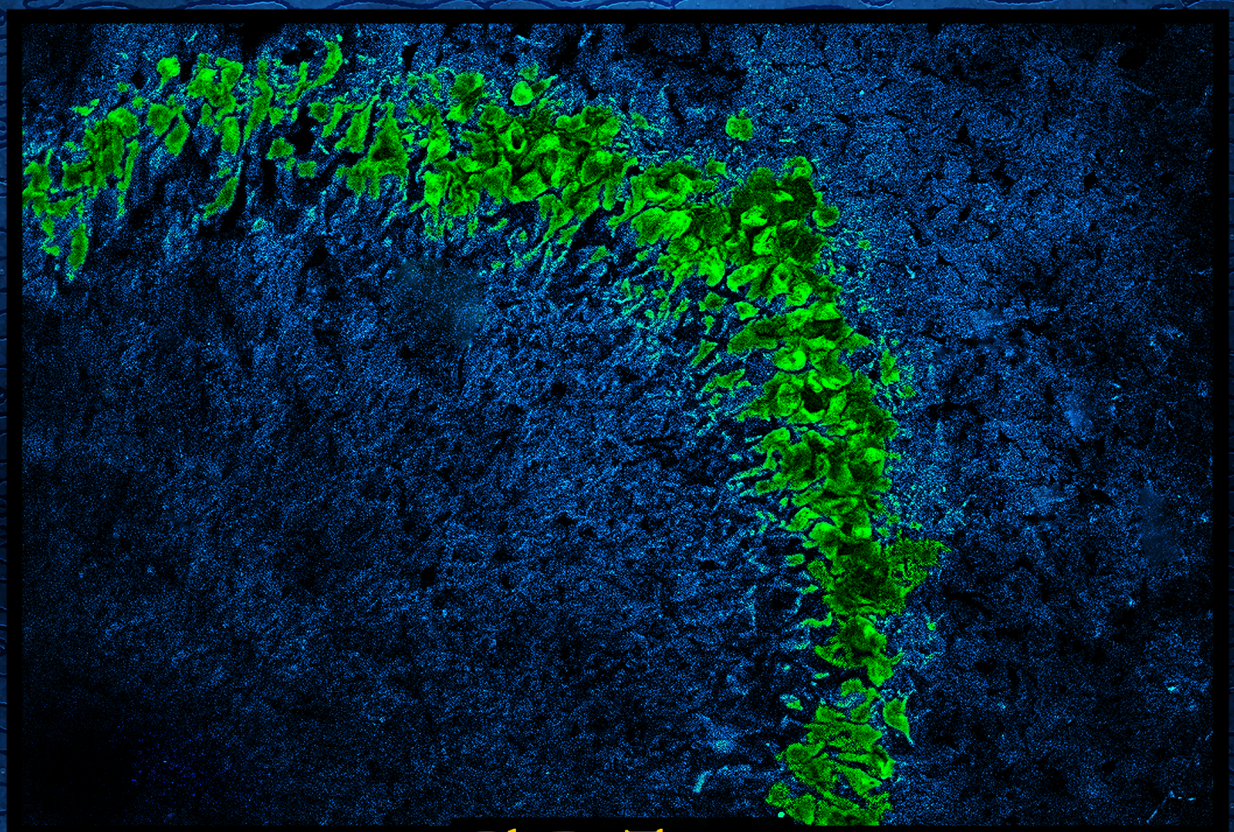
Adrenergic and muscarinic receptor subtypes functional regulation during diabetogenesis: Effect of curcumin and vitamin D_3 pre-treatment



Ph.D. Thesis

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Adrenergic and muscarinic receptor subtypes functional regulation during diabetogenesis: Effect of curcumin and vitamin D_3 pre-treatment

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BY

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CERTIFICATE

This is to certify that the thesis entitled **"Adrenergic and muscarinic receptor subtypes functional regulation during diabetogenesis: Effect of curcumin and vitamin D_3 pre-treatment"** is a bonafide record of the research work carried out by **Mr. Naijil George,** 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
May 14, 2014

(C. S. Paulose)

DECLARATION

I hereby declare that the thesis entitled "**Adrenergic and muscarinic receptor subtypes functional regulation during diabetogenesis: Effect of curcumin and vitamin D_3 pre-treatment"** 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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At times our own light goes out
and is rekindled by a spark from another person.
Each of us has cause to think with deep gratitude
of those who have lighted the flame within us.

- Albert Schweitzer

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Naijil George

Dedicated to my beloved family …

ABBREVIATIONS

1,25(OH)2D3	1α,25-dihydroxyvitamin D_3
Bax	BCL-2-associated X protein
B_{max}	Maximal binding
BSA	Bovine serum albumin
CA	Cornu Ammonis
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CPM	Counts per minute
CREB	cAMP response element-binding protein
Ct	Cycle threshold
DAG	1,2-Diacylglycerol
DAMP	Deoxy acetyl methyl piperidine
DEPC	Diethyl pyro carbonate
EDTA	Ethylene diamine tetra acetic acid
FITC	Florescent isothiocyanate
G protein	Guanosine nucleotide-binding proteins
GABA	Gamma amino butyric acid
GDM	Gestational diabetes mellitus
GLUT	Glucose transporter
GPCR	G protein-coupled receptor
GPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HBSS	Hang's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IDF	International diabetes federation
IGF	Insulin-like growth factor
IP3	Inositol trisphosphate
ITI	Inter-trial interval

K_d	Dissociation constant
LTD	Long term depression
MGB	Minor groove binding protein
MLD-STZ	Multiple low dose streptozotocin
mRNA	Messenger ribonucleic acid
nAChRs	Nicotinic acetylcholine receptors
NeuroD1	Neuronal differentiation 1
NF-κB	Nuclear factor-kappa B
Nrf2	Nuclear factor erythroid-2-related factor-2
p	Level of significance
Pax	Paired box protein
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Triton X- 100
PCR	Polymerase chain reaction
Pdx-1	Pancreatic duodenal homeobox-1
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PLC	Phospholipase C
PMT	Photomultiplier tube
PP	Pancreatic polypeptide
QNB	Quinuclidinyl benzilate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
RPMI	Roswell park memorial institute
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SOD	Superoxide dismutase
STZ	Streptozotocin
TNF-α	Tumour necrosis factor-α
VDR	Vitamin D receptor

CONTENTS

Introduction

The term "diabetes mellitus" describes a metabolic disorder of multiple aetiology, characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of diabetes mellitus include long-term damage, dysfunction and failure of various organs.

382 million people worldwide, or 8.3% of adults, were estimated to have diabetes in 2013. Globally, 175 million people with diabetes are undiagnosed. According to the Diabetes Atlas 2013 published by the International Diabetes Federation (IDF), 592 million people, or one adult in 10, will have diabetes by 2035. Total deaths from diabetes are projected to rise by more than 50% in the next 10 years. About 80% of people with diabetes live in low and middle income countries. A vast majority of people with diabetes is between 40 and 59 years of age. According to the Diabetes Atlas 2013, the number of people with diabetes in India is 65.1 million and by 2035, this is expected to rise to 109.0 million (IDF Diabetes Atlas, 2013).

The cases of diabetes fall into two broad etiopathogenetic categories. The first category, type 1 diabetes or insulin dependent diabetes, is characterized by lack of insulin production due to autoimmune destruction of pancreatic beta cells. The second and more prevalent category, type 2 diabetes or non-insulin dependent diabetes, emanates from a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (Harris, 1988). Increasing age, obesity and physical inactivity are considered as the risk factors for type 2 diabetes. Gestational diabetes (GDM) is another form of diabetes, characterized by high blood glucose level during pregnancy. Prediabetes, typically defined as blood glucose level above normal but below diabetes thresholds, is a risk state that defines a high chance of developing diabetes (Tabák *et al*., 2012).

Medical complications as well as economic consequences of diabetes management led us to focus on its preventive strategies. In diabetes prevention

trials, approaches targeting different arms of immune system were uniformly unsuccessful (Bluestone *et al*., 2010). Dietary modifications were used as typical treatment modalities for diabetes and were accredited for its protective role. Diet and nutrition that start in childhood or even before birth play an important role in the development of life style diseases including diabetes (Szczepura, 2011). Insight into the role of nutraceuticals in delaying or preventing diabetes will contribute to the development of novel therapeutics.

Turmeric (*Curcuma longa*), a rhizomatous monocotyledonous perennial herbaceous plant of the ginger family (Zingiberaceae), has been used for the treatment of diabetes in Ayurvedic and traditional Chinese medicine (Zhang *et al*., 2013). Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], is the major active component of turmeric. It has been shown to exhibit a wide range of pharmacological activities including anti-inflammatory, anti-cancer, anti-oxidant, anti-atherosclerotic, anti-microbial and wound healing effects (Maheshwari *et al*., 2006). Curcumin has been shown to delay the development of diabetes by reducing pancreatic oxidative stress and inflammation, improving beta cell function, preventing beta cell death and reducing insulin resistance in animal models (Suryanarayana *et al*., 2005; Meghana *et al*., 2007; Peeyush *et al*., 2009). Chuengsamarn *et al*., (2012) showed that curcumin extract could improve the overall function of pancreatic beta cells and effectively prevent the prediabetic population from developing diabetes.

Vitamin D is a pleiotropic secosteroid pro-hormone important for health and disease prevention. The diverse biological effects (endocrine, autocrine, paracrine) of vitamin D are mediated by the vitamin D receptor (VDR) that binds the active form of vitamin D, 1,25-dihydroxycholecalciferol [1,25(OH)2D], to induce both transcriptional and non-genomic responses (Shin *et al*., 2010). Activated VDR directly and/or indirectly regulate 0.5–5% of genes in total human genome i.e., 100–1250 genes (Hossein-nezhad *et al*., 2013). The relationship between diabetes mellitus and vitamin D deficiency has been studied extensively (Luong *et al*., 2005). Increasing evidence suggests that regular supplementation of

vitamin D has an important role in reducing the risk of diabetes (Takiishi *et al*., 2013). Hypovitaminosis D, either due to deficiency of vitamin D or relative vitamin D resistance, is known to be a risk factor for glucose intolerance (Boucher, 2012). Long-term supplementation of vitamin D_3 could decrease the incidence of insulitis in spontaneous autoimmune diabetes (Mathieu *et al*., 2005). Further, vitamin D_3 administration was proved to increase plasmatic insulin, normalise blood glucose levels and hepatic glycogen concentration in diabetic animal models (Peeyush *et al*., 2010; Takiishi *et al*., 2013). On the basis of evidence from both animal and human observational studies, curcumin and vitamin D_3 can be considered as potential risk modifiers for diabetes.

Pancreatic beta cells are highly specialized and high throughput units for the production of insulin, the key hormone for maintenance of glucose homeostasis (Magro & Solimena, 2013). Diabetes is a complex metabolic disorder characterized by hyperglycemia associated with insulin deficiency as a result of beta cell failure (Montane *et al*., 2014). Marked reduction in beta cell mass due to increased apoptosis is central to the development of all types of diabetes (Butler *et al*., 2003). During the early stages of hyperglycemia, pancreatic beta cell function is increased to meet the increased metabolic demand of the body. But sustained hyperglycemia and subsequent induction of oxidative stress lead to the reduction in beta cell mass and defects in insulin secretion that impair the regulation of blood glucose level (Jin & Patti, 2009). In the resultant 'beta cell glucose toxicity' state, hyperglycemia *per se* and subsequent induction of oxidative stress further decrease insulin biosynthesis and secretion (Prentki & Nolan, 2006). Under such conditions, nuclear expression levels of pancreatic beta cell differentiation, proliferation and maturation markers decrease, leading to the suppression of insulin biosynthesis and secretion from pancreas. In addition, expression levels of apoptotic genes increase, contributing to the dramatic decrease in beta cell mass (Kaneto & Matsuoka, 2013). Chronic hyperglycemia also induce alterations in central nervous system (CNS) and lead to an impairment of neuronal signalling to pancreas, further resulting in beta cell mass reduction (Park *et al*., 2013).

Glucose homeostasis and pancreatic beta cell mass regulation depends on signals from endocrine, neural and metabolic origins. Such signals control endogenous glucose production and utilization to maintain a physiological glycemia (Thorens, 2011). Among the regulatory signals, neurotransmitters generated by the CNS play an essential role. The pancreatic islets are richly innervated by sympathetic and parasympathetic branches of the autonomic nervous system (Ahrén, 2000). The parasympathetic efferent fibres originating from the brain stem synapse with intrapancreatic ganglionic cells and activate post-ganglionic neurons. Acetylcholine is the major neurotransmitter that modulates pancreatic insulin secretion *via* changes in parasympathetic activity (Chandra & Liddle, 2009). The islet sympathetic nerves are postganglionic with the nerve cell bodies located in ganglia outside the pancreas (Quinson *et al*., 2001). Autonomic nervous system stimulate insulin secretion by the activation of parasympathetic nerves and inhibit insulin secretion by the activation of sympathetic nerves (Hsu *et al*., 1991). Norepinephrine and acetylcholine are the major neurotransmitters released at the islet nerve terminals (Mitrani *et al*., 2007). Autonomic nervous system modulate insulin release and beta cell mass *via* muscarinic and adrenergic receptor mediated cell signalling (Hellman *et al*., 2014). Activation or inhibition of the sympathetic or parasympathetic nervous systems are controlled by glucose-excited or glucose-inhibited neurons located at different anatomical sites, mainly in brain stem, hippocampus and hypothalamus (Balkan & Li, 2000; Preitner *et al*., 2004). Activation of these neurons by hyperglycemia or hypoglycaemia is a pivotal point in the control of glucose homeostasis, islet function and beta cell mass (Thorens, 2011).

The onset of diabetes can be delayed or even prevented by activating the compensatory mechanisms to increase beta cell mass through augmentation of islet neogenesis, replication and by a reduction in the rate of beta cell death. Formation of new beta cells in pancreas by differentiation of progenitor cells is controlled by transcription factors like Pax, Pdx-1 and NeuroD1 (Sharma *et al*., 1999; Shao *et al*., 2009; Dave *et al*., 2014). Along with these transcription factors,

insulin like growth factor I, a family of peptide growth factors, play an important role in pancreatic regeneration by autocrine or paracrine mechanisms to stimulate DNA synthesis and act as a beta cell differentiation factor (Smith *et al*., 1988; George *et al*., 2002). Further downstream, this will lead to the activation of Akt pathway thereby triggering cell survival signalling and suppression of cell death pathways by reducing oxidative damage through the regulation of antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GPx) and suppression of apoptotic mediators like Bax, caspase 3, caspase 8 and TNF-α (Hers *et al*., 2011; Nakabayashi & Shimizu, 2012).

Multiple low dose streptozotocin (MLD-STZ) induced diabetic rat models have been used extensively in diabetes research (Lukić *et al*., 1998; Rees & Alcolado, 2005). Pancreatic beta cell toxicity and diabetogenic properties of streptozotocin are mediated through the targeted uptake of streptozotocin in beta cells by glucose transporter 2 (GLUT 2) receptors (Lenzen, 2008; Raza & John, 2012). The present study uses MLD-STZ induced diabetic rat models to evaluate the protective effects of curcumin and vitamin D_3 pre-treatment. The study aims to identify the regulatory pathways implicated in contributing to the anti-diabetogenesis effect of curcumin and vitamin D_3. This will help in devising a better life style management to delay or prevent diabetes.

In the present study, the initial phase was directed to confirm the effects of curcumin and vitamin D_3 in preventing or delaying diabetes onset by studying the blood glucose and insulin levels in the pre-treated and diabetic groups. Behavioural studies were conducted to evaluate the cognitive and motor function in experimental rats. The major focus of the study was to understand the cellular and neuronal mechanisms that ensure the prophylactic capability of curcumin and vitamin D_3. To elucidate the mechanisms involved in conferring the anti-diabetogenesis effect, we examined the DNA and protein profiles using radioactive incorporation studies for DNA synthesis, DNA methylation and protein synthesis. Furthermore the gene expression studies of Akt-1, Pax, Pdx-1, Neuro D1, insulin like growth factor-1 and NF-κB were done to monitor

pancreatic beta cell proliferation and differentiation. The antioxidant and anti-apoptotic actions of curcumin and vitamin D_3 were examined by studying the expression of antioxidant enzymes - SOD and GPx, and apoptotic mediators like Bax, caspase 3, caspase 8 and TNF-α. In order to understand the signalling pathways involved in curcumin and vitamin D_3 action, the second messengers, cAMP, cGMP and IP3 were studied along with the expression of vitamin D receptor in the pancreas. The neuronal regulation of pancreatic beta cell maintenance, proliferation and insulin release was studied by assessing the adrenergic and muscarinic receptor functional regulation in the pancreas, brain stem, hippocampus and hypothalamus. The receptor number and binding affinity of total muscarinic, muscarinic M1, muscarinic M3, total adrenergic, α adrenergic and β adrenergic receptor subtypes were studied in pancreas, brain stem and hippocampus of experimental rats. The mRNA expression of muscarinic and adrenergic receptor subtypes were determined using Real Time PCR. Immunohistochemistry studies using confocal microscope were carried out to confirm receptor density and gene expression results. Cell signalling alterations in the pancreas and brain regions associated with diabetogenesis and anti-diabetogenesis were assessed by examining the gene expression profiles of vitamin D receptor, CREB, phospholipase C, insulin receptor and GLUT. This study will establish the anti-diabetogenesis activity of curcumin and vitamin D_3 pre-treatment and will attempt to understand the cellular, molecular and neuronal control mechanism in the onset of diabetes.

Objectives of the present study

1. To evaluate anti-diabetogenesis property of curcumin and vitamin D_3 pre-treatment in multiple low dose streptozotocin induced diabetic rat model.
2. To investigate the behavioural changes in experimental rats using Y maze test, rotarod test and grid walk test.
3. To study the pancreatic beta cells proliferation using thymidine, leucine and methyl group incorporation.
4. To study the gene expression of beta cell regeneration markers- Akt-1, NeuroD1, Pax, Pdx-1 and cyclin D2, apoptotic markers- Bax, caspase 3, caspase 8 and TNF-α and cell signalling molecules insulin like growth factor-1 and NF-κB in the pancreas of experimental rats using Real Time PCR.
5. To measure total muscarinic, muscarinic M1, muscarinic M3, total adrenergic, α adrenergic and β adrenergic receptor subtypes binding parameters in pancreas, brain stem and hippocampus of experimental rats.
6. To study muscarinic and adrenergic receptor subtypes gene expression in pancreas, brain stem, hippocampus and hypothalamus of experimental rats.
7. To study the expression of muscarinic M1, muscarinic M3, α adrenergic and β adrenergic receptor subtypes in pancreas, brain stem and hippocampus of experimental rats using confocal microscope.
8. To analyse the gene expression of choline acetyltransferase, acetylcholinesterase and α7 nicotinic acetylcholine receptor in pancreas, brain stem, hippocampus and hypothalamus of experimental rats using Real Time PCR.
9. To investigate cell signalling alterations by gene expression studies of vitamin D receptor, CREB, phospholipase C, insulin receptor and GLUT in pancreas, brain stem, hippocampus and hypothalamus of experimental rats.

10. To examine the oxidative stress in pancreas, brain stem, hippocampus and hypothalamus of experimental rats by assessing gene expression status of antioxidant enzymes- superoxide dismutases and glutathione peroxidase.
11. To study the second messengers- cAMP, cGMP and IP3 content in pancreas, brain stem and hippocampus of experimental rats.

Literature Review

HISTORY OF DIABETES

Clinical description of complications similar to diabetes mellitus was reported 3500 years ago by the ancient Egyptians. Araetus of Cappodocia, 81-133AD coined the term "diabetes" meaning "a siphon" and in his description, "Diabetes is a dreadful affliction, not very frequent among men, being a meltdown of the flesh and limbs into urine" (Leopald, 1930). Sweetness of urine and blood of diabetic patients was first noticed by the ancient Indians. Thomas Willis in 1675, added the world "mellitus" meaning honey or sweet, reflecting the sweet taste of urine of those affected. In 1776, an English physician Mathew Dobson confirmed the presence of excess sugar in urine and blood of diabetic patients (Henry, 1811; Ahmed, 2002).

In the mid-19th century, French physiologist Claude Bernard showed that sugar present in diabetic urine was stored in the liver as glycogen. His studies on vagal motor nuclei found that the CNS was involved in blood glucose homeostasis (Robin, 1979). The role of pancreas in diabetes was first demonstrated by Joseph von Mering and Oskar Minkowski in 1889. German physician Paul Langerhans in 1869 discovered the irregularly shaped small clusters of cells scattered throughout the pancreas and named it "islands of Langerhans" (Banting *et al*., 1962). Edward Albert Sharpey-Schafer in 1910 coined the term "insulin", from the Latin word 'insula', meaning island. He hypothesized that diabetes was due to the deficiency of single chemical called 'insulin' produced by the pancreatic islands of Langerhans (Banting *et al*., 1991). In 1921, Frederick Banting and Charles Best discovered insulin and used it to reverse diabetes in dogs (Bliss, 1997).

Diabetes Mellitus is a metabolic disorder of multiple aetiology that results from defective insulin secretion, resistance to insulin and/or both (Whiting *et al*., 2011). Out of several different types of diabetes mellitus, the most important ones are type 1 and type 2 diabetes. Type 1 diabetes results from the autoimmune destruction of the pancreatic beta cells and the resultant absolute deficiency of

insulin. Diabetes types 2 are characterized by increased insulin resistance and impaired insulin secretion. As a chronic metabolic disorder, it directly or indirectly induces physiological alterations in all cells, tissues and organs in the body. All types of diabetes mellitus are associated with hyperglycaemic state. Persistent hyperglycaemia causes kidney failure, blindness, neuropathy and nontraumatic amputations and thus contributes to significant morbidity and mortality in diabetic patients (Sargis, 2014).

PREVALENCE OF DIABETES

The global burden of diabetes mellitus has reached catastrophic proportions and continues to rise at an alarming rate. In 2013, 382 million individuals were estimated to have diabetes worldwide and almost half of them remained undiagnosed (IDF Diabetes Atlas, 2013). The International Diabetes Federation (IDF) is an umbrella organization for over 230 national diabetes associations in 170 countries. According to IDF Diabetes Atlas, India had 35.5 million diabetic patients in 2003 and by 2025 it is expected to rise to 73.5 million (IDF Diabetes Atlas, 2003). Recent IDF statistics show that India has already surpassed that estimate and had 65.1 million individuals affected by diabetes in 2013 (IDF Diabetes Atlas, 2013). In 2013, 5.1 million deaths were estimated to be caused by diabetes mellitus. The financial burden of diabetes is enormous as it is taking up some USD 548 billion dollars in health spending and by 2035, it is estimated to become 627 billion USD (IDF Diabetes Atlas, 2013).

DIABETES AND PANCREAS

A Greek physician Latinized Herophilus (300 BC) first described the organ, pancreas. Rufus, an anatomist from Ephesus, coined the name “pancreas” from the Greek roots ‘pan’ meaning ‘all’ and ‘creas’ meaning ‘flesh’. Pancreas is present in all vertebrates and is located deep in the abdomen (Bujalkova, 2011; Chakraborty *et al*., 2011). The pancreas is a heterocrine gland that functions both as a digestive organ and an endocrine gland. Human pancreas is about 70-150

grams in weight and 15-25 cm in length. It is a soft, lobulated and retroperitoneal organ that lies transversely on the posterior abdominal wall behind the stomach, across the lumbar (L1-2) spine (Major, 1914). Ampulla of Vater located at the major duodenal papilla connects pancreas and gall bladder to the duodenum. During embryonic development, pancreas originates from endoderm as dorsal and ventral anlage buds (Baker & Caldwell, 1947).

The pancreas consists of morphologically and functionally distinct exocrine and endocrine glandular tissue. Exocrine pancreas is a lobulated, branched and acinar gland that secretes pancreatic juice into the small intestine. The pancreatic juice consists of digestive enzymes like trypsinogen, chymotrypsinogen, carboxypeptidase, elastases, lipase, sterol esterase, phospholipase, nucleases and amylase (Harper, 1972). Acinar cells and duct cells of exocrine pancreas constitute 95%–99% of the pancreas (Githens, 1988). Endocrine component of pancreas consist of well-organized cell clusters called islets of Langerhans and secrete hormones into the bloodstream (Orci, 1982). Pancreatic islets are scattered throughout the exocrine pancreas. Each islet of Langerhans consists of 100–500 micron spheres and contains approximately 1000 cells. Islets of Langerhans are primarily composed of glucagon-producing alpha-cells, insulin-producing beta-cells, somatostatin-producing delta-cells, pancreatic polypeptide–producing PP-cells and ghrelin producing epsilon-cells (Haist, 1971; Herrera, 2002; Rindi *et al*., 2004).

ALPHA CELLS

15–20% islets of Langerhans are composed of alpha cells in mammals (Brissova *et al*., 2005). Alpha cells of pancreas produce glucagon from preproglucagon *via* prohormone convertase 2. Glucagon helps to maintain plasma glucose levels in the post-absorptive state by stimulating hepatic gluconeogenesis and glycogenolysis. Glucagon counteracts hypoglycaemia by opposing insulin action to increase blood glucose concentrations. Glucose and paracrine factors

regulate glucagon secretion from the alpha cells by electrical machinery comprised of ion channels (Göpel *et al*., 2000).

Alpha cells are important in the pathogenesis of diabetes on account of the glycogenolytic, gluconeogenic and ketogenic actions of glucagon (Müller *et al*., 1973). Dysregulation of alpha cells and increased glucagon secretion contribute to hyperglucagonemia, unrestricted hepatic gluconeogenesis and glycogenolysis, and the development of diabetic hyperglycaemia and ketoacidosis (Shah *et al*., 2000; Bramswig & Kaestner, 2011). Diabetes is associated with a significant decrease in pancreatic beta cell mass and an expanded population of glucagon positive alpha cells (Novikova *et al*., 2013). Glucagon hypersecretion is considered as a major cause of glycemic volatility in diabetes (Unger & Cherrington, 2012).

BETA CELLS

At least 70 percent cells in the islets of Langerhans are beta cells. Approximately one billion beta cells are present in a normal adult human pancreas. Pancreatic beta cells are generated by the proliferation and differentiation of pancreatic progenitor cells. Beta cells synthesize and secrete insulin, a critical regulator of mammalian metabolism. Insulin regulates glucose homeostasis, energy homeostasis, metabolism, skeletal physiology, somatic growth and reproduction (Pagliuca & Melton, 2013). Large quantities of insulin are synthesized and stored in pancreatic beta cells and are secreted with exquisite timing and precision to maintain blood glucose levels between 4 mM and 8 mM (70–140 mg/dL) (van de Bunt & Gloyn, 2012).

Pancreatic beta cells respond to hyperglycaemia by secreting an appropriate amount of insulin to maintain normoglycaemia. In insulin-sensitive tissues, it triggers the uptake of glucose from the blood and stimulates liver to convert glucose into its storage form, glycogen (Bouwens & Rooman, 2005). The unique glucose recognition mechanism in beta cells is able to accurate sense the transient increase in blood glucose by regulated uptake of glucose *via* specific glucose transporter – GLUT 2 in rodents and GLUT 1 in humans (De Vos *et al*.,

1995). Intracellular metabolism of beta cells generates ATP from this glucose and induces a rise in ATP/ADP ratio. It leads to the closure of ATP-sensitive potassium channels and opening of voltage-dependent Ca^{2+} channels (Dukes & Philipson, 1996; Philipson, 1999). The subsequent Ca^{2+} entry into beta cells increase intracellular calcium concentration and trigger exocytosis of secretory insulin granules (MacDonald *et al*., 2005). K^{+}-ATP-dependent pathway and K^{+}-ATP independent pathway also regulate insulin secretion at the mitochondrial level (Henquin, 2011). Both sympathetic and parasympathetic branches of autonomous nervous system innervate pancreas and control pancreatic endocrine output (Renuka *et al*., 2004; Taborsky & Mundinger, 2012). Intrapanceatic neural connections induce oscillations in insulin secretion with a periodicity of about five minutes (Stagner *et al*., 1980; Song *et al*., 2000). Incretin hormones, glucagon-like peptide 1 and gastrointestinal insulinotropic peptides are other factors that regulate insulin release from beta cells (Baggio & Drucker, 2007).

Pancreatic beta cells secrete insulin in a biphasic manner with first and second phases of insulin release. Rapid, but transient first phase insulin secretion begins in response to a sudden rise in plasma glucose levels and continues for 10 to 15 minutes (Brunzell *et al*., 1976). The first phase of insulin secretion helps in rapid glucose clearance from blood; however it is impaired in the prediabetic state (Leahy *et al*., 1992). The first phase is followed by the progressive second phase of prandial insulin secretion that persists throughout the hyperglycemic state (Grodsky, 1989).

Pancreatic beta cell population exist as a dynamic mass in adults with a fine balance between beta cell birth, differentiation and death. During pregnancy and insulin resistance, there occurs a persistent increase in blood glucose levels. In response to these chronically augmented insulin demands, beta cells initiate an adaptive process called ‘beta cell compensation’ (Heit *et al*., 2006). The net insulin secretory capacity of pancreas is raised by an increase of beta cell mass to meet the physiological challenges and to maintain the metabolic balance (Bouwens & Rooman, 2005). Beta-cell mass is precisely regulated by glucose and

hormonal effects on beta-cell proliferation, size, apoptotic elimination and neogenesis from progenitor cells (Mathis *et al*., 2001). Blood glucose level is a major determinant of beta cell function and growth. Hyperglycaemia significantly increases beta cell mass by hyperplasia, hypertrophy and persistent hypoglycaemia induces beta cell atrophy (Swenne, 1982; Jonas *et al*., 1999). Failure of beta cells to adapt to the increase metabolic demand during pregnancy, obesity, insulin resistance of peripheral tissues or tissue injury lead to the development continuous hyperglycaemia or diabetes (Saltiel & Kahn, 2001).

Deficient insulin secretion and/or action are a crucial pathophysiology of all types of diabetes. In type 1 diabetes, pancreatic beta cells mass is progressively reduced due to autoimmune destruction. This decrease in insulin output from pancreas leads to the subsequent failure of the residual insulin secreting beta cells due to the increased metabolic load (Lightfoot *et al*., 2012). Pathophysiology of type 2 diabetes includes insulin resistance in peripheral tissues and pancreatic beta cell dysfunction (Fink *et al*., 1983; Kahn *et al*., 2006). Initial increase in insulin demand results in impaired fasting glucose levels and the development of a prediabetes condition. Sustained hyperglycaemia during the prediabetes state induce transient and reversible desensitization of beta cells, a transient cellular insensitivity in glucose stimulated insulin secretion (Robertson *et al*., 1992). But still, beta cell compensatory response perpetuates the prediabetic state due to the unaltered second phase of insulin release (Cerasi & Luft, 1967; Robertson *et al*., 2003).

As the prediabetes advances, persistent hyperglycaemia leads to progressive beta cell dysfunction and functional mass deterioration (Butler *et al*., 2003). This leads to beta cell exhaustion with depleted beta cell insulin stores and an impaired second phase insulin secretion. Beta cells release less insulin in response to various secretatgogue signals due to the lack of stored insulin (Ward *et al*., 1984). Beta cell desensitization and exhaustion together accounts for an 85% reduction of pancreatic insulin output due to a 50% reduction in the beta cell mass (Weir & Bonner-Weir, 2013). Glucose toxicity is a clinical condition where

chronic hyperglycaemia deteriorates beta cell insulin secretion and worsens blood glucose homeostasis (LeRoith, 2002). This vicious circle finally leads to the total incapacity of beta cells to secrete insulin (Dubois *et al*., 2007).

Hyperglycaemia induced oxidative stress is considered as the principal aetiology of glucose toxicity (Tiedge *et al*., 1997). Increased intracellular glucose content increase reactive oxygen species levels by promoting non-enzymatic glycosylation and production of superoxide anions from mitochondrial electron transfer system and hexosamine pathway (Kaneto *et al*., 1996, 2001; Sakai *et al*., 2003). Reactive oxygen species reduce insulin biosynthesis and secretion by inhibiting Pancreatic duodenal homeobox-1 (Pdx-1) (Kaneto *et al*., 1996). Pdx-1 is the chief transcriptional activator for insulin gene. Hyperglycaemia induced oxidative stress increase insulin resistance in peripheral tissues by inhibiting the translocation of glucose transporters to the plasma membrane (Rudich *et al*., 1998). Progressive insulin resistance together with insufficient insulin secretion result in the failure of beta cells to adequately compensate for the existing metabolic demand and thereby leads to an impaired glucose homeostasis and the development of diabetes (Leahy *et al*., 1986; Weir & Bonner-Weir, 2013). Thus, glucose toxicity also contributes to beta cell dysfunction and beta cell mass deterioration to cause an absolute insulin deficiency (Tanaka *et al*., 2002). Pancreatic beta cell is therefore acknowledged as a central player in the pathogenesis of diabetes. Preservation, expansion and improvement of beta cell functions will hence have a crucial role in delaying or preventing diabetes mellitus.

Insulin

Insulin is a dipeptide hormone synthesised and secreted by pancreatic beta cells in the islets of Langerhans. It regulates carbohydrate, protein and lipid metabolism in the body. It also controls cell growth, proliferation and differentiation. Insulin is composed of A and B polypeptide chains with 21 and 30 amino acids, respectively. The two polypeptide chains are joined together by two

disulphide bonds. Insulin is synthesised in the rough endoplasmic reticulum as Pre-proinsulin. Removal of signal peptide from the N-terminus of Pre-proinsulin produces proinsulin, which is transported through Golgi apparatus to storage vesicles (Bratanova-Tochkova *et al*., 2002). In immature storage vesicles, proinsulin is cleaved to produce insulin and C-peptide. Regulated exocytosis of mature storage vesicles release insulin and C-peptide to circulation. Insulin biosynthesis and secretion is controlled by blood glucose levels. Glucose induces insulin secretion by increasing ATP to ADP ratio, closure of K^+-ATP channels and production of cAMP. Other insulin secretagogue molecules are fatty acids, amino acids, acetylcholine, catecholamines, glucagon-like peptide-1, glucose-dependent insulinotropic polypeptide, somatostatin and pituitary adenylate cyclase-activating polypeptide. Cholinergic and adrenergic neuronal stimulation is one of the major non-nutrient secretagogue stimuli (Pørksen *et al*., 2002).

The physiological effects of insulin in various tissues are mediated through binding to a receptor protein tyrosine kinase called insulin receptor. This transmembrane signalling protein is mainly expressed by hepatocytes, adipocytes and skeletal muscle cells. Insulin receptor is a heterotetrameric glycoprotein present in the cell membrane. Functional insulin receptor dimers are covalently maintained by disulphide bonds (Lee & Pilch, 1994). Binding of insulin at the extracellular domain of insulin receptor stimulates phosphorylation and activation of intracellular domain. Its tyrosine kinase activity enables phosphorylation of a protein called insulin responsive substrates. Phosphorylated insulin responsive substrates proteins activate a number of downstream pathways through src-homology-2 domain proteins and Grb2 (Withers & White, 2000). Insulin responsive substrates mediated activation of phosphoinositide 3-kinase pathway promote glucose uptake through specific glucose transporters and stimulate glycogen, lipid and protein biosynthesis. Mitogenic effects of insulin are mediated by the activation of RAS pathway (Kido *et al*., 2001).

DELTA CELLS

Third most abundant cell type in the islets of Langerhans is somatostatin producing delta cells, which comprises about 10% of islet cells (Wieczorek *et al*., 1998). Apart from pancreatic delta cells, somatostatin is also produced by hypothalamus, stomach, salivary glands, urinary tract and intestine (Bloom & Polak, 1987; Davis *et al*., 2001). Neurohormone, somatostatin is one of the major regulatory hormones in the central nervous system and digestive system. In the digestive system, it inhibits the exocrine function of stomach, pancreas and gall bladder (Marteau *et al*., 1989).

Glucose is the major inducer of somatostatin release from delta cells (Grill *et al*., 1984). In hetero-cellular region of islets of Langerhans, beta cells are in direct contact with delta cells (Unger & Orci, 1977). Somatostatin controls glucose metabolism by acting as a paracrine suppressor of both insulin and glucagon secretion (Lucey, 1986). Alpha cell hormone, glucagon indirectly regulates insulin release by stimulating the release of somatostatin (Patton *et al*., 1976). As the delta cells play an important role in the regulation of insulin secretion, delta cell impairment also contributes to beta cell dysregulation. Baetens *et al*., (1976) demonstrated that delta cell degeneration is associated with hyperinsulinemic forms of diabetes. Diabetes associated hyperglycaemia is known to impair somatostatin release, which in turn alter somatostatin mediated suppression of endocrine and exocrine secretions (Segers *et al*., 1989).

PP CELLS

Pancreatic polypeptide (PP) is produced by PP cells of pancreas. The hormonal action of pancreatic polypeptide is to inhibit gall bladder contraction and pancreatic enzyme release (Greenberg *et al*., 1978). In pancreas, PP cells are primarily present in the islets of Langerhans and are partly found scattered throughout the exocrine pancreas (Larsson *et al*., 1975). PP secretion is mainly regulated by cholinergic vagal stimulation along with glucose and neuropeptides

(Tong *et al*., 2007). Kahleova *et al*., (2012) showed that during diabetes, decreased PP secretion helps to improve beta cell function significantly.

EPSILON CELLS

The peptide hormone, ghrelin is produced by epsilon cells of pancreatic islets (Kojima *et al*., 1999). Other sources of circulating ghrelin include stomach, intestine, hypothalamus and testis (Wierup *et al*., 2007). Ghrelin is a regulator of food intake, body weight and a potent stimulator of growth hormone secretion (Wiedmer *et al*., 2007). Even though numerous epsilon cells are present in foetal and neonatal pancreas, its number was significantly reduced in adult pancreas (Wierup *et al*., 2002). Ghrelin plays an important role in blood glucose homeostasis by inhibiting insulin release from beta cells (Myrsén-Axcrona *et al*., 1997).

BRAIN AND INSULIN RELEASE

Brain is the principal regulator of energy metabolism. It receives information regarding nutritional status *via* sensory and gastrointestinal afferent neurons and control energy balance by modulating key metabolic hormones. Central nervous system responds to changes in energy requirements by regulating plasma glucose concentrations. Sympathetic and parasympathetic nervous system act in concert with the hypothalamic-pituitary-adrenal axis to regulate glucose homeostasis (Eikelis & Esler, 2005). Brain stem, hippocampus and hypothalamus are the critical areas of the brain that regulate energy metabolism through neuroendocrine control of pancreatic hormones (Schwartz & Porte, 2005).

Pancreatic endocrine and exocrine secretions are regulated by an integrated array of neural and hormonal inputs. Pancreatic neural innervations include sympathetic, parasympathetic, sensory afferent, nitric oxide synthase-containing and entero-pancreatic neurons (Brunicardi *et al*., 1995). The endocrine secretions from the islets of Langerhans are mainly regulated by sympathetic and

parasympathetic branches of the autonomic nervous system (Havel & Ahren, 1997). In pancreas, autonomic neuronal terminals are located adjacent to all islet cell types. Splanchnic sympathetic neuronal stimulation leads to the glucagon secretion form the alpha cells. Insulin release from beta cells is induced by the activation of vagal parasympathetic neurons (Gotoh *et al*., 1989; Nonogaki *et al*., 2000). Pancreatic sympathetic neurons and parasympathetic fibres originate from spinal cord and brain stem respectively (Buijs *et al*., 2001).

Sympathetic regulation

The sympathetic nerves innervating the islets are postganglionic neurons having a long axon (Ahrén *et al*., 1986). Most of its cell body is located in the celiac ganglion or in the paravertebral ganglia. Postganglionic neuronal terminals release catecholamine and are in close association with pancreatic islets (Brunicardi *et al*., 1995). The neuronal input into the paravertebral or celiac ganglia is conducted by the preganglionic nerve fibres that leave the spinal cord at the level of C8 to L3. These preganglionic nerve fibres originate from the hypothalamus (Ahrén, 2000).

The precise effect of sympathetic neuronal stimulation on beta cell depends on the relative abundance and activity of α and β adrenergic receptors. Norepinephrine mediated activation of α adrenergic receptors, particularly α2 subtype, leads to the inhibition of glucose-stimulated insulin secretion (Porte & Williams, 1966; Das *et al*., 2006). Activation of α2 adrenergic receptors leads to hyperpolarisation of beta cells due to the opening of the ATP regulated K^+ channels and a reduction in cytoplasmic Ca^{2+} concentration (Nilsson *et al*., 1988). Sympathetic neuronal stimulation show more inhibitory effects in the first phase of insulin release (Ahrén & Taborsky, 1988). Noradrenaline stimulates insulin secretion from beta cells by the activation of β adrenergic receptors (Skoglund *et al*., 1988). Beta cell β adrenergic receptors stimulate the production of cAMP and induce exocytosis of insulin secretory vesicles (Plant *et al*., 1991). Catecholamine

indirectly stimulates beta cell insulin production and secretion *via* the activation of alpha cells (Ahrén *et al*., 1987).

Parasympathetic regulation

Cholinergic neuronal signalling from the vagal efferent neurons stimulates insulin secretion and beta cell compensatory response (Holst *et al*., 1981). Intrapancreatic ganglia act as the origin of cholinergic parasympathetic postganglionic nerve innervation to pancreas. The intrapancreatic ganglia are under the control of vagus efferent fibres originating from dorsal motor nucleus in brain stem (Stagner & Samols, 1985; Putti *et al*., 2000). Solitary tract in dorsal motor complex in the brain stem receive sensory neuronal input relating to glucose homeostasis from pancreas, hypothalamus and other brain regions (Berthoud & Powley, 1990). Projections of solitary tract extend onto the preganglionic motor neurons of vagus. This directs preganglionic vagal efferent fibres to intrapancreatic ganglia (Gilon *et al*., 2002).

In intrapancreatic ganglia, acetylcholine secreted by the preganglionic motor neuronal terminal of vagus nerves stimulates nicotinic acetylcholine receptors on the postganglionic neuron. Postganglionic nerve terminals also release acetylcholine at the vicinity of pancreatic beta cells and activate the muscarinic acetylcholine receptors (Boschero *et al*., 1995; Gireesh *et al*., 2008). Once activated, muscarinic receptors induce a rapid stimulation of exocytosis and insulin secretion by increasing cytosolic concentration of Ca^{2+} (Niwa *et al*., 1998).

BRAIN STEM

Brain stem is the posterior part of the brain that functions to control many vital body functions through autonomous nervous system. Brain stem is composed of three major parts: midbrain, pons and medulla. It acts as a signal integrative conduit for many ascending and descending neuronal pathways. Control of movement, autonomic reflexes, consciousness, arousal and modulation of pain are

the major functions of brain stem. Posterior cerebral artery and vertebral-basilar system provide vascular supply to the brain stem (Barber & Burks, 1987).

Sympathetic and parasympathetic pathways originating from brain stem control energy metabolism of the whole body and glucose homeostasis. Brain stem assesses the energy status by sensing circulating concentration of hormones and metabolites. Dorsal vagal complex in the brain stem integrate sensory signals from vagal inputs and signals from other areas of brain to elicit a modification in energy metabolism (Berthoud & Neuhuber, 2000). Dorsal motor nucleus of the vagus nerve and nucleus tractus solitarius are the primary components of dorsal vagal complex that play a crucial role in the parasympathetic control of pancreatic beta cells (Spanswick *et al*., 2000). It also sends regulatory signals to gastrointestinal tract and liver. Dorsal motor nucleus of the vagus nerve receives and integrates signals from the afferent fibres of cranial nerves VII, IX and X (Barber & Burks, 1987).

Brain stem responds to any alterations in blood glucose level by the modulation of vagal signalling (Balfour *et al*., 2006). Dorsal vagal complex inhibits vagal output in response to acute hyperglycaemia during the initial stages of diabetes. Vagal nerve activity is significantly increased in the later stages of chronic hyperglycaemia. Dorsal motor nucleus of the vagus nerve partakes in diabetogenesis by modulating the parasympathetic innervations to pancreas (Pocai *et al*., 2005; Zsombok *et al*., 2011). Ahrén *et al*., (1996) suggested that vagal parasympathetic stimulation protects pancreatic beta cells from glucose toxicity and reduces diabetogenesis in mice. Vagal functional alteration during diabetes leads to the inhibition of hepatic gluconeogenesis and the subsequent hepatic glucose dysregulation, gastrointestinal motility and pancreatic exocrine function (Saltzman & McCallum, 1983; Zsombok & Smith, 2009).

HIPPOCAMPUS

Hippocampus is located immediately below the floor of the temporal horn of the lateral ventricle. It has an important role in the limbic system and formation

of memory. Hippocampus is composed of dentate gyrus and the cornu ammonis. The dentate gyrus is a simple cortical region of hippocampal formation. The dentate gyrus consists of three layers- molecular layer, granule cell layer and polymorphic cell layer (Seress & Mrzljak, 1987). Adult neural stem cells present in the subgranular zone of dentate gyrus have an important role in learning and memory (D'Amour & Gage, 2003). Cornu ammonis consist of CA1 field, CA2 field and CA3 field. Neuronal signal from entorhinal cortex is conducted to hippocampal dentate gyri *via* fibers called the perforant path (Claiborne *et al*., 1990). After information processing, dentate gyri conveys it to the CA3 field of the hippocampus (Amaral *et al*., 2007).

Kuwabara *et al*., (2011) reported that gene expression pattern for pancreatic beta cells and developing neurons in the dentate gyri are remarkably similar. The parasympathetic regulation of pancreatic insulin release is influenced by hippocampus through its neuronal connections to brain stem (Wyss *et al*., 1979; Zuo *et al*., 2007; Moraes-Neto *et al*., 2014). Diabetes associated chronic hyperglycaemia causes an increase in intracellular glucose levels in the hippocampal nerve cells. It induces oxidative stress by the polyol pathway and increased lipid peroxidation (Lipinski, 2001). Diabetes induced oxidative stress exposes hippocampal neurons to high level of nitric oxide thereby resulting in apoptotic neuronal cell death (Sharma & Ebadi, 2003). This leads to alterations in hippocampal synaptic plasticity and adult dentate gyri neurogenesis leading to an impairment of hippocampal learning and memory (Greenwood & Winocur, 2005).

HYPOTHALAMUS

The hypothalamus is the ventral-most part of diencephalon responsible for the regulation of autonomic and endocrine systems. It is located between rostral limit of the optic chiasm and the caudal limit of the mammillary bodies. Functions of hypothalamus include maintenance of homeostasis, control of endocrine glands and emotional expression. Hypothalamus is divided into three regions- supraoptic, tuberal and mammillary. Supraoptic region contain supraoptic and paraventricular

nuclei and release vasopressin, oxytocin and corticotropin releasing hormone *via* supraopticohypophysial tract (Atweh & Kuhar, 1977). Ventromedial nuclei and arcuate nucleus nuclei are present in the tuberal region. They are responsible for the control of eating and endocrine regulation of adenohypophysis respectively. Mammillary region contain posterior hypothalamic and prominent mammillary nuclei involved in thermoregulation and memory (Broadwell & Brightman, 1976; Cheung *et al*., 2013).

Four directional fibre tracts connect hypothalamus to other brain regions. It include, fornix connected to hippocampus, mammillothalamic tract joined to thalamus, stria terminalis fibres to amygdala and medial forebrain bundle connecting brain stem. Hypothalamus is called the head ganglion of autonomic nervous system. Neuronal fibres originating from different parts of the hypothalamus innervate preganglionic sympathetic neurons in the lateral horn of the spinal cord and preganglionic parasympathetic neurons in the dorsal motor nucleus of vagus (Horvath *et al*., 1990).

Hypothalamus regulates energy homeostasis by adjusting body metabolism according to the peripheral nutrient status (Schwartz *et al*., 2000). In response to a hyperglycaemia, hypothalamus initiates a stimulation to increase glucose utilization in peripheral tissues and regulate food intake (Anand *et al*., 1964). Ventromedial, dorsomedial, lateral and paraventricular regions of hypothalamus are reported to have a major role in glucose homeostasis (Elmquist, 2001). The major blood glucose sensor of brain is situated in ventromedial lateral and arcuate nuclei of hypothalamus (Borg *et al*., 1999; Verberne *et al*., 2014). In response to variations in circulating glucose levels, hypothalamic glucose-excited and glucose-inhibited neurons elicit an action potential (Song *et al*., 2001). These neurons send signals to ventromedial nucleus and regulate glucose homeostasis (King, 2006). Persistent hyperglycaemia associated with diabetes decrease the excitability of glucose-excited and glucose-inhibited neurons. This reduction of the glucose sensing ability supresses the autonomic counter regulatory responses

of central nervous system and lead to hypoglycaemia-associated autonomic failure (Donnelly *et al*., 2005).

NEUROTRANSMITTERS AND DIABETES

Diabetes is associated with anatomical, functional and biochemical alterations in the central and peripheral nervous system (Tomlinson *et al*., 1992). More than 50% of diabetic patients develop diabetic polyneuropathy, a major cause of diabetes associated morbidity and mortality (Forsblom *et al*., 1998). Persistent hyperglycaemia disturb the intracellular glucose transport system of nerve cells and result in changes of intracellular glucose concentrations according to the blood glucose levels. Hyperglycaemia induced increase in intracellular glucose concentration stimulates polyol pathway, which increases susceptibility of nerve cells to intracellular oxidative stress (Lee & Chung, 1999). Improved glucose availability also increases mitochondrial oxidative metabolism and generate superoxide radicals from electron transport chain (Korshunov *et al*., 1997). Excessive generation of reactive oxygen radicals and reduced antioxidant defence disrupt neuronal electrophysiological properties and alter neurotransmitter function. Diabetes is associated with functional alteration of neurotransmitters like epinephrine, norepinephrine, acetylcholine, glutamate, dopamine, serotonin and gama-aminobutyric acid (Gupta *et al*., 1992; Lacković *et al*., 1990; Peeyush *et al*., 2010). These alterations contribute to neurological complications including cognitive dysfunction and hypoglycaemia-associated autonomic failure (Gispen & Biessels, 2000; Kodl & Seaquist, 2008). The alterations in the epinephrine and norepinephrine system impair the sympathetic control of pancreatic beta cell mass regulation and insulin release. Disturbed acetylcholine function alters the parasympathetic beta cell functional regulation in maintaining normoglycaemia.

EPINEPHRINE AND NOREPINEPHRINE

Epinephrine (4,5-β-trihydroxy-N-methylphenethylamine) and norepinephrine (4,5-β-trihydroxyphenethylamine) are two separate but related

catecholamines derived from tyrosine. Epinephrine and norepinephrine have similar chemical structures and induce similar pharmacological effects. These sympathomimetic agents act both as a hormone and a neurotransmitter. In response to stress, adrenal medulla synthesizes and release epinephrine and norepinephrine into the blood. This adrenomedullary hormonal secretion contains 80 percent epinephrine and 20 percent norepinephrine. Hence epinephrine and norepinephrine are also known as adrenaline and noradrenaline respectively. Adrenal medulla is the sole source of epinephrine. Endocrine signalling through circulating epinephrine and norepinephrine generally produce similar physiological effects (Blaschko, I939; Euler, 1946). The major outcome of these hormones is to constrict minute blood vessels, dilate skeletal muscles of blood vessels, increase cardiac output, increase blood pressure, stimulate liver glycogen breakdown and increase circulating free fatty acids. Epinephrine is the major hormone that mediate systemic response to glucoprivation and emotional distress (Euler, 1946; Fluck, 1972).

In nervous system, catecholamines act as neurotransmitters. Sympathetic nerve fibres predominantly use norepinephrine to conduct nerve impulses to effector organs (Esler & Kaye, 2000). In central nervous system, noradrenergic (norepinephrine) neurons originate from locus coeruleus. Neurons in locus coeruleus-noradrenergic system innervate brain stem, hypothalamus, cerebellum, hippocampus, spinal cord and thalamic relay nuclei. Noradrenergic neuronal signalling plays a key role in alertness, arousal and regulation of sensory responsiveness (Berridge & Waterhouse, 2003). Animal experiments demonstrate the vital role of epinephrine and norepinephrine mediated neuronal signalling in producing behavioural changes in response to constantly changing environments (Castelino & Schmidt, 2010).

Functional regulation of adrenergic receptors plays an important role in maintaining glucose homeostasis in response to varying metabolic demand. Sympathetic neuronal stimulation of insulin secretion is mediated by the activation of adrenergic receptors. Long-standing hyperglycaemia is associated with

alterations in adrenergic receptor function in central and peripheral nervous system (Bennett *et al*., 1978). Chronic hyperglycaemia during diabetes is associated with an elevated circulatory norepinephrine concentration that arise from an increased synthesis of norepinephrine in the adrenergic nerve terminals (Neubauer & Christensen, 1976). In central nervous system, hypothalamus and brain stem have been reported to show a significant increase in norepinephrine levels during persistent diabetes (Ramakrishna & Namasivayam, 1995; Ohtani *et al*., 1997). In striatum, hippocampus and hypothalamus, diabetes induces a significant increase of epinephrine levels (Ramakrishna & Namasivayam, 1995).

ADRENERGIC RECEPTORS

The biological action of both epinephrine and norepinephrine are mediated by a class of integral membrane G protein-coupled receptors called adrenergic receptors or adrenoceptors. This receptor is a member of the family of 7-transmembrane domain guanine nucleotide protein coupled receptors. Ligand binding to adrenergic receptors is rapid, saturable, stereoselective, reversible and of high affinity (Barnes, 1981).

All types of adrenergic receptors show considerable amino acid sequence homology with several important structural and functional similarities. They are single subunit proteins containing seven separate hydrophobic amino acid stretches that represent potential membrane spanning transmembrane alpha-helixes (Kassis & Fishman, 1984). For the functional regulation of adrenergic receptor, their cytoplasmic domains contain several potential sites for phosphorylation. N-linked glycosylation near the amino terminus of extracellular domains also control receptor sensitivity and signal transduction (Stiles, 1985).

Like the other transmembrane G protein-coupled receptors, adrenergic receptors have three structurally and functionally distinct regions- extracellular domains, cytoplasmic domains and transmembrane domains (Simantov & Sachs, 1978). Epinephrine or norepinephrine binding pocket in transmembrane region is stabilized by disulphide bonds formed by the cysteine residues of extracellular

domains. Loops associated with seven transmembrane regions of the adrenergic receptors form a ligand-binding pocket for the specific binding of catecholamine (Dixon *et al*., 1987). Ligand binding induced conformational changes in extracellular and transmembrane domains are converted to intracellular signals by the interaction of cytoplasmic domains with specific G proteins and various kinases (Matsui *et al*., 1989).

The initial classification of adrenergic receptors into α adrenergic and β adrenergic receptors was made by Ahlquist (1948). This was based on their pharmacological characteristics. Based on pharmacological and functional criteria, α adrenergic receptors are further subdivided into α1 and α2 receptors (Hoffman & Lefkowitz, 1980). β1, β2 and β3 receptors are the subtypes of β adrenergic receptors (Lefkowitz & Caron, 1985; Webber & Stock, 1992). α and β adrenergic receptor subtypes have different affinity for epinephrine and norepinephrine. α1 adrenergic receptor is coupled to Gq type of G protein at the cytoplasmic domain. Activated Gq stimulates the hydrolytic enzyme phospholipase C and mediate signal transduction *via* IP3 and calcium (Berridge & Irvine, 1984). Unlike α1 receptor, G-protein Gi is associated with α2 adrenergic receptor. Upon activation, Gi inhibits the hydrolytic enzyme adenyl cyclase resulting in a decrease of second massager cAMP. β1, β2 and β3 adrenergic receptors are coupled with G proteins of the type Gs. Activation of Gs leads to the stimulation of adenylate cyclase and generation of cAMP (Lefkowitz & Caron, 1987; Webber & Stock, 1992).

α1 adrenergic receptor

The most important function of α1 adrenergic receptors is to regulate muscle contraction and hypertrophic growth of smooth muscle and cardiac cells. It also plays a critical role in hepatic glucose metabolism (Koshimizu *et al*., 2002). Evidence from pharmacological and molecular cloning studies further subdivide α1 adrenergic receptor into three subtypes α1A, α1B and α1D (Bylund, 1992). All the three α1 adrenergic receptors are expressed from separate genes, but they show good homology in their transmembrane domains. All the α1 adrenergic

receptor subtype signalling is mediated through both pertussis toxin-sensitive G-proteins and G proteins of the Gq family (Wu *et al*., 1992). Binding of ligand to the α1 adrenergic receptor activates hydrolytic enzymes, phospholipases C and A2. This results in an increased intracellular calcium levels by its mobilization from intracellular calcium stores and *via* voltage-dependent and independent calcium channels (Theroux *et al*., 1996).

