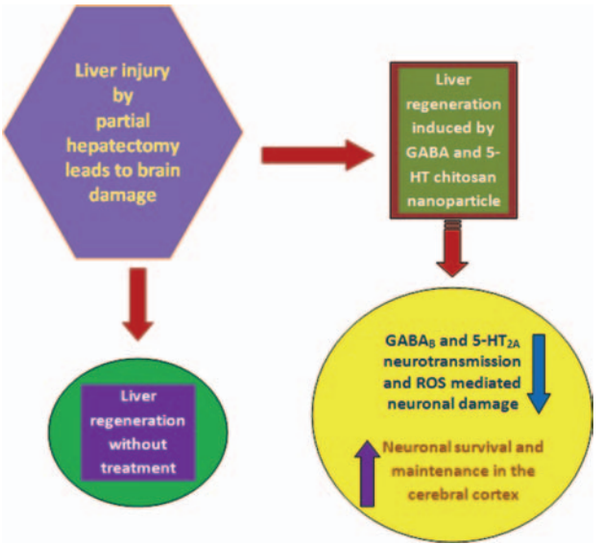


**Figure 7. The overall study for achieving an increased cortical neuronal survival and maintenance by GABA and 5-HT chitosan nanoparticle treatment in partially hepatectomised rats.**

Brain is rich in polyunsaturated fatty acids and deficient in oxidative defence mechanisms and hence is at great risk of damage mediated by reactive oxygen species (ROS) resulting in molecular and cellular dysfunction.[48] An increase in ROS levels automatically favoured an up regulation of antioxidant enzyme, SOD gene expression and its activity,[49] which resulted in subsequent suppression of ROS mediated brain damage. SOD is an essential enzyme for detoxifying superoxide radicals to hydrogen peroxide. Oxidative stress was demonstrated in cerebral cortex of hepatic failure induced hyperammonemic and hepatoencephalatic animals.[50] From our study, a significant increase in SOD activity and its gene expression in rats treated with GABA and serotonin chitosan nanoparticles individually and in combination implied that oxidative neuronal damage in the rat cerebral cortex was less when compared to PHNT.

The present study discussed and pictorially represented (Fig. 7) the potential of GABA and 5-HT, individually and in combination, encapsulated in chitosan nanoparticles for protecting the brain cortical cells during liver injury. The neuronal protection and survival in the cerebral cortex was enhanced in partially hepatectomised rats treated with a combination of GABA and 5-HT chitosan nanoparticles. Thus in addition to active hepatocyte proliferation our study provided new hopes in minimizing neuronal damage in liver based injuries and diseases by utilising nanosized polymeric carriers with active compounds.

## CONCLUSION

Hepatic insufficiency due to liver damage led to an imbalance in routine brain and body functions. This work revealed the potential of GABA and 5-HT chitosan

nanoparticle treatment in improving neuronal survival and reducing oxidative stress mediated cortical damage after partial hepatectomy and successive regeneration events in rats. The study can be further extended to evaluate the role of neurotransmitter receptor mediated functional alterations in neuronal protection and damage in other prominent brain regions like corpus striatum, hippocampus and brain stem during active hepatocyte proliferation.

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