α2 adrenergic receptor

α2 adrenergic receptors play a key role in locus coeruleus-noradrenergic system and sympathetic nervous system. On the basis of molecular cloning evidence, α2 adrenergic receptors are divided into α2A, α2B and α2C subtypes. α2 adrenergic receptor subtypes show differential distribution- α2A subtype is the main subtype in brain and in cardiovascular control centres, α2B subtype in sympathetic neurons and α2C in caudate (Kanagy, 2005). α2 adrenergic receptors are coupled to heterotrimeric G proteins of Gi/o subfamily. Binding of epinephrine or norepinephrine to α2 adrenergic receptors inhibit cAMP producing enzyme, adenylyl cyclase. Upon activation, it inhibits voltage-gated Ca^{2+} channels and cause the opening of K^{+} channels and stimulate Mitogen-activated protein kinases (MAPK) signalling cascades (Richman & Regan, 1998).

In presynaptic neurons, α2 adrenergic receptors inhibit norepinephrine release and in postsynaptic neurons, they modulate neuronal excitability (Knaus *et al*., 2007). α2 adrenergic receptors are involved in the regulation of insulin secretion and beta cell function (Padayatti & Paulose, 1999; Boesgaard *et al*., 2010). Polymorphism in α2-adrenergic receptors increase the risk of diabetes in Caucasians and African Americans by promoting obesity-related phenotypes (Li *et al*., 2006; Rosengren *et al*., 2010). Devedjian *et al*., (2000) reported that *in vivo* overexpression of α2 adrenoceptors in mouse pancreatic beta cells result in glucose intolerance. Studies on α2 adrenergic receptors in knockout mice, suggest

that α2 adrenergic receptor subtypes have a major role in the regulation of blood glucose homeostasis (Fagerholm *et al*., 2004).

β1 adrenergic receptor

β1 adrenergic receptors play a key role in regulation of cardiac excitation, cardiac muscle contraction, gene transcription and growth. Approximately 80% β adrenergic receptors present in heart are of β1subtype (Liggett, 2010). β1 adrenergic receptors control lipolysis in adipocytes and renin secretion from the juxtaglomerular cells of kidney. Gs coupled β1 adrenergic receptors induce cAMP dependent Protein kinase A activation and signal transduction (Borea *et al*., 1992). Propranolol insensitive state of β1 adrenergic receptors is sometimes referred to as β4 adrenergic receptors (Granneman, 2001).

β2 adrenergic receptor

G protein, Gs coupled β2 adrenergic receptor mediated activation of adenylate cyclase is more efficient than β1 adrenergic receptor or β3 adrenergic receptor. Activated β2 adrenergic receptor stimulates key regulatory proteins like protein kinase C and MAP kinase (Ortega *et al*., 2007). Activated protein kinase C phosphorylates several other structural and regulatory proteins. MAPKs activation has a role in regulating several fundamental cellular processes like differentiation, stress response, apoptosis and proliferation. Functions of β2 adrenergic receptor include regulation of bronchodilation, ventricular function, vasodilation, lipolysis, heart rate and cardiac contractility (Snyder *et al*., 2008; Mutlu *et al*., 2004).

Polymorphisms in the amino-terminus of β2 adrenergic receptor gene are associated with obesity and hypertriglyceridaemia. This contributes to the development of diabetes by promoting fat accumulation and peripheral insulin resistance (Ortega *et al*., 2007). Jiang *et al*., (2013) reported that β2 adrenergic receptor mediated cell signalling diminishes insulin signalling and increases cell apoptosis by inducing an increase in tumour necrosis factor-α (TNF-α) levels. β2

adrenergic receptor knockout mice developed a morphological and function damage in retina, analogous to the diabetic retinopathy in rodents (Jiang *et al*., 2013).

β3 adrenergic receptor

β3 adrenergic receptor mediated cell signalling stimulates cAMP synthesis *via* adenylate cyclase activation. Unlike the β1 and β2 receptors, negative inotropic effect of β3 adrenergic receptor activation is associated with an increased production of nitric oxide (Niu *et al*., 2012). Brown and white adipose tissues are the major sites of β3 adrenergic receptor expression. Activation of this receptor stimulates lipolysis and thermogenesis (Berkowitz *et al*., 1995). β3 adrenergic receptor is also known to have a protective role in regulating cardiovascular function during heart failure (Niu *et al*., 2012).

ACETYLCHOLINE

Acetylcholine, an ester of choline and acetic acid, is the principal neurotransmitter of neuromuscular junction and ganglionic synapses. Synaptic nerve impulse transmission mediated by acetylcholine is known as cholinergic transmission. Choline acetyltransferase catalyses the synthesis of acetylcholine from acetyl coenzyme A and choline. Cholinergic neurons synthesize and store acetylcholine in secretory vesicles of presynaptic neurons. After release, acetylcholine acts on the postsynaptic neurons through acetylcholine receptors. Cholinergic signal termination is facilitated through acetylcholinesterase by its hydrolysis into acetate and choline. Acetylcholine mediated parasympathetic vagal neuronal stimulation regulates pancreatic beta cell response (Kaneto *et al*., 1967). Cholinergic nerve impulse transmission in the central and peripheral nervous system is mediated through the activation of muscarinic and nicotinic acetylcholine receptors.

MUSCARINIC ACETYLCHOLINE RECEPTORS

Muscarinic receptors get its name from its interaction with a toxin called muscarine derived from the mushroom *Amanita muscaria*. Muscarine binding activate muscarinic receptors in the peripheral sympathetic nervous system. They are widely distributed among neuronal cells and mediate cellular signalling of their natural ligand, acetylcholine. They control numerous physiological functions of different organs in response to central and peripheral neuronal activity. The muscarinic receptors are metabotropic G protein-coupled receptors that transduce the intracellular signals through guanosine nucleotide-binding proteins (G protein) (Nathanson, 1987).

The physiological functions of muscarinic acetylcholine receptor mediated signalling include the regulation of smooth muscles of blood vessels, heart rate, detrusor relaxation and constriction of the iris sphincter and ciliary muscles of the eye. They also play a major role in regulating secretions from various organs of the gastrointestinal tract, sweat glands and endocrine pancreas. In the central nervous system, muscarinic receptors are involved in motor control, temperature regulation, cardiovascular regulation and memory (Bonner *et al*., 1989; Caulfield & Birdsall, 1998).

Molecular structure of muscarinic receptor is composed of a single glycoprotein containing seven hydrophobic transmembrane domains connected by intracellular and extracellular loops. It has an extracellular amino-terminal domain and a cytoplasmic carboxy-terminal domain. Activated muscarinic receptors initiate intracellular responses *via* interaction of its cytosolic domain with G proteins. Caulfield and Birdsall (1998) classified muscarinic acetylcholine receptors into five subtypes, referred to as M1 to M5. Molecular cloning experiments revealed that all the five muscarinic receptors display a high degree of sequence similarity and structural homology (Bonner *et al*., 1989).

The ligand binding pocket of muscarinic acetylcholine receptors reside on the extracellular surface and comprises of extracellular loops and an amino-terminal domain (Hulme *et al*., 1990). Binding of acetylcholine at the extracellular

domain induces a conformational change within the seven hydrophobic transmembrane domains. This induces a conformational change in the cytoplasmic face of receptor to enable its interaction with intracellular G proteins (Wess, 1996). Each type of muscarinic acetylcholine receptor subtype is capable activating multiple intracellular signal transduction pathways through its interaction with different G proteins (Felder, 1995; Hosey, 1992). According to the type of G protein coupled with the cytosolic domain muscarinic receptors, they can be divided into two groups. M1, M3 and M5 muscarinic receptors are coupled to heterotrimeric Gq/11-type G proteins, that activate hydrolytic enzyme phospholipase C. Gi/o type G proteins coupled with muscarinic M2 and M4 receptors inhibit adenylate cyclase enzyme (Caulfield & Birdsall, 1998; Eglen & Nahorski, 2000).

Muscarinic M1 receptor

Muscarinic M1 receptor subtype is the principal muscarinic receptor in cerebral cortex, hippocampus and corpus striatum (Gerber *et al*., 2001). During early development, M1 receptor in the forebrain regulates the expression of neurotrophins such as nerve growth factor and brain-derived neurotrophic factor to support normal development, maturation and function (Betancourt *et al*., 2006). M1 receptor impairment is associated with neurological disorders like schizophrenia, Alzheimer disease and other dementias (Piggott *et al*., 2003; Deng & Huang, 2005). Stress adaptive cardiovascular reflexes and central blood pressure control is mediated by the M1 receptors in the peripheral nervous system. M1 expressed in the pancreatic islets of Langerhans is accredited to have a major role in beta cell functional regulation (Gilon & Henquin, 2001). Pancreatic regeneration is associated with a significant reduction in the muscarinic M1 receptor function in the brain stem (Renuka *et al*., 2006; Balakrishnan *et al*., 2009).

Muscarinic M2 receptor

Muscarinic M2 receptor activation leads to the inhibition of adenylate cyclase, phosphoinositide degeneration and potassium channel mediation through the action of G proteins. In peripheral nervous system it is mainly involved in the regulation of bradycardia and cardiac contractility (Pedder *et al*., 1991). M2 acetylcholine receptors are the major muscarinic receptor subtype present in the heart, where it serves to mediate a decrease in cardiac beating frequency and a reduction in atrial contractility (Hulme *et al*., 1990). Several forms of cardiovascular diseases are associated with presence of muscarinic M2 specific autoantibodies. Gomeza *et al*., (1999) reported that in central nervous system M2 receptors play an important role in muscarinic receptor-dependent movement and temperature control as well as analgesia. Activation of M2 receptors present in the pancreatic beta cells inhibits the increase in intracellular Ca^{2+} concentration (Harden *et al*., 1986). Persistent hyperglycaemia induced oxidative stress alter the muscarinic M2 receptor by MEK/ERK induced phosphorylation of GATA binding protein 4 (Mar *et al*., 2013).

Muscarinic M3 receptor

Binding of acetylcholine to the Gq-type G protein couples M3 receptor resulting in the activation of phospholipase C. The hydrolytic enzyme phospholipase C cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate into inositol trisphosphate (IP3) and diacylglycerol. IP3 causes an increase in cytosolic Ca^{2+}. Diacylglycerol causes phosphorylation of various proteins by protein kinase and activation of nonselective cationic channels (Eglen *et al*., 1996). Muscarinic M3 receptors are involved in neuronal regulation of smooth muscles, exocrine glands, endocrine glands and lungs. In central nervous system, M3 receptor expression is mainly at dorsal vagal complex of brain stem, ventromedial hypothalamus and arcuate nuclei of hypothalamus (Zubieta & Frey, 1993).

M3 muscarinic acetylcholine receptor is the major muscarinic receptor present in pancreatic beta cells and in areas of brain that influence insulin secretion (Ahrén, 2000; Balakrishnan *et al*., 2009). Glucose mediated stimulation of parasympathetic efferent fibres originating from the brain stem release acetylcholine in the vicinity of islets. Gautam *et al*., (2006) reported that, acetylcholine mediated activation of M3 muscarinic receptors in pancreatic beta cells is essential for insulin release to maintain normal glucose homeostasis. M3 muscarinic receptors also regulate energy metabolism by controlling the release of ghrelin and cholecystokinin through cholinergic vagal innervations to stomach and intestine (Berthoud, 2008). Maintenance of glucose homeostasis by M3 receptor is also mediated through insulin-stimulated glucose uptake in adipose tissue (Yang *et al*., 2009).

Muscarinic M4 receptor

The muscarinic M4 receptor is present in different brain regions along with M1 and M3 subtypes. Activated muscarinic M4 receptors inhibit adenylate cyclase, stimulate the breakdown of phosphoinositides and modulate potassium channels (Levey *et al*., 1995). M4 acetylcholine receptors, abundantly expressed in striatum has an important role in dopamine homeostasis (Levey, 1993; Zhang *et al*., 2002). Dopamine-induced locomotory inhibition is mediated by the activation of M4 receptors in the striatum.

Muscarinic M5 receptor

M5 subtype of muscarinic acetylcholine receptor expression is very low in most brain regions. Substantia nigra *par* compacta region and ventral tegmental area of midbrain abundantly express muscarinic M5 receptor. M5 receptor regulates dopamine release from these regions (Raffa, 2009).

NICOTINIC ACETYLCHOLINE RECEPTOR

Nicotinic acetylcholine receptors are cys-loop family of cationic transmembrane ligand-gated ion channels present in the central nervous system and on presynaptic and postsynaptic sides of neuromuscular junction. Muscle-type nicotinic acetylcholine receptors consist of four subunits organized around a central pore in the plasma membrane. Several different combinations of homopentameric and heteropentameric nicotinic acetylcholine receptors are formed from 16 different subunits encoded by the human genome (Wang, 2003). Binding of acetylcholine activates this receptor to transport sodium ions into the cell and release intracellular potassium ions outside the cell (Albuquerque *et al*., 1995). This membrane depolarization induces an action potential in the postsynaptic neurons or in muscle fibres. Nicotinic acetylcholine receptor mediated cell signalling contributes to the psychoactive properties of drugs like nicotine. Activation of muscarinic acetylcholine receptor is relatively slow when compared to nicotinic acetylcholine whose activity is induced in sub-microsecond range (Karlin *et al*., 1986).

Nicotinic acetylcholine receptor mediated nerve impulse transmission is predominantly seen between motor nerves and muscle fibres and in autonomic ganglia. Wide distribution of structurally and functionally diverse isoforms nicotinic acetylcholine receptor in the central nervous system carries out many modulatory functions (Wada *et al*., 1989). Nicotinic receptors in the presynaptic neuronal terminals enhance neurotransmitter release to synaptic cleft. Fast excitatory transmission is a key feature of postsynaptic neuronal nicotinic receptors (Dani & Bertrand, 2007). The involvement of nicotinic receptor function is necessary for learning, memory, embryonic development and synaptic plasticity. Out of the different nicotinic acetylcholine receptor subunits, α7 subunit is essential for the vagus nerve stimulated cholinergic suppression (Wang *et al*., 2003). Stimulation of the nicotinic cholinergic pathway is known to induce insulin secretion from the beta cells of pancreas (Ilcol *et al*., 2008). Neuropathology of Alzheimer's disease, Parkinson's disease, schizophrenia, lung cancer, epilepsy,

autism and addiction is associated with the impairment of acetylcholine mediated cell signalling *via* nicotinic acetylcholine receptors.

PREVENTION OF DIABETES

Diabetes is a multisystem disease, characterized by the dysregulation of glucose homeostasis resulting in hyperglycaemia. Chronic hyperglycaemia, a characteristic future of uncontrolled diabetes, is associated with long-term microvascular and macrovascular damages. These damages lead to dysfunction and failure of various organs- a root cause of diabetes associated morbidity and mortality. Efforts to prevent or delay the incidence of diabetes are necessary to minimize the individual, familial and public health burden. Long term pancreatic beta cell dysfunction and insulin resistance herald the onset of all types of diabetes (Tuomilehto & Wolf, 1987; Schulze & Hu, 2005). Changing these modifiable risk factors of diabetes, therefore represent a good strategy for preventing or delaying the onset of diabetes.

Risk factors for the development of diabetes can be broadly classified as genetic or environmental. Modifiable behavioural and environmental risk factors such as overweight, unhealthy diet, sedentary lifestyle and perinatal factors act along with unmodifiable genetic predisposition to prompt beta cell dysfunction and insulin resistance (Hamman, 1992). Genetic basis of diabetogenesis is yet to be established. However, the current global epidemic of diabetes can be ascribed to an increased prevalence of sedentary lifestyle and an unhealthy diet (Schulze & Hu, 2005).

Evidence from many prospective cohort studies and randomized clinical trials have demonstrated that diabetes can be prevented or delayed with regular physical activity, maintenance of a healthy body weight, healthy food habits and avoidance of sedentary behaviours (Schulze & Hu, 2005). There is increasing evidence for the fact that nutritional therapy can be used for the prevention of diabetes. Primary prevention is attained by targeting or controlling modifiable risk factors in a population (Psaltopoulou *et al*., 2010). Based on these modifiable risk

factors, many diabetes prevention programs have focussed on the use of phytochemicals and vitamins to improve beta cell function and/or insulin sensitivity. Diabetes prevention strategies focusing on phytochemicals and vitamins are simple, efficient and cost-effective as well (Sacco *et al*., 2003). Curcumin, a diarylheptanoid in turmeric, and vitamin D_3, a secosteroid, is accredited to have potential therapeutic and preventive effects on diabetes (Badenhoop *et al*., 2012; Sahebkar, 2013). A study of the anti-diabetogenesis properties of curcumin and vitamin D_3 will therefore help in the development of effective strategies to delay or prevent the onset of diabetes.

CURCUMIN

Rhizome of turmeric (*Curcuma longa*) is a widely used spice and a colouring agent used in Indian cuisine. Curcumin, the active component of turmeric, has been shown to possess significant antioxidant, anti-inflammatory, anticarcinogenic, antiviral, hypoglycaemic, antibacterial, wound healing, hypolipidemic, anticancer and anti-infectious properties (Brondino *et al*., 2014). Curcumin also has chemopreventive, chemosensitization, chemotherapeutic and radio-sensitization capabilities. Lampe and Milobedzka (1913) identified the chemical structure of curcumin as diferuloylmethane or 1,6-heptadiene-3,5-dione-1,7-bis (4-hydroxy-3-methoxyphenyl)-(1E, 6E).

Traditional Indian medicine considers this polyphenolic curcuminoid compound as an effective drug for various pathological conditions such as asthma, epilepsy, gall stone, bronchial hyperactivity, allergy, anorexia, coryza, cough, hepatic diseases and sinusitis (Prasad *et al*., 2014). In last decades, accumulating scientific evidence from preclinical and clinical studies have propounded the use of curcumin for treatment of various diseases of nervous system, immune system, cardiovascular system, respiratory system, gastrointestinal system and endocrine system (Aggarwal *et al*., 2007).

Taxonomic classification of turmeric is as follows

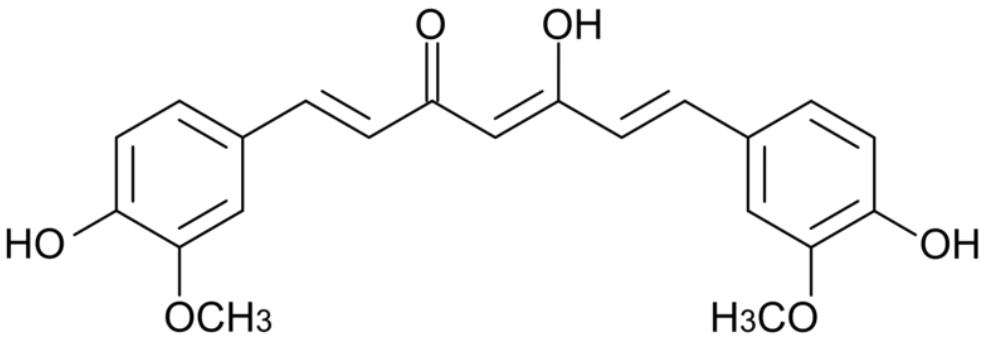
Division	Magnoliophyta
Class	Liliopsida
Subclass	Zingiberidae
Order	Zingiberales
Family	Zingiberaceae
Genus	Curcuma L.
Species	*Curcuma longa* L.

Curcuma longa L. is a perennial herb that has a short stem with large oblong leaves and bears ovate, pyriform or oblong rhizomes. Turmeric is widely cultivated in tropical countries such as India, Sri Lanka, China, Australia, Taiwan, Africa, Peru and Indonesia. In India, the main growing states are Tamil Nadu, Karnataka, Kerala, Andhra Pradesh, Maharashtra and Orissa. Out of the many different components present in the turmeric, volatile oils are the main constituents. The main volatile oils present are sesquiterpenes, d-sabinene, borneol, d-α-phellandrene, zingiberene and cinol, many of which are specific for turmeric. Zingiberene, arturmerone and turmerone are responsible for the characteristic aroma of turmeric (Masuda *et al*., 1999; Aggarwal, 2010). The yellow pigmentation of the rhizomes of turmeric is due to the presence of curcuminoids. The three major components of curcuminoids are curcumin (diferuloylmethane), demethoxycurcumin and bisdemethoxycurcumin. In curcumin biosynthesis, bisdemethoxycurcumin is converted to curcumin through demethoxycurcumin (Ammon & Wahl, 1991; Kita *et al*., 2008). Curcumin constitute 2 to 8% of the spice and 75% curcuminoids in the rhizomes of turmeric.

Curcumin is a lipophilic and photosensitive molecule with two unsaturated carbonyl groups connecting the two aromatic rings. Molecular mass of curcumin is 368.39 g/mol and has the chemical composition $C_{21}H_{20}O_6$. Its molecular structure resembles ubiquinols and diarylheptanoids. The unique

structure of curcumin has two isomers, β-diketone form and enol form (Aggarwal *et al*., 2007). Due to keto-enol tautomerism, curcumin is mainly present in keto form under acidic conditions and under alkaline conditions, it prefers the enol form (Balasubramanian, 2006). The enol form of curcumin is more energetically stable in the solid phase as well as in solution. The most important functional site in the molecule is the central OH group. Hydrogen bonding associated with this OH group is known to stabilize the molecule (Priyadarsini *et al*., 2003). Presence of hydroxyl groups and β-dicarbonylic system in the molecule provide anti-inflammatory and antiparasitic activities to curcumin (Kiuchi *et al*., 1982; Claeson *et al*., 1996). Phenolic OH of curcumin is essential for its antioxidant, hydrogen donating and redox properties (Priyadarsini *et al*., 2003).

Structure curcumin (Enol form)



Turmeric and its constituents are reported to be an effective, inexpensive, safe and easy to access medication for impeding the development of chronic diseases such as diabetes, obesity, Alzheimer's disease, atherosclerosis and cataract (Grynkiewicz & Ślifirsk, 2012). Oxidative damage and inflammation are the root cause of diabetes and associated complications. Several animal, epidemiological and clinical studies have established that diabetes can be prevented, managed or delayed by treatment with anti-oxidant and anti-inflammatory drugs (Basnet & Skalko-Basnet, 2011). Thus curcumin with its

strong anti-oxidant and anti-inflammatory properties, can be deemed as a potential candidate for the prevention and/or treatment of diabetes.

Srinivasan (1972) first reported the anti-hyperglycaemic property of curcumin. Recently studies in rodent models have revealed the therapeutic potential of curcumin in experimental diabetes and in the treatment of diabetes associated long term complications (Pérez-Torres *et al*., 2013; Zhang *et al*., 2013). The reports on molecular mechanisms of the anti-hyperglycaemic effect of curcumin in diabetes models are manifold. The actions proposed include inhibition of lipid peroxidation and reduction of the levels of thiobarbituric acid reactive substances (TBARS) (Murugan & Pari, 2007). Curcumin is regarded to prevent cell damage arising from persistent hyperglycaemia by the attenuation of TNF-α, nuclear factor-kappa B (NF-κB) and lysosomal enzyme activities (El-Azab *et al*., 2011; Soetikno *et al*., 2011). It boosts the induction of peroxisome proliferator-activated receptor-gamma (PPAR-γ) activation, lipoprotein lipase (LPL) activity and nuclear factor erythroid-2-related factor-2 (Nrf2) function (Nishiyama *et al*., 2005). Liver enzymes associated with glycolysis, gluconeogenic and lipid metabolic processes are activated by curcumin treatment. Further, in plasma, it significantly increases circulating insulin levels and decreases plasma free fatty acids (Seo *et al*., 2008; Peeyush *et al*., 2011). Hence, the ability of curcumin to treat diabetes associated complications and to improve the overall function of pancreatic beta cells helps to prevent or delay diabetogenesis. As the strong antioxidant property of curcumin has been shown to reduce inflammation and oxidative damage in different tissues (Nishiyama *et al*., 2005), its administration can be proposed to increase resistance to the disease. Curcumin is also reported to have neuro-protective and neuro-modulatory properties. The therapeutic value of curcumin is established in neurodegenerative diseases like Alzheimer's and Parkinson's (Darvesh *et al*., 2012).

VITAMIN D_3

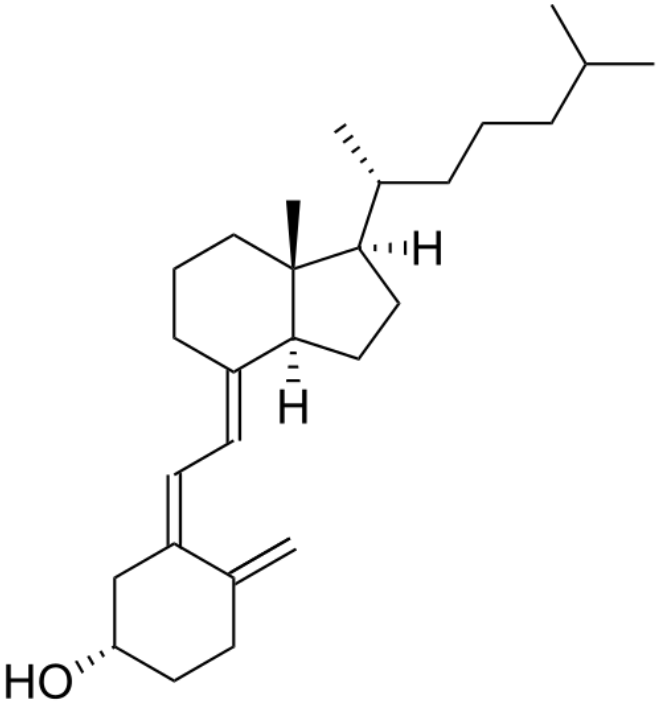
Vitamin D is fat-soluble secosteroid. It plays a key role in calcium and phosphate homeostasis and is critical for bone development and maintenance. Unlike other vitamins, it can be photochemically synthesised in the skin under the influence of UV light. The ring structure of vitamin D_3 contains a cyclopentano-perhydro-phenanthrene, similar to the structure of steroid hormones. So technically, it can be considered as a prohormone (Walters, 1992).

The major physiological function of Vitamin D is stimulation of calcium absorption in the gut to maintain sufficient circulating calcium and phosphate concentrations and to aid bone growth and remodelling. In children, vitamin D deficiency leads to rickets and in adults, it causes osteomalacia. Several other functions of vitamin D like regulation of cell proliferation, differentiation and apoptosis, neuromuscular modulation and control of immune response have been reported in recent decades. Serum concentration of 25-hydroxyvitamin D is used as a biomarker to measure the vitamin D produced cutaneously and that obtained from diet. Around 50 nmol/L of serum 25-hydroxyvitamin D is considered as optimal for bone and overall health. 25-hydroxyvitamin D levels below 30 nmol/L are considered as deficiency and above 125 nmol/L are associated with potential adverse effects. According to Dietary Reference Intakes (DRIs) developed by the Food and Nutrition Board (FNB) of The National Academies, USA 40 IU of vitamin D is equal to 1 µg. Recommended Dietary Allowances for human adults of 1-70 years of age are 600 IU (Holick, 2007; Institute of Medicine, 2010).

Vitamin D belongs to a family of hydrophobic lipids called secosteroids. Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) are the two major physiologically relevant forms of vitamin D. Vitamin D without any subscript refers to either D_2 or D_3 or both and is biologically inert without two obligate hydroxylation. Structurally, vitamin D_2 and D_3 differ in their side chains. Vitamin D_2 has a double bond between carbons 22 and 23, and a methyl group on carbon 24 (Okamura *et al*., 1974). Vitamin D_3 is photosynthesized in the skin of vertebrates while Vitamin D_2 originates from a yeast and plant sterol. Vitamin D_3

is more effective and potent than Vitamin D_2 in raising serum 25-hydroxyvitamin D levels (Trang *et al*., 1998). Rapidly equilibrating chair conformers of the A-ring in Vitamin D is necessary for its optimum biological activity (Hollis *et al*., 1986). Cholecalciferol has a molecular weight of 384.64 g/mol with a chemical composition of $C_{27}H_{44}O$.

Structure of vitamin D_3



Two sources of vitamin D are diet and its synthesis by the action of sunlight on the skin. Dietary sources of vitamin D_2 or vitamin D_3 include fish oils, egg yolks and liver. Generally, adequate quantities vitamin D are not present in natural diets, therefore exposure to sunlight is necessary to prevent deficiency diseases. The first step in the endogenous production of Vitamin D_3 is the conversion of 7-dehydrocholesterol to pre-vitamin D_3 by ultraviolet B with a wavelength of 290–320 nanometres. This radiation penetrates uncovered skin to induce photolytic cleavage of secosteroid B ring. Pre-vitamin D_3 then isomerizes to D_3 in a thermo-sensitive but noncatalytic process (Hanewald *et al*., 1961;

Holick *et al*., 1977). Rate of pre-vitamin D_3 production in skin depends on the intensity of ultraviolet radiation and level of skin pigmentation. Other factors that influence production are season of the year, time of day, length of daytime, cloud cover and sunscreen. Photoconversion of Pre-vitamin D_3 to lumisterol and tachysterol prevents accumulation of toxic amounts of vitamin D_3 during prolonged exposure to sunlight (Webb *et al*., 1988; Holick, 2007). Vitamin D_3 deficiencies mainly result from dietary inadequacy, impaired absorption from digestive tract and limited exposure to sunlight.

The vitamin D_3 and D_2, either made in skin or ingested in diet undergo a two-step activation in liver and kidney. The first step of metabolic activation that occurs in the liver involves cytochromeP-450 monooxygenase. It catalyses hydroxylation of carbon 25 of vitamin D to produce 25-hydroxyvitamin D (Fraser & Kodicek, 1970). The more important second step of activation takes place in the kidney, where 25-hydroxyvitamin D-1α-hydroxylase, a cytochrome P-450 monooxygenase requiring enzyme, converts 25-hydroxyvitamin D to 1,25, dihydroxyvitamin D (Knutson & DeLuca, 1974). Circulating levels of active vitamin D_3 are tightly regulated by reciprocal changes in the rates of synthesis and degradation. Plasma levels of vitamin D_3 are principally regulated by parathyroid hormone and calcium. Vitamin D-24-hydroxylase is a mitochondrial cytochrome P-450 that requires molecular oxygen and reduced ferredoxin. The vitamin is catabolised by oxidation of its side chain. Subsequent oxidative cleavage of the side chains result in the production of biologically inert calcitroic acid (Reddy & Tserng, 1989).

1,25, dihydroxyvitamin D generate physiological responses by regulation of gene transcription and activation of several signal transduction pathways. Most biological activities of 1,25, dihydroxyvitamin D are mediated by a class II steroid hormones receptor called vitamin D receptor. They are high-affinity receptors that acts as transcription factors upon binding 1,25, dihydroxyvitamin D. Vitamin D receptors show sequential and structural resemblance to retinoic acid receptor, peroxisome proliferator activator receptor and thyroid hormone receptors (Mellon

& DeLuca, 1979; Jones *et al*., 1998). They also show considerable sequence similarity among different species. Rat vitamin D receptor is about 50 kDa in size and contains 423 amino acids. In humans, the N terminus of the protein contains additional 4 amino acids and has 427 amino acids in total (DeLuca, 2004). Like other proteins in the superfamily of nuclear receptors, vitamin D receptor contains several functional domains. Truncated A/B domain is present at the N terminus of the protein. DNA-binding region is present between amino acids 20 and 90 and is called the C domain. It is followed by the D or hinge domain and the ligand-binding C terminal E domain. Activation of vitamin D receptor is mediated by the F domain (Brown & DeLuca, 1990; Jones *et al*., 1998).

1,25, dihydroxyvitamin D binds to the ligand binding domain of vitamin D receptor with high affinity. Ligand binding allows the ligand-receptor complex to interact with many proteins of transcriptional machinery. The ligand-receptor complex mainly binds to a cofactor called retinoid X receptor to form a heterodimer (Haussler *et al*., 1997). Activated vitamin D receptor-retinoid X receptor heterodimer mediate its gene regulatory functions through vitamin D-responsive elements. It usually contains repeat sequences of 6 nucleotides, separated by 3 nonspecified bases present within 1 kilobase of the start site of the target gene (Rachez *et al*., 1998). Heterodimer binding induces a bend in the vitamin D-responsive element within the promoter. Further, 1,25, dihydroxyvitamin D binding permits the interaction of vitamin D receptor coactivator proteins. Two major coactivator complexes associated with activated vitamin D receptor are steroid receptor activator complex and vitamin D receptor interacting protein complex (Evans, 1988; Smith *et al*., 1996; Rachez & Freedman, 2000). Upon DNA binding, the coactivators help to remodel DNA structure through their histone acetylase activity. Thus, binding of ligand receptor complex to vitamin D-responsive element of an up regulated target promoter result in an increased production of the corresponding protein. In human genome, it is estimated that vitamin D receptor regulates the expression of 100–1250 genes

(Dusso *et al*., 2005). Non-genomic effects of vitamin D_3 are rapid and are mediated by specific plasma membrane receptors (Garcion *et al*., 2002).

Emerging epidemiological and experimental data indicate that vitamin D is a potential risk modifier for diabetes and associated complications (Pittas *et al*., 2007; George *et al*., 2012). Several observational studies show that vitamin D insufficiency is associated with impaired insulin synthesis and secretion due to impaired beta cell function (Mathieu & Badenhoop, 2005). Pancreatic beta cells express the enzyme 1-alpha-hydroxylase necessary for the production of 1,25, dihydroxyvitamin D. So beta cells produce the biologically active form of vitamin D from circulating pre-vitamin D_3. Vitamin D receptor expressed by beta cells is thought to have a role in the maintenance of normoglycaemia by modulating pancreatic beta-cell function and insulin release (van Etten & Mathieu, 2005). Insulin secretion and sensitivity is indirectly affected by vitamin D on account of its role in calcium homeostasis and regulation of calcium flux through cell membranes in beta cells and peripheral insulin-target tissues. Immunomodulatory properties of vitamin D help to protect pancreatic beta cells from autoimmune destruction (Pittas & Dawson-Hughes, 2010). Consistent association between low vitamin D status and prevalence of diabetes has been reported in cross-sectional clinical studies (Nemerovski *et al*., 2009).

1,25, dihydroxyvitamin D can cross the blood brain barrier and can be synthesised in brain by the action of 1-alpha-hydroxylase. Most of the brain regions express vitamin D receptor in nerve cells, microglial cells, astrocytes, oligodendrocytes and Schwann cells. Vitamin D plays a key role in brain development and function by mediating neuronal differentiation, axonal connectivity and structural and functional development of brain (Eyles *et al*., 2013). Vitamin D stimulates neuronal growth by increasing the expression of major neurotropic factors including glial cell-derived neurotrophic factor, nerve growth factor and transforming growth factor (Airavaara *et al*., 2012). Vitamin D deficiency is associated with developmental physiological disorders like autistic spectrum disorder, schizophrenia and depression (Keeney *et al*., 2013). 1,25,

dihydroxyvitamin D protects nerve cells by inhibiting the synthesis of reactive oxygen radicals and increasing the antioxidant levels. Vitamin D deficiency increases the production of reactive oxygen radicals in the brain and promotes cognitive decline and memory loss (Garcion *et al*., 2002). The extensively reported neuro-protective and neuro-modulatory action of vitamin D_3 probably has a significant role in the pathophysiology of diabetogenesis (Peeyush *et al*., 2010).

STREPTOZOTOCIN INDUCED DIABETIC RAT MODEL

In laboratory animals, several methods are used to induce experimental diabetes mellitus. Out of these, the most useful rodent model of human diabetes is induced by streptozotocin (Wei *et al*., 2003). Streptozotocin diabetic rat model is demonstrative, simple and convenient to use. Streptozotocin injection leads to the specific dysfunction and destruction of pancreatic beta cells in the islets of Langerhans. Streptozotocin diabetic rat model mimics pathogenesis and complications seen in humans (Zhang *et al*., 2003). This well-documented model of experimental diabetes is widely used to study the molecular mechanisms of diabetes and to test therapeutic innovations for the amelioration of diabetes (Fox *et al*., 1999).

Streptozotocin (2-deoxy-2-(3-(methyl-3- nitrosoureido)-D-glucopyranose) is an antibiotic synthesized by the bacteria, *Streptomycetes achromogenes*. Streptozotocin is a pancreatic beta cell specific toxin. It impairs blood glucose homeostasis by decreasing insulin biosynthesis and secretion. Pancreatic beta cells specifically transport streptozotocin into cell *via* GLUT 2 on account of its structural homology to glucose (Thulesen *et al*., 1997). Streptozotocin promotes beta cell death and dysfunction by its DNA alkylating properties. Indirect toxicity of streptozotocin is through the production of free radicals like nitrogen oxide and reactive oxygen species. Toxicity of streptozotocin first disrupts glucose sensing ability of beta cells, followed by permanent dysfunction and death of cells (West *et al*., 1996).

Nitrosourea moiety of streptozotocin catalyses alkylation and subsequent fragmentation of DNA. As streptozotocin is a potent nitrogen oxide donor, it contributes to free radical mediated DNA damage (Sofue *et al*., 1991). Streptozotocin induces generation of superoxide anions by accelerating the xanthine oxidase activity. Superoxide anion in turn stimulates production of hydrogen peroxide and hydroxyl radicals and fuel beta cell damage. Stimulated DNA damage activates cellular poly ADP-ribosylation processes, which exhaust NAD^+ and ATP content. This depletion of energy suppresses insulin synthesis and secretion (Sandler & Swenne, 1983).

Out of several methods available for creating streptozotocin induced diabetic rat models, multiple low dose streptozotocin induced diabetes is most useful for preventive studies (Song *et al*., 2003; Meghana *et al*., 2007; Shehata *et al*., 2011). Administration of multiple low dose streptozotocin produces a gradual and progressive beta cell dysfunction and death. Like natural development of diabetes in humans, diabetogenesis in this model involve beta cell destruction by both nitric oxide dependent and nonnitric oxide-mediated mechanisms (Lukić *et al*., 1998). When compared to single dose streptozotocin induced diabetic rat models, multiple low dose streptozotocin model is more comprehensive and shows characteristics of progressiveness of human diabetes (Arora *et al*., 2009).

BEHAVIOURAL RESPONSE

Diabetes associated central and peripheral neuronal damage result in motor and cognitive deficits. These alterations are studied in rats using different behavioural tests. Rats show several behavioural responses analogous to human diabetic subjects. A wide variety of behavioural response models are available to assess diabetes associated motor and cognitive impairments. The Y maze test is used to study the cognitive functions such as exploratory behaviour, learning and memory. Motor coordination and motor learning of rodents are assessed using rotarod test. Grid walk test helps in the precise evaluation of sensory motor

coordination in rats (Fernández-Guasti *et al*., 2001; Chathu *et al*., 2008; Sherin *et al*., 2010).

CELL FUNCTIONAL MARKERS

Progressive decline in the beta cell mass and function in response to elevated insulin resistance is a key feature of diabetes. Pancreatic tissue is known to have the potential to maintain or increase its beta cell mass in response to metabolic demands. The proliferation, differentiation and maintenance of pancreatic beta cells are associated with modifications in the expression pattern of some key regeneration markers. Apoptosis plays a critical role in removing damaged cells. But inappropriate activation of apoptosis pathways lead to tissue degeneration and dysfunction. Increased apoptotic destruction of pancreatic beta cells is a major cause of decrease of pancreatic secretions. The balance between beta cell regeneration and degeneration helps to maintain glucose homeostasis and any deviation in this leads to the development of diabetes. Beta cell function and mass regulation is controlled by central nervous system through neuronal innervations to pancreas. So, cell signalling alterations in the brain regions also influence the beta cell mass balance. Following are the major markers associated with cell regeneration, degeneration and cell signalling.

Akt

Akt, also known as protein kinase B, is a serine/threonine protein kinase that responds to growth stimulation mediated by growth factors, cytokines, hormones and several nutrients. It is one of the principal regulators of intracellular signalling systems for cell growth, proliferation, apoptosis, genome stability, cellular migration and angiogenesis (Xu *et al*., 2012). Insulin, insulin like growth factor-I, epidermal growth factor, vascular endothelial growth factor and nerve growth factor use Akt mediated cell signalling pathway for their endocrine/paracrine action. Binding of these ligands to the specific plasma membrane receptors induce phosphorylation and activation of Akt. Activated Akt

phosphorylates RXRXX(S/T) sequence surrounded by hydrophobic residues in the target proteins (Kumar *et al*., 2013). Insulin receptor substrate 2 mediates cell signalling *via* Akt and plays a central role in controlling pancreatic beta cell growth and survival (Dickson & Rhodes, 2004).

NeuroD1

Neuronal differentiation 1 (NeuroD1) is a cell type-restricted helix-loop-helix protein essential for normal pancreatic development and maintenance of glucose homeostasis. It is expressed in pancreatic endocrine cells, brain and intestine. The major functions of NeuroD1 are to activate transcription of insulin gene in beta cells and induce differentiation of nerve cells (Gao *et al*., 2009). NeuroD1 activates transcription of E-box sequence containing genes by forming heterodimers with other restricted helix-loop-helix proteins. NeuroD1 mediate cellular differentiation of pancreatic islets, enteroendocrine cells and granule cells. NeuroD1 null mice develop severe diabetes due to significant reduction in the number of insulin-producing beta cells. It is also crucial for survival and maturation of adult-born neurons (Naya *et al*., 1995; Pleasure *et al*., 2000).

Pax

Paired box protein (Pax) is a family of nuclear transcription factors, involved in organogenesis. It plays a critical role in the development and differentiation of beta and alpha cells of pancreas. Characteristic feature of Pax is the presence of a highly conserved DNA binding, paired domain and in most isoforms, a homeodomain and an octapeptide are also present (Dahl *et al*., 1997). Embryonic development of eyes, central nervous system, kidney, immune system and the pancreas are regulated by Pax family of transcription factors. They also regulate production of endocrine hormones like glucagon, insulin and somatostatin by binding to a common sequence in their promoters. Abnormalities in the expression of Pax genes are associated with beta cell dysfunction and loss of insulin producing beta cells (Dohrmann *et al*., 2000).

Pdx-1

Pancreatic duodenal homeobox 1, also known as insulin promoter factor-1 (Pdx-1) is an orphan homeodomain containing transcription factor of paramount significance in the development of endocrine and exocrine pancreas. It is required for the development, differentiation and maintenance of insulin producing beta cells (Offield *et al*., 1996). Pdx-1 regulates the expression of insulin, somatostatin, islet amyloid polypeptide, GLUT 2 and glucokinase in the pancreas. Pdx-1 stimulates insulin expression by binding to the highly conserved regulatory sequence, A-box sites of insulin gene (Hay & Docherty, 2006). Impaired Pdx-1 gene expression contributes to the development of diabetes by reducing pancreatic insulin output and altering beta cell glucose sensing (Melloul *et al*., 2002).

Insulin like growth factor-I

Insulin like growth factor-I (IGF-I) is a polypeptide hormone with endocrine, paracrine and autocrine functions. Though mainly produced by the liver, it is also secreted by other organs like pancreas for autocrine/paracrine purposes. It shows structural and functional homology to insulin and upon binding insulin receptor with a lower affinity, mediates some of its metabolic functions. Insulin like growth factor-I receptor is a protein tyrosine kinase, expressed in a wide verity of tissues. In pancreas, activation of this receptor is known to trigger cell survival, growth, proliferation and inhibit apoptosis *via* Akt pathway (Conti *et al*., 2002). Reduced insulin like growth factor-I secretion from pancreas and liver are associated with glucose intolerance and diabetes. It is also reported that hyperglycaemia induces a mitogenic response in pancreatic beta cells *via* insulin like growth factor-I mediated pathway (Rhodes, 2000). Significant reduction in pancreatic insulin like growth factor-I expression has also been observed in the pathogenesis of diabetes mellitus.

NF-κB

Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) is a protein family that comprises of DNA-binding pleiotropic transcription factors controlling inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. Upon activation, dimeric NF-κB bind at kappa-B sites in the DNA of their target genes. Homo- or heterodimeric NF-κB complexes act like transcriptional activators or repressors, respectively (Giannoukakis *et al*., 2000). NF-κB mediate insulin resistance triggered pancreatic beta cell compensatory response by inducing cell proliferation. Glucose-stimulated insulin secretion from beta cells is partially controlled by NF-κB (Norlin *et al*., 2005). Significant alterations or reduction in NF-κB expression in beta cells will have deleterious effects on beta cells and thereby, promote diabetogenesis.

Cyclin D2

G1/S transition is very critical in the progression of cell cycle for cell proliferation. Cyclin D2, a regulator of cyclin-dependent kinases, has an instrumental role in facilitating this G1/S transition. Cyclin D2 binds to cyclin-dependent kinases and act as integrators of various exogenous and endogenous mitogenenic and antimitogenic signals. Once activated, it phosphorylates and inhibits retinoblastoma protein 1 and activates transcription factor, E2F to stimulate G1 progression (Bouchard *et al*., 1999). In response to the increased metabolic demand, cyclin D2 in the pancreatic beta cells facilitates re-entry to proliferative face and helps to remodel beta cell mass (Kushner *et al*., 2005).

Bax

BCL-2-associated X protein (Bax) is a chief pro-apoptotic member of the BCL-2 family that promotes programmed cell death. It is a fundamental mediator of apoptosis, which helps to maintain tissue homeostasis by regulating the balance between cellular life and death. Apoptotic stimuli induced conformation changes

in cytosolic Bax protein allow its mitochondria translocation (Oltvai *et al*., 1993). Bax permeabilize the outer mitochondrial membrane and stimulate cytochrome c release to cytoplasm. It brings about apoptotic cell death by activating caspase 3. There are several reports on diabetes associated pancreatic beta cell death, mediated through the activation of Bax (Koh, 2007).

Caspases

Caspases are a large family of cysteine-dependent aspartate-directed proteases responsible for programmed disassembly of the cells during apoptosis. Caspases are synthesized as inactive zymogens and are termed procaspases. In response to apoptotic signals, procaspases are converted into active caspases by proteolytic cleavage or by scaffold-mediated transactivation. Caspases initiate and execute intrinsic cell suicide program by the cleavage of numerous proteins including major structural elements, DNA repair proteins and regulatory protein kinases (Earnshaw *et al*., 1999).

Caspase 3 is a member of the CED-3 subfamily of caspases. It is a frequently activated executioner protease in apoptotic cell death. Caspase 3 is activated by caspase 9 mediated proteolytic processing or by mitochondrial cytochrome c. Active caspase 3 drives apoptotic cell death through the subsequent activation of caspase 2 and caspase 9 (Porter & Jänicke, 1999). Caspase 8 plays a key role in the extrinsic pathway of Fas-induced apoptosis. Death-inducing signal complex formed by the cross-linking of the Fas receptor by Fas ligand, initiate activation of caspase 8. This, in turn leads to the activation of downstream effector caspases and trigger mitochondrial damage (Li *et al*., 1998).

TNF-α

TNF-α is a multifunctional cytokine that has a major role in inflammation, immune response, regulation of bone homeostasis, lipid and protein metabolism, haematopoiesis and malignancy. It is synthesised as a transmembrane protein and

a proteolytic cleavage releases the soluble form of TNF-α. TNF-α signalling activate programmed cell death in tumour cells. Activated TNF-α exerts its inflammatory and apoptotic effects through the activation of NF-κB mediated pathway. It reduces tyrosine kinase activity of the insulin receptor and mediates insulin resistance (Hotamisligil *et al*., 1996).

CREB

Genomic action of the second messenger, cyclic adenosine 3',5'-monophosphate (cAMP) is mediated by cAMP response element-binding protein (CREB). Elevated intracellular cAMP levels lead to the activation of a cAMP-dependent protein kinase called protein kinase A. It phosphorylates and regulates several proteins involved in transcription, metabolism, cell cycle progression and apoptosis. Phosphorylation of the transcription factor CREB stimulates its dimerization and nuclear localization. It specifically binds to the cAMP response element (CRE) present in the promoter region of cAMP response gene and stimulates transcription (Kwok *et al*., 1994). CREB induced gene expression plays a critical role in glucose homeostasis and growth-factor-dependent pancreatic beta cell survival by the activation of Akt and Insulin like growth factor-1 (Jhala *et al*., 2003).

Phospholipase C

Phospholipase C (PLC) plays a key role in the signalling pathways of many receptors to induce cellular responses such as proliferation or secretion. It conducts several extracellular signals such as hormones, neurotransmitters and growth factors to induce a cellular response. Activated Phospholipase C catalyses hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). Both of them act as intracellular second messengers. 1,2-diacylglycerol activates protein kinase-C and IP3 induces release of intracellular calcium (Cifuentes *et al*., 1994). It also modulates cell survival and

growth by regulating the GTPase activity of Ras protein through Ras guanine-exchange factor (Falasca *et al*., 1998).

GLUT

Glucose transporters (GLUT) are membrane associated carrier proteins involved in energy-independent glucose transport. Based on substrate specificity, glucose transport kinetics and tissue distribution, GLUTs are classified into 14 isoforms. All GLUTs show a high degree of homology in sequence and structure and comprise 12 transmembrane domains. Differential tissue expression of GLUT helps to regulate glucose uptake according to metabolic characteristics (Scheepers *et al*., 2004). The low affinity transporter, GLUT 2 is expressed mainly in liver, pancreatic beta cells, kidney and small intestine. Its ability to facilitate bidirectional glucose transport supports glucose-sensing mechanism of pancreatic beta cells. Impaired GLUT 2 expression leads to inappropriate glucose sensing and inadequate insulin production (Bady *et al*., 2006). High affinity transporter, GLUT 3 is mainly expressed in nerve cells of brain. Its low Km helps in the efficient transport of glucose at low circulating concentrations. It also mediates transport of other sugars like galactose, maltose, mannose and xylose (Ferreira *et al*., 2011).

ANTIOXIDANT ENZYMES

Oxidative stress mediated pancreatic beta cell dysfunction is climacteric in the pathogenesis of diabetes. Elevated reactive oxygen species, accompanied by a weak cellular antioxidant defence system leads to free radical mediated beta cell damage and activation of apoptosis. During the initial stages of diabetes, hyperglycaemia induced reactive oxygen species production contributes to the progression of diabetes by decreasing pancreatic insulin output and alters glucose-stimulated insulin secretion (Maritim *et al*., 2003). In peripheral tissues, elevated oxidative stress contributes to the development of insulin resistance. Chronic hyperglycaemia associated with diabetes produce glucotoxicity and lipotoxicity in

most of the cells. It has a primary role in the pathogenesis complications during diabetes (Baynes & Thorpe, 1999). Antioxidant enzymes like superoxide dismutases and glutathione peroxidises are used as biomarkers for assessing changes in oxidative stress. Superoxide dismutases are the first line of cellular defence against reactive oxygen species. Superoxide dismutases serve the function of reducing production of reactive peroxynitrite by conversion of superoxide anion radicals to hydrogen peroxide (Zelko *et al*., 2002). Glutathione peroxidases catalyse reduction of this hydrogen peroxide to water. This enzyme also helps in the reduction of lipid peroxides and organic hydroperoxides. Reduced activity of these antioxidant enzymes increase free radical mediated activation of apoptotic pathways (Espinoza *et al*., 2008).

SECOND MESSENGERS

Second messengers are small molecules that mediate intracellular transmission of biological information. The major function of second messenger is to conduct and amplify signals from activated receptors to multiple targets. Intracellular production of second messengers is triggered by the receptor binding to first messengers like hormones and growth factors. Its mass production and small size helps to transmit signal throughout the cell with an amplified strength. Second messengers act as integration points for many independent cell signalling pathways. Second messengers activate multiple downstream signal transduction cascades to regulate all biochemical and physiological activities of cells. The synthesis, activation, targeting, regulation and removal of different second messengers are highly diverse (Rasmussen, 1977; Monick & Hunninghake, 2002).

Cyclic nucleotides such as cAMP and cyclic guanosine monophosphate (cGMP) are important group of second messengers. They are produced by G-protein mediated activation of enzymes like adenylyl cyclases and guanylyl cyclases. Elevated levels of cAMP and cGMP facilitate cell signalling by activating cAMP-dependent protein kinases and cGMP-dependent protein kinases, respectively. Cyclic nucleotides also produce rapid changes in cells through

cyclic-nucleotide gated ion channels (Voorhees *et al*., 1976; Ahern *et al*., 2002). Another important second messenger, IP3 (inositol trisphosphate) is derived from membrane phosphide phosphatidylinositol bisphosphate. The enzyme phospholipase C cleaves phosphatidylinositol bisphosphate to IP3 and diacylglycerol. Once produced, IP3 is rapidly diffused within the cytosol and this triggers calcium release from the endoplasmic reticulum through IP3 receptors (Bruch, 1996; da Silva & Guse, 2000).

In the present study, we investigated the anti-diabetogenesis property of curcumin and vitamin D_3 pre-treatment in Wistar rats. The ability of curcumin and vitamin D_3 to stimulate beta cell proliferation was studied by radio ligand incorporation studies. The molecular mechanism behind pre-treatment induced beta cell compensatory response against hyperglycemia was evaluated by studying the gene expression of key markers of beta cell survival, regeneration and differentiation. The adrenergic and muscarinic receptor subtype functional regulation in the pancreas, brain stem, hippocampus and hypothalamus were studied to understand the sympathetic and parasympathetic neuronal signalling during diabetogenesis and anti-diabetogenesis. The second messenger levels and expression of key cell signalling proteins and antioxidant enzymes were assessed to find intercellular signalling mechanisms present in pancreas and brain regions. Elucidating the adrenergic and muscarinic receptor subtype functional regulation associated with the curcumin and vitamin D_3 mediated anti-diabetogenesis effect, will provide a key therapeutic target to prevent and/or to delay the onset of diabetes.

Materials and Methods

CHEMICALS USED IN THE STUDY AND THEIR SOURCES

Biochemicals

Curcumin, cholecalciferol (vitamin D_3), streptozotocin, epinephrine, phentolamine, propranolol, atropine, pirenzepine, 4-DAMP mustard (4-deoxy acetyl methyl piperidine mustard), fetal calf serum (heat inactivated), poly L-lysine, collagenase type XI, bovine serum albumin fraction V, RPMI-1640 medium, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Hanks Balanced Salt Solution (HBSS), ethylenediaminetetraacetic acid (EDTA), Triton X-100, Tris HCl, sucrose, D-glucose, citric acid, magnesium chloride, calcium chloride and paraformaldehyde (PFA) were purchased from Sigma Chemical Co., St. Louis, USA. Tissue freezing medium Jung was purchased from Leica Microsystems Nussloch GmbH, Heidelberger, Germany. CpG Methyl transferase enzyme was purchased from New England Biolabs, Beverly, USA. Glucose estimation kit was obtained from Merck, New Jersey, USA. All the other analytical grade reagents were purchased locally from SRL, Mumbai, India.

Radiochemicals

Levo-[N-methyl-^{3}H] Epinephrine (Sp. activity 68.6 Ci/mmol), Quinuclidinyl benzilate L-[Benzilic-4,4'-^{3}H]-[4-^{3}H] (Sp. activity 42.0 Ci/mmol), ans 4-DAMP [N-methyl-^{3}H] (Sp. activity 83.0 Ci/mmol), were purchased from NEN Life Sciences products Inc., Boston, USA. [*o-methyl*-^{3}H] Yohimbine (Sp. activity 83.0 Ci/mmol), DL-[4-^{3}H] Propranolol (Sp. activity 29.0 Ci/mmol), [^{3}H] thymidine (Sp. activity 18.0 Ci/mmol) and [^{3}H] leucine (Sp. activity 63.0 Ci/mmol) were obtained from Amersham Life science, Buckinghamshire, UK. [^{3}H] methyl S-adenosylmethionine (Sp. activity 80.0 Ci/mmol), [^{3}H] cAMP, [^{3}H] cGMP and [^{3}H] IP3 kits were purchased from American Radiolabeled Chemicals, St. Louis, USA. Radioimmunoassay kit for insulin was purchased from Baba Atomic Research Centre (BARC), Mumbai, India.

Molecular Biology Chemicals

TRI-reagent 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, USA.

Akt (Rn_00583646), Neuro-D1 (Rn_00824571), Pax (Rn_00582529), PDX-1(Rn_00755591), Insulin like growth factor-1 (Rn_99999087), NF-κB (Rn_01399583), cyclin D2 (Rn_01492401), Bax (Rn_01480160), caspase 3 (Rn_00563902), caspase-8 (Rn_00574069), TNFα (Rn_99999017), α2A adrenergic receptor (Rn_00562488), β2 adrenergic receptor (Rn_00560650), muscarinic M1 receptor (Rn_00589936), muscarinic M3 receptor (Rn_00788315), choline acetyltransferase (Rn_01453446), acetylcholinesterase (Rn_00596883), muscarinic M2 receptor (Rn_02532311), α7 nicotinic acetylcholine receptor (Rn_01644792), vitamin D receptor (Rn_00566976), CREB (Rn_00561126), phospholipase C (Rn_01647142), insulin receptor (Rn_00567070), GLUT 2 (Rn_00563565), GLUT 3 (Rn_00567331), superoxide dismutases (Rn_01477289) and glutathione peroxidase (Rn_00577994) primers were used for the gene expression studies.

Confocal Dyes

Rat primary antibody for Caspase 3 (BD Biosciences, San Jose, USA), Phosphor-Akt (Cell signaling technology, Danvers, USA), vitamin D receptor (Cell signaling technology, Danvers, USA), α2A adrenergic receptor, β2 adrenergic receptor, muscarinic M1 receptor and muscarinic M3 receptor (abcam, Cambridge, England) and secondary antibody of either FITC (Chemicon, Temecula, USA), Alexa Fluor 546 (Invitrogen, Carlsbad, USA) or Alexa Fluor 594 (Invitrogen, Carlsbad, USA) were used for the immunohistochemistry studies using confocal microscope.

ANIMALS

Male Wistar rats of 90-100 g body weight purchased from Kerala Agricultural University, Mannuthy, India and Amrita Institute of Medical Sciences, Kochi, India were 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 procedures were in accordance with Institutional, Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA – Reg. No: 383/01/a/CPSCEA) and the National Institute of Health guidelines.

DIABETES INDUCTION

Diabetes was induced in rats by multiple low dose streptozotocin (MLD-STZ) administration according to the procedure of Like and Rossini (1976). Intra-peritoneal injection of 40 mg/kg body weight streptozotocin was given for five consecutive days. Fresh solution of streptozotocin was prepared in citrate buffer, pH 4.5 (Junod *et al*., 1969). Control rats were injected with citrate buffer.

DETERMINATION OF ANTI-DIABETOGENESIS POTENTIAL OF CURCUMIN AND VITAMIN D_3

Animals used in this study were randomly divided into the following groups. Each group consisted of 6-8 animals.

a) Group 1: Control (C)
b) Group 2: Diabetic (D)
c) Group 3: Rats pre-treated with curcumin and injected MLD-STZ (C + D)
d) Group 4: Rats pre-treated with vitamin D_3 and injected MLD-STZ (V + D)

The 2nd group of rats were made diabetic using MLD-STZ. Curcumin was given orally to the 3rd group of rats in the dose of 7.5 mg/kg body weight at 24 hr intervals for 60 days (Kanitkar *et al*., 2008). Curcumin suspended in 0.5% sodium

carboxymethylcellulose was administered at a constant volume of 5 ml/kg body weight (Sharma *et al*., 2007). Cholecalciferol (vitamin D_3) dissolved in 0.3 mL of coconut oil was administered orally to the 4th group of rats in the dosage of 1 µg/kg (40 IU/kg) body weight for a period of 60 days (de Souza Santos & Vianna, 2005). After 60 days of pre-treatment, MLD-STZ was injected in the 3rd and 4th groups. Blood samples were collected from the tail vein and glucose levels were estimated. Changes in the body weight of animals were also monitored.

SACRIFICE AND TISSUE PREPARATION

The animals were sacrificed on the 15th day after the beginning of MLD-STZ injection by decapitation. The brain stem and hypothalamus were dissected out quickly over ice according to the procedure of Glowinski and Iversen (1966) and the pancreas was dissected quickly over ice. Hippocampus was dissected according to the procedure of Heffner *et al*., (1980). 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.

ESTIMATION OF BLOOD GLUCOSE

Blood glucose was estimated using glucose estimation kit that employs the principle of oxidase-peroxidase reactions. Glucose oxidase catalyses the oxidation of glucose into gluconic acid and hydrogen peroxide. The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase to form a dye called N-(-4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 510 nm in spectrophotometer (Shimadzu UV-1700 pharmaSPEC, Kyoto. Japan).

ESTIMATION OF CIRCULATING INSULIN BY RADIOIMMUNOASSAY

Principle of the assay

The assay was done according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method is based on the competition between unlabelled insulin in the standard or samples and [^{125}I] insulin for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound and free insulin were separated by the second antibody-polyethylene glycol (PEG) aided separation method. Measuring the radioactivity associated with bound fraction of sample and standards quantitates insulin concentration of samples.

Assay Protocol

Standards, ranging from 0 to 200 µU/mL, insulin free serum and insulin antiserum (50 µL each) were added together and the volume was made up to 250 µL with assay buffer. Samples of appropriate concentration were used for the assay. They were incubated overnight at 2 ºC. Then [^{125}I] insulin (50 µL) was added and incubated at room temperature for 3 hours. The second antibody was added (50 µL) along with 500 µL of PEG. The tubes were then vortexed and incubated for 20 minutes and centrifuged at 1500 x g for 20 minutes. The supernatant was aspirated out and the radioactivity in the pellet was determined in a gamma counter.

A standard curve was plotted with % B/Bo on the Y-axis and insulin concentration/mL on the X-axis of a log-logit graph. % B/Bo was calculated as:

$$\frac{\text{Corrected average count of standard or sample}}{\text{Corrected average count of zero standard}} \times 100$$

Insulin concentration in the samples were determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

BEHAVIOURAL STUDIES

Animals were observed every day for any overt abnormal activity.

Y-Maze Test

The Y-maze was made of grey wood, covered with black paper and consisted of three arms (width x length x height: 8 x 30 x 15) with 120 degrees angles between each arms. Each arm was 8 cm width × 30 cm length × 15 cm height. The three identical arms were designated randomly: Start arm, in which the rat started to explore (always open); Novel arm, which was blocked at the 1st trial, but open at the 2nd trial; and the other arm (always open). The maze was placed in a separate room with enough light. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. Visual cues were placed on the walls of the maze. The Y-maze test consisted of two trials separated by an inter-trial interval (ITI). The first trial (training) was of 10 minutes duration and allowed the rat to explore only two arms (start arm and the other arm) of the maze, with the third arm (novel arm) blocked. After a 1 hour ITI (Ma *et al*., 2007), the second trial (retention) was conducted, during which all three arms were accessible and novelty versus familiarity was analysed by comparing the behaviour in all three arms. For the second trial, the rat was placed back in the maze in the same starting arm, with free access to all three arms for 5 minutes. The time spent in each arm was analysed. Data was expressed as percentage of performance in all three arms during the five minutes of test (Mathew *et al*., 2010).

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. The rod was placed at a height of 50 cm to discourage the jumping of animals from the rotating rod. Each rat was given five trials before the actual reading was taken. Fifteen days after the beginning of MLD-STZ administration, the readings were taken at 10, 15 and 25 RPM.

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 to 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) were counted in each crossing for 3 minutes (Chao *et al*., 2012).

ISOLATION OF PANCREATIC ISLETS

Fifteen days after the beginning of MLD-STZ treatment, pancreatic islets were isolated from all the experimental groups by standard collagenase digestion procedure using aseptic techniques (Howell & Taylor, 1968). The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) (Pipeleers *et al*., 1985). The pancreas from the rats were aseptically dissected out into a sterile Petri dish containing ice cold HBSS and excess fat and blood vessels were removed. The pancreas was cut into small pieces and transferred to a sterile glass vial containing 2 mL collagenase type XI solution (1.5 mg/mL in HBSS, pH 7.4). The collagenase digestion was carried out for 15 minutes at 37°C in a shaker with vigorous shaking (300 RPM/minute). The tissue digest was filtered through a 500 μm nylon screen and the filtrate was washed thrice by successive centrifugation and re-suspension in cold HBSS. The pancreatic islet preparation with a viability of >90%, as assessed by trypan blue exclusion was chosen for experiments.

[^{3}H] THYMIDINE AND [^{3}H] LEUCINE INCORPORATION STUDIES

150 μL of pancreatic beta cell suspension (cell density of 1.6×10^5 cells/cm^2) was added to a poly L-lysine coated glass slide. 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 2000 x g 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 Perkin Elmer Tri-Carb 2810 TR liquid scintillation analyser.

DNA METHYLATION STUDY

The ability of DNA to incorporate [^{3}H] methyl groups *in vitro* is inversely related to endogenous DNA methylation. The DNA was isolated from the pancreatic beta cells 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, [^{3}H] methyl *S*-adenosylmethionine, 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 hr and placed on ice for 5 minutes. The reaction mixture was loaded onto a 2.5 cm, round, Whatman DE81 ion-exchange paper filter. The filter was washed 3 times successively with 7.5 mLof 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 Perkin Elmer Tri-Carb 2810 TR liquid scintillation analyser.

TBARS ASSAY

As an index of lipid peroxidation the level thiobarbituric acid reactive substances (TBARS) was measured in the pancreas of experimental rats. TBARS are the products of oxidative degradation of polyunsaturated fatty acids, in particular malonaldialdehyde (MDA). The reaction mixture contained 0.2 mL of pancreatic homogenate, 1.5 mL of acetic acid (pH 3.5, 20%), 1.5 mL of thiobarbituric acid (0.8% w/v) and 0.2 mL of SDS. The absorbance of the sample was read spectrophotometrically at 532 nm (Heinecke *et al*., 1987).

GENE EXPRESSION STUDIES IN PANCREAS AND DIFFERENT BRAIN REGIONS OF EXPERIMENTAL RATS

Preparation of RNA

RNA was isolated from pancreas and different brain regions - brain stem, hippocampus and hypothalamus of experimental rats using TRI reagent from Sigma Chemical Co., St. Louis, USA.

Isolation of RNA

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

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

cDNA Synthesis

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

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, mRNA levels were quantified by TaqMan quantitative 2-step reverse transcriptase polymerase chain reaction (RT-PCR). First-strand cDNA was synthesized using 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 labelled with FAM at the 5' end and a quencher (Minor Groove Binding Protein - MGB) at the 3' end. The Real Time PCR data was analysed with Sequence Detection Systems software version 1.7. All the reactions were performed in duplicate.

The TaqMan reaction mixture contained 25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer and TaqMan probes, endogenous control (β-actin) and 12.5 µL of TaqMan 2X Universal PCR Master Mix and RNase free water to a total volume of 20 µL. Each run contained both negative (no template) and positive controls.

The thermo-cycling profile conditions were as follows:

50 ºC	--	2 minutes	--- Activation	
95 ºC	--	10 minutes	--- Initial Denaturation	
95 ºC	--	15 seconds	--- Denaturation	} 40 cycles
60 ºC	--	1 minute	--- 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 ($2^{-\Delta\Delta CT}$).

ADRENERGIC AND MUSCARINIC RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS

Total adrenergic, α2 adrenergic and β adrenergic receptor binding studies

[^{3}H] Epinephrine, [^{3}H] Yohimbine and [^{3}H] Propranolol binding assay in pancreas, brain stem and hippocampus were done according to the modified procedure of U'Prichard and Snyder (1977). The pancreas and brain tissues were

homogenized in 20 volumes of cold 50 mM Tris-HCl buffer, pH 7.7 in a polytron homogeniser. The homogenate was centrifuged twice at 50,000 x g for 10 minutes. The pellet was resuspended in an appropriate volume of incubation buffer containing 0.1% ascorbic acid, 1 mM catechol, 0.1 mM EDTA-Na_2, 10 µM dithiothreitol, 50 mM Tris-HCl and 10 mM magnesium chloride of pH 7.7. Binding assay was performed using different concentrations of radioligands.

Total adrenergic receptor binding parameter assays were done using [^{3}H] Epinephrine (0.5 - 24 nM), α2 adrenergic receptor assays using [^{3}H] yohimbine (0.1 - 6 nM) and β adrenergic receptor assays using [^{3}H] propranolol (0.5 - 6 nM) in the incubation buffer, pH 7.7 in a total volume of 250 µL containing 200-250 µg protein concentration. The non-specific binding was determined using 100 µM unlabelled epinephrine for total adrenergic receptor, phentolamine for α2 adrenergic receptor and propranolol for β adrenergic receptor. The reaction volume of 250 µL contained 200-250 µg protein concentration. Tubes were incubated at 37 °C for 15 minutes. After incubation the mixture was filtered rapidly through GF/B filters (Whatman, Little Chalfont, UK) for total adrenergic receptor assay and GF/C filters (Whatman) for α2 adrenergic receptor and β adrenergic receptor binding studies. The filters were washed thrice rapidly with 5 mLof ice cold 50 mM Tris-HCl buffer, pH 7.7. Bound radioactivity was counted with cocktail-T in a Perkin Elmer Tri-Carb 2810 TR liquid scintillation analyser. The non-specific binding determined showed 30 - 40% in all our experiments.

Total muscarinic, muscarinic M1 and M3 receptor binding studies

[^{3}H] QNB and [^{3}H] DAMP binding assay in pancreas, brain stem and hippocampus were done according to the modified procedure of Yamamura and Snyder (1981). Tissues were homogenised in a polytron homogeniser with 20 volumes of cold 50 mM Tris-HCl buffer, containing 1 mM EDTA (pH 7.4). The supernatant was centrifuged at 30,000 x g for 30 minutes and the pellets were resuspended in an appropriate volume of Tris-HCl-EDTA buffer.

Total muscarinic and muscarinic M1 receptor binding parameter assays were done using [^{3}H] QNB (0.1 - 5 nM) and M3 receptor using [^{3}H] DAMP (0.1 - 5 nM) in the incubation buffer, pH 7.4 in a total reaction volume of 250 µL containing appropriate protein concentration (200-250 µg). The non-specific binding was determined using 100 µM atropine for total muscarinic receptor, pirenzepine for muscarinic M1 receptor and 4-DAMP mustard for muscarinic M3 receptor. Total incubation volume of 250 µL contains 200-250 µg protein concentration. Tubes were incubated at 22 °C for 60 minutes and filtered rapidly through GF/C filters. The filters were given three quick successive washes with 5 mL of ice cold 50 mM Tris-HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Perkin Elmer Tri-Carb 2810 TR liquid scintillation analyser. The non-specific binding determined showed 10% in all our experiments.

Protein determination

Protein was measured according to 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.

IMMUNOCYTOCHEMISTRY OF α2 ADRENERGIC, β ADRENERGIC, MUSCARINIC M1 AND MUSCARINIC M3 RECEPTORS IN THE PANCREAS AND BRAIN REGIONS OF EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

The experimental rats were deeply anesthetized and were transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS (Chen *et al*., 2007). After perfusion, pancreas and brain regions from each experimental group was dissected out and fixed in 4% paraformaldehyde for 1 hour and then equilibrated with 30% sucrose solution in PBS. 10 μm sections of pancreas and brain regions were cut using Cryostat (Leica, CM1510 S). The sections were washed with PBS and then blocked with Phosphate buffered saline with Triton X-100 (PBST) containing 5% normal goat serum for 1 hour. The primary antibodies of α2 adrenergic receptor, β2 adrenergic receptor, muscarinic M1 receptor and muscarinic M3 receptor (1:400 dilution in PBST with 5% normal goat serum) were added to the respective sections and incubated overnight at 4 °C. After overnight incubation, the tissue slices were rinsed with PBS and incubated with fluorescent labelled secondary antibody prepared in PBST with 5% normal goat serum at 1:1000 dilution for 2 hours in room temperature. The sections were observed and photographed using confocal imaging system (Leica TCS SP5 laser scanning confocal microscope). 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 photomultiplier tube (PMT), pinhole and zoom factor were kept the same for imaging the sections of all experimental groups.

cAMP CONTENT IN THE PANCREAS AND BRAIN REGIONS OF EXPERIMENTAL RATS

Pancreas, brain stem and hippocampus were homogenised in a polytron homogeniser with cold 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was centrifuged at 40,000 x g for 15 minutes 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 labelled 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 calculation of the amount of unlabelled cAMP in the sample.

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 was then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample was 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 unknown cAMP concentrations. The mixture was incubated at 2 °C for 2 hours. Cold

charcoal reagent was added to the tubes and the tubes were immediately centrifuged at 12,000 x g for 2 minutes at 2 °C. Aliquots of the supernatant were transferred into scintillation vials and mixed with cocktail-T immediately and counted in a Perkin Elmer Tri-Carb 2810 TR liquid scintillation analyser.

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

cGMP CONTENT IN THE PANCREAS AND BRAIN REGIONS OF EXPERIMENTAL RATS

Pancreas, brain stem and hippocampus were homogenised in a polytron homogeniser with cold 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was centrifuged at 40,000 x g for 15 minutes 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 calculation of amount of unlabelled cGMP in the sample. Separation of the antibody bound cGMP from the unbound nucleotide is done by ammonium sulphate precipitation, followed by centrifugation. The precipitate which contains the antibody bound complex is 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 4.0 pmol/tube and [^{3}H] cGMP were added together and the volume was made up to 100 µL with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The antiserum was added to all the assay tubes and the tubes were vortexed well. They were incubated for at 2 – 8 °C 90 minutes. Ammonium sulphate was added to all tubes, mixed and allowed to stand for 5 minutes in ice bath. The tubes were centrifuged at 12000 x g for 2 minutes 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 (pmol/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 were determined by interpolation from the plotted standard curve.

IP3 CONTENT IN THE PANCREAS AND BRAIN REGIONS OF EXPERIMENTAL RATS

Pancreas, brain stem and hippocampus were 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 centrifuged at 40,000 x g for 15 minutes and the supernatant was transferred to fresh tubes for IP_3 assay using [^{3}H] IP3 Biotrak Assay System kit.

Principle of the assay

The assay is based on competition between [^{3}H] IP3 and unlabelled IP3 in the standard or samples for binding to binding protein prepared from bovine adrenal cortex. The bound IP3 was then separated from the free IP3 by centrifugation. The free IP3 in the supernatant was discarded by simple

decantation, leaving the bound fraction adhering to the tube. Measurement of the radioactivity in the tube enables the calculation of the amount of unlabelled IP3 in the sample.

Assay Protocol

Standards, ranging from 0.19 to 25 pmol/tube, [^{3}H] IP3 and binding protein were added together and the volume was made up to 100 μL with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The tubes were vortexed and incubated on ice for 15 minutes and they centrifuged at 2000 x g for 10 minutes at 4 °C. The supernatant was aspirated out and the pellet was resuspended in water and incubated at room temperature for 10 minutes. The tubes were 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 IP3 concentration (pmol/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. IP3 concentration in the samples was determined by interpolation from the plotted standard curve.

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). Relative Quantification Software was used for analyzing Real Time PCR results.

Results

Fasting blood glucose level on final day (Day 14) after MLD-STZ injection in experimental rats

Fourteen days after MLD-STZ administration, diabetic group showed a significant ($p<0.001$) increase in fasting blood glucose level when compared with control. Pre-treated rats (C + D and V + D) showed a significant ($p<0.01$) increase in blood glucose when compared with control. When compared with diabetic group, fasting blood glucose levels of rats pre-treated with curcumin (C + D) and vitamin D_3 (V + D) were significantly ($p<0.001$) decreased (Figure-1, Table-1).

Random blood glucose level after MLD-STZ injection in experimental rats

Random blood glucose level of all rats before MLD-STZ administration was within the normal range. MLD-STZ injection led to an increase in random blood glucose level of diabetic group. Fourteen days after MLD-STZ administration diabetic group showed a significant ($p<0.001$) increase in random blood glucose level when compared with control. However, curcumin and vitamin D_3 pre-treated rats developed only a prediabetes condition after MLD-STZ injection. 14^{th} day after MLD-STZ administration, curcumin and vitamin D_3 pre-treatments were able to significantly ($p<0.001$) retained a near control level of random blood glucose level when compared with diabetic group (Figure-2, Table-2).

Circulating insulin level on final day (Day 14) after MLD-STZ injection in experimental rats

Circulating insulin level was significantly ($p<0.001$) decreased in diabetic group when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.01$) prevented the decrease in plasma insulin level when compared with diabetic group (Figure-3, Table-3).

Body weight of experimental rats

On 0^{th} day, pre-treated group showed a significant ($p<0.05$) increase in body weight when compared with both control and diabetic rats. 7^{th} day after MLD-STZ injection, body weight of curcumin and vitamin D_3 pre-treated rats were significantly ($p<0.05$) increase when compared with both control and diabetic rats. On 14^{th} day, diabetic group showed a significant ($p<0.05$) decrease in body weight when compared with control and pre-treated groups (Figure-4, Table-4).

Figure-1

Fasting blood glucose level on final day (Day 14) after MLD-STZ injection in experimental rats

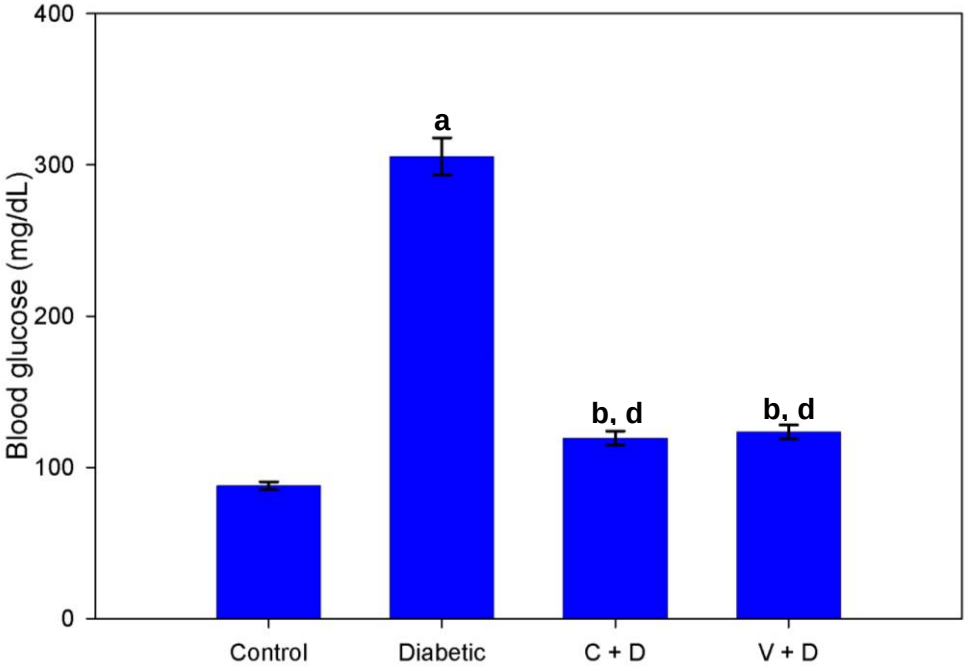


Table-1

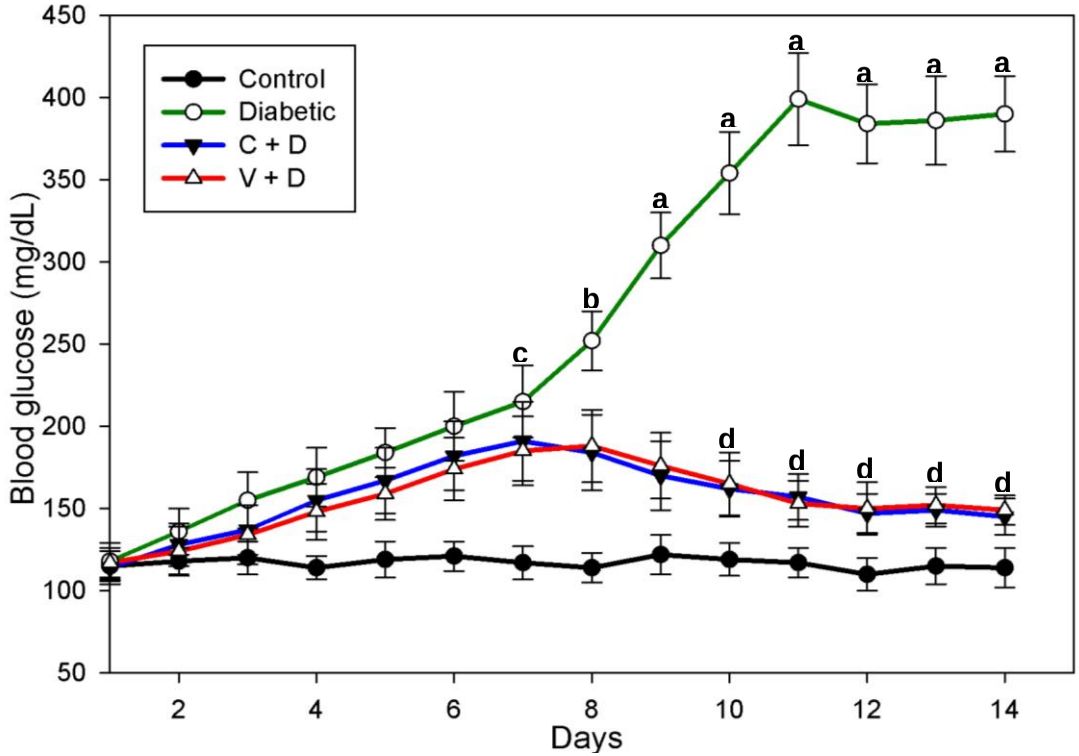
Fasting blood glucose level on final day (Day 14) after MLD-STZ injection in experimental rats

Experimental groups	Blood glucose (mg/dL)
Control	87.83 ± 2.61
Diabetic	305.50 ± 12.32 **a**
C + D	119.33 ± 4.65 **b, d**
V + D	123.50 ± 4.55 **b, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **b** $p<0.01$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-2

Random blood glucose level after MLD-STZ injection in experimental rats



Values are mean ± S.E.M of 3-4 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Table-2

Random blood glucose level after MLD-STZ injection in experimental rats

Experimental groups	Blood glucose (mg/dL)						
	1st Day	**2ed Day**	**3ed Day**	**4th Day**	**5th Day**	**6th Day**	**7th Day**
Control	115 ± 9	118 ± 8	120 ± 10	114 ± 7	119 ± 11	121 ± 9	117 ± 10
Diabetic	118 ± 11	136 ± 14	155 ± 17	169 ± 18	184 ± 15	200 ± 21	215 ± 22 **c**
C + D	114 ± 10	128 ±13	137 ± 15	155 ± 19	167 ± 20	182 ± 21	191 ± 24
V + D	117 ± 9	124 ± 15	134 ± 18	148 ± 17	159 ± 16	174 ± 19	185 ± 21

Experimental groups	Blood glucose (mg/dL)						
	8th Day	**9th Day**	**10th Day**	**11th Day**	**12th Day**	**13th Day**	**14th Day**
Control	114 ± 9	122 ± 12	119 ± 10	117 ± 9	110 ± 10	115 ± 11	114 ± 12
Diabetic	252 ± 18 **b**	310 ± 20 **a**	354 ± 25 **a**	399 ± 28 **a**	384 ± 24 **a**	386 ± 27 **a**	390 ± 23 **a**
C + D	184 ± 23	170 ± 21 **e**	162 ± 17 **d**	157± 14 **d**	147 ± 12 **d**	149 ± 10 **d**	145 ± 11 **d**
V + D	188 ± 22	176 ± 20 **d**	165 ± 19 **d**	153 ± 14 **d**	150 ± 16 **d**	152 ± 11 **d**	149 ± 9 **d**

Values are mean ± S.E.M of 3-4 separate experiments. Each group consist of 4-6 rats.

a $p<0.001$, **b** $p<0.01$, **c** $p<0.05$ when compared to Control. **d** $p<0.001$, **e** $p<0.01$ when compared to Diabetic group.

C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-3

Circulating insulin level on final day (Day 14) after MLD-STZ injection in experimental rats

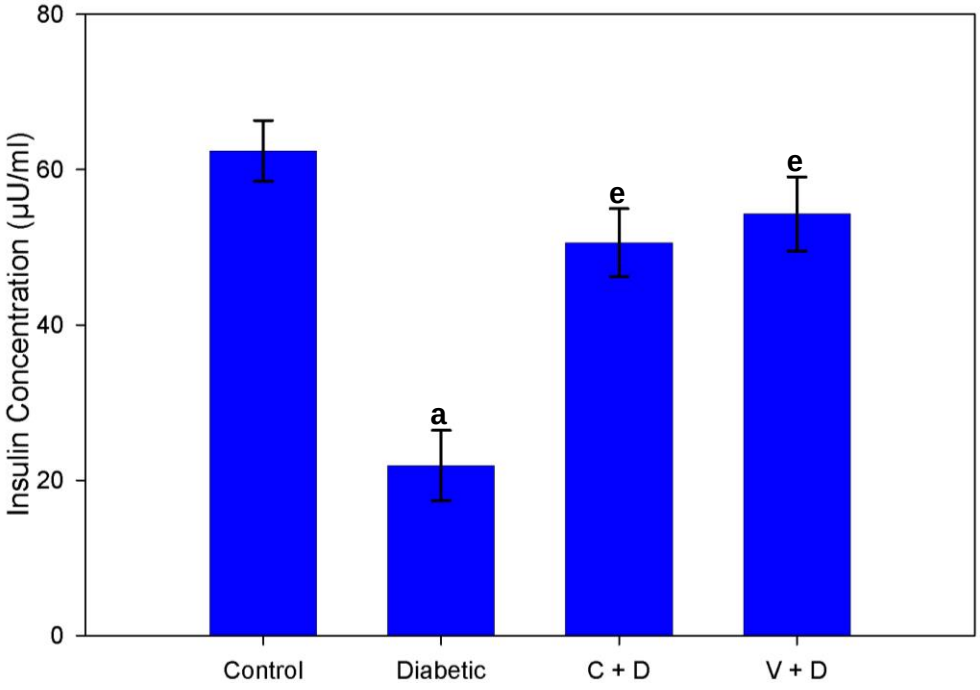


Table-3

Circulating insulin level on final day (Day 14) after MLD-STZ injection in experimental rats

Experimental groups	Insulin Concentration (µU/ml)
Control	62.38 ± 3.91
Diabetic	21.90 ± 4.53 [a]
C + D	50.60 ± 4.37 [e]
V + D	54.29 ± 4.76 [e]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
[a] $p<0.001$ when compared to Control. [e] $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-4

Body weight of experimental rats

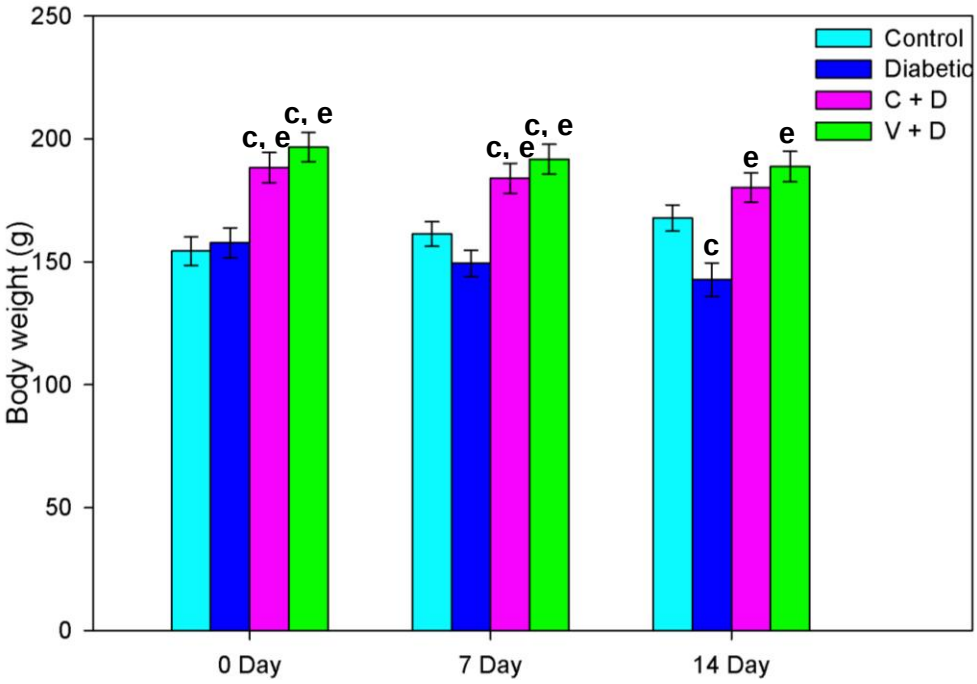


Table-4

Body weight of experimental rats

Experimental groups	Body weight (g)		
	0th Day	**7th Day**	**14th Day**
Control	154.32 ± 5.78	161.27 ± 4.98	167.76 ± 5.33
Diabetic	157.17 ± 6.12	149.30 ± 5.36	142.65 ± 6.80 **c**
C + D	188.33 ± 6.17 **c, e**	183.97 ± 6.08 **c, e**	180.17 ± 5.97 **e**
V + D	196.68 ± 5.92 **c, e**	191.71 ± 6.02 **c, e**	188.74 ± 6.23 **e**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
c $p<0.05$ when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

BEHAVIOURAL STUDIES

Behavioural response of experimental rats in Y maze test

The percentage of visits to novel arm was found to be significantly decreased ($p<0.001$) in diabetic rats when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a near control percentage of visits to novel arm when compared with diabetic group ($p<0.001$; Figure- 5, Table- 5).

Behavioural response of experimental rats in rotarod test

In rotarod experiment diabetic rats showed a significant ($p<0.01$) decrease in the retention time on rotating rod at 10, 15 and 25 RPM when compared with control. Pre-treatment groups maintained the retention time to near control value with a significant ($p<0.01$) increase when compared with diabetic group (Figure-6, Table-6).

Behavioural response of experimental rats in grid walk test

There was a significant increase ($p<0.001$) in the foot slips in diabetic rats when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significantly ($p<0.001$) low foot slips when compared with diabetic group and retained the level near to control (Figure-7, Table-7).

Figure-5

Behavioural response of experimental rats in Y maze test

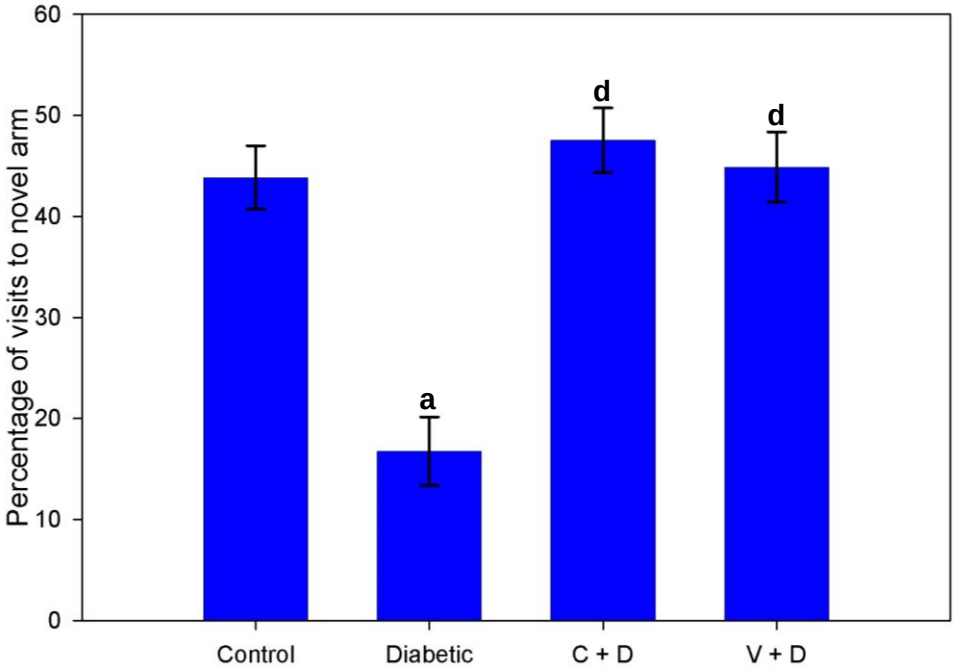


Table-5

Behavioural response of experimental rats in Y maze test

Experimental groups	Percentage of visits to novel arm
Control	43.84 ± 3.14
Diabetic	16.76 ± 3.40 **a**
C + D	47.53 ± 3.21 **d**
V + D	44.88 ± 3.48 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-6

Behavioural response of experimental rats in rotarod test

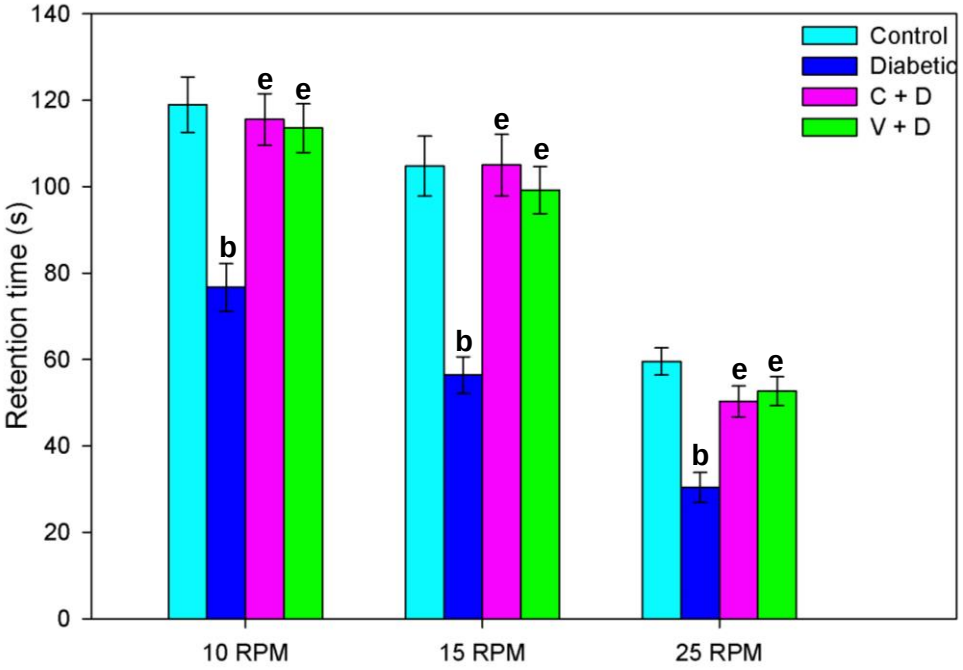


Table-6

Behavioural response of experimental rats in rotarod test

Experimental groups	Retention time (s)		
	10 RPM	**15 RPM**	**25 RPM**
Control	118.97 ± 6.42	104.76 ± 6.92	59.52 ± 3.13
Diabetic	76.69 ± 5.51 **b**	56.37 ± 4.25 **b**	30.36 ± 3.47 **b**
C + D	115.53 ± 5.96 **e**	105.00 ± 7.12 **e**	50.32 ± 3.56 **e**
V + D	113.54 ± 5.62 **e**	99.22 ± 5.46 **e**	52.65 ± 3.36 **e**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$ when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-7

Behavioural response of experimental rats in grid walk test

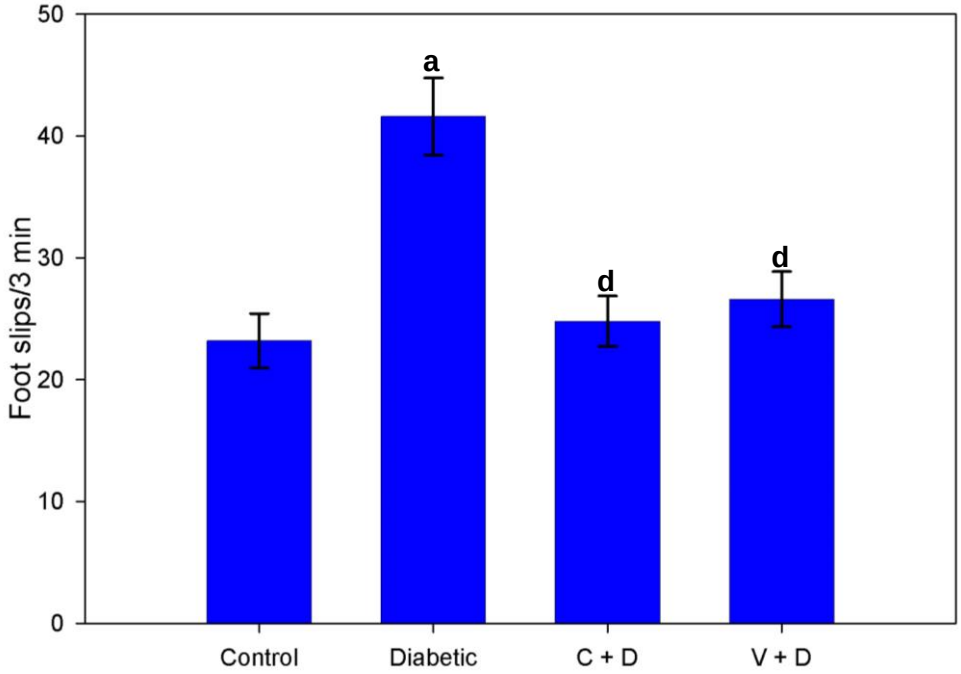


Table-7

Behavioural response of experimental rats in grid walk test

Experimental groups	Foot slips per 3 minutes
Control	23.20 ± 2.22
Diabetic	41.60 ± 3.17 **a**
C + D	24.8 ± 2.06 **d**
V + D	26.60 ± 2.27 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

PANCREAS

[^{3}H] Thymidine incorporation in the pancreatic beta cells of experimental rats

Pancreatic beta cells isolated from diabetic rats showed a significant ($p<0.05$) decrease in [^{3}H] thymidine incorporation when compared with control. While, beta cells from curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) increase in [^{3}H] thymidine incorporation when compared with both control and diabetic group (Figure- 8, Table- 8).

[^{3}H] Leucine incorporation in the pancreatic beta cells of experimental rats

[^{3}H] Leucine incorporation in the pancreatic beta cells were significantly ($p<0.01$) reduced in diabetic group when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) increased [^{3}H] Leucine incorporation when compared with both control and diabetic group (Figure- 9, Table- 9).

[^{3}H] Methyl group incorporation in the pancreatic beta cells of experimental rats

Diabetic ($p<0.01$) and pre-treated ($p<0.001$) groups showed a significant increase in [^{3}H] methyl group incorporation in the pancreatic beta cells when compared with control. [^{3}H] methyl group incorporation in curcumin and vitamin D_3 pre-treated rats were significantly ($p<0.001$) increased when compared with diabetic group (Figure- 10, Table- 10).

Lipid peroxidation in the pancreatic beta cells of experimental rats

Malondialdehyde concentration in the pancreas of diabetic ($p<0.001$) and pre-treated ($p<0.05$) rats showed a significant increase when compared with control. Pre-treatment with curcumin and vitamin D_3 significantly ($p<0.001$) decreased the malondialdehyde concentration when compared with diabetic group (Figure- 11, Table- 11).

Real Time PCR amplification of Akt mRNA in the pancreas of experimental rats

Gene expression of Akt mRNA showed a significant ($p<0.001$) down regulation in the pancreas of diabetic rats when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) up regulated the Akt mRNA expression when compared with both control and diabetic group. There was also a significant ($p<0.05$) up regulation in the Akt expression in V + D when compared with C + D (Figure- 12, Table- 12).

Confocal imaging of Akt in the pancreas of experimental rats

Akt antibody staining in the pancreas showed a significant ($p<0.01$) decrease in mean pixel intensity value of diabetic rats when compared with control. Pre-treated group showed a significant ($p<0.05$) increase when compared with control. Curcumin and vitamin D_3 pre-treatment significantly ($p<0.001$) increased the mean pixel intensity when compared with diabetic (Figure- 13, Table- 13).

Real Time PCR amplification of NeuroD1 mRNA in the pancreas of experimental rats

The gene expression studies showed that NeuroD1 mRNA was significantly ($p<0.001$) down regulated in diabetic group when compared with control. In C + D and V + D there was a significant ($p<0.001$) up regulation of the NeuroD1 expression when compared with both control and diabetic group. In pre-treated groups, vitamin D_3 pre-treated group showed a significant ($p<0.001$) up regulation when compared with curcumin pre-treated group (Figure- 14, Table- 14).

Real Time PCR amplification of Pax mRNA in the pancreas of experimental rats

Real time PCR gene expression of Pax showed a significant up regulation in the pancreas of diabetic ($p<0.05$) and pre-treated ($p<0.001$) rats when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) increase when compared with diabetic group. Further, a significant ($p<0.01$) increase was observed in V + D when compared with C + D (Figure- 15, Table- 15).

Real Time PCR amplification of Pdx-1 mRNA in the pancreas of experimental rats

Pdx-1 gene expression showed a significant ($p<0.01$) down regulation in the pancreas of diabetic rats when compared with control. A significant ($p<0.001$) up regulation in the Pdx-1 mRNA was observed in the pre-treated groups when compared with both control and diabetic group (Figure- 16, Table- 16).

Real Time PCR amplification of insulin like growth factor-I mRNA in the pancreas of experimental rats

The gene expression of insulin like growth factor-I showed a significant ($p<0.001$) up regulation in diabetic and pre-treated groups when compared with control. When compared with diabetic, C + D and V + D showed a significant ($p<0.001$) increase in insulin like growth factor-1 mRNA expression (Figure- 17, Table- 17).

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

Real-time PCR gene expression of NF-κB showed a significant ($p<0.001$) up regulation in the pancreas of diabetic and pre-treated rats when compared with control. When compared with diabetic group, pre-treated rats showed a significant ($p<0.001$) decrease in the gene expression (Figure- 18, Table- 18).

Real Time PCR amplification of cyclin D2 mRNA in the pancreas of experimental rats

mRNA level expression of cyclin D2 showed a significant ($p<0.05$) down regulation in the pancreas of diabetic group when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) up regulation in cyclin D2 mRNA expression when compared with both control and diabetic group (Figure- 19, Table- 19).

Real Time PCR amplification of Bax mRNA in the pancreas of experimental rats

Bax gene expression showed a significant up regulation in the pancreas of diabetic ($p<0.001$), C + D ($p<0.05$) and V + D ($p<0.01$) rats when compared with control. When compared with diabetic group, a significant ($p<0.001$) decrease in Bax mRNA expression was observed in the pre-treated groups. In pre-treated groups, curcumin pre-treated group showed a significant ($p<0.05$) decrease when compared with vitamin D_3 pre-treated group (Figure- 20, Table- 20).

Real Time PCR amplification of caspase 3 mRNA in the pancreas of experimental rats

The gene expression studies showed that caspase 3 mRNA was significantly up regulated in diabetic ($p<0.001$) and pre-treated ($p<0.01$) group when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) decreased the gene expression when compared with diabetic group (Figure- 21, Table- 21).

Confocal imaging of caspase 3 in the pancreas of experimental rats

Confocal imaging of caspase 3 in the pancreas of diabetic ($p<0.001$) and pre-treated ($p<0.05$) rats showed a significant increase in mean pixel intensity when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.01$) increased the mean pixel intensity when compared with diabetic group (Figure- 22, Table- 22).

Real Time PCR amplification of caspase 8 mRNA in the pancreas of experimental rats

Caspase 8 gene expression showed a significant up regulation in the pancreas of diabetic ($p<0.001$) and pre-treated ($p<0.01$) rats when compared with control. When compared with diabetic, a significant ($p<0.001$) decrease of caspase 8 mRNA was observed in pre-treated groups (Figure- 23, Table- 23).

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

Gene expression of TNF-α showed a significant up regulation in the pancreas of diabetic ($p<0.001$), C + D ($p<0.001$) and V + D ($p<0.01$) groups when compared with control. When compared with diabetic, C + D and V + D showed a significant ($p<0.001$) decrease in expression (Figure- 24, Table- 24).

Figure-8

[^{3}H] Thymidine incorporation in the pancreatic beta cells of experimental rats

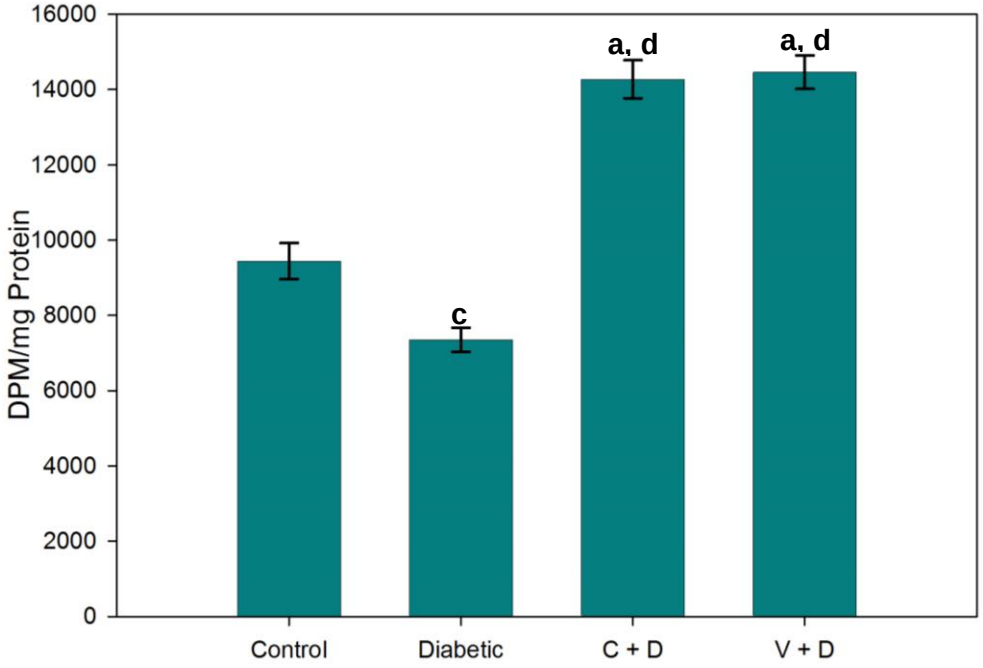


Table-8

[^{3}H] Thymidine incorporation in the pancreatic beta cells of experimental rats

Experimental groups	DPM/mg Protein
Control	9441 ± 480
Diabetic	7350 ± 318 **c**
C + D	14272 ± 504 **a, d**
V + D	14458 ± 446 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **c** $p<0.05$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-9

[^{3}H] Leucine incorporation in the pancreatic beta cells of experimental rats

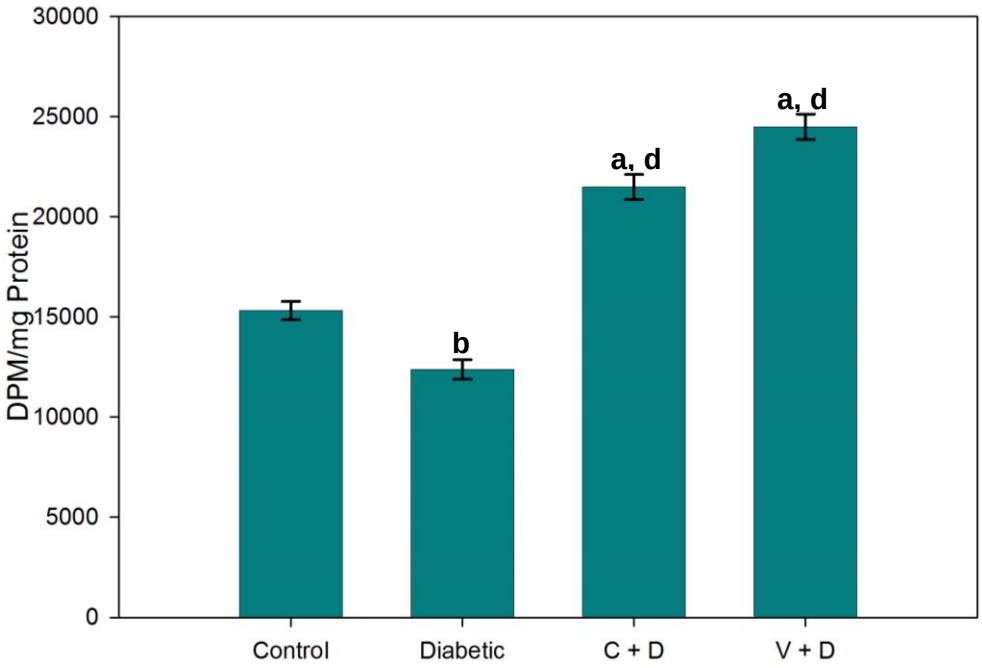


Table-9

[^{3}H] Leucine incorporation in the pancreatic beta cells of experimental rats

Experimental groups	DPM/mg Protein
Control	15319 ± 460
Diabetic	12377 ± 492 **b**
C + D	21493 ± 618 **a, d**
V + D	24491 ± 636 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a p<0.001, **b** p<0.01 when compared to Control. **d** p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-10

[^{3}H] Methyl group incorporation in the pancreatic beta cells of experimental rats

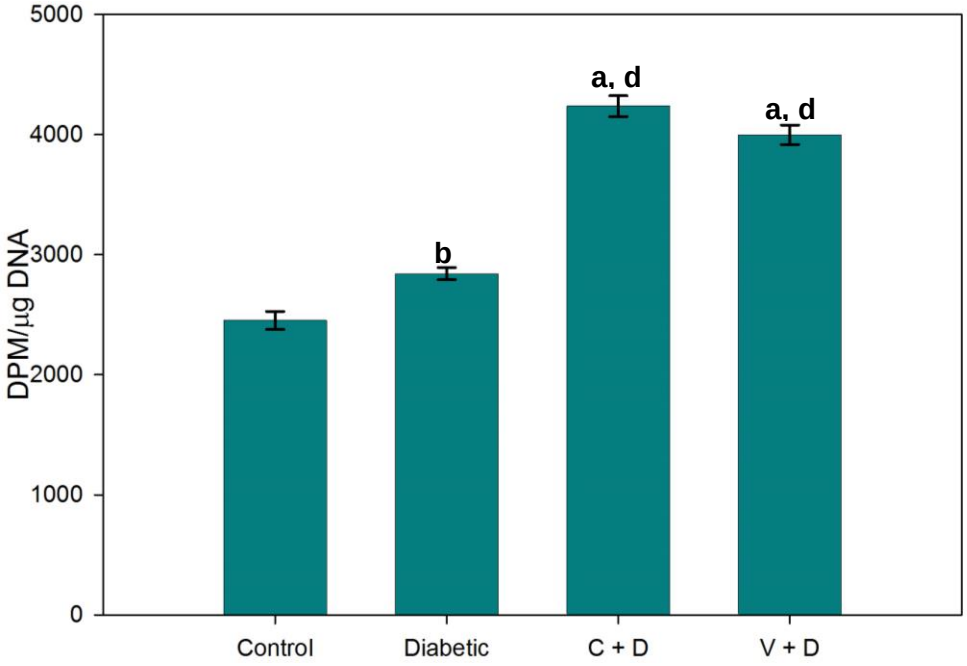


Table-10

[^{3}H] Methyl group incorporation in the pancreatic beta cells of experimental rats

Experimental groups	DPM/µg DNA
Control	2452 ± 75
Diabetic	2841 ± 50 **b**
C + D	4236 ± 86 **a, d**
V + D	3998 ± 82 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **b** $p<0.01$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-11

Lipid peroxidation in the pancreatic beta cells of experimental rats

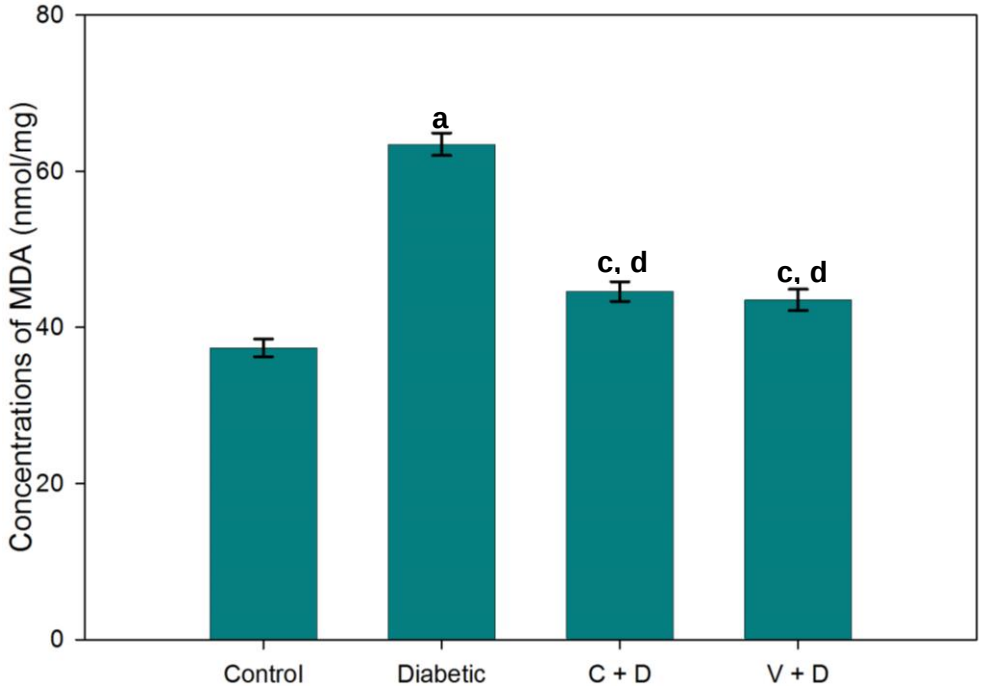


Table-11

Lipid peroxidation in the pancreatic beta cells of experimental rats

Experimental groups	Concentration of MDA (nmol/mg)
Control	37.37 ± 1.14
Diabetic	63.44 ± 1.43 **a**
C + D	44.59 ± 1.26 **c, d**
V + D	43.52 ± 1.35 **c, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **c** $p<0.05$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-12

Real Time PCR amplification of Akt mRNA in the pancreas of experimental rats

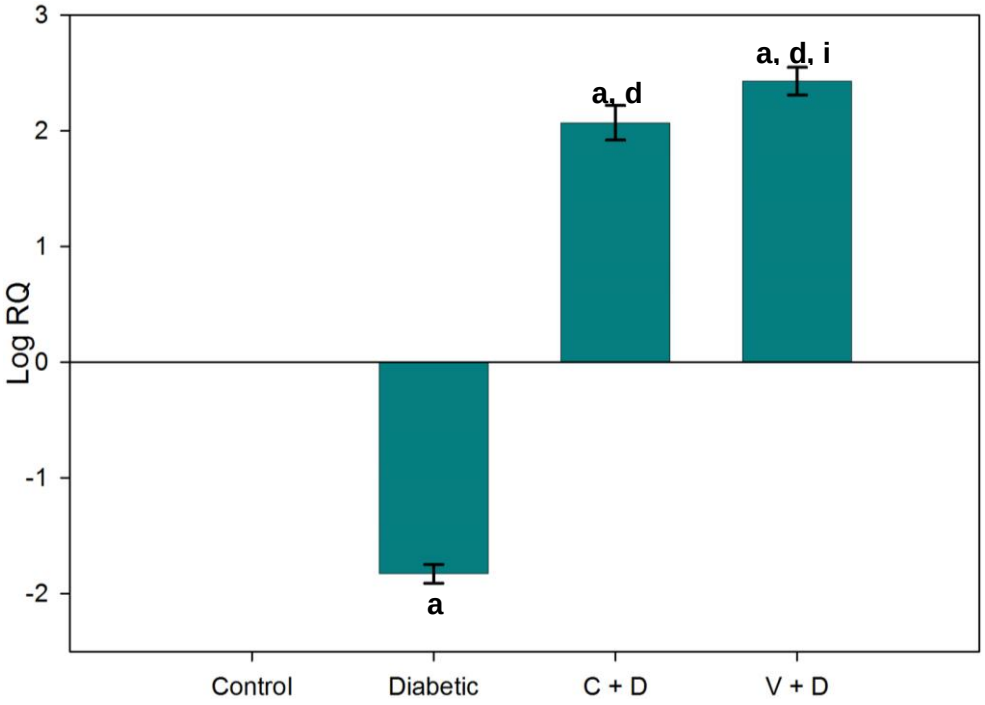


Table-12

Real Time PCR amplification of Akt mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.83 ± 0.08 **a**
C + D	2.07 ± 0.15 **a, d**
V + D	2.43 ± 0.12 **a, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. **i** $p<0.05$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-13
Confocal imaging of Akt in the pancreas of experimental rats

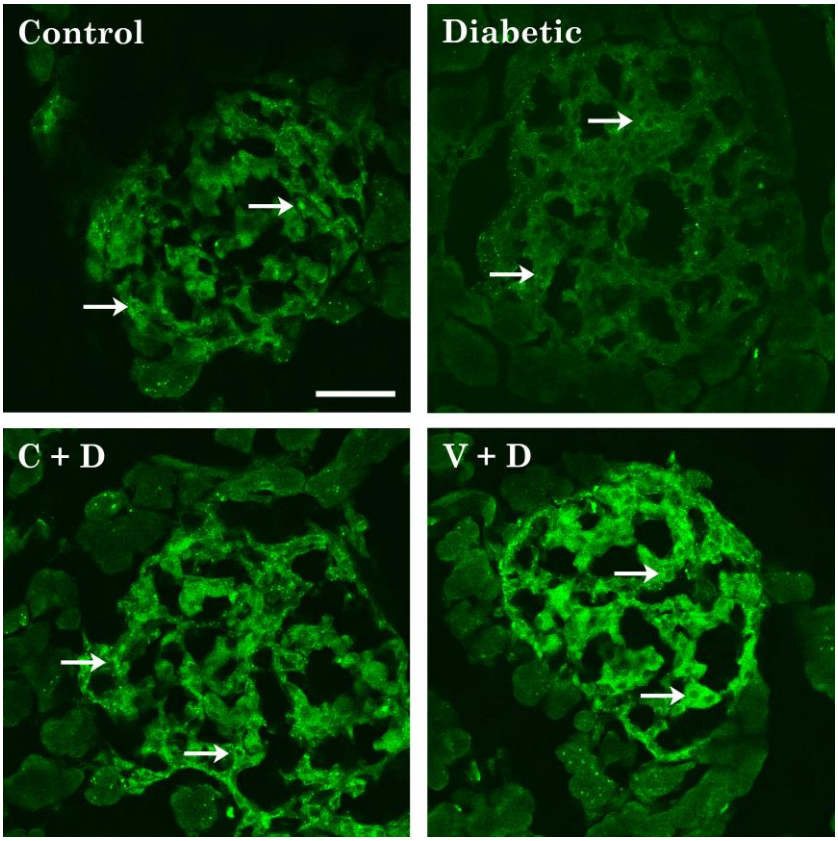


Table-13
Confocal imaging of Akt in the pancreas of experimental rats

Experimental groups	Mean Pixel Intensity
Control	38.57 ± 2.04
Diabetic	27.84 ± 1.69 [b]
C + D	47.83 ± 2.14 [c, d]
V + D	49.66 ± 2.59 [c, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
[b]$p<0.01$, [c]$p<0.05$ when compared to Control. [d]$p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⟹ shows Akt. Scale bar represents 50µm.

Figure-14

Real Time PCR amplification of NeuroD1 mRNA in the pancreas of experimental rats

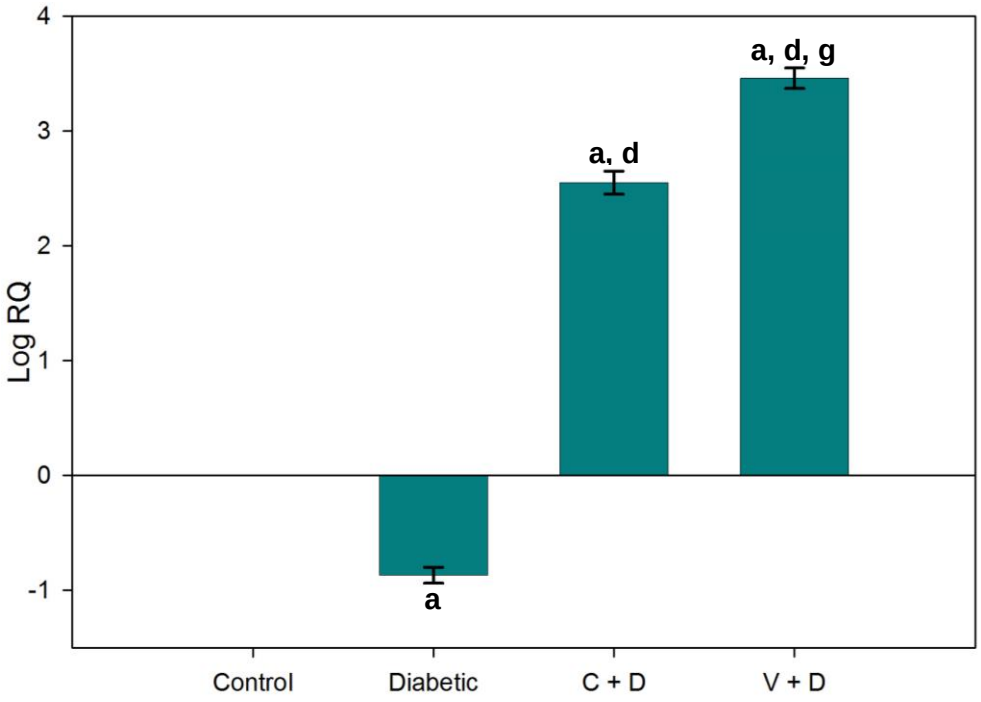


Table-14

Real Time PCR amplification of NeuroD1 mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.87 ± 0.07 **a**
C + D	2.55 ± 0.10 **a, d**
V + D	3.46 ± 0.09 **a, d, g**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. **g** $p<0.001$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-15

Real Time PCR amplification of Pax mRNA in the pancreas of experimental rats

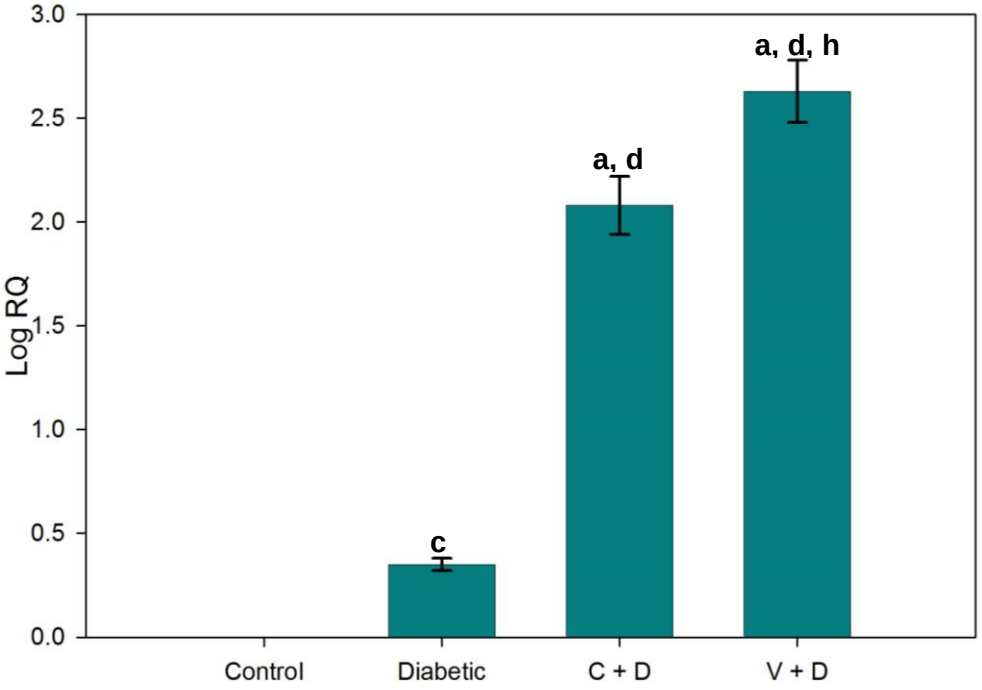


Table-15

Real Time PCR amplification of Pax mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.35 ± 0.03 **c**
C + D	2.08 ± 0.14 **a, d**
V + D	2.63 ± 0.15 **a, d, h**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **c** $p<0.05$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. **h** $p<0.01$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-16

Real Time PCR amplification of Pdx-1 mRNA in the pancreas of experimental rats

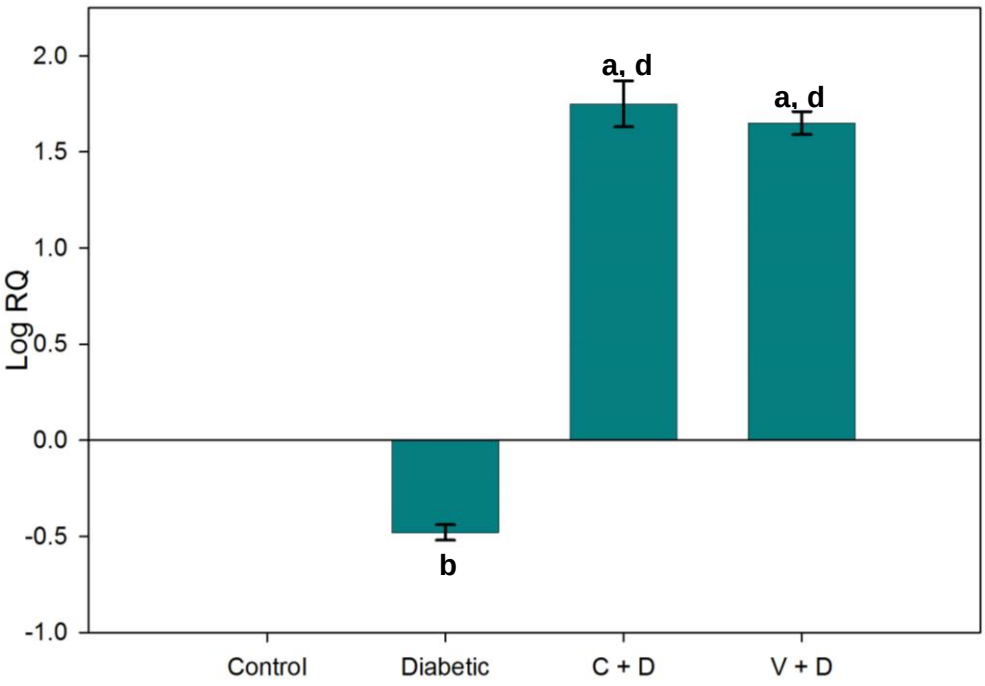


Table-16

Real Time PCR amplification of Pdx-1 mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.48 ± 0.04 **b**
C + D	1.75 ± 0.12 **a, d**
V + D	1.65 ± 0.06 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a p<0.001, **b** p<0.01 when compared to Control. **d** p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-17

Real Time PCR amplification of insulin like growth factor-1 mRNA in the pancreas of experimental rats

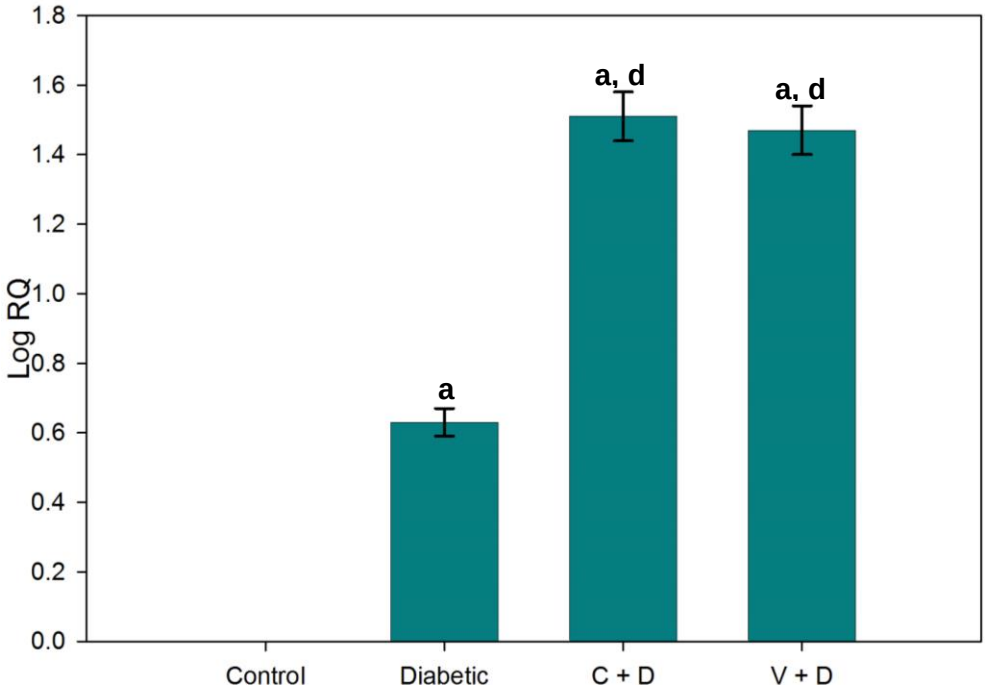


Table-17

Real Time PCR amplification of insulin like growth factor-1 mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.63 ± 0.04 **a**
C + D	1.51 ± 0.07 **a, d**
V + D	1.47 ± 0.07 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-18

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

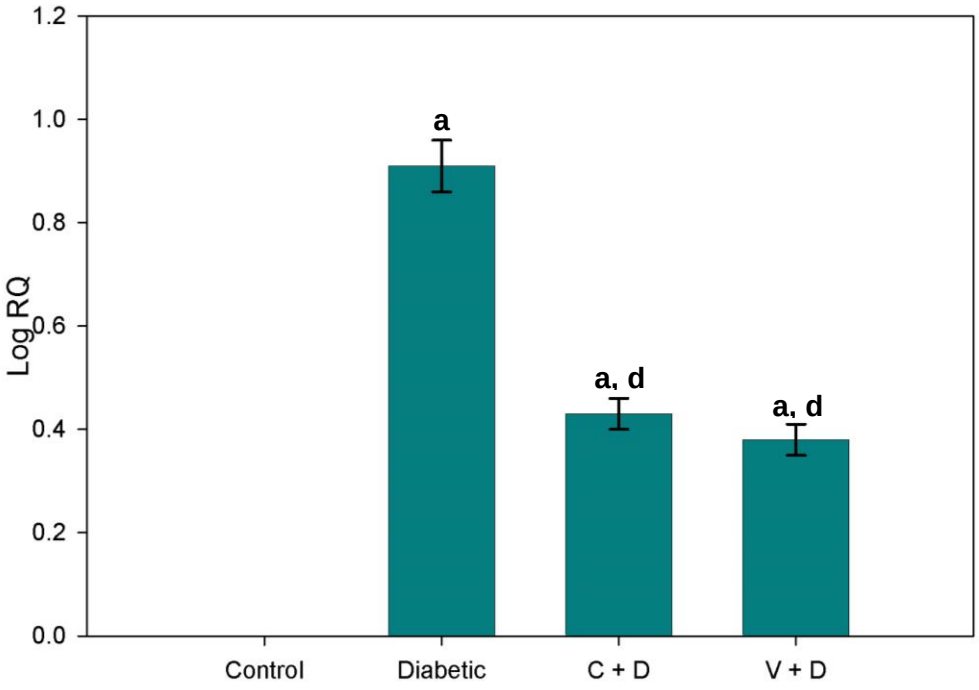


Table-18

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

Experimental groups	Log RQ
Control	0
Diabetic	0.91 ± 0.06 **a**
C + D	0.39 ± 0.04 **a, d**
V + D	0.45 ± 0.03 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-19

Real Time PCR amplification of cyclin D2 mRNA in the pancreas of experimental rats

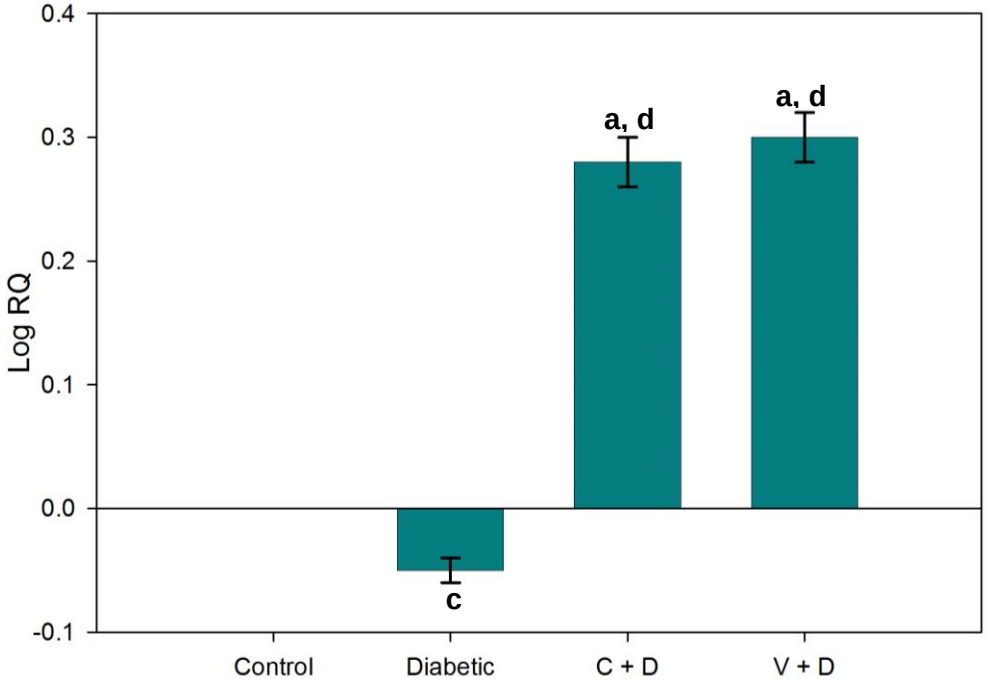


Table-19

Real Time PCR amplification of cyclin D2 mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.05 ± 0.01 **c**
C + D	0.28 ± 0.02 **a, d**
V + D	0.30 ± 0.02 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **c** $p<0.05$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-20

Real Time PCR amplification of Bax mRNA in the pancreas of experimental rats

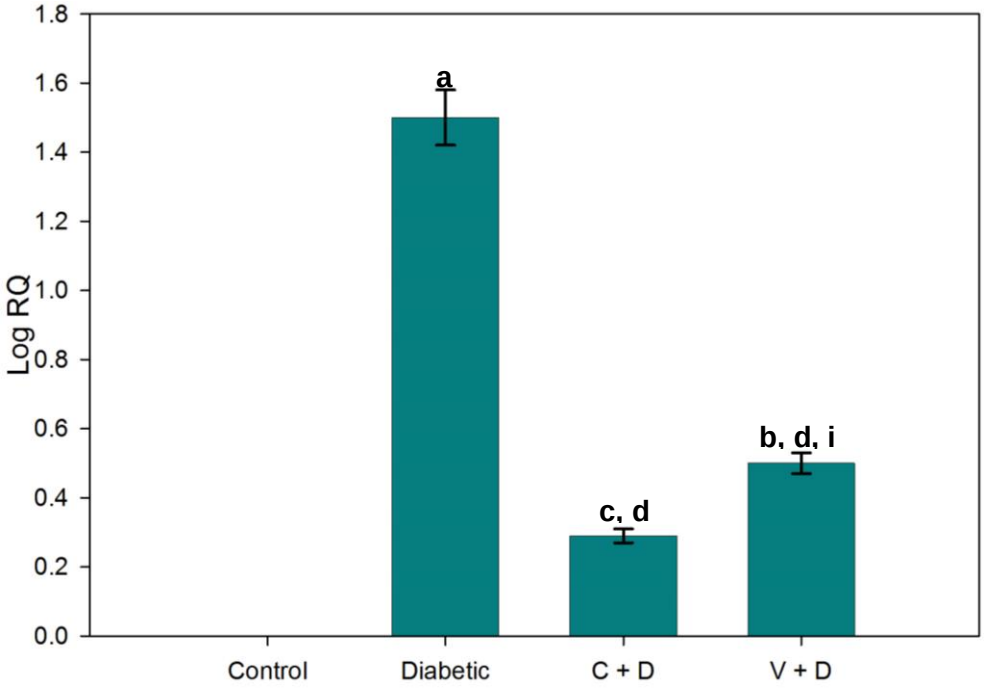


Table-20

Real Time PCR amplification of Bax mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.50 ± 0.08 **a**
C + D	0.29 ± 0.02 **c, d**
V + D	0.50 ± 0.03 **b, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **b** $p<0.01$, **c** $p<0.05$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. **i** $p<0.05$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-21

Real Time PCR amplification of caspase 3 mRNA in the pancreas of experimental rats

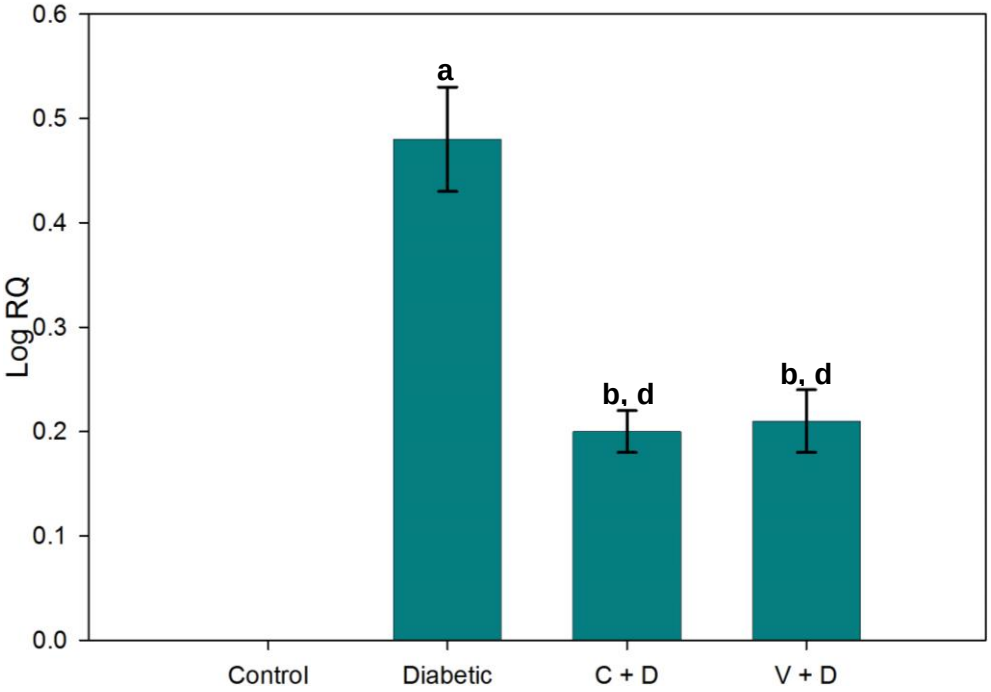


Table-21

Real Time PCR amplification of caspase 3 mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.48 ± 0.05 **a**
C + D	0.20 ± 0.02 **b, d**
V + D	0.21 ± 0.03 **b, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **b** $p<0.01$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-22
Confocal imaging of caspase 3 in the pancreas of experimental rats

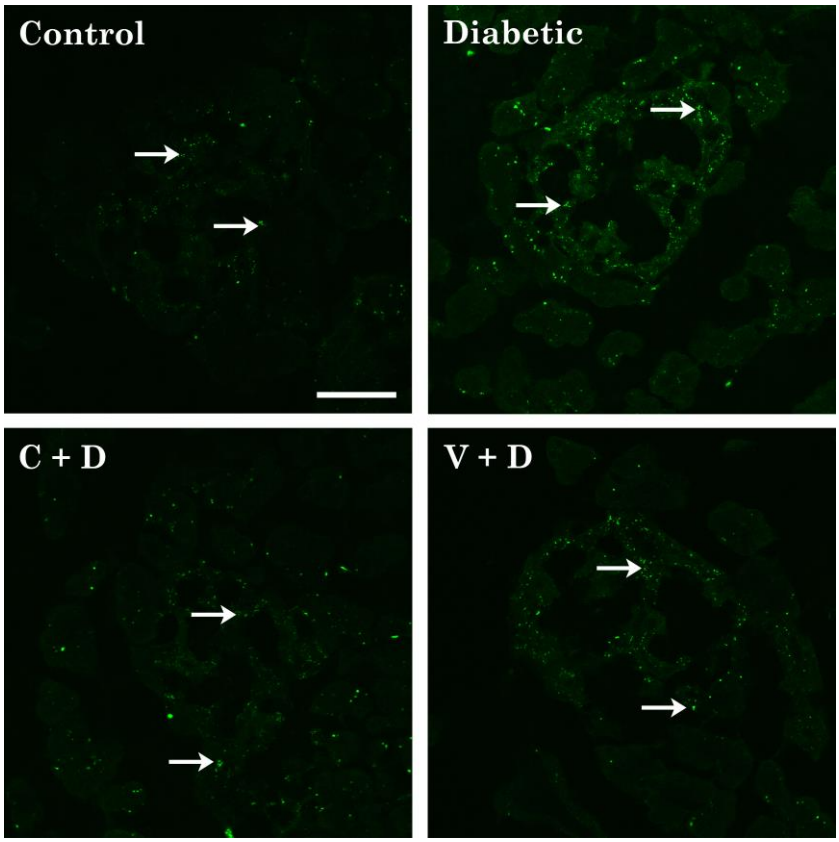


Table-22
Confocal imaging of caspase 3 in the pancreas of experimental rats

Experimental groups	Mean Pixel Intensity
Control	19.94 ± 1.47
Diabetic	33.93 ± 1.36 **a**
C + D	25.67 ± 1.42 **c, e**
V + D	26.20 ± 1.15 **c, e**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **c** $p<0.05$ when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⟹ shows caspase 3. Scale bar represents 50µm.

Figure-23

Real Time PCR amplification of caspase 8 mRNA in the pancreas of experimental rats

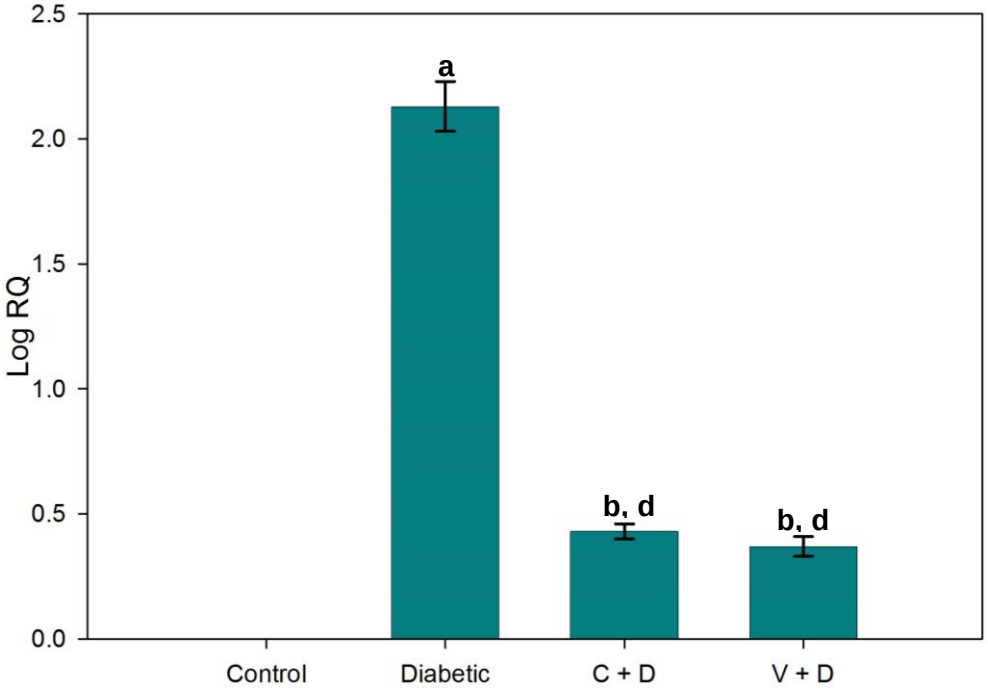


Table-23

Real Time PCR amplification of caspase 8 mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	2.13 ± 0.10 **a**
C + D	0.43 ± 0.03 **b, d**
V + D	0.37 ± 0.04 **b, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **b** $p<0.01$when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-24

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

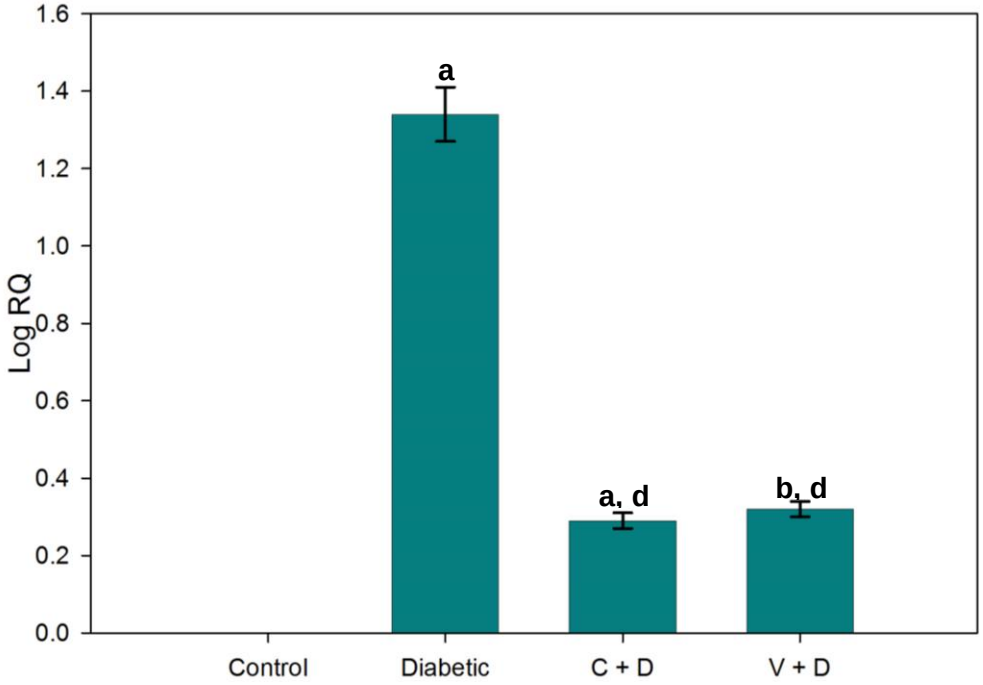


Table-24

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

Experimental groups	Log RQ
Control	0
Diabetic	1.34 ± 0.07 **a**
C + D	0.29 ± 0.02 **a, d**
V + D	0.32 ± 0.02 **b, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **b** $p<0.01$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

PANCREAS

Scatchard analysis of total adrenergic receptor using [^{3}H] epinephrine binding against epinephrine in the pancreas of experimental rats

Scatchard analysis of total adrenergic receptor using [^{3}H] epinephrine binding against epinephrine in the pancreas of diabetic rats showed a significant ($p<0.01$) increase in B_{max} when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.01$) retained the B_{max} to near control when compared with diabetic group. There was no significant change in K_d in all experimental groups of rats (Figure- 25, Table- 25).

Scatchard analysis of α2 adrenergic receptor using [^{3}H] yohimbine binding against phentolamine in the pancreas of experimental rats

α2 adrenergic receptors B_{max} was significantly increased ($p<0.001$) in diabetic group when compared with control. Pre-treatment using curcumin ($p<0.001$) and vitamin D_3 ($p<0.01$) significantly increased B_{max} when compared with diabetic group. In receptor studies, there was no significant change in the K_d values of experimental rats (Figure- 26, Table- 26).

Real Time PCR amplification of α2 adrenergic receptor mRNA in the pancreas of experimental rats

The gene expression studies showed that α2 adrenergic receptor mRNA was significantly up regulated in diabetic ($p<0.001$) and pre-treated ($p<0.01$) group when compared with control. When compared with diabetic group, α2 adrenergic receptor expression was significantly ($p<0.001$) decreased in C + D and V + D groups (Figure- 27, Table- 27).

Confocal imaging of α2 adrenergic receptor in the pancreas of experimental rats

α adrenergic receptor specific antibody staining in the pancreas showed a significant increase ($p<0.01$) in the mean pixel intensity of diabetic rats when

compared with control. Curcumin ($p<0.01$) and vitamin D_3 ($p<0.05$) pre-treatment significantly retained the mean pixel value to near control when compared with diabetic group (Figure- 28, Table- 28).

Scatchard analysis of β adrenergic receptor using [^{3}H] propranolol binding against propranolol in the pancreas of experimental rats

In diabetic group, β adrenergic receptors B_{max} and K_d were significantly ($p<0.01$) decreased when compared with control. Rats pre-treated with curcumin showed a significant increase in B_{max} and K_d when compared with control ($p<0.05$) and diabetic ($p<0.001$) rats. B_{max} of vitamin D_3 pre-treated group showed a significant ($p<0.01$) increase when compared with diabetic group. V + D group showed a significant increase in K_d when compared with diabetic group ($p<0.01$) and a significant decrease when compared with C + D group ($p<0.05$) (Figure- 29, Table- 29).

Real Time PCR amplification of β2 adrenergic receptor mRNA in the pancreas of experimental rats

Gene expression of β2 adrenergic receptor mRNA showed a significant down regulation ($p<0.001$) in the pancreas of diabetic rats when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) up regulated the β2 adrenergic receptor gene expression when compared with both control and diabetic group (Figure- 30, Table- 30).

Confocal imaging of β2 adrenergic receptor in the pancreas of experimental rats

Confocal microscopic image of β2 adrenergic receptors in the pancreas of diabetic group showed a significant ($p<0.001$) decrease in the mean pixel intensity when compared with control. V + D rats showed a significant ($p<0.05$) increase in mean pixel intensity when compared with control. When compared with diabetic group, the mean pixel intensity was significantly ($p<0.001$) increased in pre-treated rats (Figure- 31, Table- 31).

Scatchard analysis of total muscarinic receptor using [^{3}H] QNB binding against atropine in the pancreas of experimental rats

In diabetic group, B_{max} of total muscarinic receptor was significantly ($p<0.001$) decreased when compared with control. Pre-treatment with curcumin was able to maintain a near control level of B_{max} when compared with diabetic group. V + D rats showed a significant ($p<0.05$) decrease in B_{max} when compared with control and C + D rats. When compared with diabetic rats V + D rats showed a significant ($p<0.01$) increase in B_{max}. K_d of V + D rats were significantly ($p<0.05$) decreased when compared with C + D (Figure- 32, Table- 32).

Scatchard analysis of muscarinic M1 receptor using [^{3}H] QNB binding against pirenzepine in the pancreas of experimental rats

Scatchard analysis of muscarinic M1 receptor using [^{3}H] QNB binding against pirenzepine in the pancreas of diabetic rats showed a significant ($p<0.01$) decrease in B_{max} when compared with control. Curcumin and vitamin D_3 pre-treatments were able to maintain the B_{max} to near control when compared with diabetic rats ($p<0.01$). There was no significant change in K_d in all experimental groups of rats (Figure- 33, Table- 33).

Real Time PCR amplification of muscarinic M1 receptor mRNA in the pancreas of experimental rats

Gene expression of muscarinic M1 receptor mRNA showed a significant down regulation ($p<0.001$) in the pancreas of diabetic rats when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) up regulated the gene expression when compared with both control and diabetic group (Figure- 34, Table- 34).

Confocal imaging of muscarinic M1 receptor in the pancreas of experimental rats

Muscarinic M1 receptor antibody staining in the pancreas showed a significant ($p<0.001$) decrease in mean pixel intensity value of diabetic rats when compared with control. Curcumin and vitamin D_3 pre-treatment significantly ($p<0.001$) increased the mean pixel intensity when compared with diabetic (Figure- 35, Table- 35).

Scatchard analysis of muscarinic M3 receptor using [^{3}H] DAMP binding against 4-DAMP mustard in the pancreas of experimental rats

Binding studies of [^{3}H] DAMP against 4-DAMP mustard for muscarinic M3 receptors showed that the binding parameter B_{max} ($p<0.01$) was significantly decreased in the pancreas of diabetic group when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.01$) increased the B_{max} when compared with diabetic group. No significant change was observed in the K_d of experimental groups of rats (Figure- 36, Table- 36).

Real Time PCR amplification of muscarinic M3 receptor mRNA in the pancreas of experimental rats

Real time PCR gene expression of muscarinic M3 receptors mRNA showed a significant ($p<0.001$) down regulation in the pancreas of diabetic rats when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) up regulation when compared with both control and diabetic group (Figure- 37, Table- 37).

Confocal imaging of muscarinic M3 receptor in the pancreas of experimental rats

Confocal imaging of muscarinic M3 receptor in the pancreas of diabetic rats showed a significant ($p<0.01$) decrease in mean pixel intensity when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly

increased the mean pixel intensity when compared with both control ($p<0.05$) and diabetic group ($p<0.001$; Figure- 38, Table- 38).

Real Time PCR amplification of choline acetyltransferase mRNA in the pancreas of experimental rats

Choline acetyltransferase gene expression showed a significant ($p<0.001$) down regulation in the pancreas of diabetic rats when compared with control. A significant ($p<0.001$) up regulation in the choline acetyltransferase mRNA was observed in pre-treated groups when compared with both control and diabetic group. Further, a significant ($p<0.001$) up regulation was observed in V + D when compared with C + D (Figure- 39, Table- 39).

Real Time PCR amplification of acetylcholinesterase mRNA in the pancreas of experimental rats

Acetylcholinesterase gene expression showed a significant ($p<0.001$) down regulation in the pancreas of pre-treated rats when compared with both control and diabetic. In pre-treated groups, curcumin pre-treated group showed a significant ($p<0.05$) down regulation when compared with vitamin D_3 pre-treated group (Figure- 40, Table- 40).

Real Time PCR amplification of muscarinic M2 receptor mRNA in the pancreas of experimental rats

Real-time PCR gene expression of muscarinic M2 receptor showed a significant ($p<0.001$) up regulation in the pancreas of diabetic rats when compared with control. Curcumin and vitamin D_3 pre-treatment significantly ($p<0.001$) retained the gene expression to near control when compared with diabetic group (Figure- 41, Table- 41).

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the pancreas of experimental rats

The mRNA level expression of α7 nicotinic acetylcholine receptor showed a significant ($p<0.001$) up regulation in the pancreas of diabetic, C +D and V + D groups when compared with control. When compared with diabetic group, pre-treated rats showed a significant ($p<0.001$) decrease in α7 nicotinic acetylcholine receptor mRNA expression (Figure- 42, Table- 42).

Real Time PCR amplification of vitamin D receptor mRNA in the pancreas of experimental rats

Vitamin D receptor gene expression showed a significant up regulation in the pancreas of diabetic ($p<0.01$), C + D ($p<0.001$) and V + D ($p<0.001$) rats when compared with control. When compared with diabetic group, curcumin ($p<0.05$) and vitamin D_3 ($p<0.001$) pre-treated rats showed a significant increase in gene expression. Among pre-treated groups, V + D showed a significant ($p<0.001$) increase when compared with C + D (Figure- 43, Table- 43).

Confocal imaging of vitamin D receptor in the pancreas of experimental rats

Confocal microscopic image of vitamin D receptor in the pancreas of vitamin D_3 pre-treated group showed a significant ($p<0.01$) increase in the mean pixel intensity when compared with control, diabetic and C + D (Figure- 44, Table- 44).

Real Time PCR amplification of CREB mRNA in the pancreas of experimental rats

Gene expression of CREB mRNA showed a significant ($p<0.001$) down regulation in the pancreas of diabetic rats when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) up regulated the CREB mRNA gene expression when compared with both control and diabetic group (Figure- 45, Table- 45).

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

The gene expression studies showed that phospholipase C mRNA was significantly down regulated ($p<0.05$) in diabetic group when compared with control. In C + D and V + D, there was a significant up regulation ($p<0.001$) of phospholipase C expression when compared with both control and diabetic group (Figure- 46, Table- 46).

Real Time PCR amplification of insulin receptor mRNA in the pancreas of experimental rats

Real time PCR gene expression of insulin receptor showed a significant ($p<0.001$) down regulation in the pancreas of diabetic rats when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) up regulation when compared with both control and diabetic group. Further, a significant ($p<0.05$) decrease was observed in the V + D when compared with C + D (Figure- 47, Table- 47).

Real Time PCR amplification of GLUT 2 mRNA in the pancreas of experimental rats

GLUT 2 gene expression showed a significant ($p<0.001$) down regulation in the pancreas of diabetic rats when compared with control. A significant up regulation in the GLUT 2 mRNA was observed in the curcumin ($p<0.001$) and vitamin D_3 ($p<0.01$) pre-treated rats when compared with control. In C + D and V + D there was a significant ($p<0.001$) up regulation of gene expression when compared with diabetic (Figure- 48, Table- 48).

Real Time PCR amplification of superoxide dismutases mRNA in the pancreas of experimental rats

Real-time PCR gene expression of superoxide dismutases showed a significant ($p<0.01$) up regulation in the pancreas of diabetic rats when compared with control. When compared with both control and diabetic, pre-treated rats

showed a significant ($p<0.001$) increase. Further, superoxide dismutases expression was significantly ($p<0.01$) decreased in the V + D group when compared with C + D (Figure- 49, Table- 49).

Real Time PCR amplification of glutathione peroxidase mRNA in the pancreas of experimental rats

mRNA level expression of glutathione peroxidase showed a significant ($p<0.001$) up regulation in the pancreas of diabetic group when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) increase in the glutathione peroxidase mRNA expression when compared with both control and diabetic group (Figure- 50, Table- 50).

cAMP content in the pancreas of experimental rats

The cAMP content in the pancreas of diabetic rats showed a significant decrease ($p<0.001$) when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) retained a near control level of cAMP when compared with diabetic group (Figure- 51, Table- 51).

cGMP content in the pancreas of experimental rats

Diabetic group showed a significant ($p<0.01$) decrease in cGMP content in the pancreas when compared with control. Curcumin and vitamin D_3 pre-treated rats were able to maintain a near control level of cGMP in there pancreas when compared with diabetic group ($p<0.01$) (Figure- 52, Table- 52).

IP3 content in the pancreas of experimental rats

IP3 content was significantly ($p<0.001$) decreased in the pancreas of diabetic group when compared with control. Curcumin and vitamin D_3 pre-treatment kept at a near control level of IP3 content when compared with diabetic group ($p<0.001$) (Figure- 53, Table- 53).

Figure-25

Scatchard analysis of total adrenergic receptor using [^{3}H] epinephrine binding against epinephrine in the pancreas of experimental rats

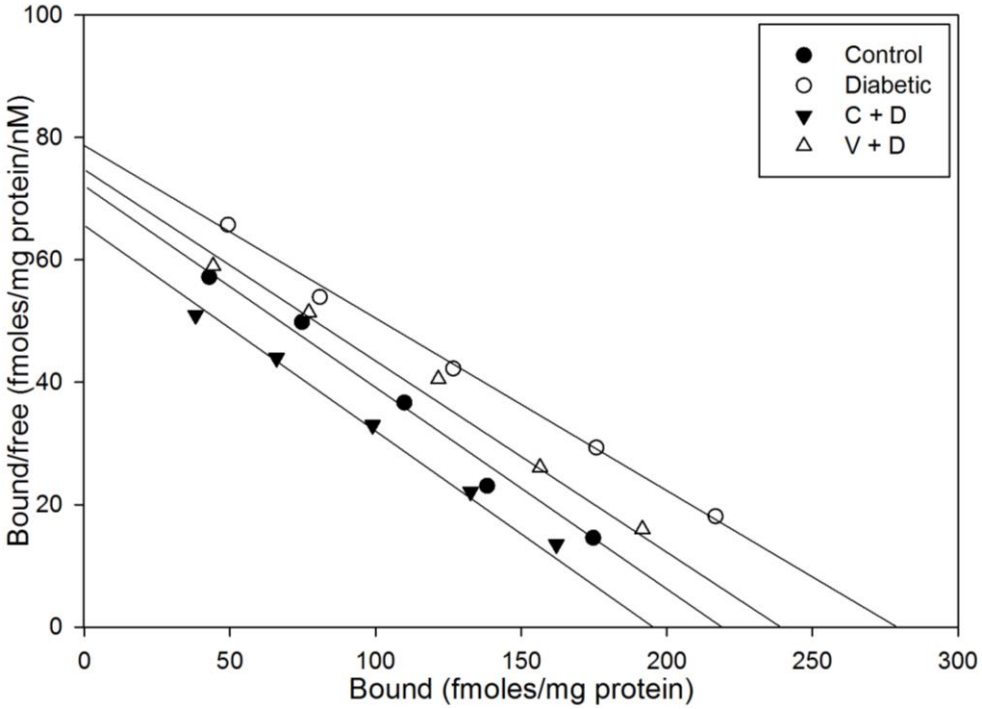


Table-25

Scatchard analysis of total adrenergic receptor using [^{3}H] epinephrine binding against epinephrine in the pancreas of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	221.28 ± 10.23	3.03 ± 0.13
Diabetic	284.83 ± 10.24 **b**	3.63 ± 0.18
C + D	192.75 ± 11.62 **e**	3.03 ± 0.16
V + D	227.73 ± 9.54 **e**	3.26 ± 0.17

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$ when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-26

Scatchard analysis of α2 adrenergic receptor using [^{3}H] yohimbine binding against phentolamine in the pancreas of experimental rats

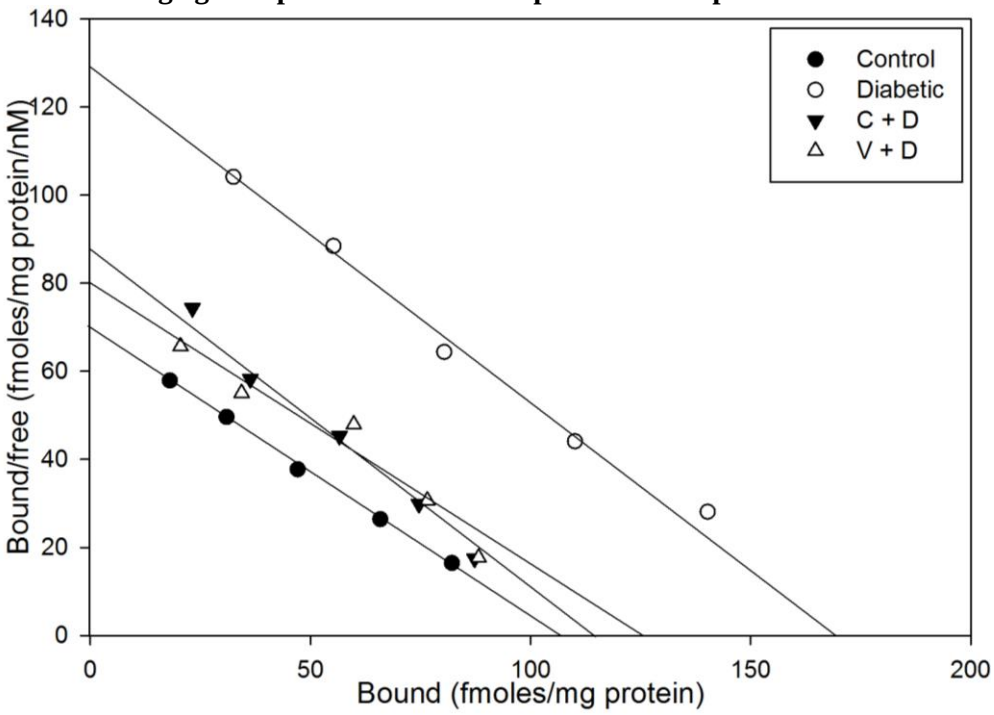


Table-26

Scatchard analysis of α2 adrenergic receptor using [^{3}H] yohimbine binding against phentolamine in the pancreas of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	105.45 ± 5.77	1.53 ± 0.03
Diabetic	174.37 ± 8.09 [a]	1.32 ± 0.05
C + D	112.96 ± 7.12 [d]	1.30 ± 0.06
V + D	125.69 ± 6.98 [e]	1.53 ± 0.07

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$, [e]$p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-27

Real Time PCR amplification of α2 adrenergic receptor mRNA in the pancreas of experimental rats

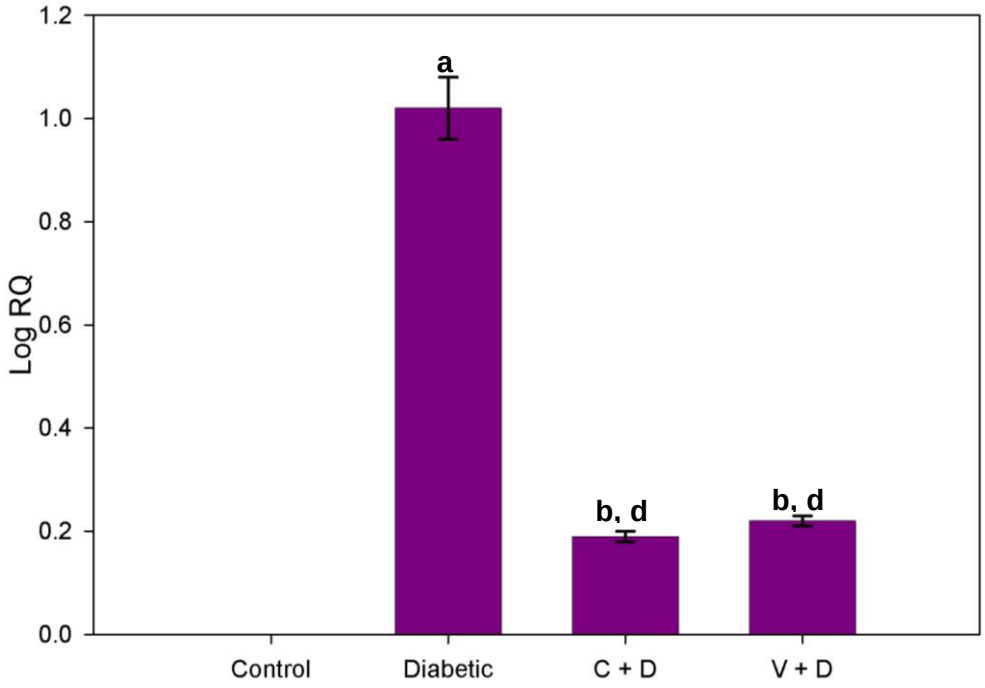


Table-27

Real Time PCR amplification of α2 adrenergic receptor mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.02 ± 0.06 **a**
C + D	0.19 ± 0.01 **b, d**
V + D	0.22 ± 0.01 **b, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001, **b**p<0.01 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-28

Confocal imaging of α2 adrenergic receptor in the pancreas of experimental rats

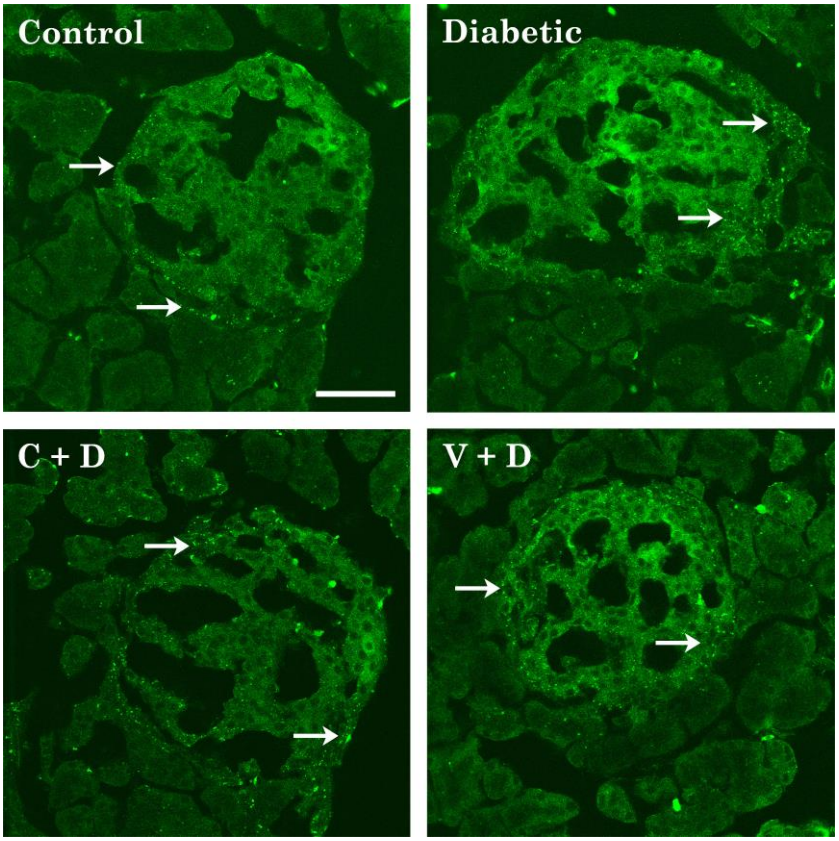


Table-28
Confocal imaging of α2 adrenergic receptor in the pancreas of experimental rats

Experimental groups	Mean Pixel Intensity
Control	27.66 ± 1.13
Diabetic	38.62 ± 1.32 **b**
C + D	30.71 ± 1.29 **e**
V + D	32.52 ± 1.56 **f**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **b** $p<0.01$, when compared to Control. **e** $p<0.01$, **f** $p<0.05$ when compared to Diabetic group. C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats. ⟹ shows α2 adrenergic receptors. Scale bar represents 50µm.

Figure-29
Scatchard analysis of β adrenergic receptor using [^{3}H] propranolol binding against propranolol in the pancreas of experimental rats

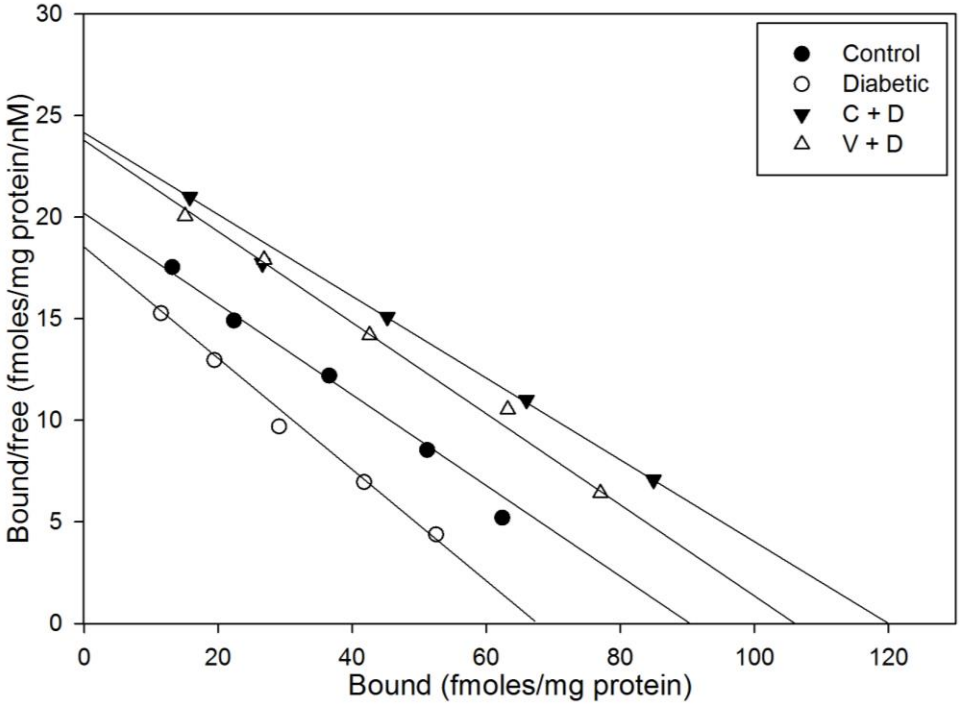


Table-29
Scatchard analysis of β adrenergic receptor using [^{3}H] propranolol binding against propranolol in the pancreas of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	90.60 ± 5.46	4.56 ± 0.27
Diabetic	66.32 ± 3.75 **b**	3.62 ± 0.22 **b**
C + D	116.13 ± 5.52 **c, d**	5.01 ± 0.25 **c, d**
V + D	104.78 ± 5.20 **e**	4.43 ± 0.29 **e, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b$p<0.01$, **c**$p<0.05$ when compared to Control. **d**$p<0.001$, **e**$p<0.01$ when compared to Diabetic group. **i** $p<0.05$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-30

Real Time PCR amplification of β2 adrenergic receptor mRNA in the pancreas of experimental rats

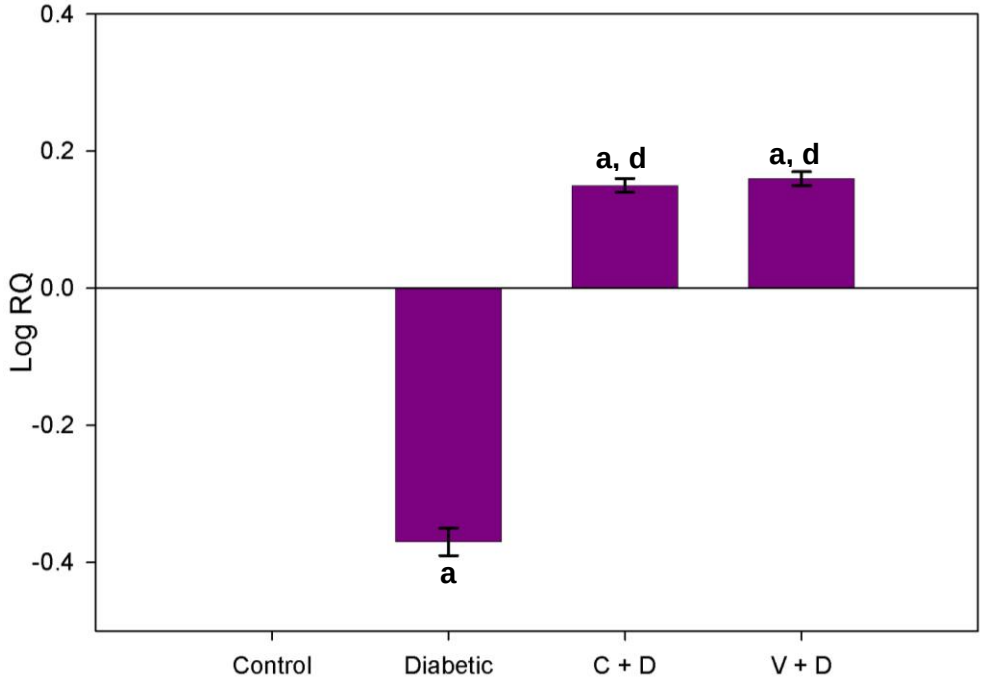


Table-30

Real Time PCR amplification of β2 adrenergic receptor mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.37 ± 0.02 **a**
C + D	0.15 ± 0.01 **a, d**
V + D	0.16 ± 0.01 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-31
Confocal imaging of β2 adrenergic receptor in the pancreas of experimental rats

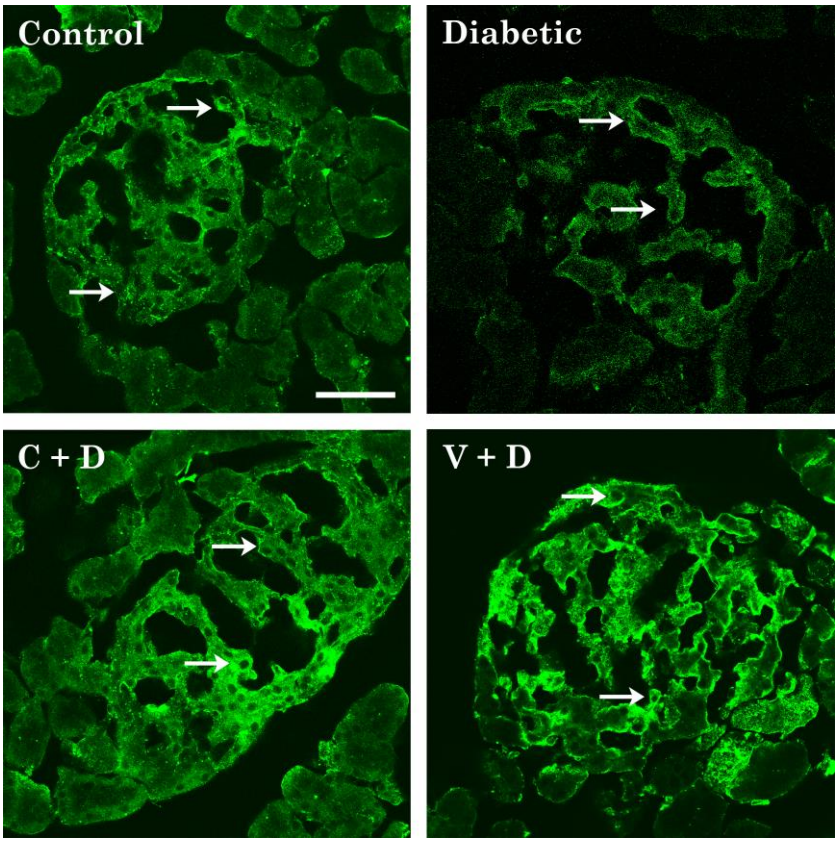


Table-31
Confocal imaging of β2 adrenergic receptor in the pancreas of experimental rats

Experimental groups	Mean Pixel Intensity
Control	36.56 ± 1.42
Diabetic	22.91 ± 1.37 [a]
C + D	39.27 ± 1.70 [d]
V + D	43.85 ± 2.19 [c, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
[a]$p<0.001$, [c]$p<0.05$ when compared to Control. [d]$p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⇨ shows β2 adrenergic receptors. Scale bar represents 50µm.

Figure-32

Scatchard analysis of total muscarinic receptor using [^{3}H] QNB binding against atropine in the pancreas of experimental rats

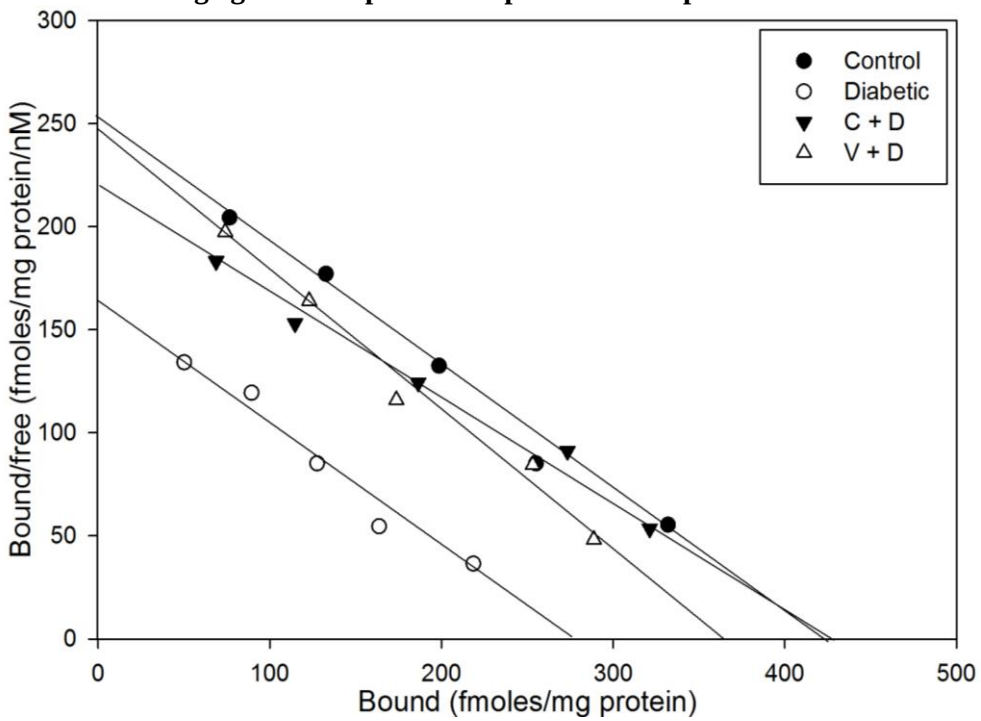


Table-32

Scatchard analysis of total muscarinic receptor using [^{3}H] QNB binding against atropine in the pancreas of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	425.08 ± 19.60	1.65 ± 0.08
Diabetic	273.35 ± 15.69 [a]	1.66 ± 0.07
C + D	429.11 ± 17.92 [d]	1.93 ± 0.11
V + D	357.16 ± 13.18 [c, e, i]	1.46 ± 0.09 [i]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. [a]$p<0.001$, [c]$p<0.05$ when compared to Control. [d]$p<0.001$, [e]$p<0.01$ when compared to Diabetic group. [i] $p<0.05$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-33

Scatchard analysis of muscarinic M1 receptor using [^{3}H] QNB binding against pirenzepine in the pancreas of experimental rats

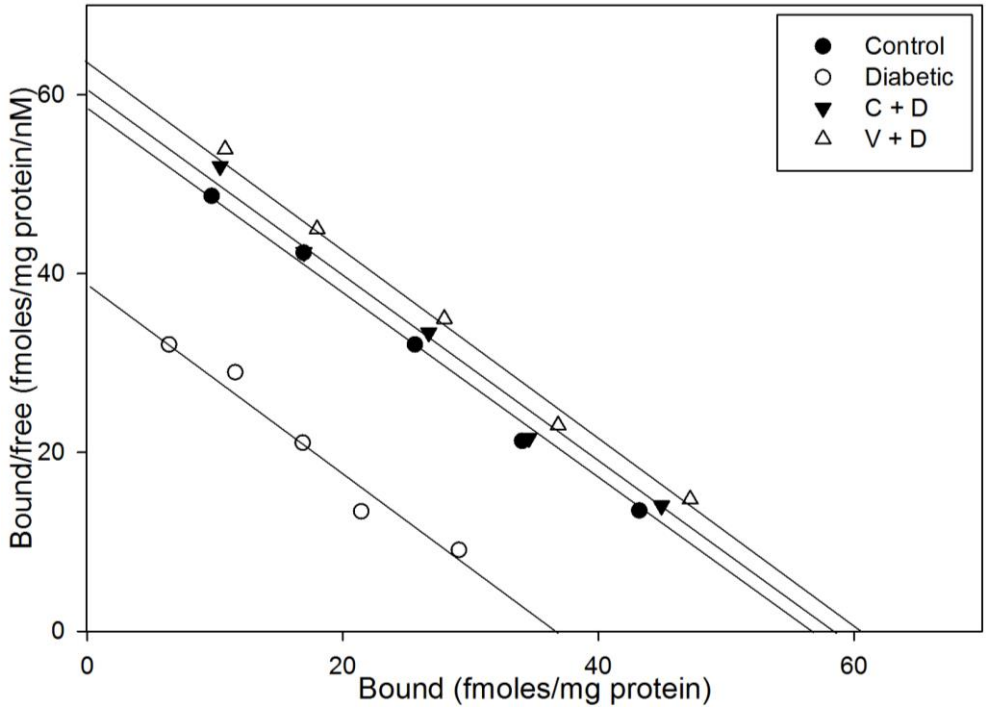


Table-33

Scatchard analysis of muscarinic M1 receptor using [^{3}H] QNB binding against pirenzepine in the pancreas of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	56.07 ± 2.61	0.96 ± 0.03
Diabetic	38.27 ± 1.64 **b**	0.93 ± 0.04
C + D	57.88 ± 2.62 **e**	0.93 ± 0.05
V + D	60.35 ± 2.99 **e**	0.96 ± 0.05

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$ when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-34

Real Time PCR amplification of muscarinic M1 receptor mRNA in the pancreas of experimental rats

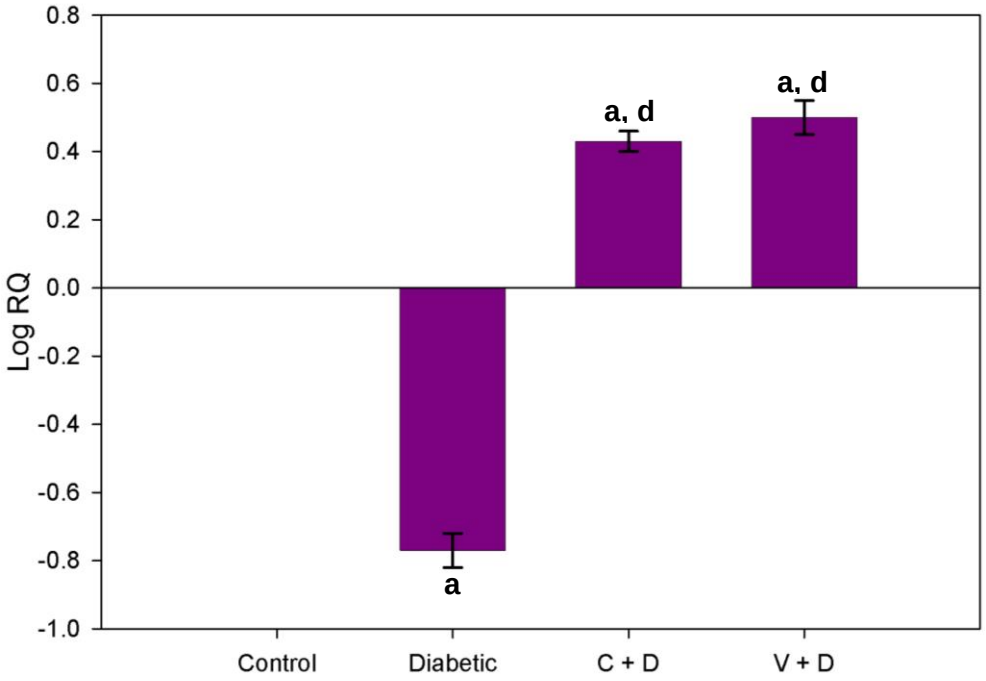


Table-34

Real Time PCR amplification of muscarinic M1 receptor mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.77 ± 0.05 **a**
C + D	0.43 ± 0.03 **a, d**
V + D	0.50 ± 0.05 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-35
Confocal imaging of muscarinic M1 receptor in the pancreas of experimental rats

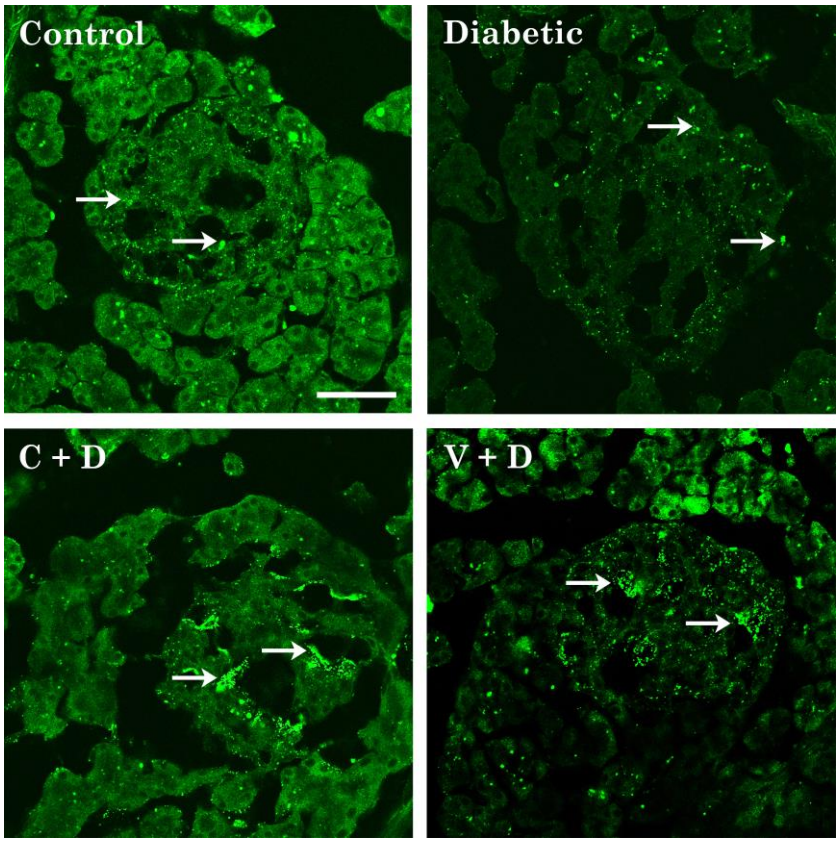


Table-35
Confocal imaging of muscarinic M1 receptor in the pancreas of experimental rats

Experimental groups	Mean Pixel Intensity
Control	28.01 ± 1.51
Diabetic	17.00 ± 0.89 **a**
C + D	31.13 ± 1.09 **d**
V + D	32.88 ± 1.32 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⇨ shows muscarinic M1 receptor. Scale bar represents 50µm.

Figure-36

Scatchard analysis of muscarinic M3 receptor using [^{3}H] DAMP binding against 4-DAMP mustard in the pancreas of experimental rats

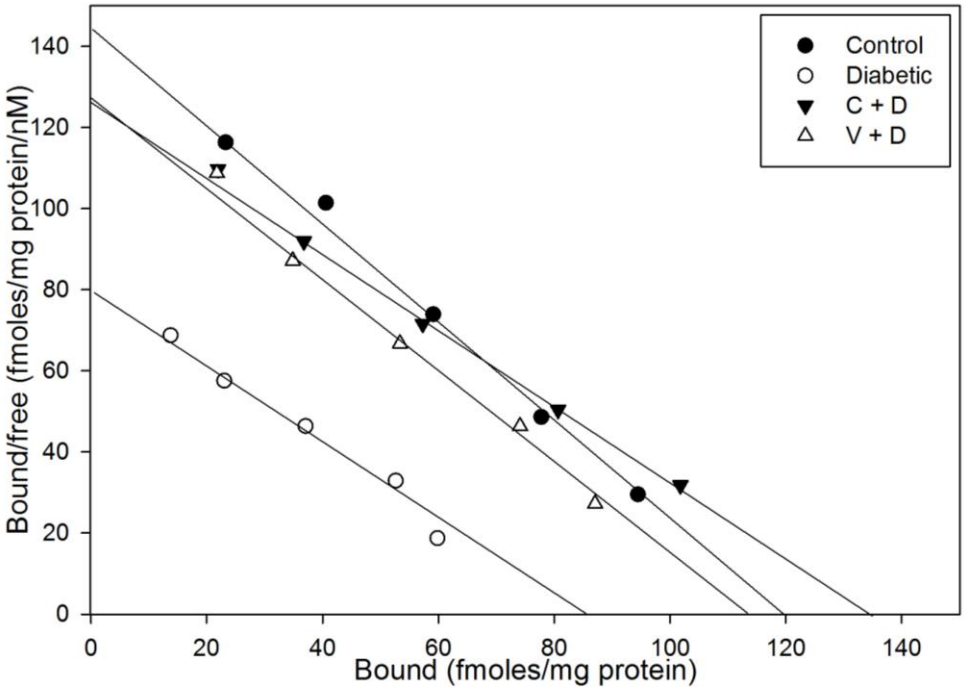


Table-36

Scatchard analysis of muscarinic M3 receptor using [3H] DAMP binding against 4-DAMP mustard in the pancreas of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	119.96 ± 6.08	0.84 ± 0.03
Diabetic	85.43 ± 4.27 **b**	1.00 ± 0.05
C + D	134.22 ± 6.29 **e**	1.07 ± 0.06
V + D	115.06 ± 6.10 **e**	0.95 ± 0.06

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$ when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-37

Real Time PCR amplification of muscarinic M3 receptor mRNA in the pancreas of experimental rats

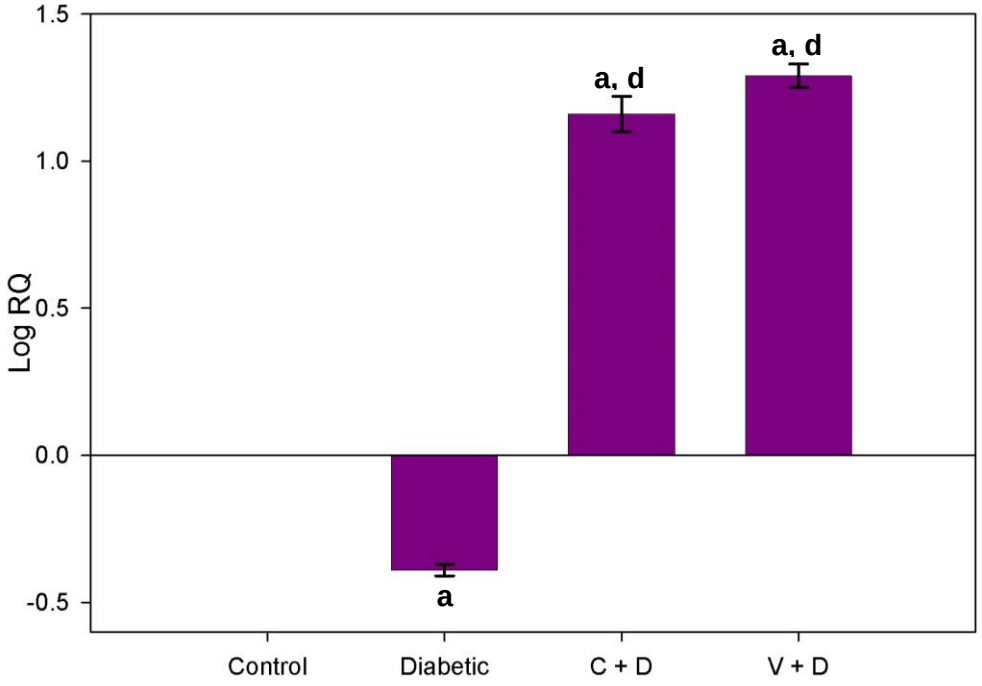


Table-37

Real Time PCR amplification of muscarinic M3 receptor mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.39 ± 0.02 **a**
C + D	1.16 ± 0.06 **a, d**
V + D	1.29 ± 0.04 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group. C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-38
Confocal imaging of muscarinic M3 receptor in the pancreas of experimental rats

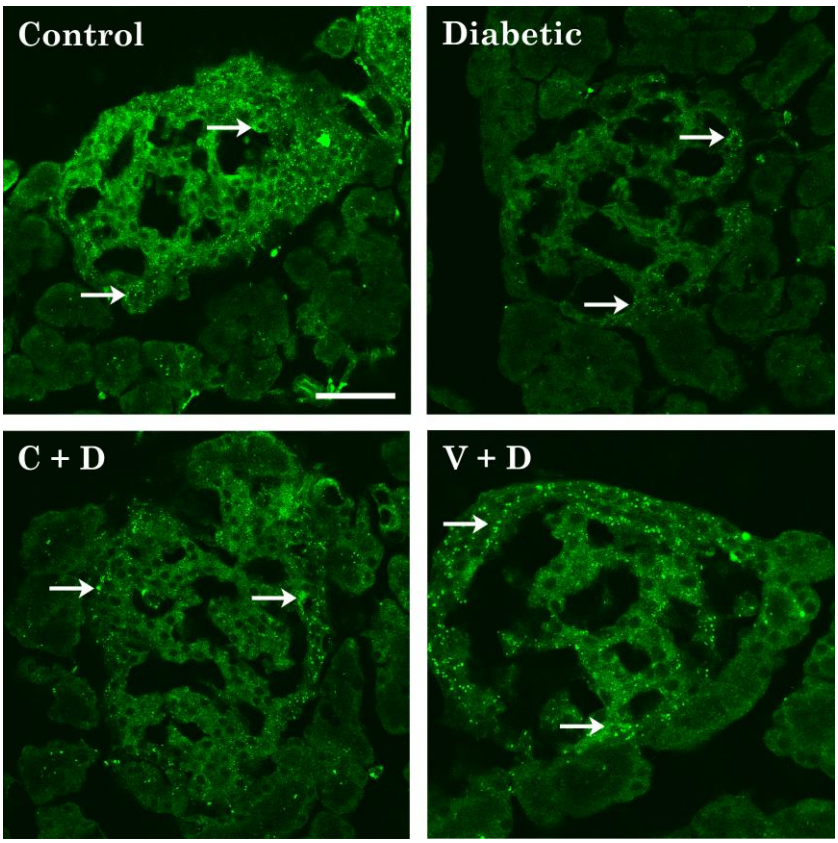


Table-38
Confocal imaging of muscarinic M3 receptor in the pancreas of experimental rats

Experimental groups	Mean Pixel Intensity
Control	30.59 ± 1.51
Diabetic	22.47 ± 1.32 **b**
C + D	36.36 ± 1.95 **c, d**
V + D	38.43 ± 1.86 **c, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$, **c** $p<0.05$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⇨ shows muscarinic M3 receptor. Scale bar represents 50µm.

Figure-39

Real Time PCR amplification of choline acetyltransferase mRNA in the pancreas of experimental rats

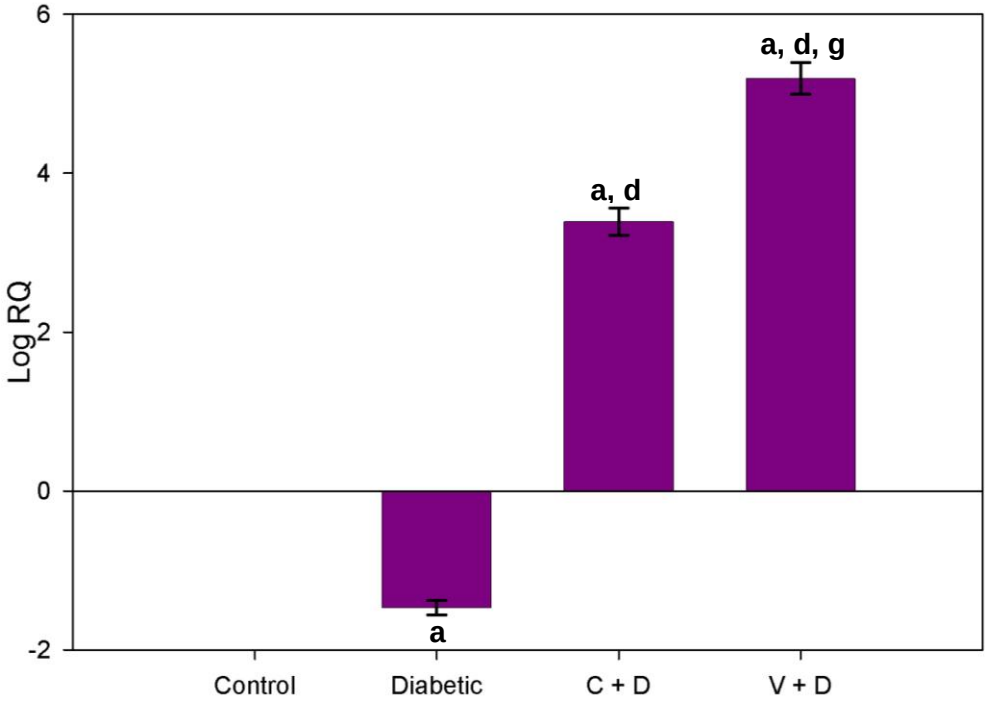


Table-39

Real Time PCR amplification of choline acetyltransferase mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.47 ± 0.09 **a**
C + D	3.39 ± 0.17 **a, d**
V + D	5.19 ± 0.20 **a, d, g**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. **g** $p<0.001$ when compared to C + D group.

C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-40

Real Time PCR amplification of acetylcholinesterase mRNA in the pancreas of experimental rats

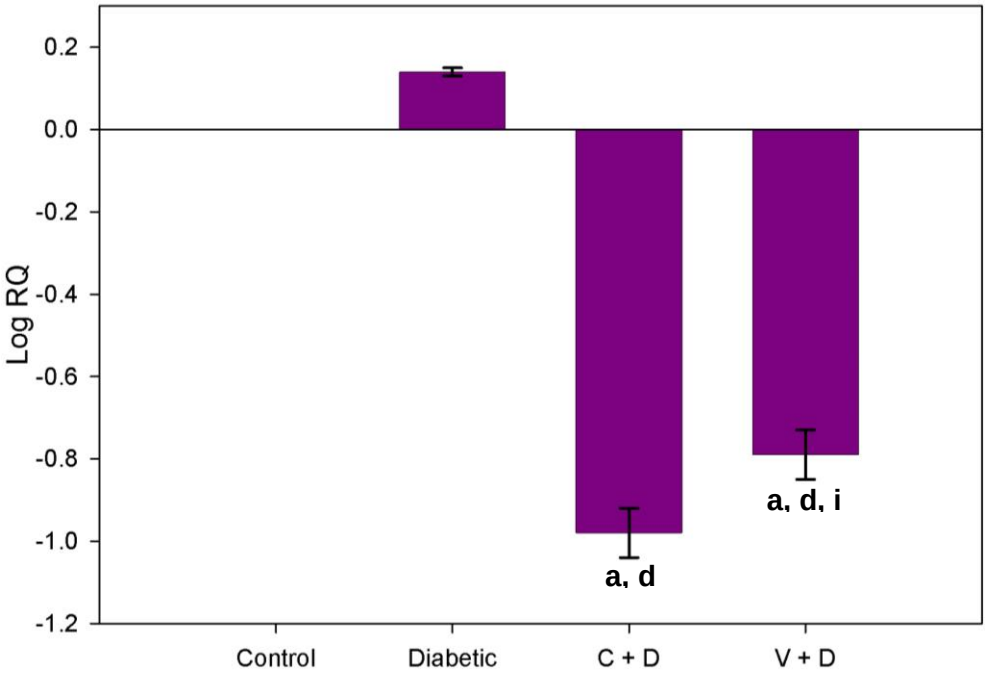


Table-40

Real Time PCR amplification of acetylcholinesterase mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.14 ± 0.01
C + D	-0.98 ± 0.06 **a, d**
V + D	-0.79 ± 0.06 **a, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. **i** $p<0.05$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-41

Real Time PCR amplification of muscarinic M2 receptor mRNA in the pancreas of experimental rats

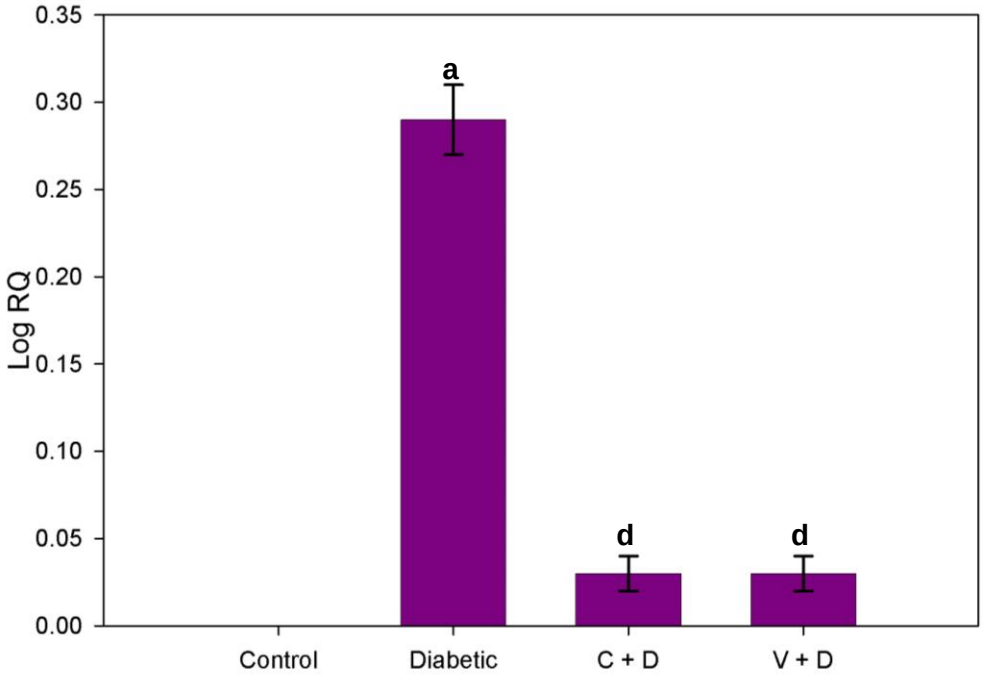


Table-41

Real Time PCR amplification of muscarinic M2 receptor mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.29 ± 0.02 **a**
C + D	0.03 ± 0.01 **d**
V + D	0.03 ± 0.01 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-42

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the pancreas of experimental rats

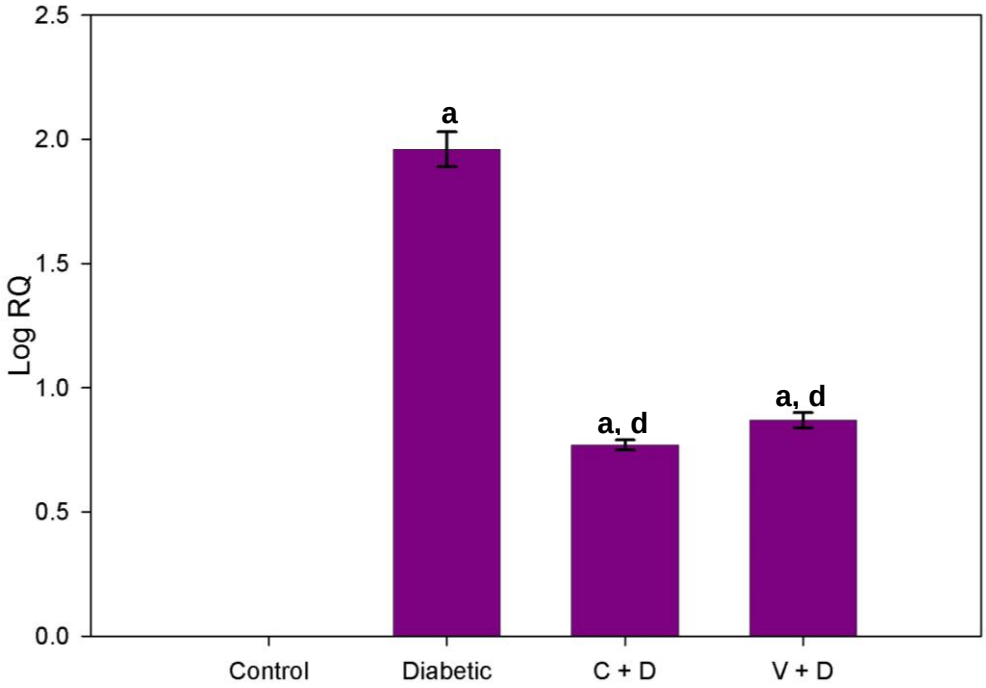


Table-42

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.96 ± 0.07 **a**
C + D	0.77 ± 0.02 **a, d**
V + D	0.87 ± 0.03 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-43

Real Time PCR amplification of vitamin D receptor mRNA in the pancreas of experimental rats

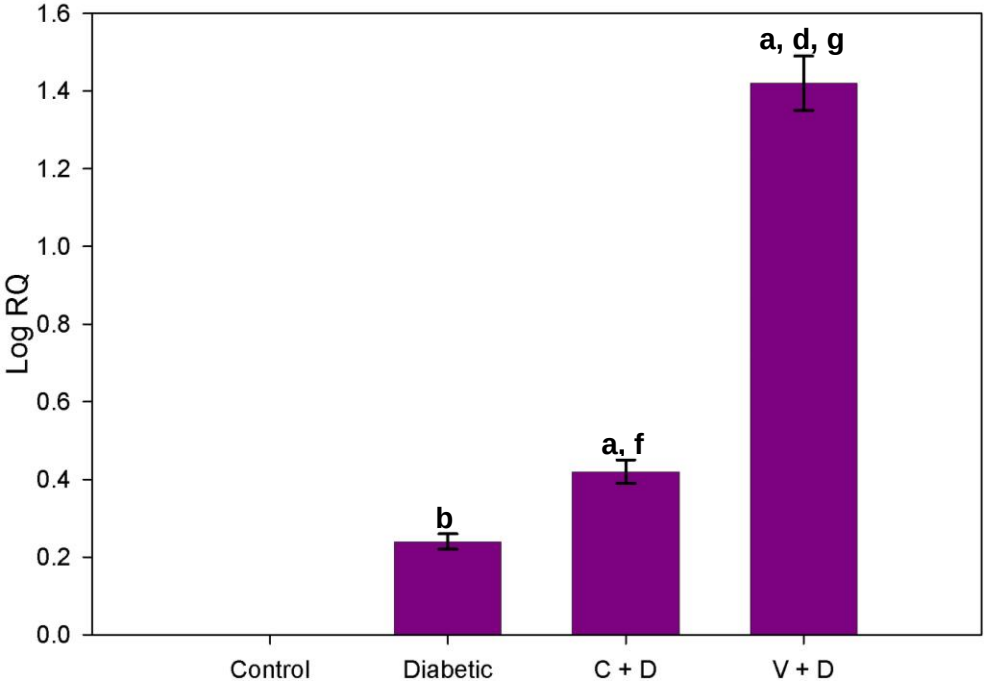


Table-43

Real Time PCR amplification of vitamin D receptor mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.24 ± 0.02 **b**
C + D	0.42 ± 0.03 **a, f**
V + D	1.42 ± 0.07 **a, d, g**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001, **b**p<0.01 when compared to Control. **d**p<0.001, **f**p<0.05 when compared to Diabetic group. **g** p<0.001 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-44
Confocal imaging of vitamin D receptor in the pancreas of experimental rats

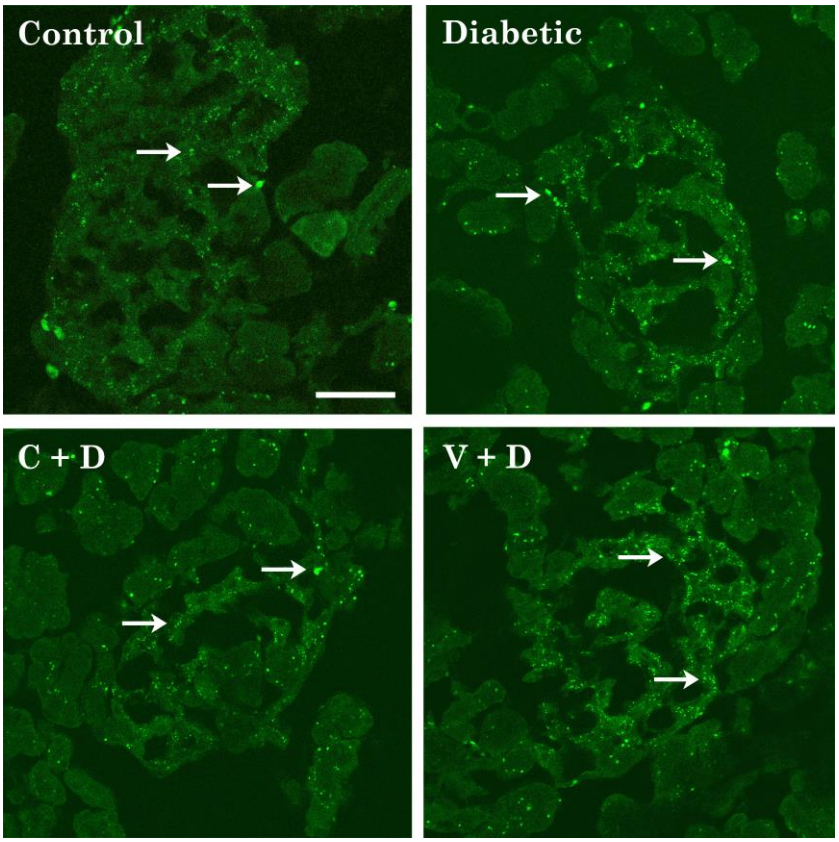


Table-44
Confocal imaging of vitamin D receptor in the pancreas of experimental rats

Experimental groups	Mean Pixel Intensity
Control	15.46 ± 0.98
Diabetic	18.21 ± 1.10
C + D	19.50 ± 0.95
V + D	24.76 ± 1.33 **b, e, h**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **b** $p<0.01$when compared to Control. **e** $p<0.01$ when compared to Diabetic group. **h** $p<0.01$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⟹ shows vitamin D receptor. Scale bar represents 50µm.

Figure-45

Real Time PCR amplification of CREB mRNA in the pancreas of experimental rats

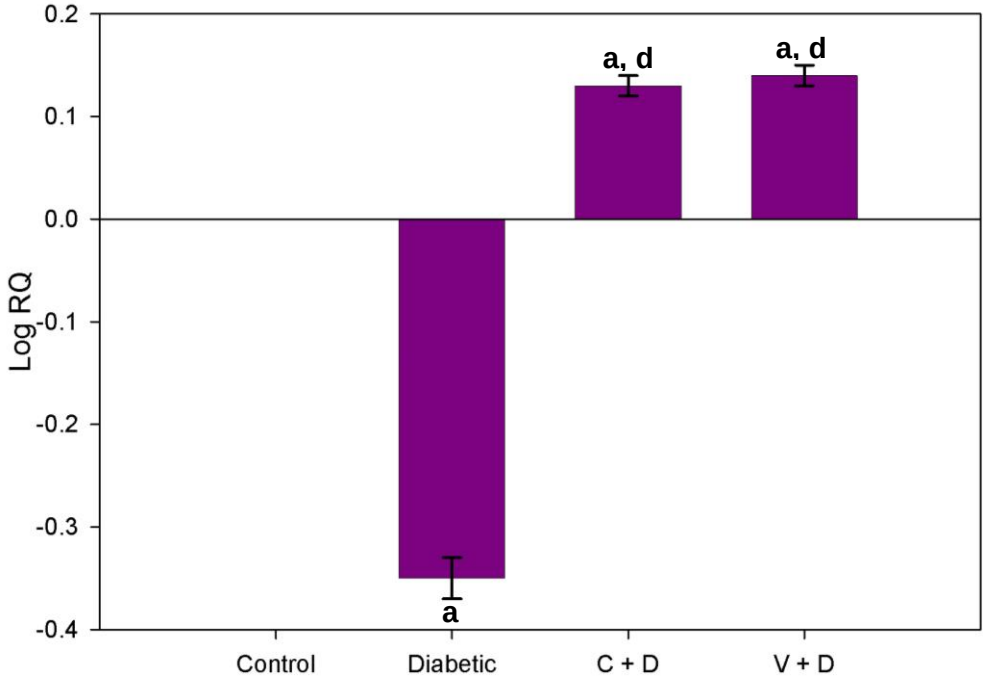


Table-45

Real Time PCR amplification of CREB mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.35 ± 0.02 **a**
C + D	0.13 ± 0.01 **a, d**
V + D	0.14 ± 0.01 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-46

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

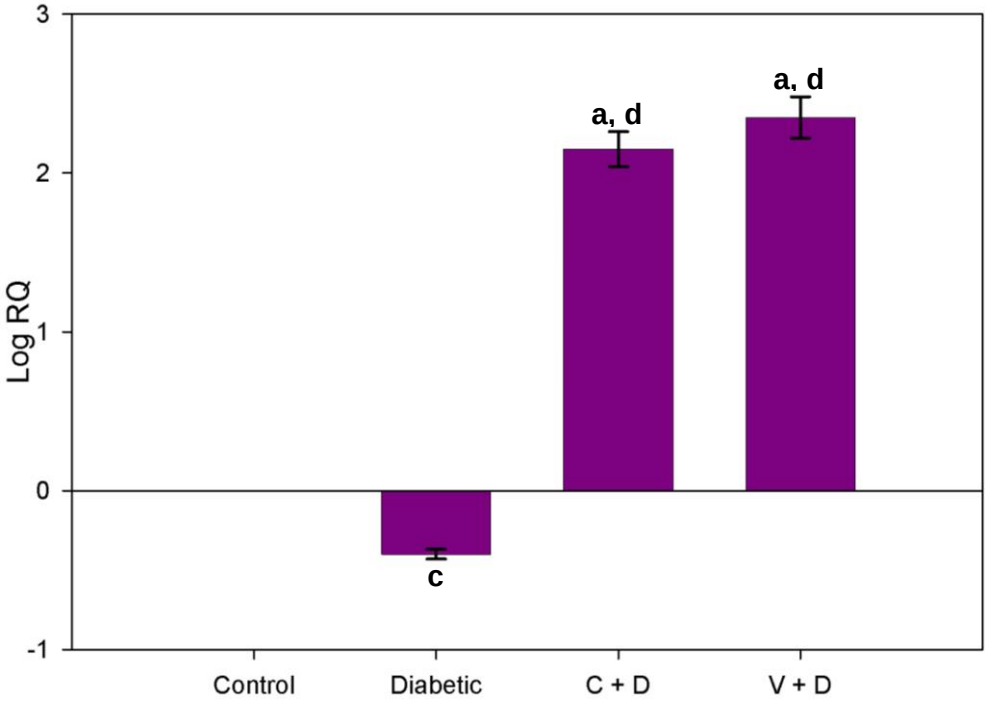


Table-46

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

Experimental groups	Log RQ
Control	0
Diabetic	-0.40 ± 0.03 **c**
C + D	2.15 ± 0.11 **a, d**
V + D	2.35 ± 0.13 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001, **c**p<0.05 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-47

Real Time PCR amplification of insulin receptor mRNA in the pancreas of experimental rats

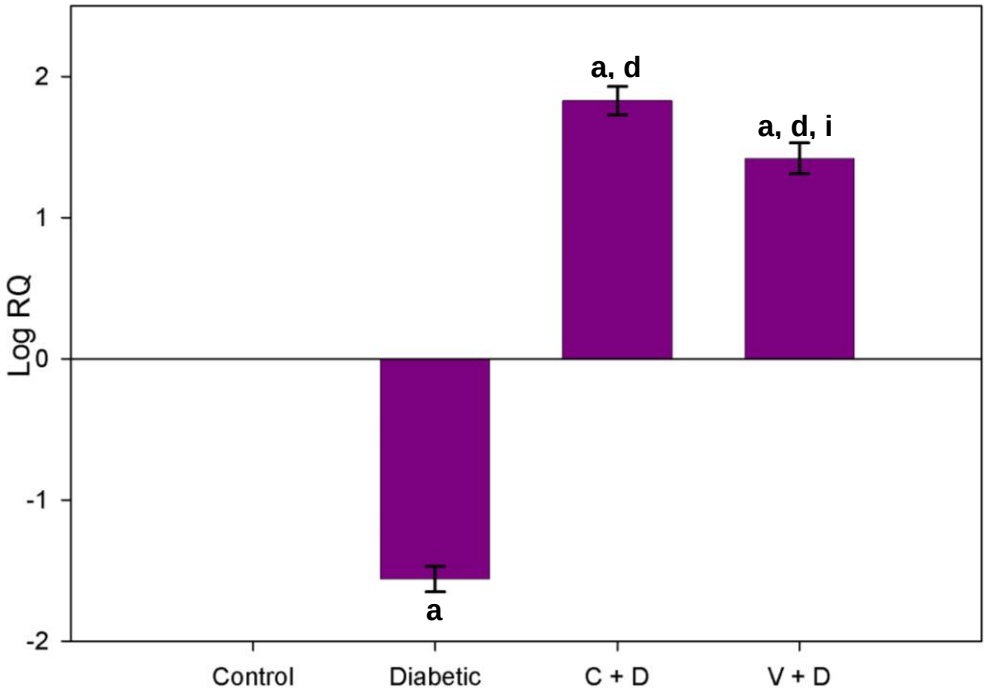


Table-47

Real Time PCR amplification of insulin receptor mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.56 ± 0.09 **a**
C + D	1.83 ± 0.10 **a, d**
V + D	1.42 ± 0.11 **a, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. **i** $p<0.05$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-48

Real Time PCR amplification of GLUT 2 mRNA in the pancreas of experimental rats

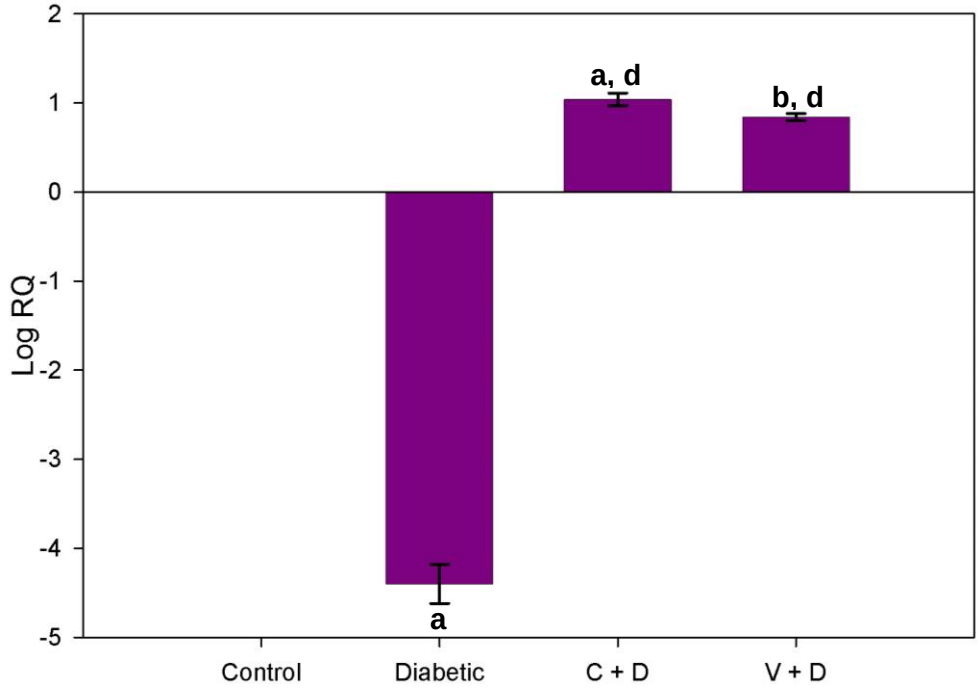


Table-48

Real Time PCR amplification of GLUT 2 mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-4.40 ± 0.22 **a**
C + D	1.04 ± 0.07 **a, d**
V + D	0.84 ± 0.04 **b, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$, **b** $p<0.01$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.

C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-49

Real Time PCR amplification of superoxide dismutases mRNA in the pancreas of experimental rats

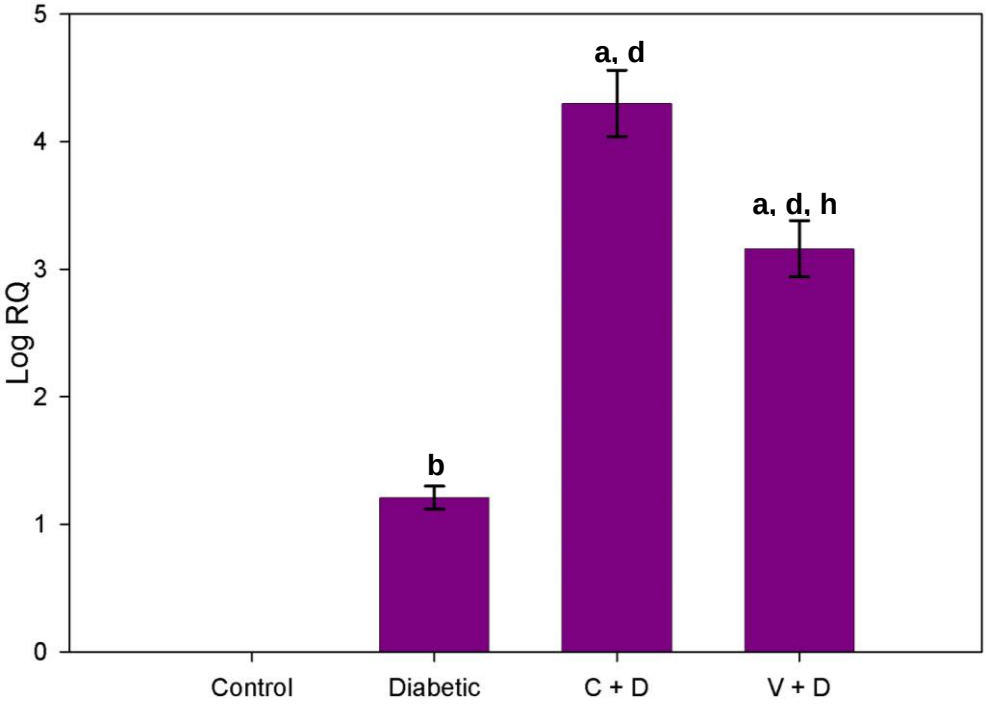


Table-49

Real Time PCR amplification of superoxide dismutases mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.21 ± 0.09 **b**
C + D	4.30 ± 0.26 **a, d**
V + D	3.16 ± 0.22 **a, d, h**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$, **b** $p<0.01$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. **h** $p<0.01$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-50

Real Time PCR amplification of glutathione peroxidase mRNA in the pancreas of experimental rats

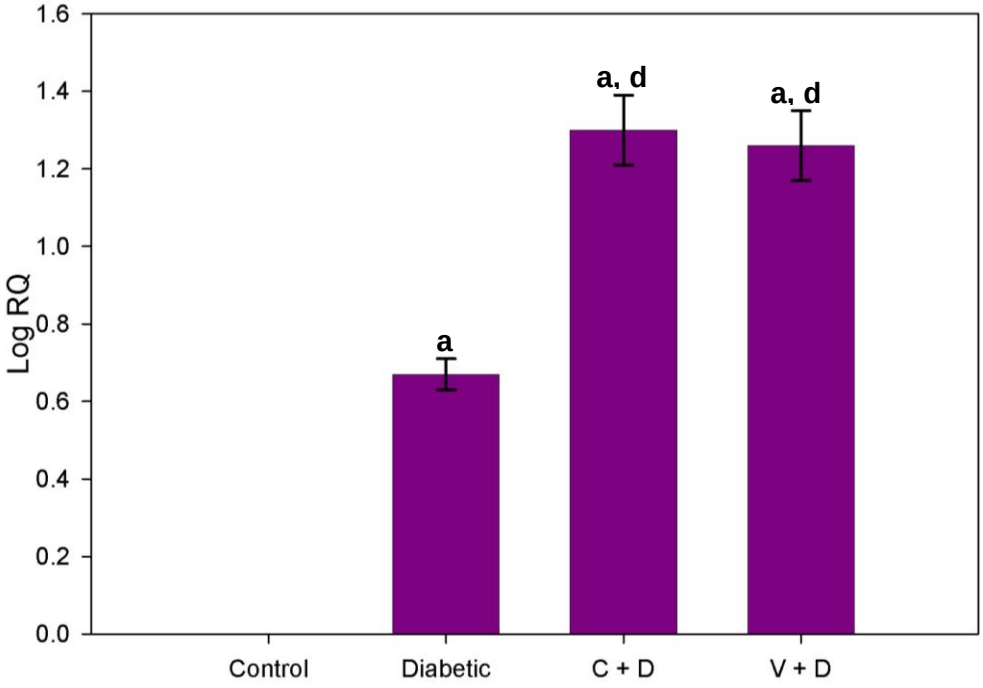


Table-50

Real Time PCR amplification of glutathione peroxidase mRNA in the pancreas of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.67 ± 0.04 **a**
C + D	1.30 ± 0.09 **a, d**
V + D	1.26 ± 0.09 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-51

cAMP content in the pancreas of experimental rats

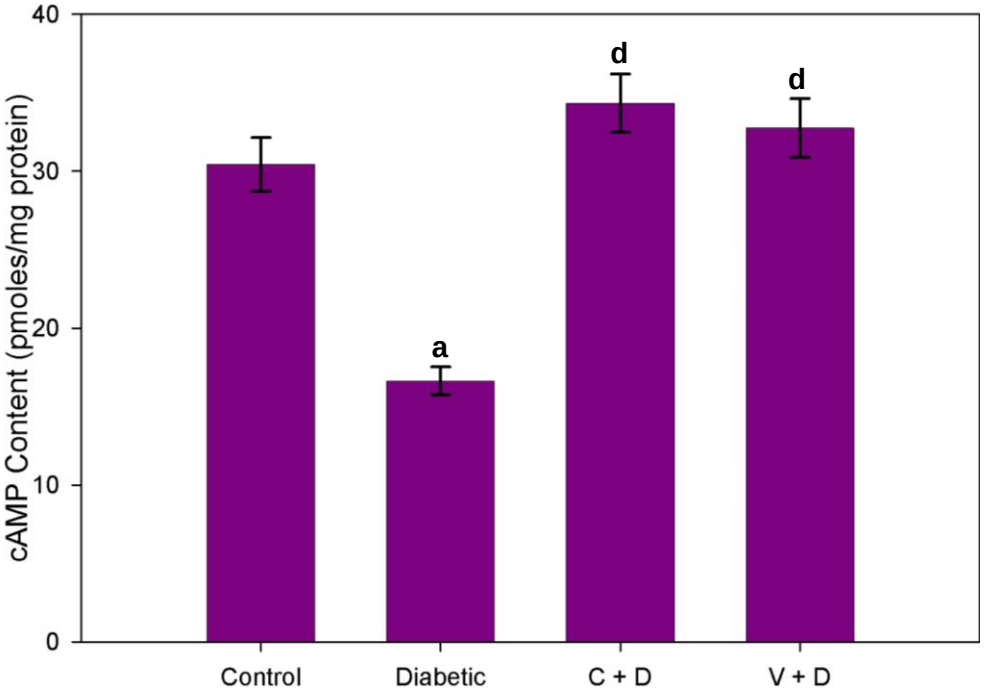


Table-51

cAMP content in the pancreas of experimental rats

Experimental groups	cAMP content (pmoles/mg protein)
Control	30.43 ± 1.71
Diabetic	16.64 ± 0.89 **a**
C + D	34.33 ± 1.86 **d**
V + D	32.75 ± 1.87 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-52

cGMP content in the pancreas of experimental rats

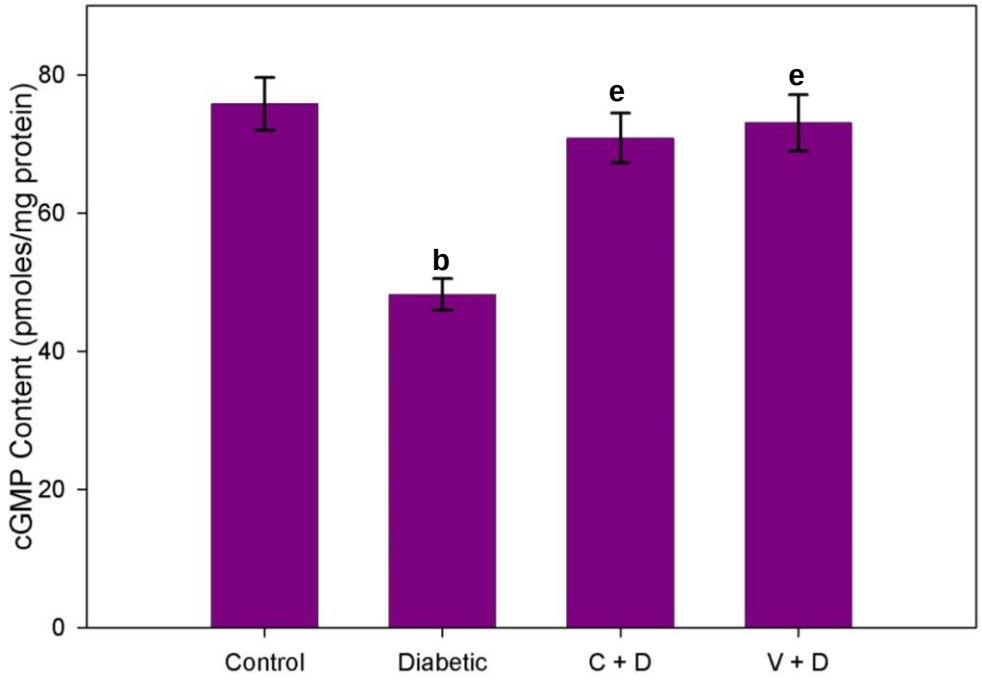


Table-52

cGMP content in the pancreas of experimental rats

Experimental groups	cGMP content (pmoles/mg protein)
Control	75.81 ± 3.81
Diabetic	48.22 ± 2.29 **b**
C + D	70.86 ± 3.60 **e**
V + D	73.06 ± 4.04 **e**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$ when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-53

IP3 content in the pancreas of experimental rats

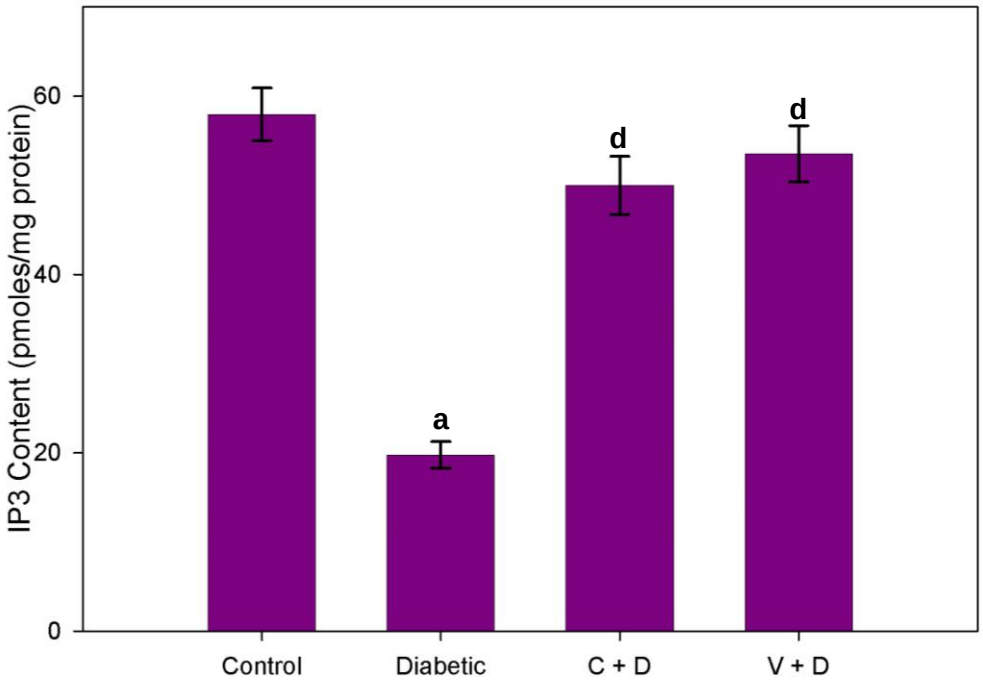


Table-53

IP3 content in the pancreas of experimental rats

Experimental groups	IP3 content (pmoles/mg protein)
Control	57.95 ± 2.95
Diabetic	19.77 ± 1.49 **a**
C + D	49.99 ± 3.25 **d**
V + D	53.54 ± 3.16 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a$p<0.001$ when compared to Control. **d**$p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

BRAIN STEM

Scatchard analysis of total adrenergic receptor using [^{3}H] epinephrine binding against epinephrine in the brain stem of experimental rats

Scatchard analysis of total adrenergic receptor using [^{3}H] epinephrine binding against epinephrine in the brain stem of diabetic rats showed a significant ($p<0.001$) decrease in B_{max} and K_d when compared with control. Pre-treatment with curcumin was able to maintain a near control B_{max} and K_d when compared with diabetic rats ($p<0.001$). B_{max} of vitamin D_3 pre-treated group was retained to near control when compared with diabetic group ($p<0.001$). K_d of V + D group showed a significant increase when compared with control ($p<0.01$), diabetic ($p<0.001$) and C + D ($p<0.01$) groups (Figure- 54, Table- 54).

Scatchard analysis of α2 adrenergic receptor using [^{3}H] yohimbine binding against phentolamine in the brain stem of experimental rats

In diabetic group, B_{max} of α2 adrenergic receptor was significantly decreased ($p<0.001$) compared to control. Pre-treatment with curcumin and vitamin D_3 were able to maintain a near control B_{max} when compared with diabetic group ($p<0.001$). Diabetic rats showed a significant ($p<0.01$) decrease in K_d of α2 adrenergic receptors in the brain stem when compared with control. Curcumin pre-treatment helps to significantly ($p<0.01$) increase the K_d when compared with diabetic. In V + D rats K_d was significantly ($p<0.001$) increased when compared with control, diabetic and C + D groups (Figure- 55, Table- 55).

Real Time PCR amplification of α2 adrenergic receptor mRNA in the brain stem of experimental rats

Gene expression of α2 adrenergic receptors mRNA showed a significant down regulation ($p<0.001$) in the brain stem of diabetic rats when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) increased the mRNA levels when compared with diabetic group. Further, the

V + D group showed a significant ($p<0.01$) increase in mRNA levels when compared with control and C + D (Figure- 56, Table- 56).

Confocal imaging of α2 adrenergic receptor in the brain stem of experimental rats

α2 adrenergic receptor antibody staining in the brain stem showed a significant ($p<0.01$) decrease in mean pixel intensity value of diabetic rats when compared with control. Curcumin and vitamin D_3 pre-treatment significantly ($p<0.01$) decrease the pixel intensity value to near control when compared with diabetic group (Figure- 57, Table- 57).

Scatchard analysis of β adrenergic receptor using [^{3}H] propranolol binding against propranolol in the brain stem of experimental rats

Binding studies of [^{3}H] propranolol against propranolol for β adrenergic receptor showed that the binding parameter B_{max} ($p<0.01$) significantly decreased in the brain stem of diabetic group when compared with control. Pre-treatment using curcumin ($p<0.001$) and vitamin D_3 ($p<0.01$) kept the B_{max} to near control when compared with diabetic group. V + D group showed a significant ($p<0.05$) increase in B_{max} when compare to C + D group. Diabetic group showed a significant ($p<0.001$) decrease in K_d when compared with control. Pre-treatments were able to retain a near control level of K_d when compared with diabetic group ($p<0.001$; Figure- 58, Table- 58).

Real Time PCR amplification of β2 adrenergic receptor mRNA in the brain stem of experimental rats

Real time PCR gene expression of β2 adrenergic receptor showed a significant ($p<0.001$) down regulation in the brain stem of diabetic rats when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant up regulation when compared with both control ($p<0.05$) and diabetic group ($p<0.001$; Figure- 59, Table- 59).

Confocal imaging of β2 adrenergic receptor in the brain stem of experimental rats

Confocal imaging of β2 adrenergic receptor in the brain stem of diabetic rats showed a significant ($p<0.001$) decrease in mean pixel intensity of diabetic rats when compared with control. Pre-treatments using curcumin and vitamin D_3 retain the mean pixel intensity to near control when compared with diabetic group ($p<0.001$; Figure- 60, Table- 60).

Scatchard analysis of total muscarinic receptor using [^{3}H] QNB binding against atropine in the brain stem of experimental rats

Scatchard analysis of total muscarinic receptor using [^{3}H] QNB binding against atropine in the brain stem of diabetic rats showed a significant ($p<0.01$) decrease in B_{max} when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.01$) retained the B_{max} to near control when compared with diabetic group. There was no significant change in K_d in all experimental groups of rats (Figure- 61, Table- 61).

Scatchard analysis of muscarinic M1 receptor using [^{3}H] QNB binding against pirenzepine in the brain stem of experimental rats

Muscarinic M1 receptors B_{max} was significantly decreased ($p<0.001$) in diabetic group when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) increase the B_{max} towards near control level when compared with diabetic group. K_d value of muscarinic M1 receptor biding of V + D group showed a significant increase when compared with control ($p<0.05$), diabetic ($p<0.01$) and C + D ($p<0.05$) groups (Figure- 62, Table- 62).

Real Time PCR amplification of muscarinic M1 receptor mRNA in the brain stem of experimental rats

The gene expression studies showed that muscarinic M1 receptor mRNA was significantly down regulated ($p<0.001$) in diabetic group when compared with control. In C + D and V + D, there was a significant up regulation ($p<0.001$) of

muscarinic M1 receptor gene expression when compared with both control and diabetic group (Figure- 63, Table- 63).

Confocal imaging of muscarinic M1 receptor in the brain stem of experimental rats

Muscarinic M1 receptor specific antibody staining in the brain stem showed a significant decrease ($p<0.001$) in mean pixel intensity of diabetic rats when compared with control. Curcumin and vitamin D_3 pre-treatment significantly ($p<0.001$) retained a near control level of mean pixel intensity when compared with diabetic group (Figure- 64, Table- 64).

Scatchard analysis of muscarinic M3 receptor using [^{3}H] DAMP binding against 4-DAMP mustard in the brain stem of experimental rats

In diabetic group, muscarinic M3 receptors B_{max} was significantly ($p<0.001$) decreased when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) increased the B_{max} when compared with diabetic group. There was no significant change in the K_d values of all groups in receptor studies (Figure- 65, Table- 65).

Real Time PCR amplification of muscarinic M3 receptor mRNA in the brain stem of experimental rats

The gene expression studies showed that muscarinic M3 receptor mRNA was significantly down regulated ($p<0.001$) in diabetic group when compared with control. In C + D, there was a significant ($p<0.001$) up regulation of muscarinic M3 receptor gene expression when compared with both control and diabetic group. V + D group showed a significant ($p<0.001$) increase in the mRNA levels when compared with diabetic and a significant ($p<0.001$) down regulation when compared with C + D (Figure- 66, Table- 66).

Confocal imaging of muscarinic M3 receptor in the brain stem of experimental rats

Muscarinic M3 receptor specific antibody staining in the brain stem showed a significant decrease ($p<0.01$) in mean pixel intensity of diabetic rats when compared with control. Curcumin ($p<0.01$) and vitamin D_3 ($p<0.05$) pre-treatment significantly retained the mean pixel value to near control when compared with diabetic group (Figure- 67, Table- 67).

Real Time PCR amplification of choline acetyltransferase mRNA in the brain stem of experimental rats

mRNA level expression of choline acetyltransferase showed a significant ($p<0.01$) up regulation in the brain stem of diabetic group when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) increase in the choline acetyltransferase mRNA expression when compared with both control and diabetic group. Further, choline acetyltransferase expression was significantly ($p<0.05$) increase in the V + D group when compared with C + D (Figure- 68, Table- 68).

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

Real-time PCR gene expression of acetylcholinesterase showed a significant ($p<0.001$) up regulation in the brain stem of diabetic rats compared to control. Pre-treatment with curcumin and vitamin D_3 helps to maintain a near control level of mRNA expression when compared with diabetic group ($p<0.001$; Figure- 69, Table- 69).

Real Time PCR amplification of muscarinic M2 receptor mRNA in the brain stem of experimental rats

Muscarinic M2 receptor gene expression showed a significant ($p<0.001$) down regulation in brain stem of diabetic, C + D and V + D rats when compared with control. When compared with diabetic rats, a significant increase in the

muscarinic M2 receptor mRNA was observed in curcumin ($p<0.05$) and vitamin D_3 ($p<0.01$) pre-treated rats (Figure- 70, Table- 70).

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the brain stem of experimental rats

Real time PCR gene expression of α7 nicotinic acetylcholine receptor showed a significant ($p<0.001$) up regulation in the brain stem of diabetic, C + D and V + D rats when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) down regulation when compared with diabetic group (Figure- 71, Table- 71).

Real Time PCR amplification of vitamin D receptor mRNA in the brain stem of experimental rats

The gene expression studies showed that vitamin D receptor mRNA was significantly up regulated in diabetic ($p<0.001$) and C + D ($p<0.01$) groups when compared with control. In C + D and V + D there was a significant ($p<0.001$) decrease of vitamin D receptor expression when compared with diabetic group. V + D group showed a significant ($p<0.05$) decrease when compared with C + D group (Figure- 72, Table- 72).

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

Gene expression of CREB mRNA showed a significant ($p<0.001$) down regulation in the brain stem of diabetic rats when compared with control. Pre-treatment using curcumin significantly ($p<0.001$) up regulated the gene expression when compared control. When compared with diabetic group, pre-treated groups showed a significant ($p<0.001$) up regulation. Vitamin D_3 pre-treated group showed a significant ($p<0.01$) decrease when compared with curcumin pre-treated group (Figure- 73, Table- 73).

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

Phospholipase C gene expression showed a significant down regulation in the brain stem of diabetic ($p<0.001$) and V + D ($p<0.01$) rats when compared with control. C + D group showed a significant ($p<0.001$) up regulation when compared with control. When compared with diabetic group, curcumin and vitamin D_3 pre-treatments showed a significant ($p<0.001$) increase in the mRNA levels. V + D group showed a significant ($p<0.001$) down regulation of phospholipase C mRNA when compared with C + D (Figure- 74, Table- 74).

Real Time PCR amplification of insulin receptor mRNA in the brain stem of experimental rats

mRNA level expression of insulin receptor showed a significant up regulation in the brain stem of diabetic ($p<0.001$) and pre-treated ($p<0.05$) groups when compared with control. When compared with diabetic group, curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) decrease in insulin receptor mRNA expression (Figure- 75, Table- 75).

Real Time PCR amplification of GLUT 3 mRNA in the brain stem of experimental rats

Real-time PCR gene expression of GLUT 3 showed a significant up regulation in the brain stem of diabetic ($p<0.001$), C + D ($p<0.01$) and V + D ($p<0.001$) rats when compared with control. Curcumin and vitamin D_3 pre-treatment significantly ($p<0.001$) decreased the mRNA expression when compared with diabetic group. Among pre-treated groups, vitamin D_3 pre-treated group showed a significant ($p<0.01$) increase when compared with curcumin pre-treated group (Figure- 76, Table- 76).

Real Time PCR amplification of superoxide dismutases mRNA in the brain stem of experimental rats

Superoxide dismutases gene expression showed a significant up regulation in the brain stem of diabetic ($p<0.001$) and pre-treated ($p<0.01$) groups when

compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) decrease in superoxide dismutases mRNA expression when compared with diabetic group (Figure- 77, Table- 77).

Real Time PCR amplification of glutathione peroxidase mRNA in the brain stem of experimental rats

Glutathione peroxidase gene expression showed a significant ($p<0.001$) up regulation in the brain stem of diabetic and pre-treated rats when compared with control. When compared with diabetic group, a significant ($p<0.001$) decrease in the glutathione peroxidase mRNA was observed in the pre-treated groups (Figure- 78, Table- 78).

cAMP content in the brain stem of experimental rats

cAMP content significantly ($p<0.001$) increased in diabetic group when compared with control. cAMP content in the brain stem were kept at a near control level ($p<0.001$) by the Curcumin and vitamin D_3 pre-treatment (Figure- 79, Table- 79).

cGMP content in the brain stem of experimental rats

Compared to control, diabetic group showed a significant ($p<0.01$) increase in the cGMP content in the brain stem. Curcumin and vitamin D_3 pre-treated rats were able to maintain a near control level of cGMP in there brain stem when compared with diabetic group ($p<0.01$; Figure- 80, Table- 80).

IP3 content in the brain stem of experimental rats

The IP3 content in the brain stem of diabetic rats showed a significant decrease ($p<0.001$) when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) retained a near control level of IP3 when compared with diabetic group (Figure- 81, Table- 81).

Figure-54

Scatchard analysis of total adrenergic receptor using [^{3}H] epinephrine binding against epinephrine in the brain stem of experimental rats

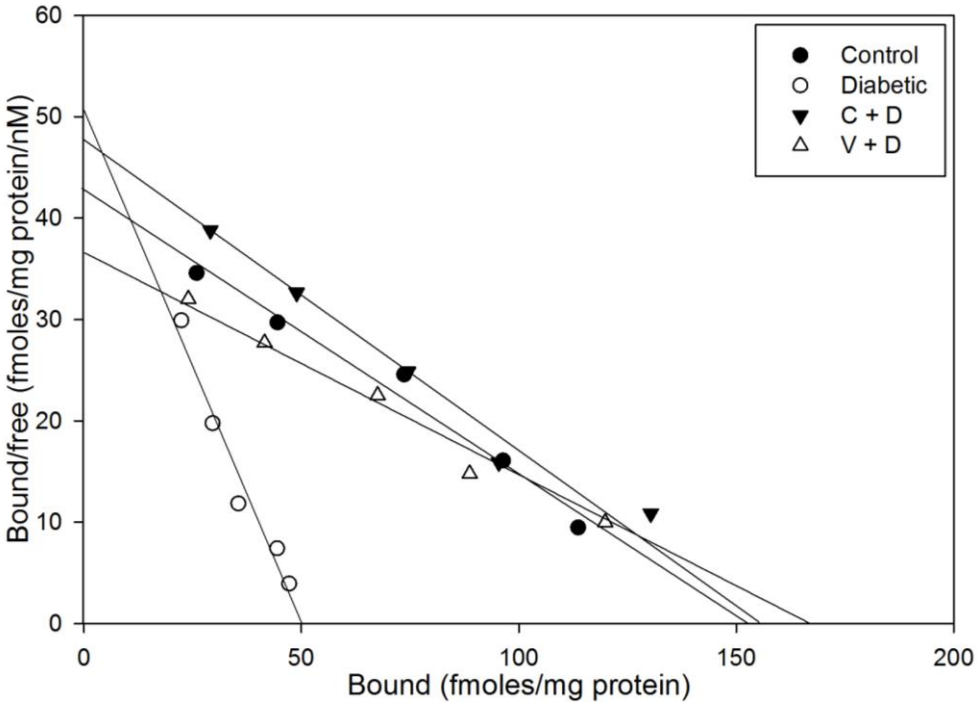


Table-54

Scatchard analysis of total adrenergic receptor using [^{3}H] epinephrine binding against epinephrine in the brain stem of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	152.14 ± 6.73	3.58 ± 0.17
Diabetic	50.48 ± 2.98 **a**	0.99 ± 0.06 **a**
C + D	156.58 ± 8.67 **d**	3.26 ± 0.15 **d**
V + D	165.81 ± 6.94 **d**	4.52 ± 0.22 **b, d, h**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **b** $p<0.01$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. **h** $p<0.01$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-55

Scatchard analysis of α2 adrenergic receptor using [^{3}H] yohimbine binding against phentolamine in the brain stem of experimental rats

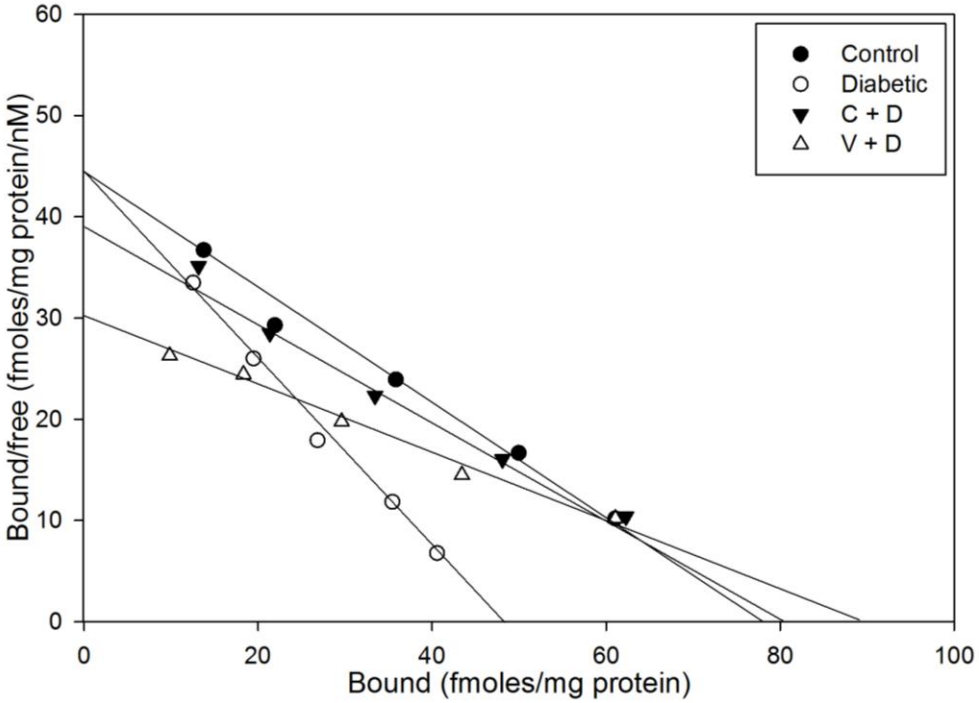


Table-55

Scatchard analysis of α2 adrenergic receptor using [^{3}H] yohimbine binding against phentolamine in the brain stem of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	77.04 ± 3.70	1.77 ± 0.05
Diabetic	47.74 ± 2.08 **a**	1.15 ± 0.08 **b**
C + D	79.61 ± 3.14 **d**	2.06 ± 0.14 **e**
V + D	90.97 ± 5.52 **d**	3.06 ± 0.19 **a, d, g**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$, **b** $p<0.01$ when compared to Control. **d** $p<0.001$, **e** $p<0.01$ when compared to Diabetic group. **g** $p<0.001$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-56

Real Time PCR amplification of α2 adrenergic receptor mRNA in the brain stem of experimental rats

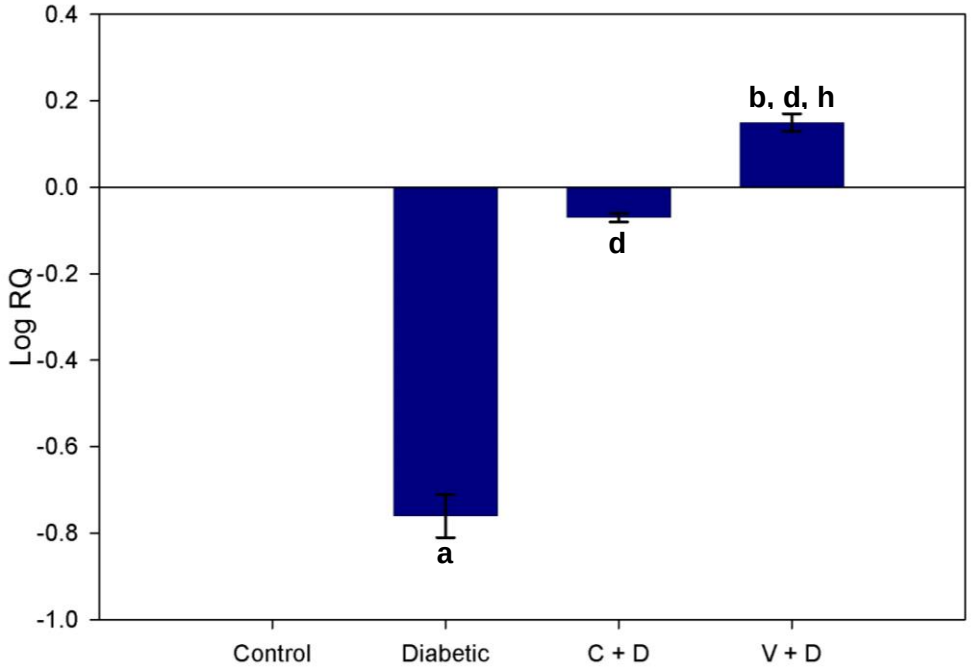


Table-56

Real Time PCR amplification of α2 adrenergic receptor mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.76 ± 0.05 **a**
C + D	-0.07 ± 0.01 **d**
V + D	0.15 ± 0.02 **b, d, h**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001, **b**p<0.01 when compared to Control. **d**p<0.001 when compared to Diabetic group. **h**p<0.01 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-57

Confocal imaging of α2 adrenergic receptor in the brain stem of experimental rats

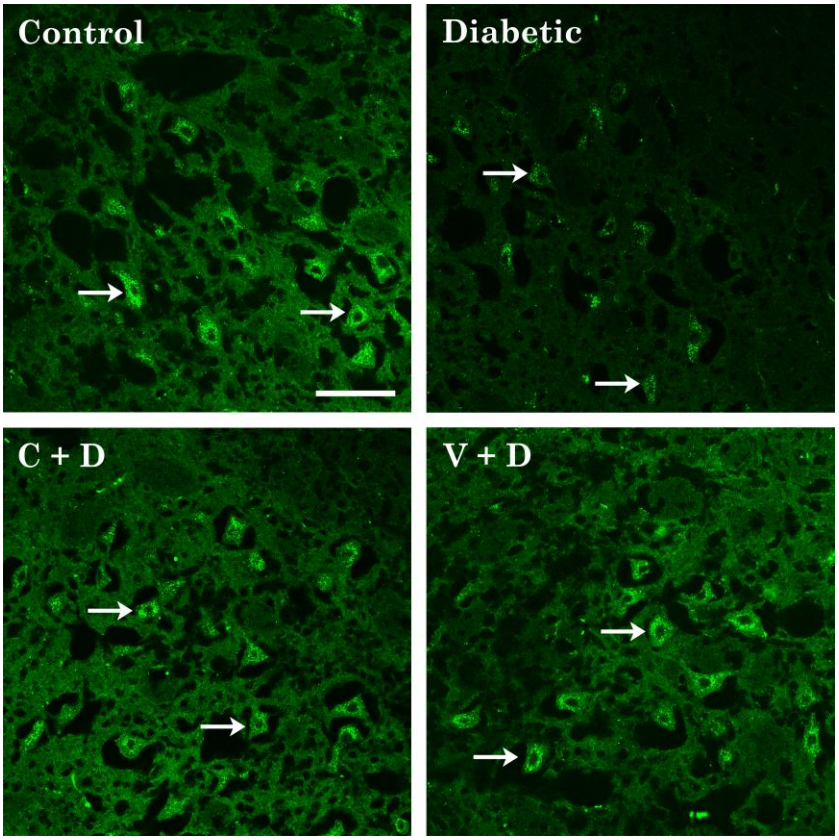


Table-57

Confocal imaging of α2 adrenergic receptor in the brain stem of experimental rats

Experimental groups	Mean Pixel Intensity
Control	29.12 ± 1.59
Diabetic	19.86 ± 1.16 **b**
C + D	29.59 ± 1.13 **e**
V + D	30.49 ± 1.28 **e**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$, when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⇨ shows α2 adrenergic receptors. Scale bar represents 50µm.

Figure-58
Scatchard analysis of β adrenergic receptor using [^{3}H] propranolol binding against propranolol in the brain stem of experimental rats

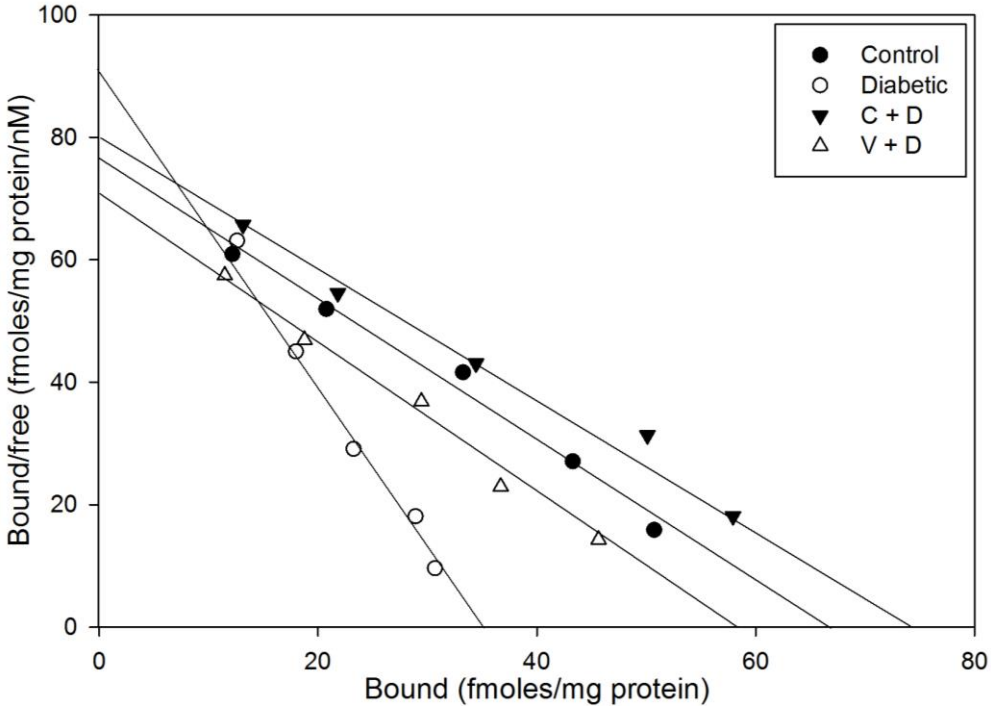


Table-58

Scatchard analysis of β adrenergic receptor using [^{3}H] propranolol binding against propranolol in the brain stem of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	65.68 ± 3.88	0.88 ± 0.04
Diabetic	36.56 ± 2.79 **b**	0.40 ± 0.03 **a**
C + D	73.83 ± 4.30 **d**	0.92 ± 0.04 **d**
V + D	57.35 ± 3.09 **e, i**	0.83 ± 0.04 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001, **b**p<0.01 when compared to Control. **d**p<0.001, **e**p<0.01 when compared to Diabetic group. **i**p<0.05 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-59

Real Time PCR amplification of β2 adrenergic receptor mRNA in the brain stem of experimental rats

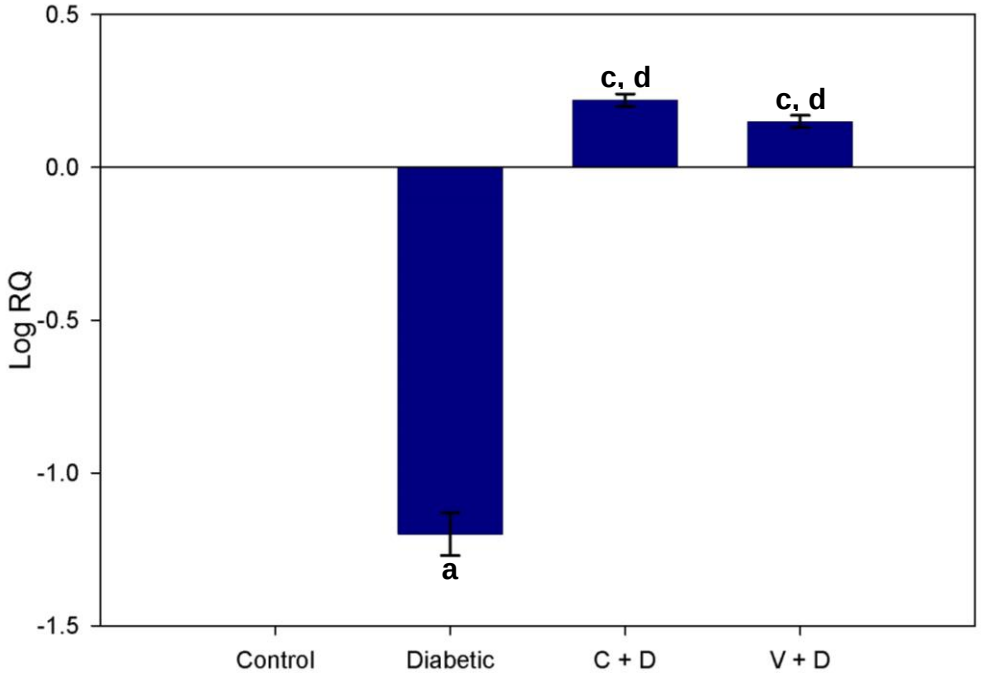


Table-59

Real Time PCR amplification of β2 adrenergic receptor mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.20 ± 0.07 **a**
C + D	0.22 ± 0.02 **c, d**
V + D	0.15 ± 0.02 **c, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, **c** $p<0.05$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-60
Confocal imaging of β2 adrenergic receptor in the brain stem of experimental rats

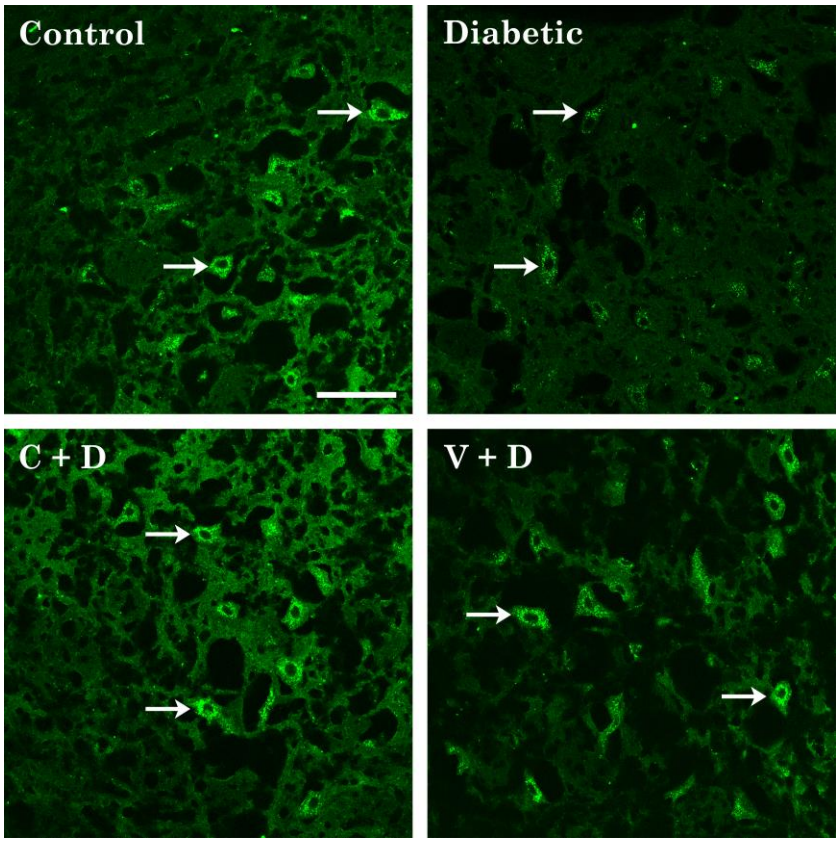


Table-60
Confocal imaging of β2 adrenergic receptor in the brain stem of experimental rats

Experimental groups	Mean Pixel Intensity
Control	25.89 ± 1.21
Diabetic	15.29 ± 1.16 **a**
C + D	26.37 ± 1.01 **d**
V + D	30.12 ± 1.10 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⟹ shows β2 adrenergic receptors. Scale bar represents 50µm.

Figure-61
Scatchard analysis of total muscarinic receptor using [^{3}H] QNB binding against atropine in the brain stem of experimental rats

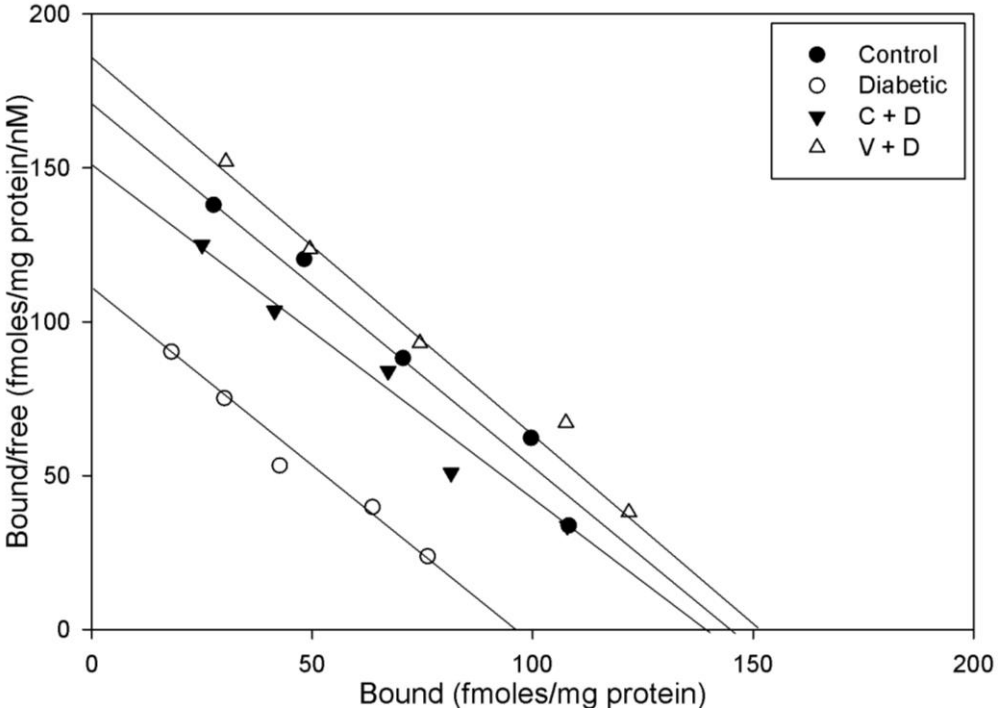


Table-61

Scatchard analysis of total muscarinic receptor using [^{3}H] QNB binding against atropine in the brain stem of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	145.58 ± 6.62	0.86 ± 0.04
Diabetic	94.76 ± 6.69 **b**	0.89 ± 0.02
C + D	139.51 ± 5.90 **e**	0.93 ± 0.04
V + D	150.66 ± 8.72 **e**	0.81 ± 0.04

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$ when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-62

Scatchard analysis of muscarinic M1 receptor using [^{3}H] QNB binding against pirenzepine in the brain stem of experimental rats

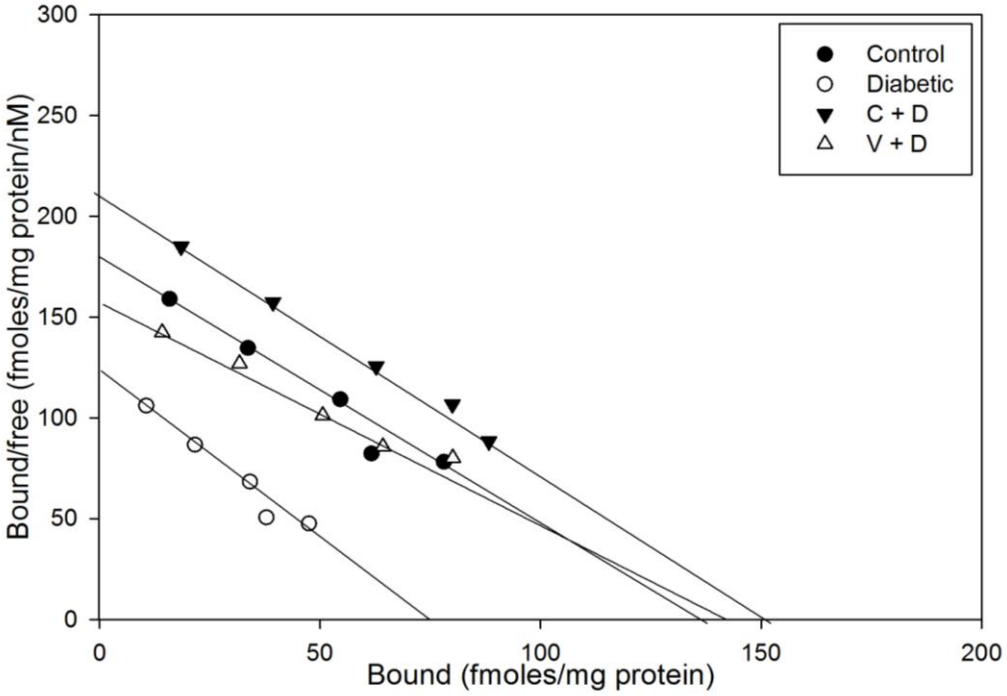


Table-62

Scatchard analysis of muscarinic M1 receptor using [^{3}H] QNB binding against pirenzepine in the brain stem of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	136.93 ± 7.34	0.77 ± 0.04
Diabetic	74.60 ± 3.76 [a]	0.61 ± 0.03
C + D	152.15 ± 8.40 [d]	0.72 ± 0.03
V + D	143.35 ± 7.34 [d]	0.92 ± 0.05 [c, e, i]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
[a]$p<0.001$, [c]$p<0.05$ when compared to Control. [d]$p<0.001$, [e]$p<0.01$ when compared to Diabetic group. [i]$p<0.05$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-63

Real Time PCR amplification of muscarinic M1 receptor mRNA in the brain stem of experimental rats

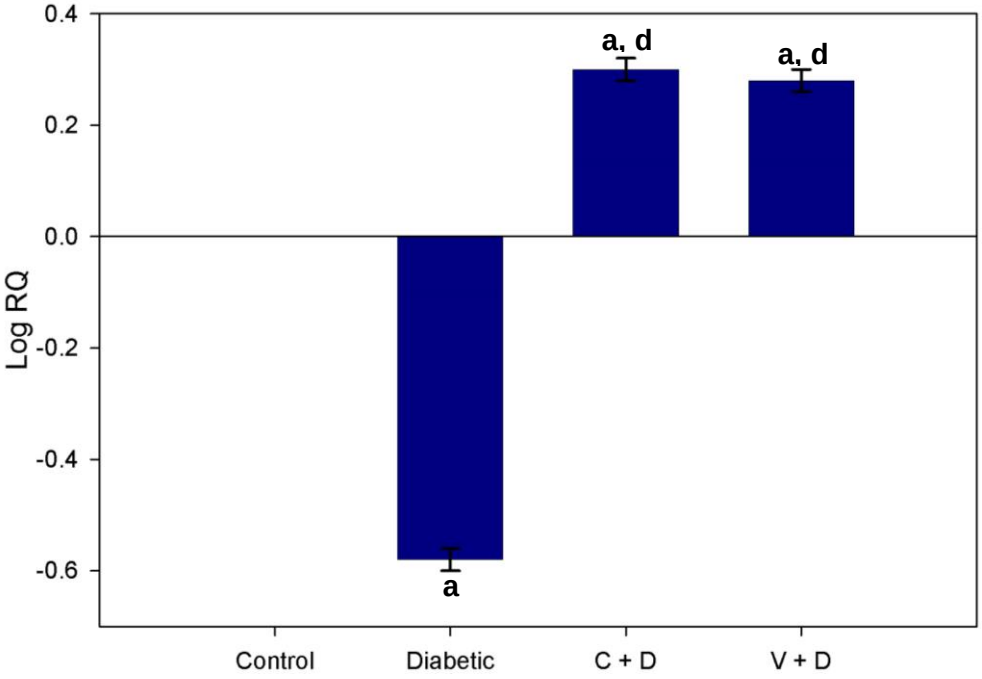


Table-63

Real Time PCR amplification of muscarinic M1 receptor mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.58 ± 0.02 **a**
C + D	0.30 ± 0.02 **a, d**
V + D	0.28 ± 0.02 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-64
Confocal imaging of muscarinic M1 receptor in the brain stem of experimental rats

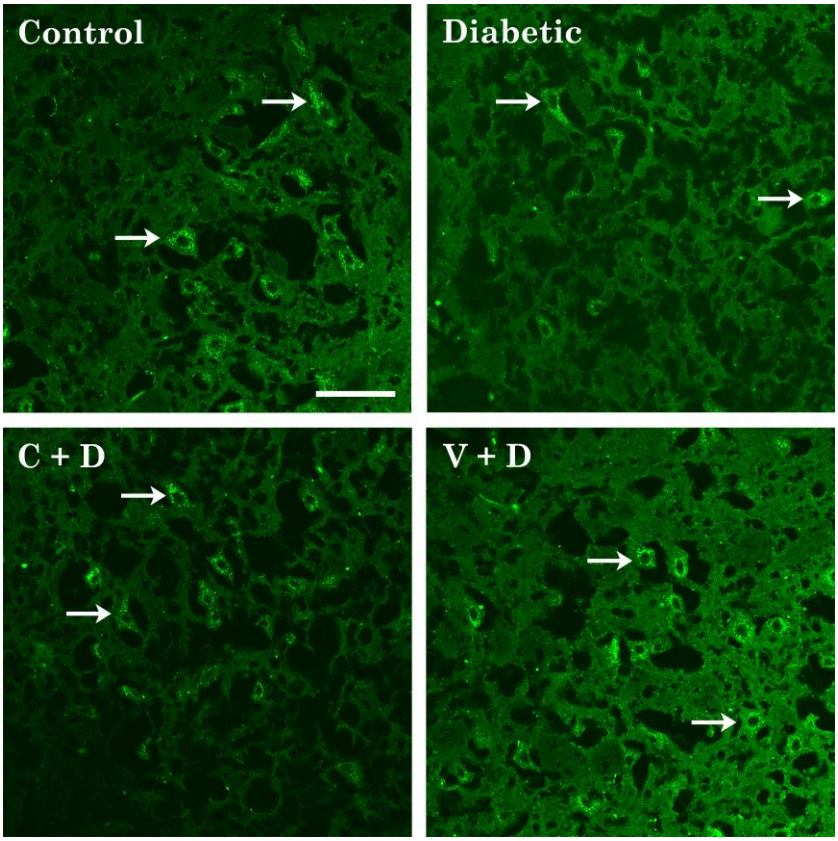


Table-64
Confocal imaging of muscarinic M1 receptor in the brain stem of experimental rats

Experimental groups	Mean Pixel Intensity
Control	28.35 ± 1.05
Diabetic	13.61 ± 0.72 **a**
C + D	25.98 ± 1.41 **d**
V + D	26.36 ± 1.19 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⇨ shows muscarinic M1 receptors. Scale bar represents 50µm.

Figure-65
Scatchard analysis of muscarinic M3 receptor using [^{3}H] DAMP binding against 4-DAMP mustard in the brain stem of experimental rats

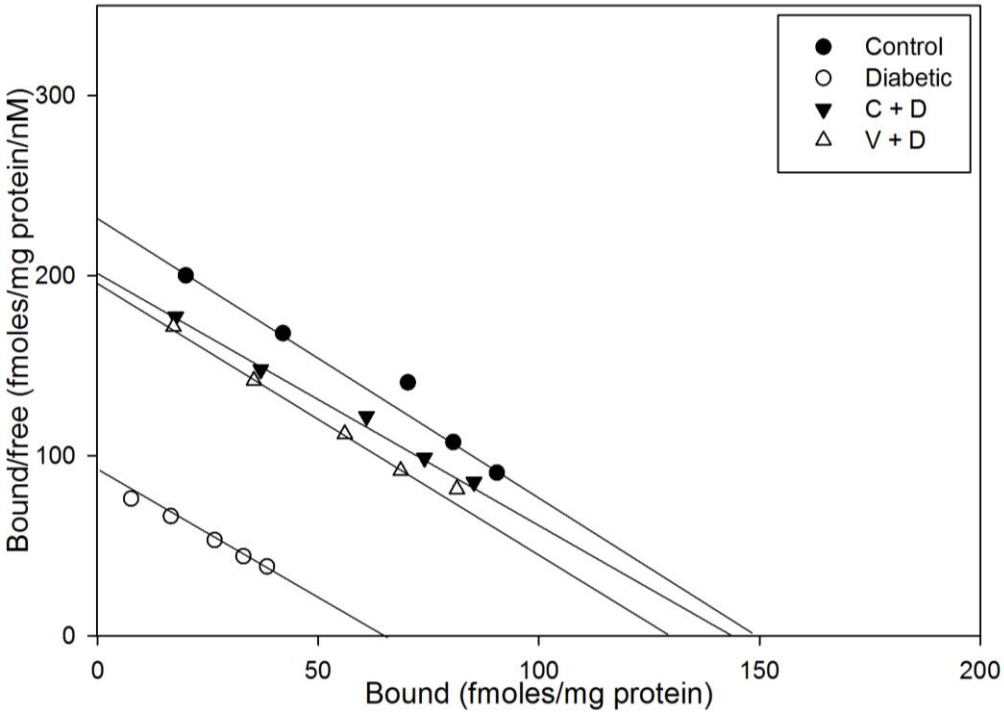


Table-65

Scatchard analysis of muscarinic M3 receptor using [^{3}H] DAMP binding against 4-DAMP mustard in the brain stem of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	148.72 ± 8.05	0.63 ± 0.04
Diabetic	63.13 ± 4.92 [a]	0.69 ± 0.04
C + D	143.40 ± 6.74 [d]	0.71 ± 0.03
V + D	128.23 ± 7.50 [d]	0.67 ± 0.04

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-66

Real Time PCR amplification of muscarinic M3 receptor mRNA in the brain stem of experimental rats

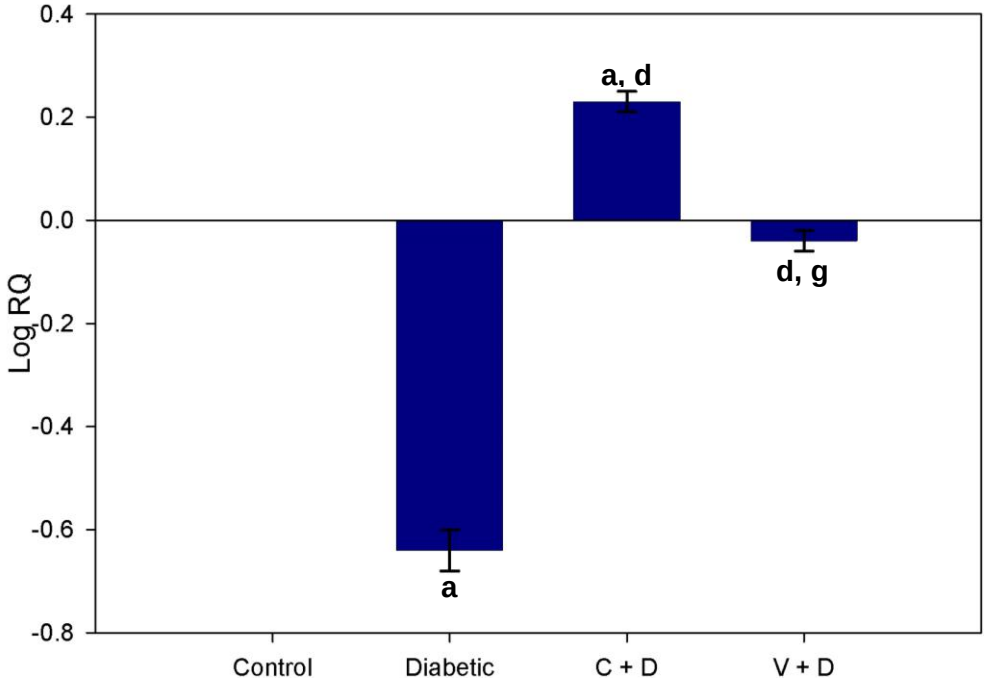


Table-66

Real Time PCR amplification of muscarinic M3 receptor mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.64 ± 0.04 **a**
C + D	0.23 ± 0.02 **a, d**
V + D	-0.04 ± 0.02 **d, g**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group. **g**p<0.001 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-67
Confocal imaging of muscarinic M3 receptor in the brain stem of experimental rats

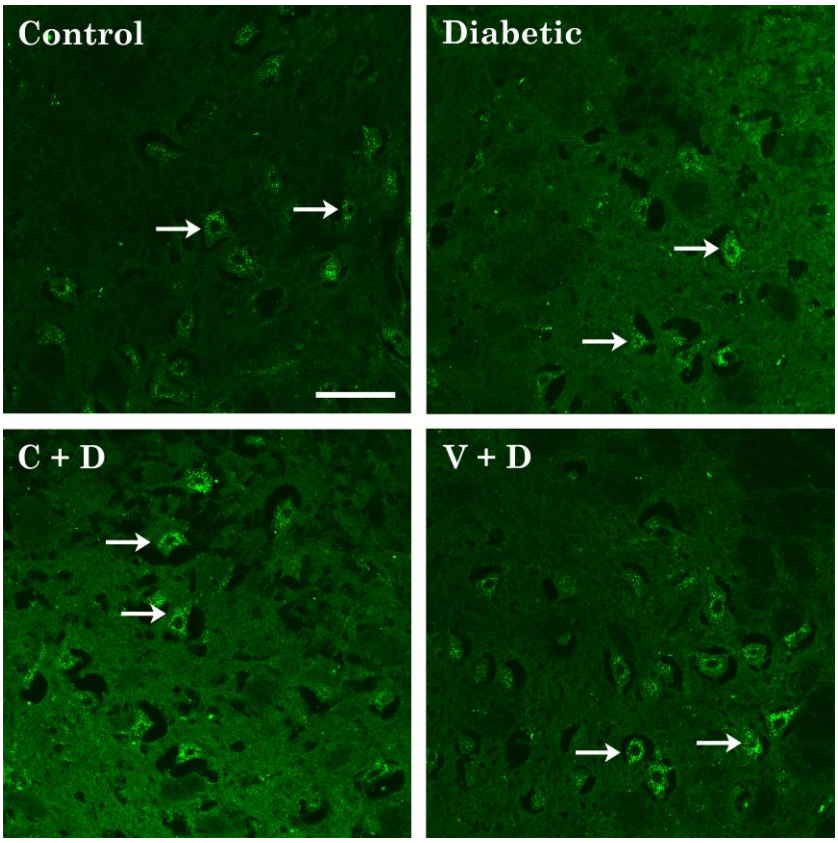


Table-67
Confocal imaging of muscarinic M3 receptor in the brain stem of experimental rats

Experimental groups	Mean Pixel Intensity
Control	22.06 ± 1.23
Diabetic	16.23 ± 1.16 **b**
C + D	26.17 ± 1.30 **e**
V + D	22.84 ± 1.22 **f**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$, when compared to Control. **e** $p<0.01$, **f** $p<0.05$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⇨ shows muscarinic M3 receptors. Scale bar represents 50µm.

Figure-68

Real Time PCR amplification of choline acetyltransferase mRNA in the brain stem of experimental rats

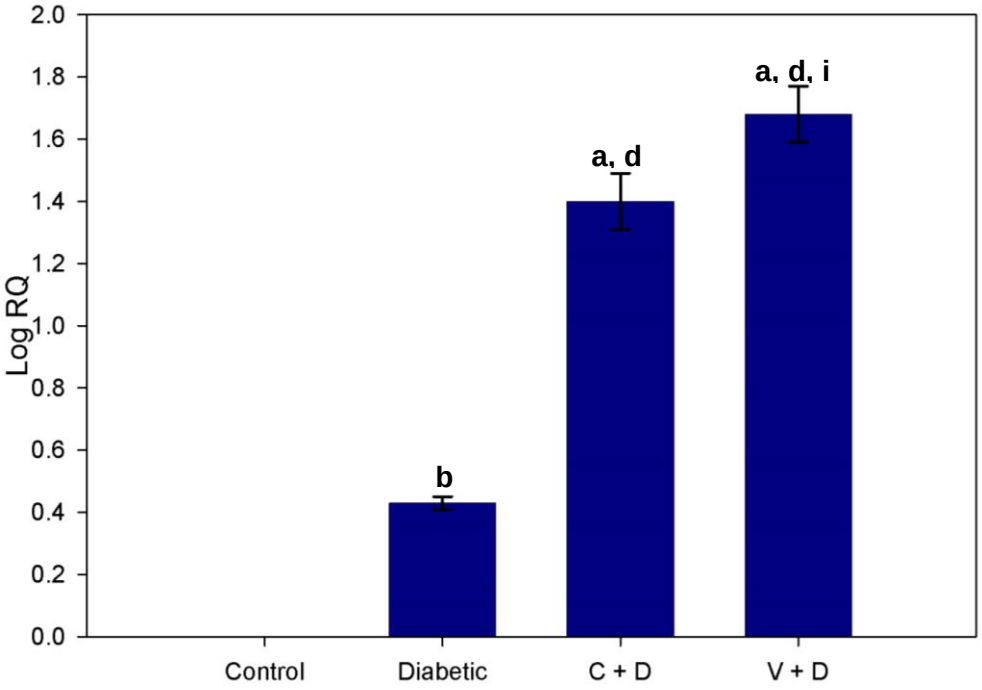


Table-68

Real Time PCR amplification of choline acetyltransferase mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.43 ± 0.02 **b**
C + D	1.40 ± 0.09 **a, d**
V + D	1.68 ± 0.09 **a, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**$p<0.001$, **b**$p<0.01$ when compared to Control. **d**$p<0.001$ when compared to Diabetic group. **i**$p<0.05$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-69

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

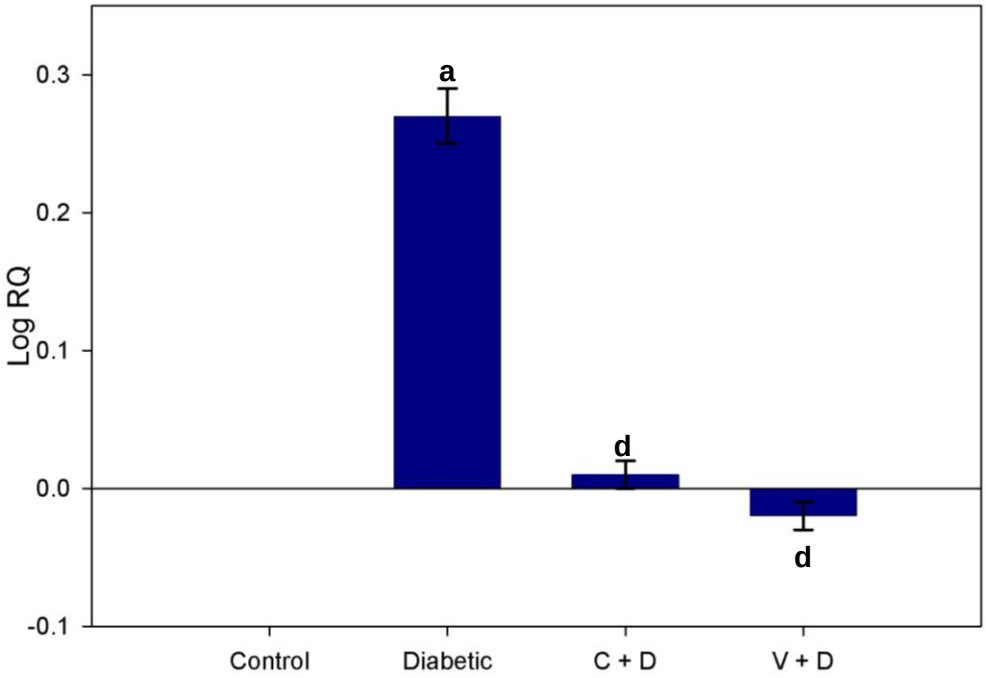


Table-69

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

Experimental groups	Log RQ
Control	0
Diabetic	0.27 ± 0.02 **a**
C + D	0.01 ± 0.01 **d**
V + D	-0.02 ± 0.01 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-70

Real Time PCR amplification of muscarinic M2 receptor mRNA in the brain stem of experimental rats

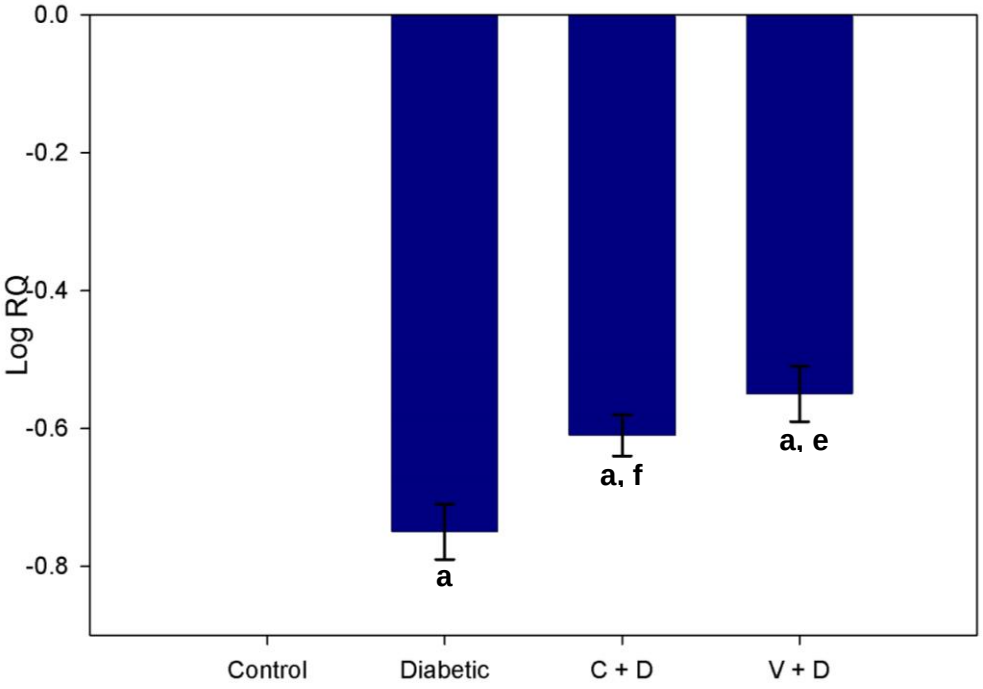


Table-70

Real Time PCR amplification of muscarinic M2 receptor mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.75 ± 0.04 **a**
C + D	-0.61 ± 0.03 **a, f**
V + D	-0.55 ± 0.04 **a, e**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$ when compared to Control. **e** $p<0.01$, **f** $p<0.05$ when compared to Diabetic group. C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-71

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the brain stem of experimental rats

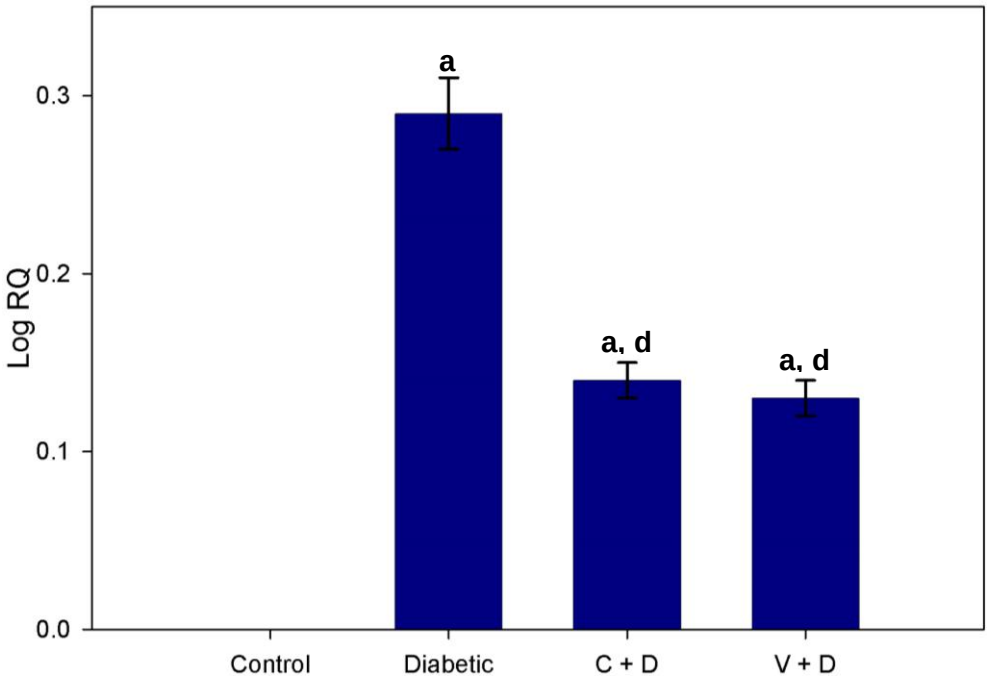


Table-71

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.29 ± 0.02 **a**
C + D	0.14 ± 0.01 **a, d**
V + D	0.13 ± 0.01 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-72

Real Time PCR amplification of vitamin D receptor mRNA in the brain stem of experimental rats

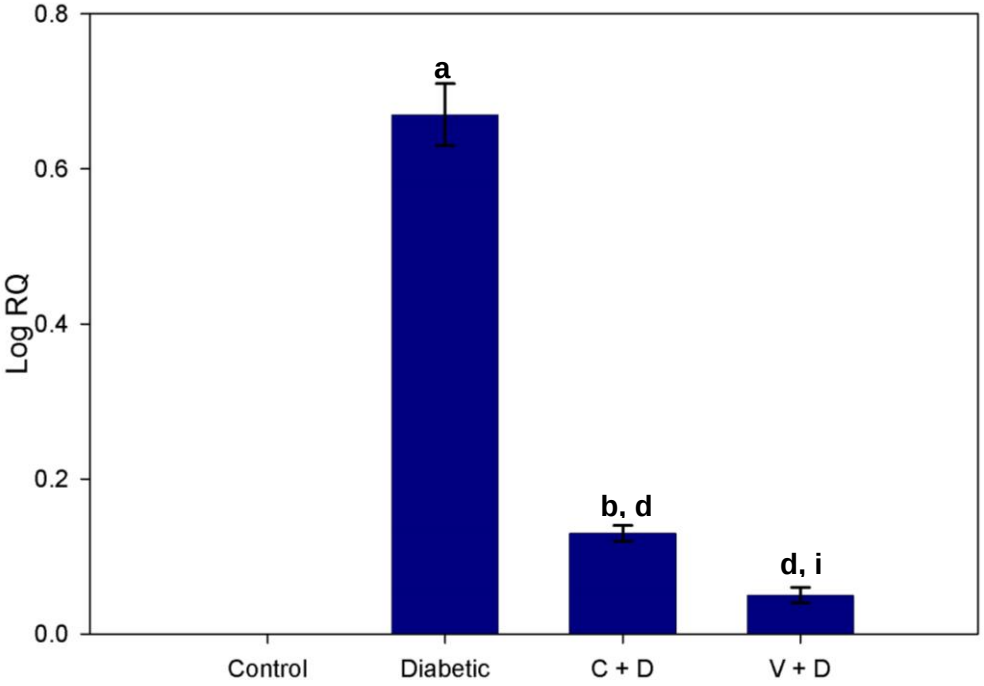


Table-72

Real Time PCR amplification of vitamin D receptor mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.67 ± 0.04 **a**
C + D	0.13 ± 0.01 **b, d**
V + D	0.05 ± 0.01 **d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001, **b**p<0.01 when compared to Control. **d**p<0.001 when compared to Diabetic group. **i**p<0.05 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-73

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

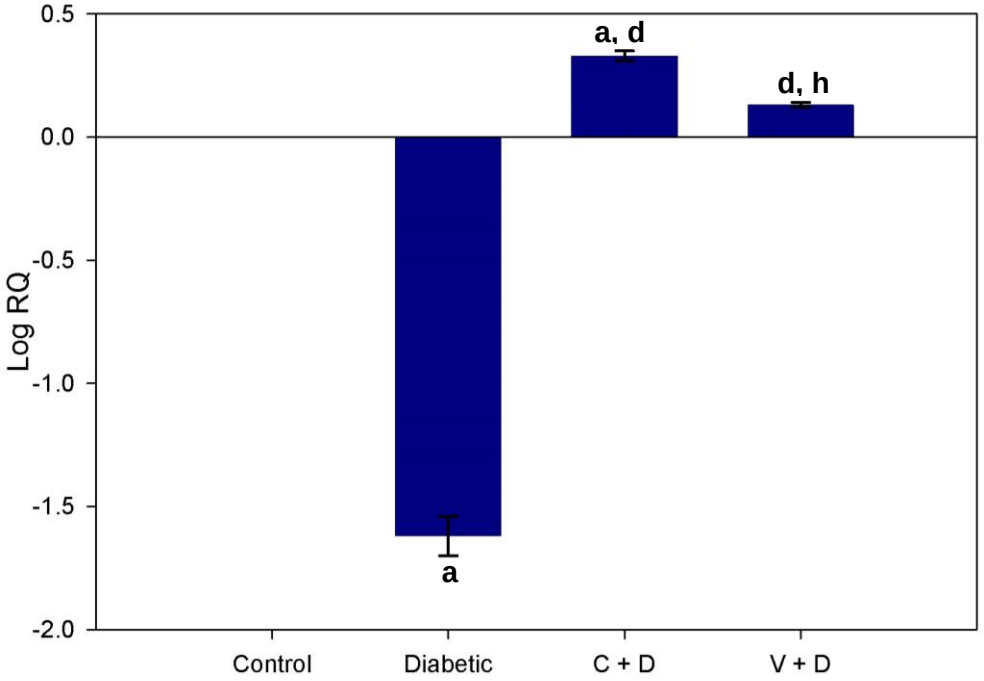


Table-73

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

Experimental groups	Log RQ
Control	0
Diabetic	-1.62 ± 0.08 **a**
C + D	0.33 ± 0.02 **a, d**
V + D	0.13 ± 0.01 **d, h**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group. **h**p<0.01 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-74

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

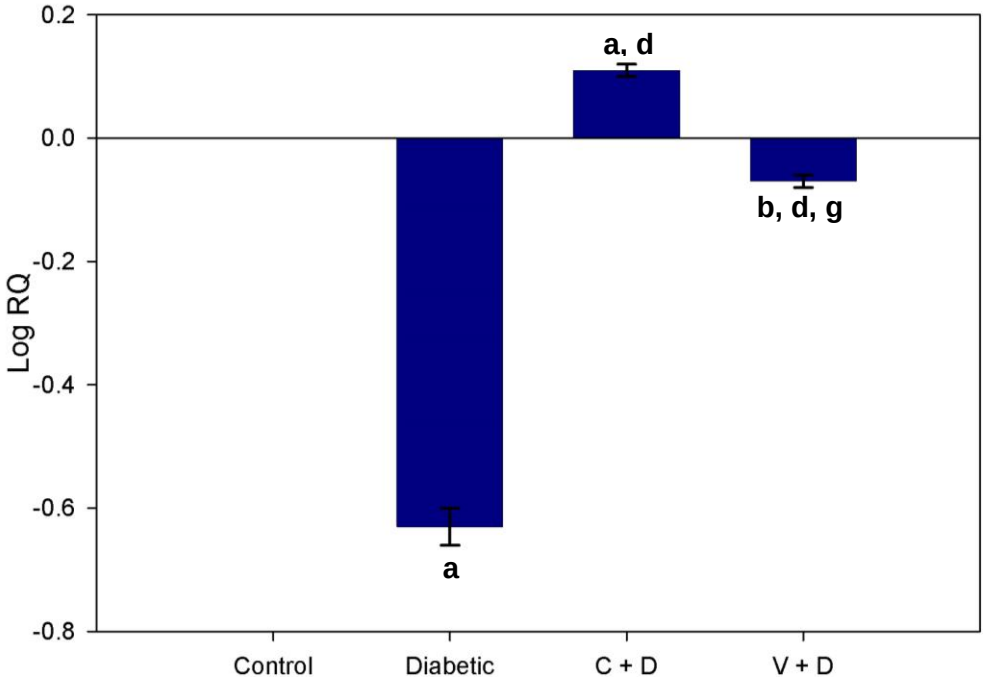


Table-74

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

Experimental groups	Log RQ
Control	0
Diabetic	-0.63 ± 0.03 **a**
C + D	0.11 ± 0.01 **a, d**
V + D	-0.07 ± 0.01 **b, d, g**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**$p<0.001$, **b**$p<0.01$ when compared to Control. **d**$p<0.001$ when compared to Diabetic group. **g**$p<0.001$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-75

Real Time PCR amplification of insulin receptor mRNA in the brain stem of experimental rats

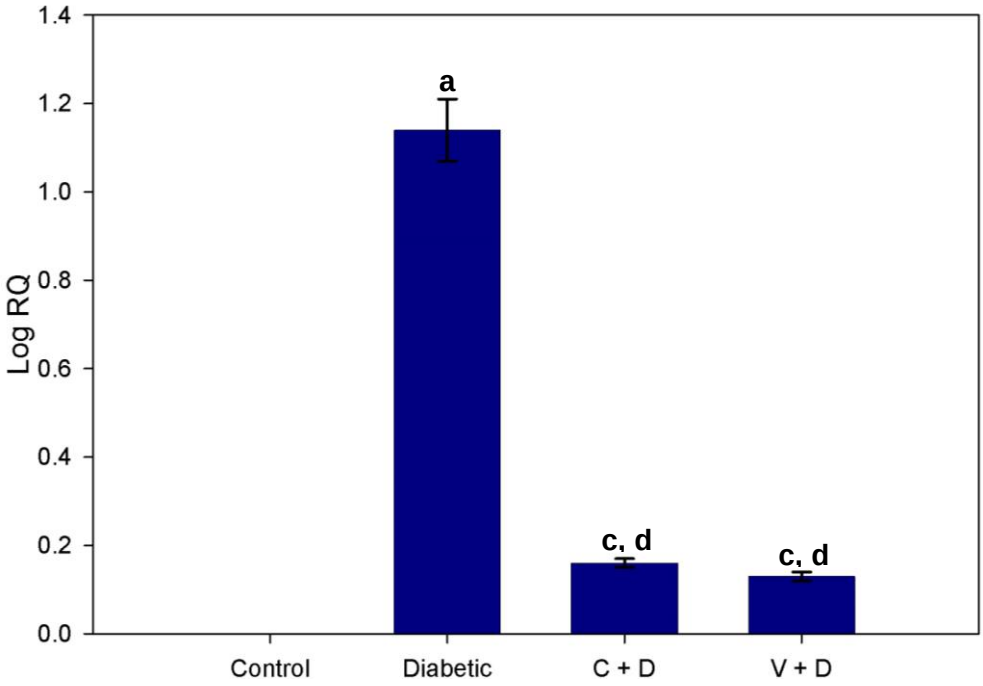


Table-75

Real Time PCR amplification of insulin receptor mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.14 ± 0.07 **a**
C + D	0.16 ± 0.01 **c, d**
V + D	0.13 ± 0.01 **c, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a$p<0.001$, **c**$p<0.01$ when compared to Control. **d**$p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-76

Real Time PCR amplification of GLUT 3 mRNA in the brain stem of experimental rats

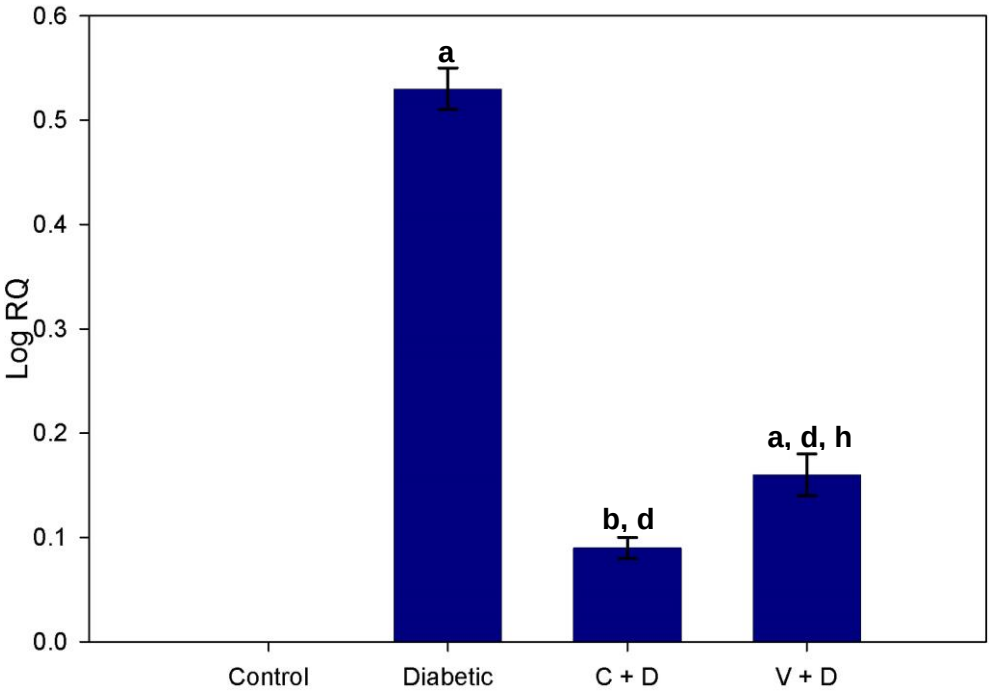


Table-76

Real Time PCR amplification of GLUT 3 mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.53 ± 0.02 **a**
C + D	0.09 ± 0.01 **b, d**
V + D	0.16 ± 0.02 **a, d, h**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**$p<0.001$, **b**$p<0.01$ when compared to Control. **d**$p<0.001$ when compared to Diabetic group. **h**$p<0.01$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-77

Real Time PCR amplification of superoxide dismutases mRNA in the brain stem of experimental rats

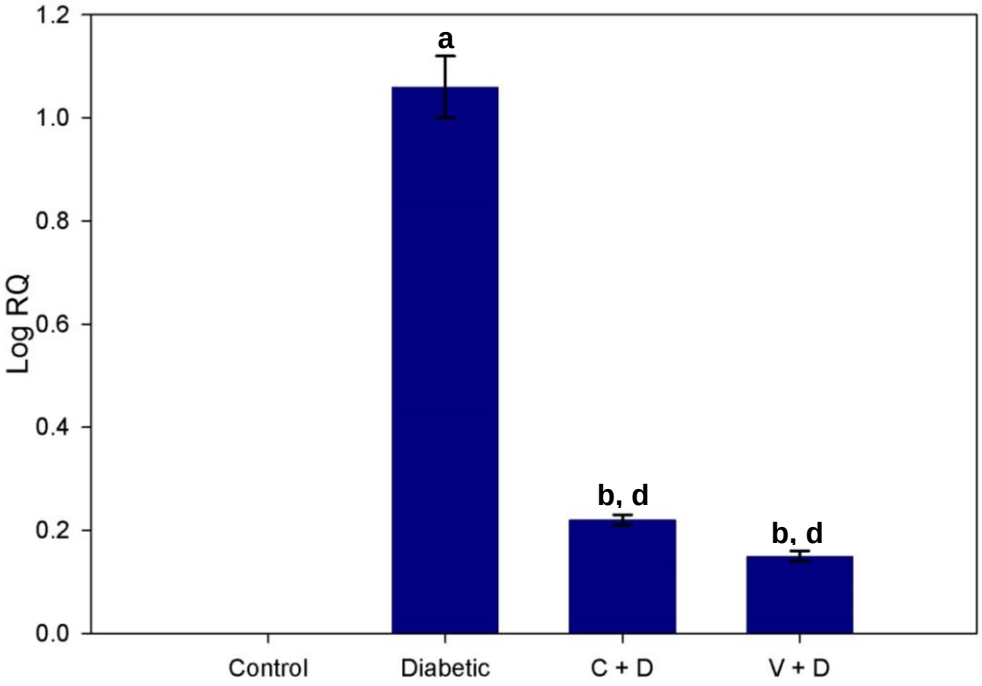


Table-77

Real Time PCR amplification of superoxide dismutases mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.06 ± 0.06 **a**
C + D	0.22 ± 0.01 **b, d**
V + D	0.15 ± 0.01 **b, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$, **b** $p<0.01$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-78

Real Time PCR amplification of glutathione peroxidase mRNA in the brain stem of experimental rats

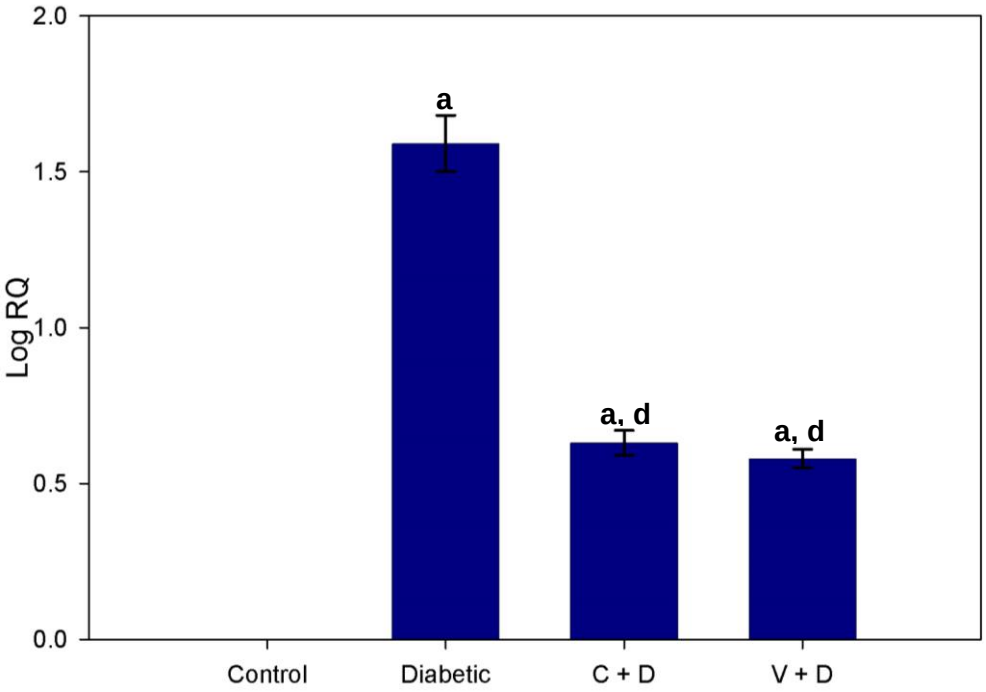


Table-78

Real Time PCR amplification of glutathione peroxidase mRNA in the brain stem of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.59 ± 0.09 **a**
C + D	0.63 ± 0.04 **a, d**
V + D	0.58 ± 0.03 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-79

cAMP content in the brain stem of experimental rats

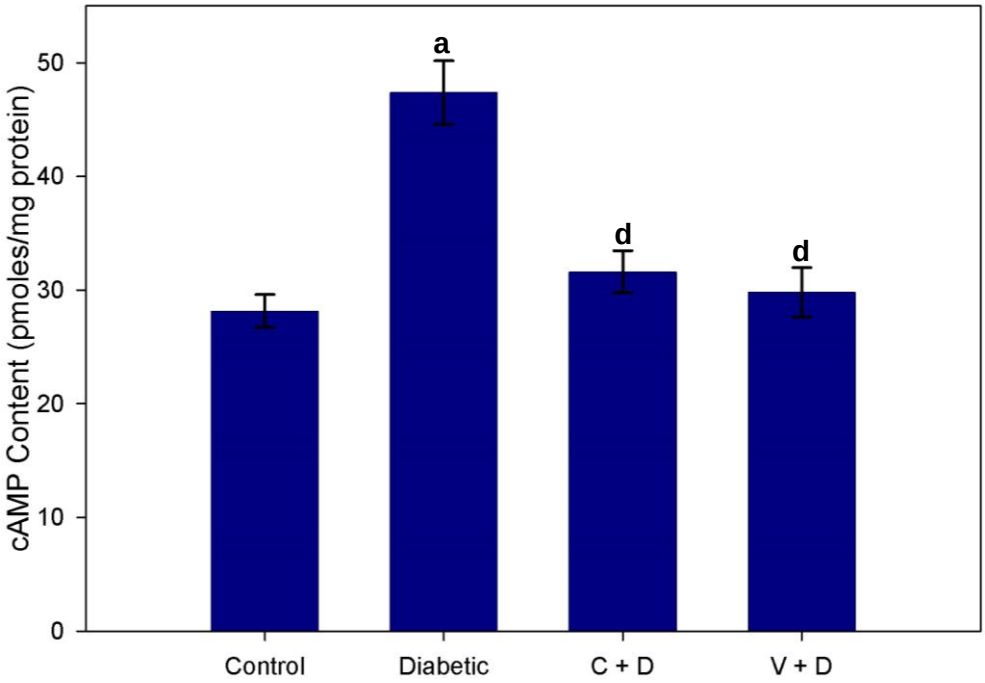


Table-79

cAMP content in the brain stem of experimental rats

Experimental groups	cAMP content (pmoles/mg protein)
Control	28.17 ± 1.45
Diabetic	47.38 ± 2.79 **a**
C + D	31.61 ± 1.86 **d**
V + D	29.82 ± 2.17 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-80

cGMP content in the brain stem of experimental rats

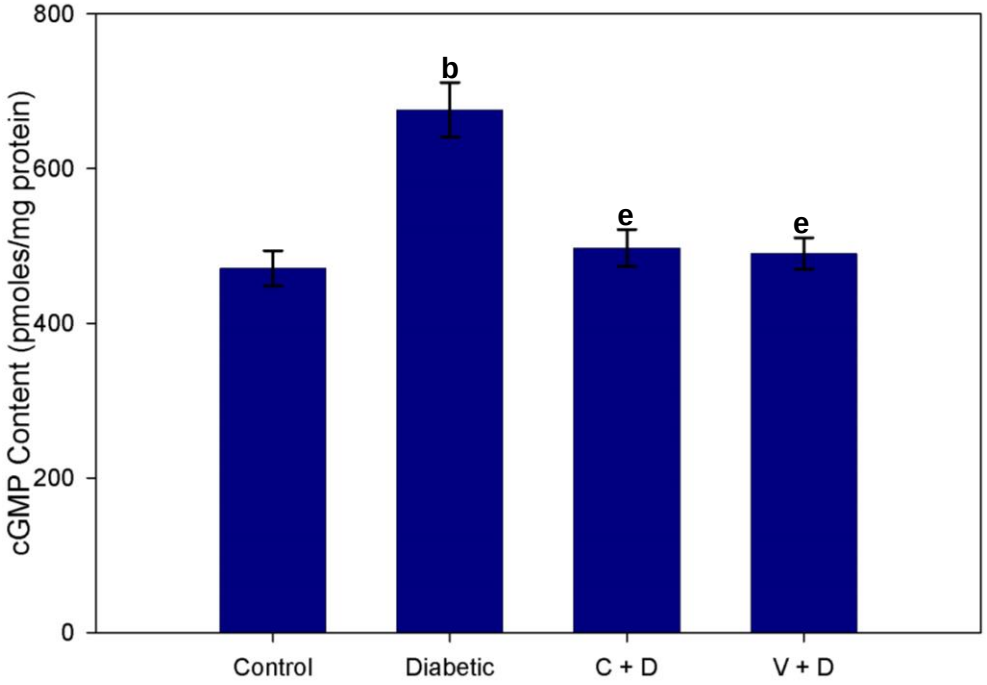


Table-80

cGMP content in the brain stem of experimental rats

Experimental groups	cGMP content (pmoles/mg protein)
Control	470.92 ± 22.65
Diabetic	675.89 ± 35.39 **b**
C + D	497.29 ± 23.92 **e**
V + D	490.18 ± 20.25 **e**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$ when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-81

IP3 content in the brain stem of experimental rats

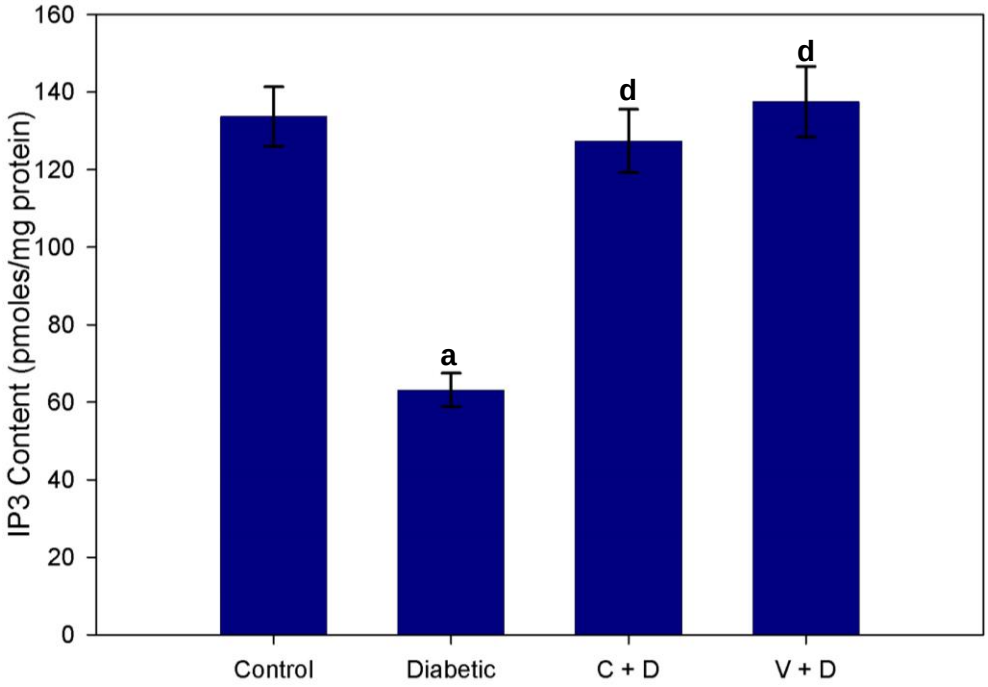


Table-81

IP3 content in the brain stem of experimental rats

Experimental groups	IP3 content (pmoles/mg protein)
Control	133.72 ± 7.67
Diabetic	63.17 ± 4.35 **a**
C + D	127.41 ± 8.15 **d**
V + D	137.55 ± 9.10 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a$p<0.001$ when compared to Control. **d**$p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

HIPPOCAMPUS

Scatchard analysis of total adrenergic receptor using [^{3}H] epinephrine binding against epinephrine in the hippocampus of experimental rats

In diabetic group, total adrenergic receptors B_{max} and K_d were significantly ($p<0.001$) increased when compared with control. Rats pre-treated with curcumin and vitamin D_3 showed a significant ($p<0.001$) decrease in B_{max} and K_d when compared with diabetic rats. K_d of V + D showed a significant ($p<0.05$) increase when compared with C + D (Figure- 82, Table- 82).

Scatchard analysis of α2 adrenergic receptor using [^{3}H] yohimbine binding against phentolamine in the hippocampus of experimental rats

α2 adrenergic receptors B_{max} and K_d were significantly ($p<0.001$) increased in diabetic group when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) retained the B_{max} and K_d to near control level when compared with diabetic group (Figure- 83, Table- 83).

Real Time PCR amplification of α2 adrenergic receptor mRNA in the hippocampus of experimental rats

Gene expression of α2 adrenergic receptor mRNA showed a significant up regulation in the hippocampus of diabetic ($p<0.001$), C + D ($p<0.001$) and V + D ($p<0.01$) when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) decreased the gene expression when compared with diabetic group. V + D rats also showed a significant ($p<0.05$) decrease in α2 adrenergic receptor mRNA levels in the hippocampus when compared with C + D (Figure- 84, Table- 84).

Confocal imaging of α2 adrenergic receptor in the hippocampus of experimental rats

Confocal microscopic image of α adrenergic receptor in the hippocampus of diabetic group showed a significant ($p<0.001$) increase in the mean pixel

intensity when compared with control. The mean pixel intensity was significantly ($p<0.001$) retained at near control in pre-treated rats when compared with diabetic group (Figure- 85, Table- 85).

Scatchard analysis of β adrenergic receptor using [^{3}H] propranolol binding against propranolol in the hippocampus of experimental rats

In diabetic group, B_{max} and K_d of β adrenergic receptors were significantly increased ($p<0.001$) when compared with control. Vitamin D_3 pre-treated group also showed a significant ($p<0.05$) increase in B_{max} when compared with control. When compared with diabetic group, C + D ($p<0.01$) and V + D ($p<0.05$) groups showed a significant decrease in B_{max}. K_d of pre-treated groups were significantly ($p<0.001$) decreased towards near control when compared with diabetic group (Figure- 86, Table- 86).

Real Time PCR amplification of β2 adrenergic receptor mRNA in the hippocampus of experimental rats

Gene expression of β2 adrenergic receptor mRNA showed a significant up regulation in the hippocampus of diabetic ($p<0.001$), C + D ($p<0.001$) and V + D ($p<0.01$) rats when compared with control. When compared with diabetic group, pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) decreased the β2 adrenergic receptor expression (Figure- 87, Table- 87).

Confocal imaging of β2 adrenergic receptor in the hippocampus of experimental rats

β adrenergic receptor antibody staining in the hippocampus of diabetic rats showed a significant ($p<0.001$) increase in mean pixel intensity values when compared with control. Curcumin and vitamin D_3 pre-treatment significantly ($p<0.001$) maintained the mean pixel intensity values to near control when compared with diabetic group (Figure- 88, Table- 88).

Scatchard analysis of total muscarinic receptor using [^{3}H] QNB binding against atropine in the hippocampus of experimental rats

Scatchard analysis of total muscarinic receptor using [^{3}H] QNB binding against atropine in the hippocampus of diabetic rats showed a significant ($p<0.001$) decrease in B_{max} and K_d when compared with control. Curcumin pre-treated group showed a significant ($p<0.05$) decrease in K_d when compared with control. Curcumin and vitamin D_3 pre-treatments were able to significantly ($p<0.001$) increased B_{max} when compared with diabetic group. In C + D ($p<0.01$) and V + D ($p<0.001$) groups total muscarinic receptors K_d was significantly increased when compared with diabetic group (Figure- 89, Table- 89).

Scatchard analysis of muscarinic M1 receptor using [^{3}H] QNB binding against pirenzepine in the hippocampus of experimental rats

Binding studies of [^{3}H] QNB against pirenzepine for muscarinic M1 receptor showed that the binding parameters B_{max} ($p<0.001$) and K_d ($p<0.01$) were significantly decreased in the hippocampus of diabetic group when compared with control. Pre-treatment using curcumin and vitamin D_3 kept the B_{max} ($p<0.001$) to near control when compared with diabetic group. K_d of C + D ($p<0.01$) and V + D ($p<0.05$) groups were significantly increased when compared with diabetic group (Figure- 90, Table- 90).

Real Time PCR amplification of muscarinic M1 receptor mRNA in the hippocampus of experimental rats

Real time PCR gene expression of muscarinic M1 receptor showed a significant down regulation in the hippocampus of diabetic ($p<0.001$), C + D ($p<0.01$) and V + D ($p<0.05$) rats when compared with control. When compared with diabetic group, curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) increase in the muscarinic M1 receptor mRNA in the hippocampus (Figure- 91, Table- 91).

Confocal imaging of muscarinic M1 receptor in the hippocampus of experimental rats

Confocal imaging of muscarinic M1 receptor in the hippocampus of diabetic rats showed a significant ($p<0.001$) decrease in mean pixel intensity when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.01$) increased the mean pixel intensity value when compared with diabetic group (Figure- 92, Table- 92).

Scatchard analysis of muscarinic M3 receptor using [^{3}H] DAMP binding against 4-DAMP mustard in the hippocampus of experimental rats

Scatchard analysis of muscarinic M3 receptor using [^{3}H] DAMP binding against 4-DAMP mustard in the hippocampus of diabetic rats showed a significant ($p<0.001$) increase in B_{max} when compared with control. Compared to control, C + D also showed a significant ($p<0.05$) increase in B_{max}. Curcumin ($p<0.01$) and vitamin D_3 ($p<0.001$) pre-treated groups showed a significant decrease in B_{max} when compared with diabetic group. When compared with C + D, B_{max} of V + D showed a significant ($p<0.05$) decrease. Diabetic, C + D and V + D groups showed a significant ($p<0.01$) increase in K_d when compared with control group (Figure- 93, Table- 93).

Real Time PCR amplification of muscarinic M3 receptor mRNA in the hippocampus of experimental rats

Muscarinic M3 receptor gene expression showed a significant ($p<0.001$) up regulation in the hippocampus of diabetic, C + D and V + D groups when compared with control. A significant ($p<0.001$) decrease in the muscarinic M3 receptor mRNA was observed in pre-treated groups when compared with diabetic group (Figure- 94, Table- 94).

Confocal imaging of muscarinic M3 receptor in the hippocampus of experimental rats

Muscarinic M3 receptor specific antibody staining in the hippocampus showed a significant increase ($p<0.01$) in mean pixel intensity of diabetic rats when compared with control. Curcumin ($p<0.001$) and vitamin D_3 ($p<0.01$) pre-treatment significantly decreased the mean pixel intensity to near control when compared with diabetic group (Figure- 95, Table- 95).

Real Time PCR amplification of choline acetyltransferase mRNA in the hippocampus of experimental rats

Choline acetyltransferase gene expression showed a significant up regulation in the hippocampus of diabetic ($p<0.001$), C + D ($p<0.01$) and V + D ($p<0.001$) rats when compared with control. When compared with diabetic group, pre-treated groups showed a significant ($p<0.001$) decrease in choline acetyltransferase mRNA expression (Figure- 96, Table- 96).

Real Time PCR amplification of acetylcholinesterase mRNA in the hippocampus of experimental rats

mRNA level expression of acetylcholinesterase showed a significant ($p<0.001$) up regulation in the hippocampus of diabetic group when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) retained the acetylcholinesterase expression to near control when compared with diabetic group (Figure- 97, Table- 97).

Real Time PCR amplification of muscarinic M2 receptor mRNA in the hippocampus of experimental rats

Muscarinic M2 receptor gene expression showed a significant up regulation in the hippocampus of diabetic ($p<0.001$), C + D ($p<0.01$) and V + D ($p<0.001$) groups when compared with control. In pre-treated groups there was a significant ($p<0.001$) decrease when compared with diabetic group. Further, a

significant ($p<0.05$) up regulation was observed in V + D when compared with C + D (Figure- 98, Table- 98).

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the hippocampus of experimental rats

Real time PCR gene expression of α7 nicotinic acetylcholine receptor showed a significant ($p<0.001$) up regulation in the hippocampus of diabetic and pre-treated rats when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) decrease when compared with diabetic group (Figure- 99, Table- 99).

Real Time PCR amplification of vitamin D receptor mRNA in the hippocampus of experimental rats

mRNA level expression of vitamin D receptor showed a significant up regulation in the hippocampus of diabetic ($p<0.01$) and pre-treated ($p<0.001$) groups when compared with control. When compared with diabetic group, curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) increase in vitamin D receptor mRNA expression. Further, vitamin D receptor expression was significantly ($p<0.05$) increased in V + D group when compared with C + D (Figure- 100, Table- 100).

Real Time PCR amplification of CREB mRNA in the hippocampus of experimental rats

Real-time PCR gene expression of CREB showed a significant ($p<0.001$) down regulation in the hippocampus of diabetic and pre-treated rats when compared with control. When compared with diabetic group, curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) increase in CREB mRNA (Figure- 101, Table- 101).

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

Phospholipase C gene expression showed a significant down regulation in the hippocampus of diabetic ($p<0.001$) and pre-treated ($p<0.01$) rats when compared with control. A significant ($p<0.001$) increase in the phospholipase C mRNA was observed in curcumin and vitamin D_3 pre-treated groups when compared with diabetic (Figure- 102, Table- 102).

Real Time PCR amplification of insulin receptor mRNA in the hippocampus of experimental rats

Real time PCR gene expression of insulin receptor showed a significant ($p<0.001$) up regulation in the hippocampus of diabetic and pre-treated rats when compared with control. When compared with diabetic group, curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) decrease in the insulin receptor mRNA expression (Figure- 103, Table- 103).

Real Time PCR amplification of GLUT 3 mRNA in the hippocampus of experimental rats

The gene expression studies showed that GLUT 3 mRNA was significantly up regulated in diabetic ($p<0.001$), C + D ($p<0.001$) and V + D ($p<0.01$) groups when compared with control. In C + D and V + D there was a significant ($p<0.001$) decrease of the gene expression of GLUT 3 when compared with diabetic group. A significant ($p<0.05$) decrease was also observed in V + D when compared with C + D (Figure- 104, Table- 104).

Real Time PCR amplification of superoxide dismutases mRNA in the hippocampus of experimental rats

Gene expression of superoxide dismutases mRNA showed a significant ($p<0.001$) up regulation in the hippocampus of diabetic and pre-treated rats when compared with control. When compared with diabetic group, pre-treatment using

curcumin and vitamin D_3 significantly ($p<0.001$) decreased the superoxide dismutases mRNA expression (Figure- 105, Table- 105).

Real Time PCR amplification of glutathione peroxidase mRNA in the hippocampus of experimental rats

Glutathione peroxidase gene expression showed a significant ($p<0.001$) up regulation in the hippocampus of diabetic, C + D and V + D rats when compared with control. When compared with diabetic group, pre-treated groups showed a significant ($p<0.001$) decrease (Figure- 106, Table- 106).

cAMP content in the hippocampus of experimental rats

There was a significant ($p<0.01$) increase in the cAMP content of diabetic rats when compared with control. Pre-treatment with curcumin and vitamin D_3 were able to retain the cAMP content to near control when compared with diabetic group ($p<0.01$; Figure- 107, Table- 107).

cGMP content in the hippocampus of experimental rats

The cGMP content in the hippocampus of diabetic rats showed a significant decrease ($p<0.01$) when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.01$) retained a near control level of cGMP when compared with diabetic group (Figure- 108, Table- 108).

IP3 content in the hippocampus of experimental rats

Compared to control, diabetic group showed a significant ($p<0.001$) increase in the hippocampal IP3 content. Curcumin and vitamin D_3 pre-treated rats were able to maintain a near control level of IP3 in there hippocampus when compared with diabetic group ($p<0.001$) (Figure- 109, Table- 109).

Figure-82

Scatchard analysis of total adrenergic receptor using [^{3}H] epinephrine binding against epinephrine in the hippocampus of experimental rats

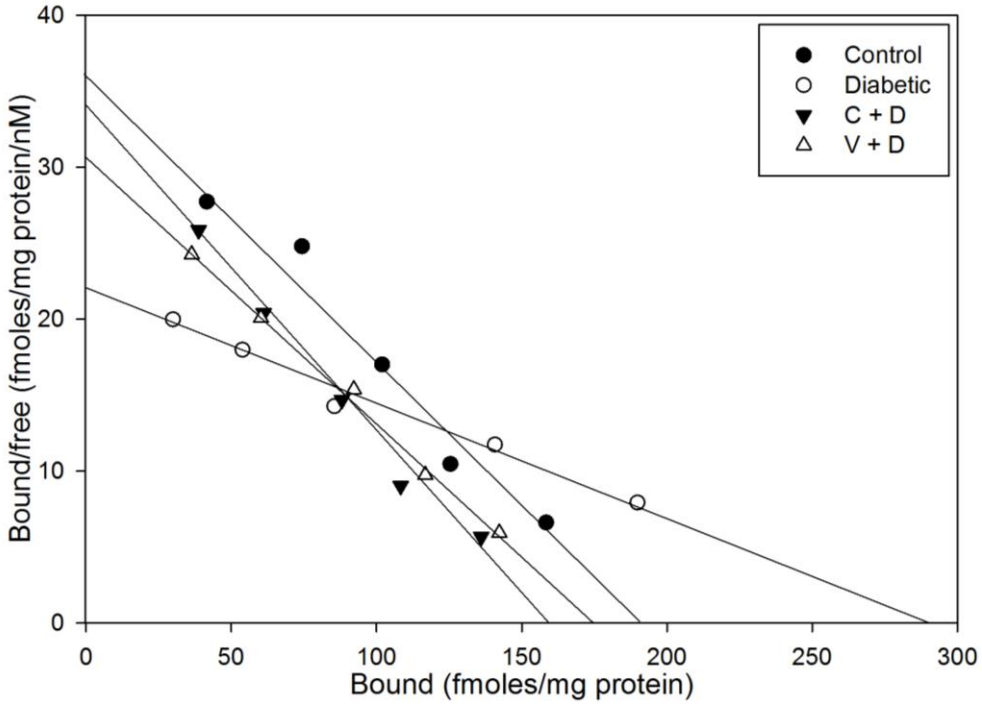


Table-82

Scatchard analysis of total adrenergic receptor using [^{3}H] epinephrine binding against epinephrine in the hippocampus of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	194.12 ± 7.99	5.22 ± 0.16
Diabetic	288.46 ± 11.97 **a**	13.16 ± 0.45 **a**
C + D	160.40 ± 7.36 **d**	4.69 ± 0.14 **d**
V + D	172.30 ± 8.59 **d**	5.81 ± 0.16 **d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. **i** $p<0.05$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-83

Scatchard analysis of α2 adrenergic receptor using [^{3}H] yohimbine binding against phentolamine in the hippocampus of experimental rats

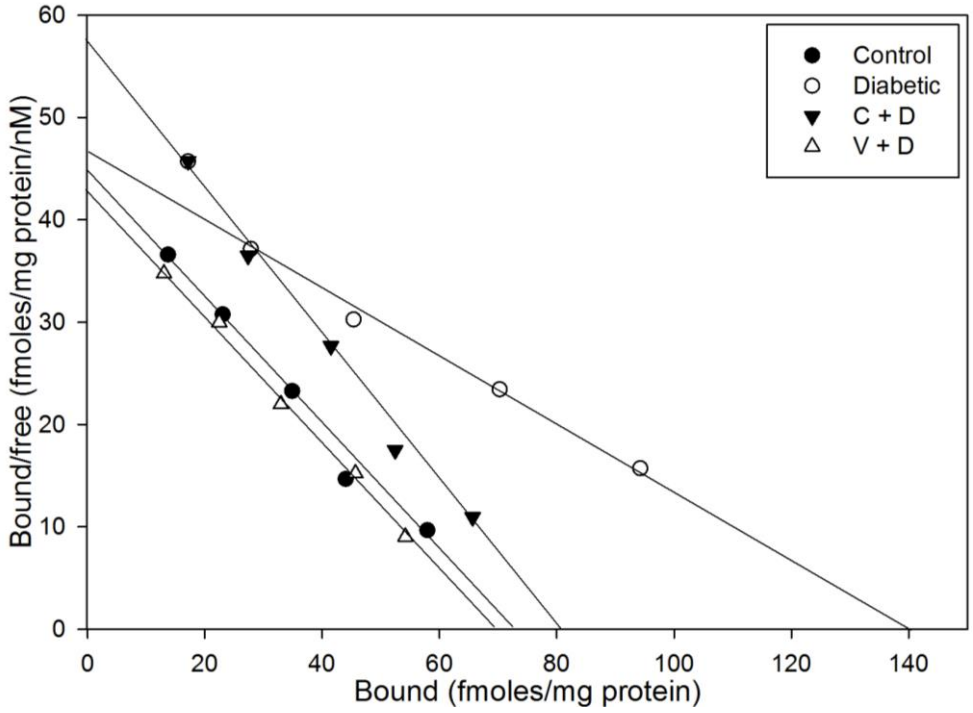


Table-83

Scatchard analysis of α2 adrenergic receptor using [^{3}H] yohimbine binding against phentolamine in the hippocampus of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	72.19 ± 4.50	1.60 ± 0.07
Diabetic	139.37 ± 7.19 **a**	3.01 ± 0.16 **a**
C + D	80.56 ± 5.17 **d**	1.38 ± 0.10 **d**
V + D	69.00 ± 4.19 **d**	1.65 ± 0.10 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-84
Real Time PCR amplification of α2 adrenergic receptor mRNA in the hippocampus of experimental rats

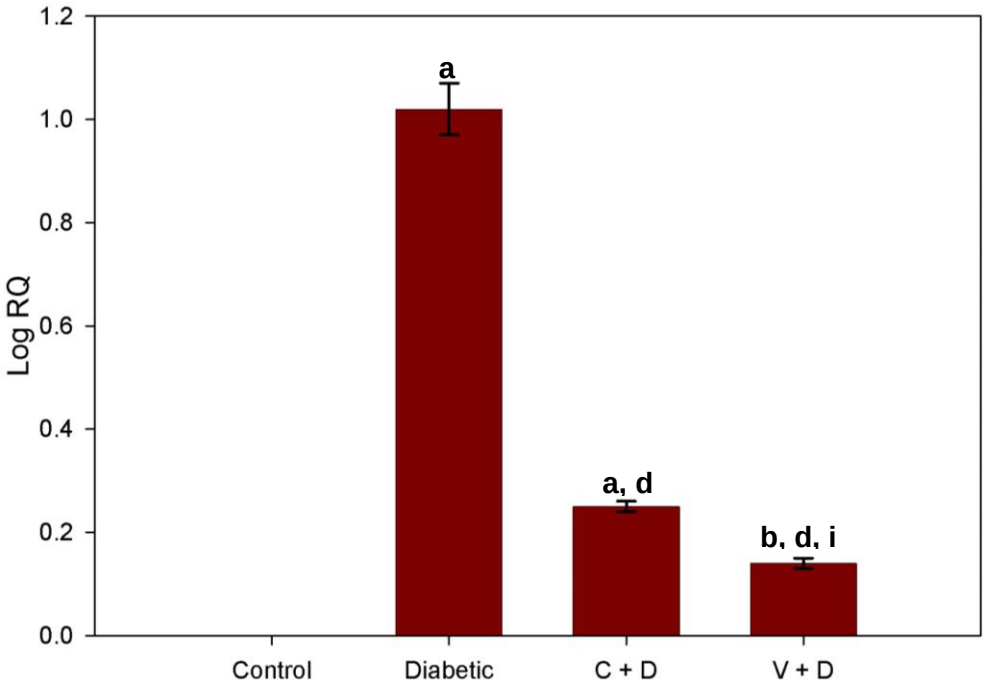


Table-84
Real Time PCR amplification of α2 adrenergic receptor mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.02 ± 0.05 **a**
C + D	0.25 ± 0.01 **a, d**
V + D	0.14 ± 0.01 **b, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001, **b**p<0.01 when compared to Control. **d**p<0.001 when compared to Diabetic group.
ip<0.05 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-85
Confocal imaging of α2 adrenergic receptor in the hippocampus of experimental rats

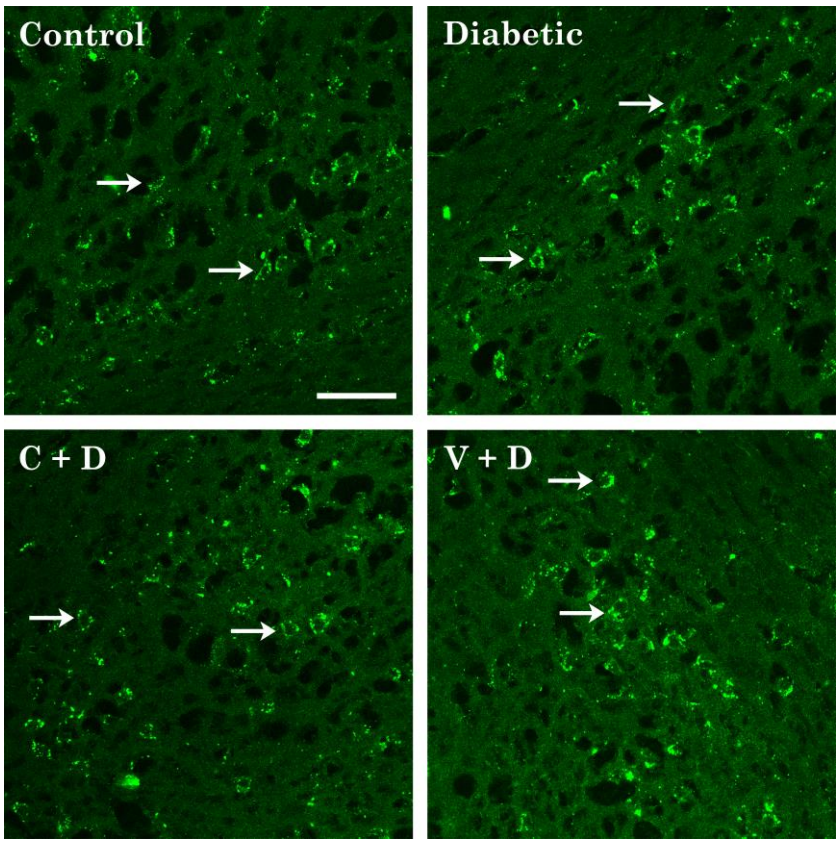


Table-85
Confocal imaging of α2 adrenergic receptor in the hippocampus of experimental rats

Experimental groups	Mean Pixel Intensity
Control	24.67 ± 0.88
Diabetic	35.33 ± 1.20 **a**
C + D	27.67 ± 0.88 **d**
V + D	24.00 ± 1.15 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$, when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⇨ shows α2 adrenergic receptors. Scale bar represents 50µm.

Figure-86

Scatchard analysis of β adrenergic receptor using [^{3}H] propranolol binding against propranolol in the hippocampus of experimental rats

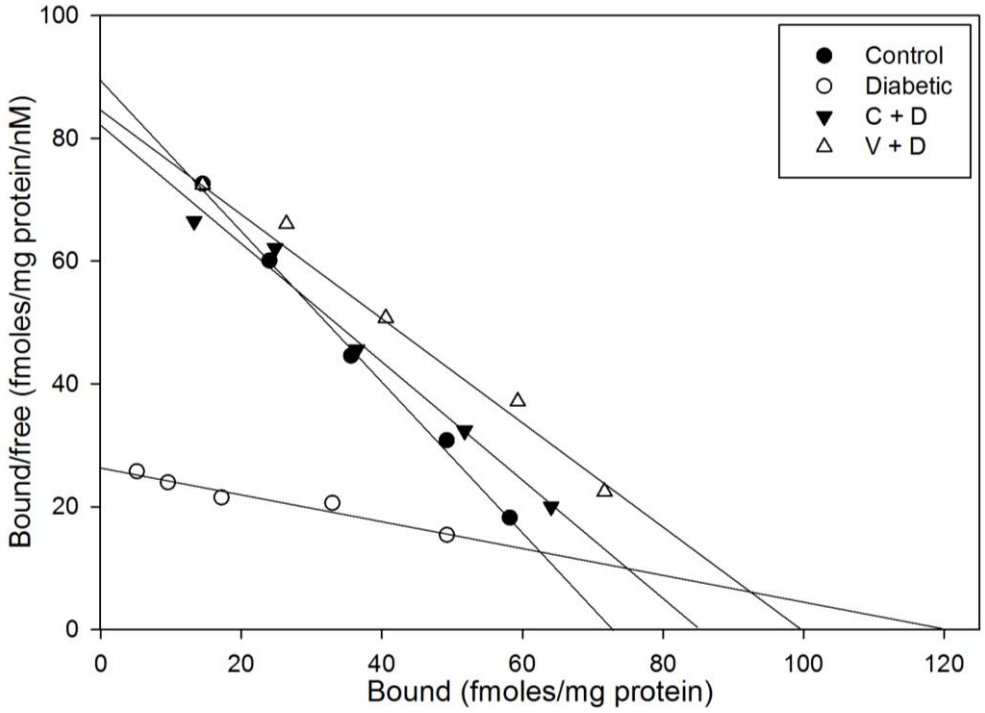


Table-86

Scatchard analysis of β adrenergic receptor using [^{3}H] propranolol binding against propranolol in the hippocampus of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	72.50 ± 3.69	0.82 ± 0.04
Diabetic	120.96 ± 5.46 [a]	4.58 ± 0.18 [a]
C + D	85.02 ± 3.73 [e]	1.06 ± 0.07 [d]
V + D	99.91 ± 6.20 [c, f]	1.16 ± 0.07 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
[a]$p<0.001$, [c]$p<0.05$ when compared to Control. [d]$p<0.001$, [e]$p<0.01$, [f]$p<0.05$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-87
Real Time PCR amplification of β2 adrenergic receptor mRNA in the hippocampus of experimental rats

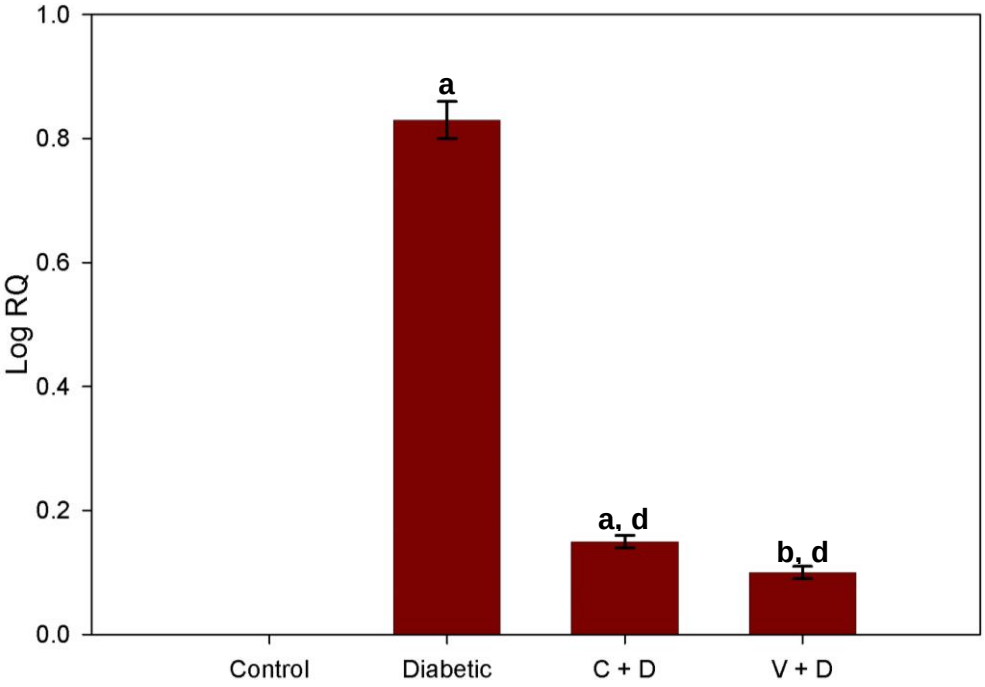


Table-87
Real Time PCR amplification of β2 adrenergic receptor mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.83 ± 0.03 **a**
C + D	0.15 ± 0.01 **a, d**
V + D	0.10 ± 0.01 **b, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$, **b** $p<0.01$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-88
Confocal imaging of β2 adrenergic receptor in the hippocampus of experimental rats

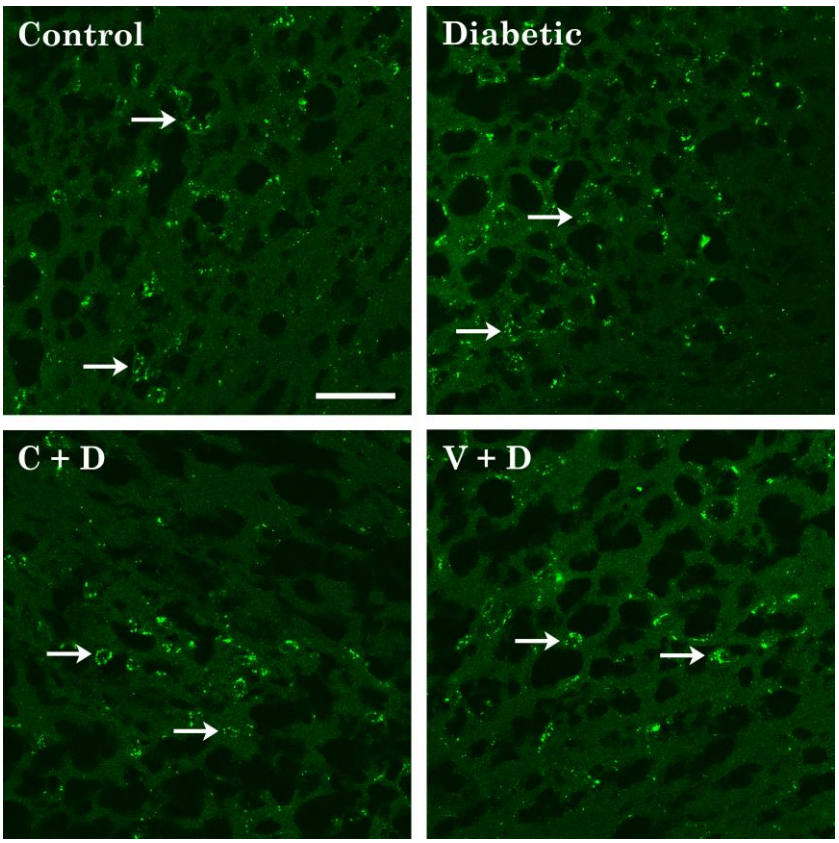


Table-88
Confocal imaging of β2 adrenergic receptor in the hippocampus of experimental rats

Experimental groups	Mean Pixel Intensity
Control	20.67 ± 0.88
Diabetic	33.25 ± 0.95 **a**
C + D	21.17 ± 0.60 **d**
V + D	22.67 ± 1.45 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a$p<0.001$, when compared to Control. **d**$p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⇨ shows β2 adrenergic receptors. Scale bar represents 50µm.

Figure-89

Scatchard analysis of total muscarinic receptor using [^{3}H] QNB binding against atropine in the hippocampus of experimental rats

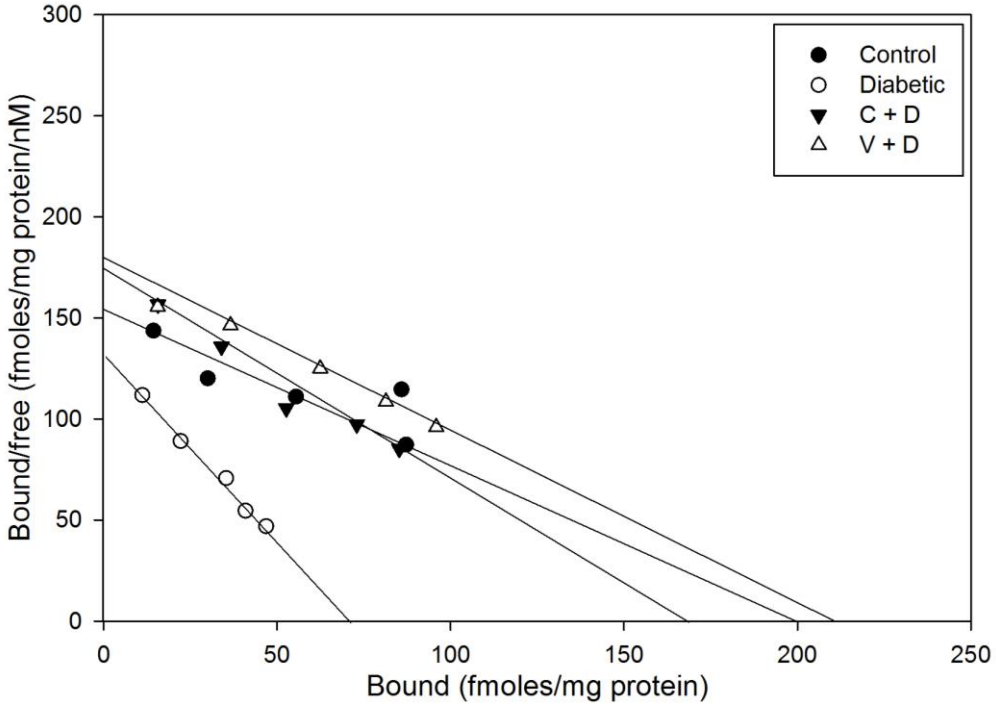


Table-89

Scatchard analysis of total muscarinic receptor using [^{3}H] QNB binding against atropine in the hippocampus of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	196.17 ± 13.02	1.27 ± 0.09
Diabetic	70.80 ± 4.94 [a]	0.53 ± 0.04 [a]
C + D	166.91 ± 9.39 [d]	0.97 ± 0.05 [c, e]
V + D	208.48 ± 13.13 [d]	1.16 ± 0.08 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
[a]$p<0.001$, [c]$p<0.05$ when compared to Control. [d]$p<0.001$, [e]$p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-90
Scatchard analysis of muscarinic M1 receptor using [^{3}H] QNB binding against pirenzepine in the hippocampus of experimental rats

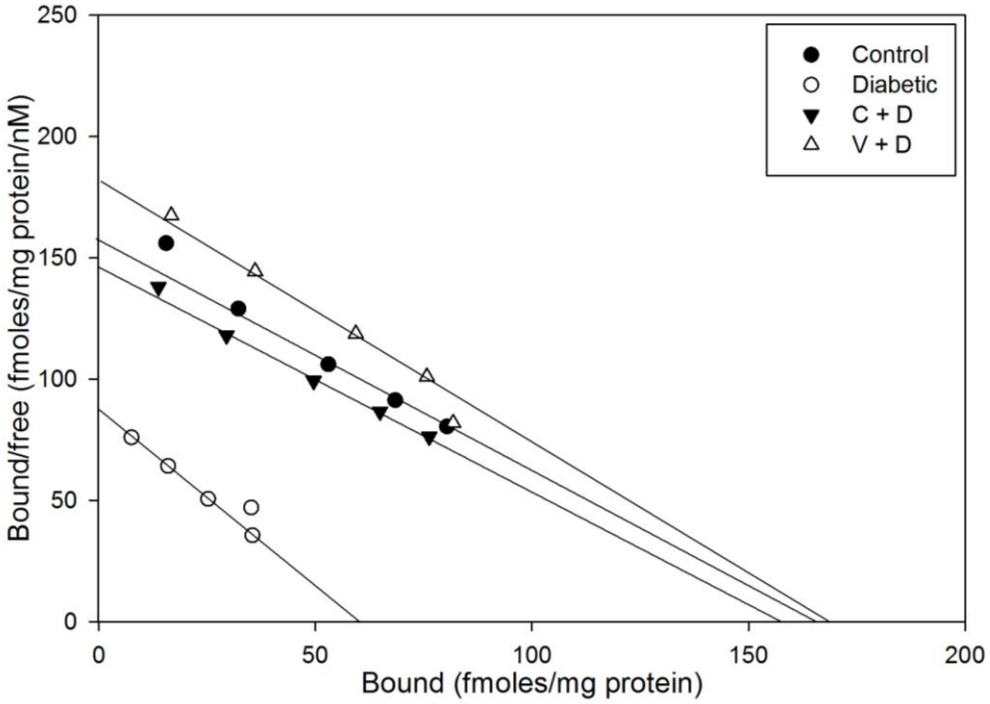


Table-90
Scatchard analysis of muscarinic M1 receptor using [^{3}H] QNB binding against pirenzepine in the hippocampus of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	165.25 ± 9.64	1.05 ± 0.07
Diabetic	59.56 ± 4.04 [a]	0.67 ± 0.04 [b]
C + D	155.69 ± 8.94 [d]	1.08 ± 0.07 [e]
V + D	168.62 ± 9.42 [d]	0.93 ± 0.06 [f]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
[a]$p<0.001$, [b]$p<0.01$ when compared to Control. [d]$p<0.001$, [e]$p<0.01$, [f]$p<0.05$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-91
Real Time PCR amplification of muscarinic M1 receptor mRNA in the hippocampus of experimental rats

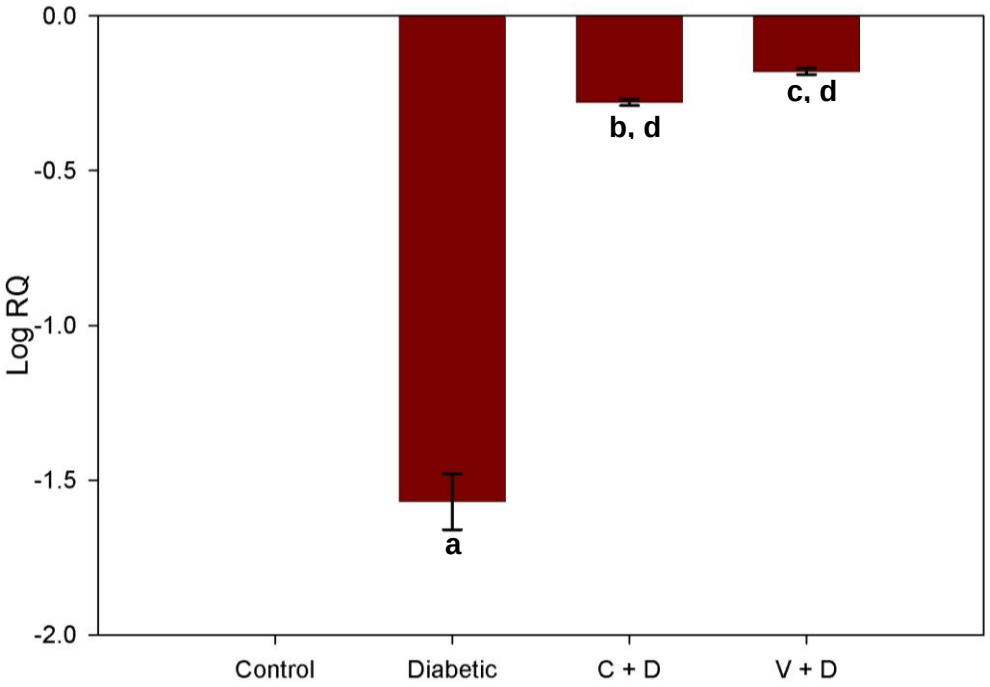


Table-91
Real Time PCR amplification of muscarinic M1 receptor mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.57 ± 0.09 **a**
C + D	-0.28 ± 0.01 **b, d**
V + D	-0.18 ± 0.01 **c, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a$p<0.001$, **b**$p<0.01$, **c**$p<0.05$ when compared to Control. **d**$p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-92
Confocal imaging of muscarinic M1 receptor in the hippocampus of experimental rats

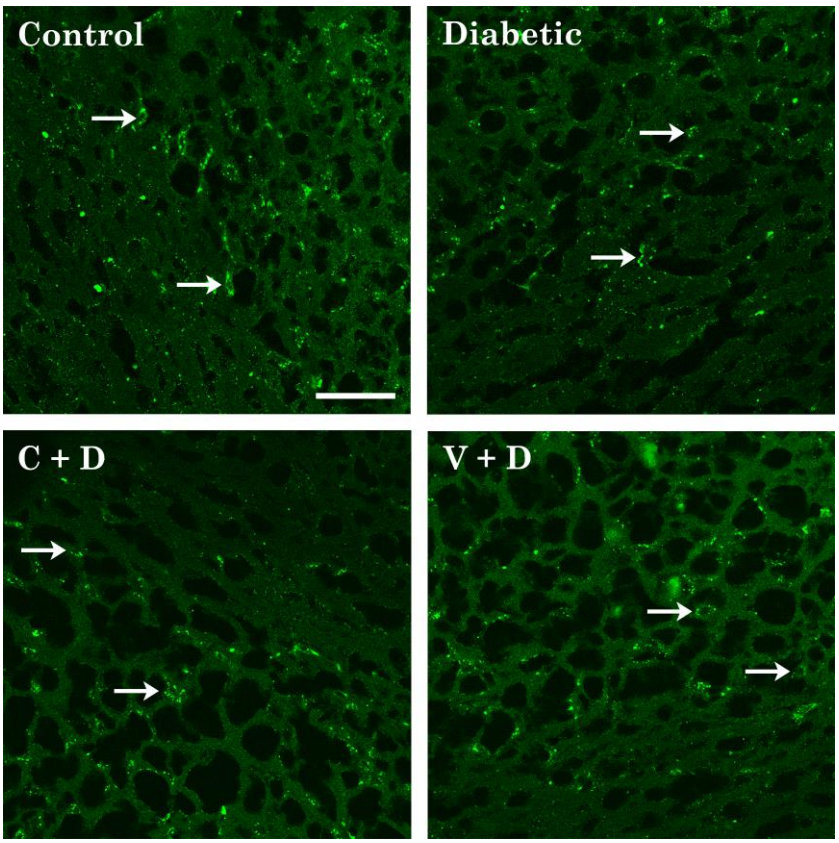


Table-92
Confocal imaging of muscarinic M1 receptor in the hippocampus of experimental rats

Experimental groups	Mean Pixel Intensity
Control	20.90 ± 1.01
Diabetic	12.48 ± 0.55 [a]
C + D	19.29 ± 0.84 [e]
V + D	18.22 ± 1.10 [e]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
[a]$p<0.001$, when compared to Control. [e]$p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⇨ shows muscarinic M1 receptors. Scale bar represents 50µm.

Figure-93
Scatchard analysis of muscarinic M3 receptor using [^{3}H] DAMP binding against 4-DAMP mustard in the hippocampus of experimental rats

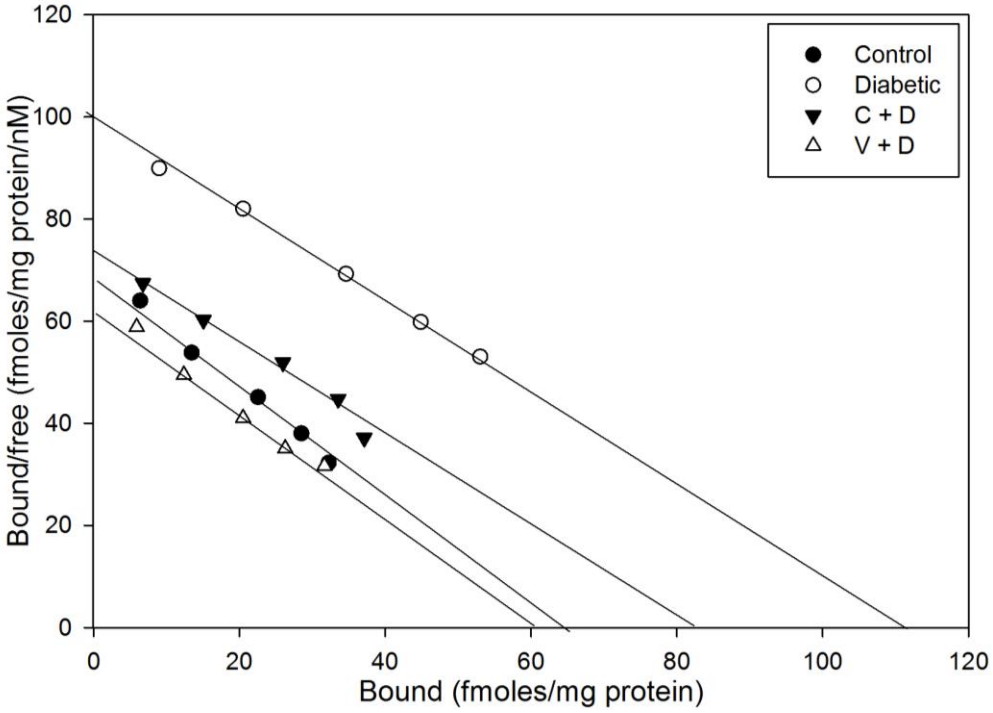


Table-93
Scatchard analysis of muscarinic M3 receptor using [^{3}H] DAMP binding against 4-DAMP mustard in the hippocampus of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	64.62 ± 3.75	0.66 ± 0.04
Diabetic	110.34 ± 7.34 [a]	1.09 ± 0.06 [b]
C + D	82.67 ± 5.11 [c, e]	1.10 ± 0.07 [b]
V + D	60.77 ± 4.09 [d, i]	0.96 ± 0.06 [b]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
[a]$p<0.001$, [b]$p<0.01$, [c]$p<0.05$ when compared to Control. [d]$p<0.001$, [e]$p<0.01$ when compared to Diabetic group. [i]$p<0.05$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-94
Real Time PCR amplification of muscarinic M3 receptor mRNA in the hippocampus of experimental rats

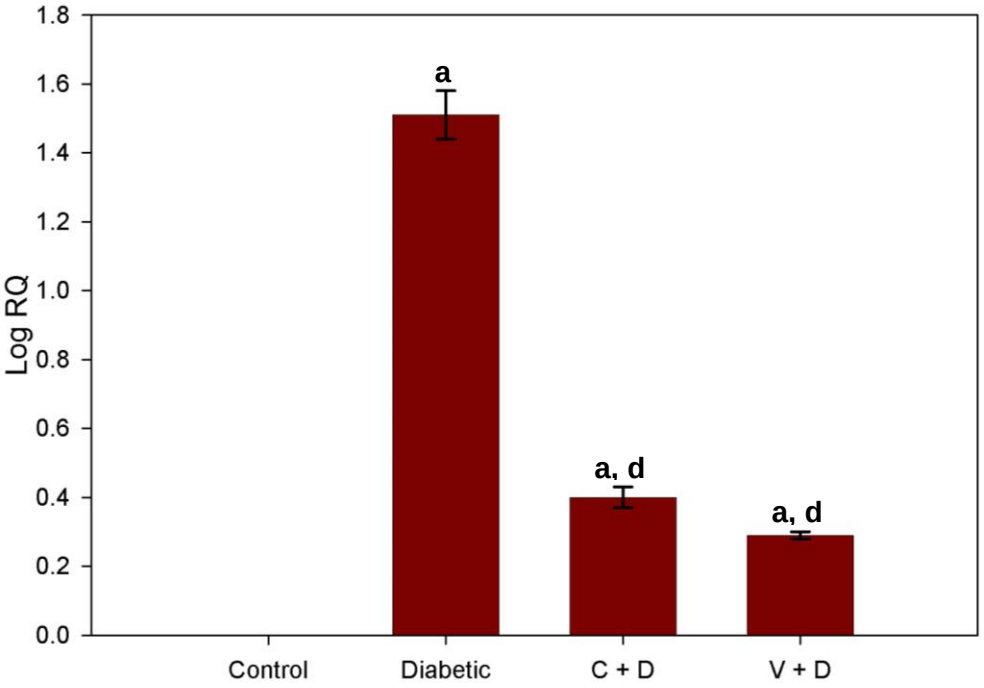


Table-94
Real Time PCR amplification of muscarinic M3 receptor mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.51 ± 0.07 **a**
C + D	0.40 ± 0.03 **a, d**
V + D	0.29 ± 0.01 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-95
Confocal imaging of muscarinic M3 receptor in the hippocampus of experimental rats

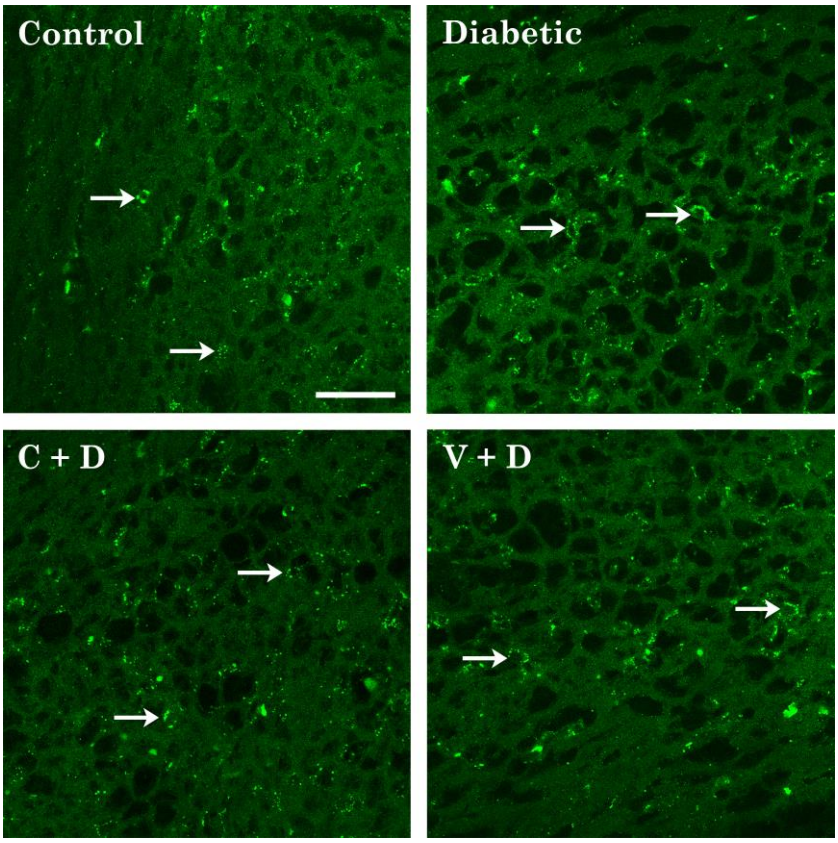


Table-95
Confocal imaging of muscarinic M3 receptor in the hippocampus of experimental rats

Experimental groups	Mean Pixel Intensity
Control	16.38 ± 0.83
Diabetic	23.34 ± 0.96 **b**
C + D	13.81 ± 1.22 **d**
V + D	16.79 ± 0.61 **e**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b$p<0.001$, when compared to Control. **d**$p<0.001$, **e**$p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.
⇨ shows muscarinic M3 receptors. Scale bar represents 50µm.

Figure-96
Real Time PCR amplification of choline acetyltransferase mRNA in the hippocampus of experimental rats

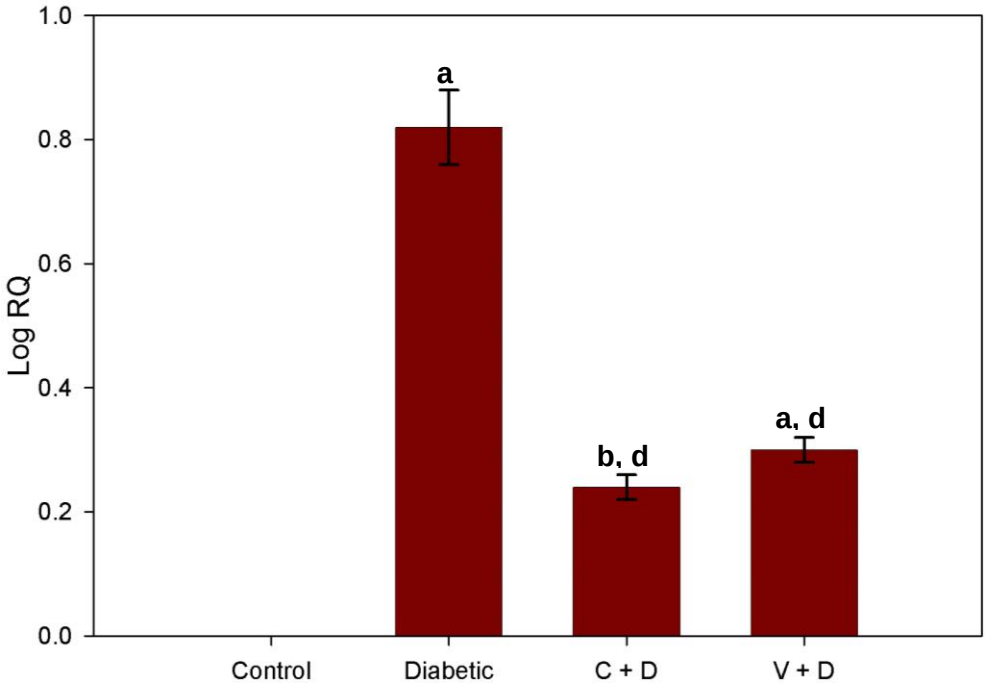


Table-96
Real Time PCR amplification of choline acetyltransferase mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.82 ± 0.06 **a**
C + D	0.24 ± 0.02 **b, d**
V + D	0.30 ± 0.02 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a** $p<0.001$, **b** $p<0.01$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group. C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-97
Real Time PCR amplification of acetylcholinesterase mRNA in the hippocampus of experimental rats

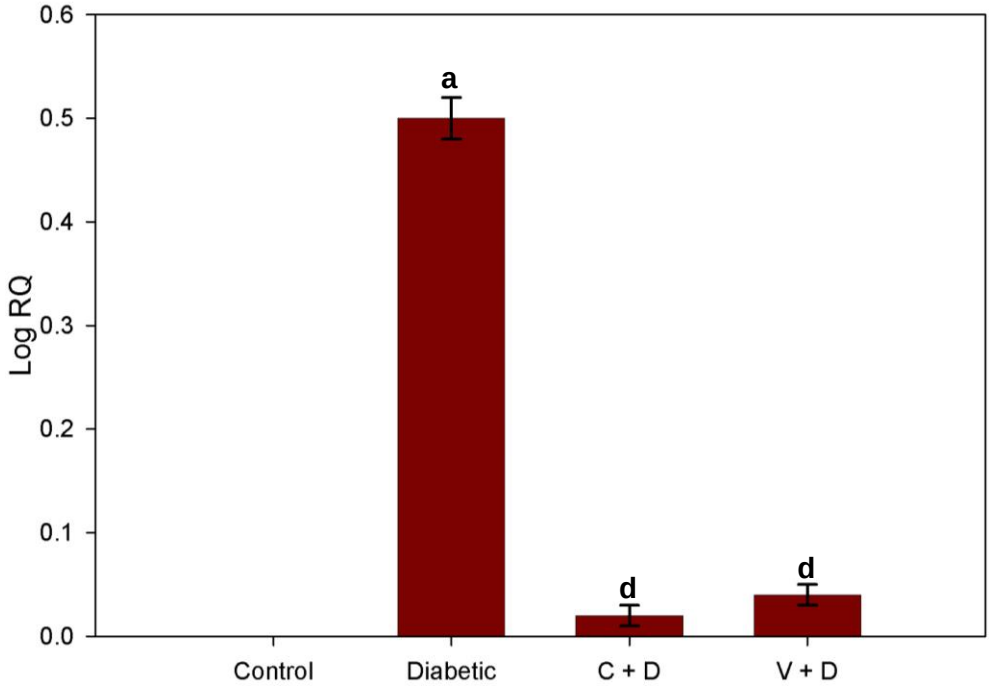


Table-97
Real Time PCR amplification of acetylcholinesterase mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.50 ± 0.02 **a**
C + D	0.02 ± 0.01 **d**
V + D	0.04 ± 0.01 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-98
Real Time PCR amplification of muscarinic M2 receptor mRNA in the hippocampus of experimental rats

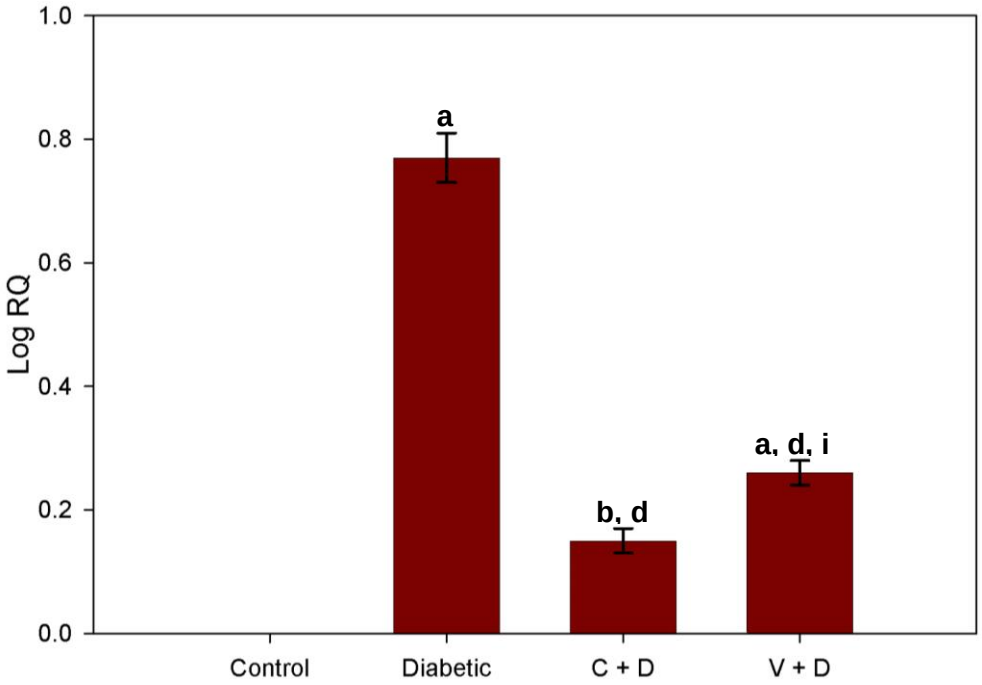


Table-98
Real Time PCR amplification of muscarinic M2 receptor mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.77 ± 0.04 **a**
C + D	0.15 ± 0.02 **b, d**
V + D	0.26 ± 0.02 **a, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001, **b**p<0.01 when compared to Control. **d**p<0.001 when compared to Diabetic group.
ip<0.05 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-99
Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the hippocampus of experimental rats

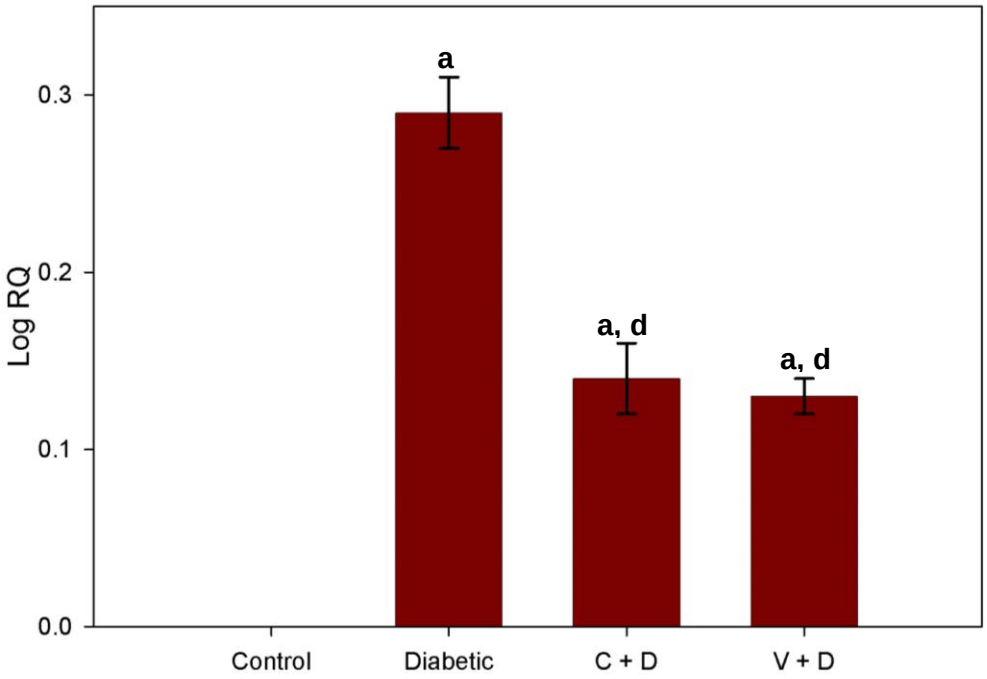


Table-99
Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.29 ± 0.02 **a**
C + D	0.14 ± 0.02 **a, d**
V + D	0.13 ± 0.01 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-100
Real Time PCR amplification of vitamin D receptor mRNA in the hippocampus of experimental rats

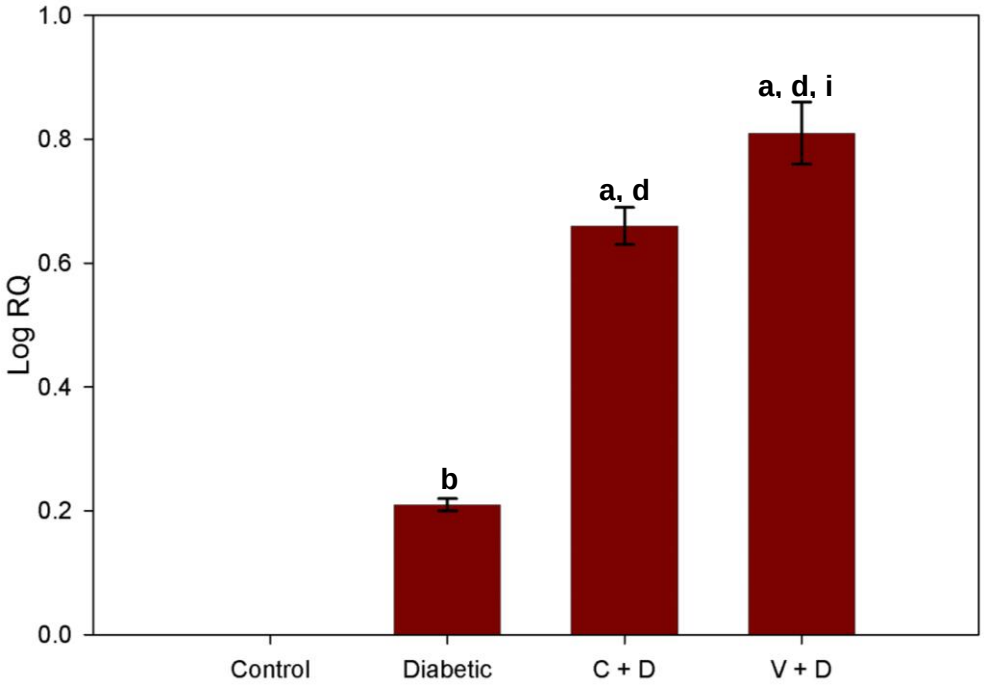


Table-100
Real Time PCR amplification of vitamin D receptor mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.21 ± 0.01 **b**
C + D	0.66 ± 0.03 **a, d**
V + D	0.81 ± 0.05 **a, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001, **b**p<0.01 when compared to Control. **d**p<0.001 when compared to Diabetic group.
ip<0.05 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-101
Real Time PCR amplification of CREB mRNA in the hippocampus of experimental rats

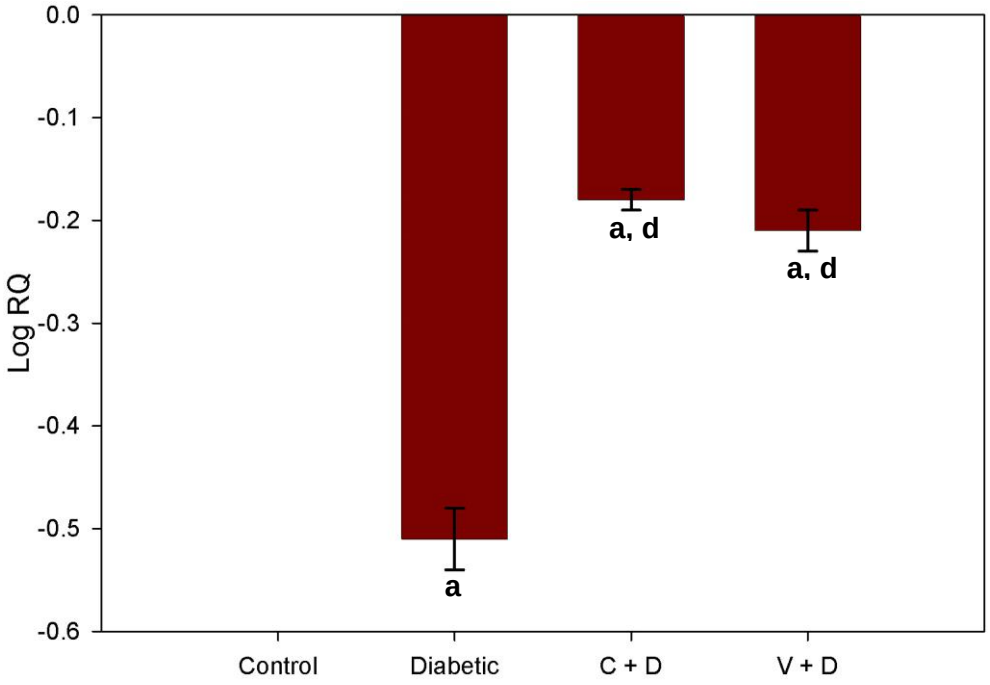


Table-101
Real Time PCR amplification of CREB mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.51 ± 0.03 **a**
C + D	-0.18 ± 0.01 **a, d**
V + D	-0.21 ± 0.02 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-102
Real Time PCR amplification of phospholipase C mRNA in the hippocampus of experimental rats

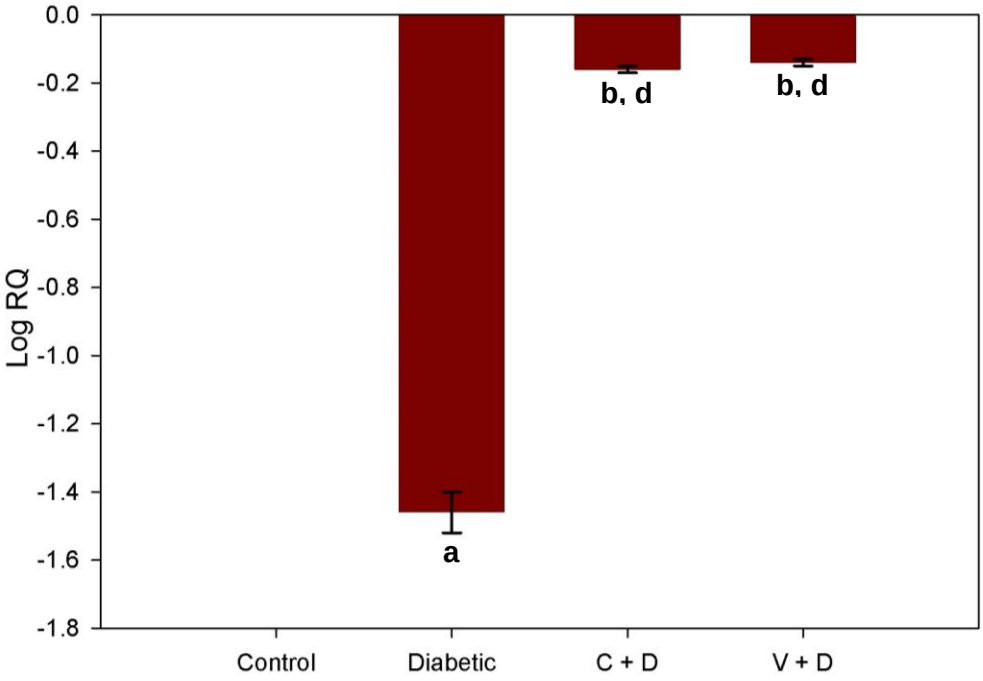


Table-102
Real Time PCR amplification of phospholipase C mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.46 ± 0.06 **a**
C + D	-0.16 ± 0.01 **b, d**
V + D	-0.14 ± 0.01 **b, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001, **b**p<0.01 when compared to Control. **d**p<0.001 when compared to Diabetic group. C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-103
Real Time PCR amplification of insulin receptor mRNA in the hippocampus of experimental rats

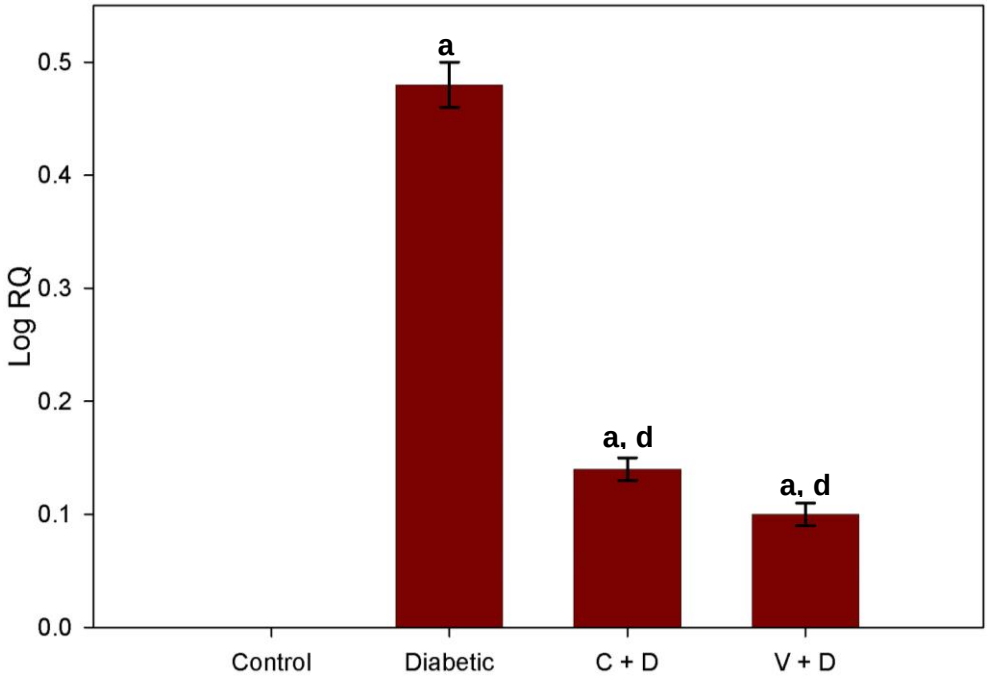


Table-103
Real Time PCR amplification of insulin receptor mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.48 ± 0.02 **a**
C + D	0.14 ± 0.01 **a, d**
V + D	0.10 ± 0.01 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-104
Real Time PCR amplification of GLUT 3 mRNA in the hippocampus of experimental rats

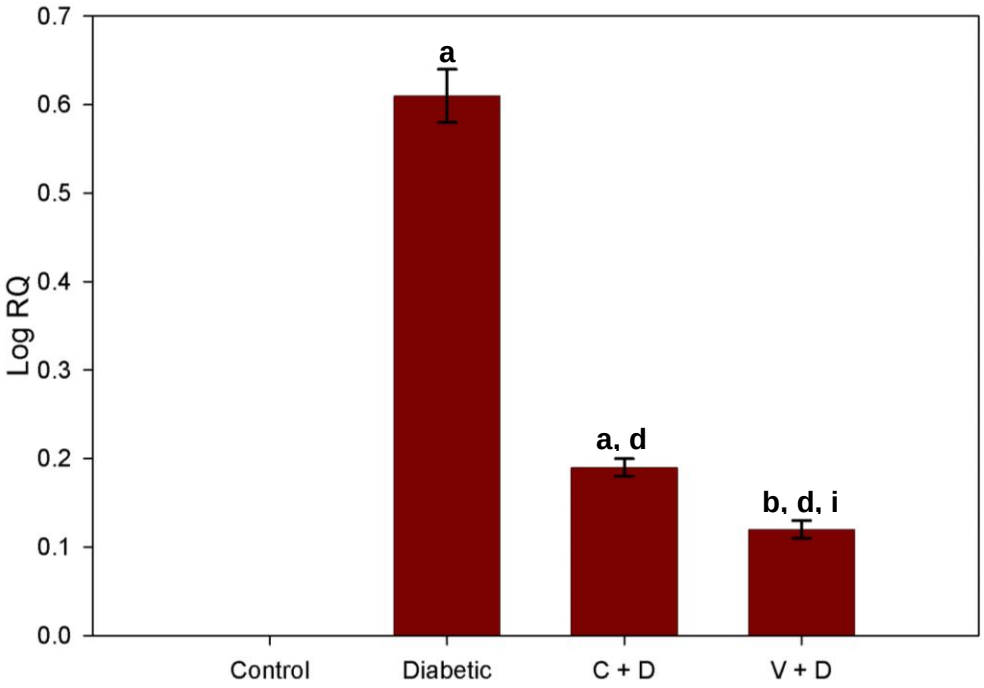


Table-104
Real Time PCR amplification of GLUT 3 mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.61 ± 0.03 **a**
C + D	0.19 ± 0.01 **a, d**
V + D	0.12 ± 0.01 **b, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001, **b**p<0.01 when compared to Control. **d**p<0.001 when compared to Diabetic group.
ip<0.05 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-105
Real Time PCR amplification of superoxide dismutases mRNA in the hippocampus of experimental rats

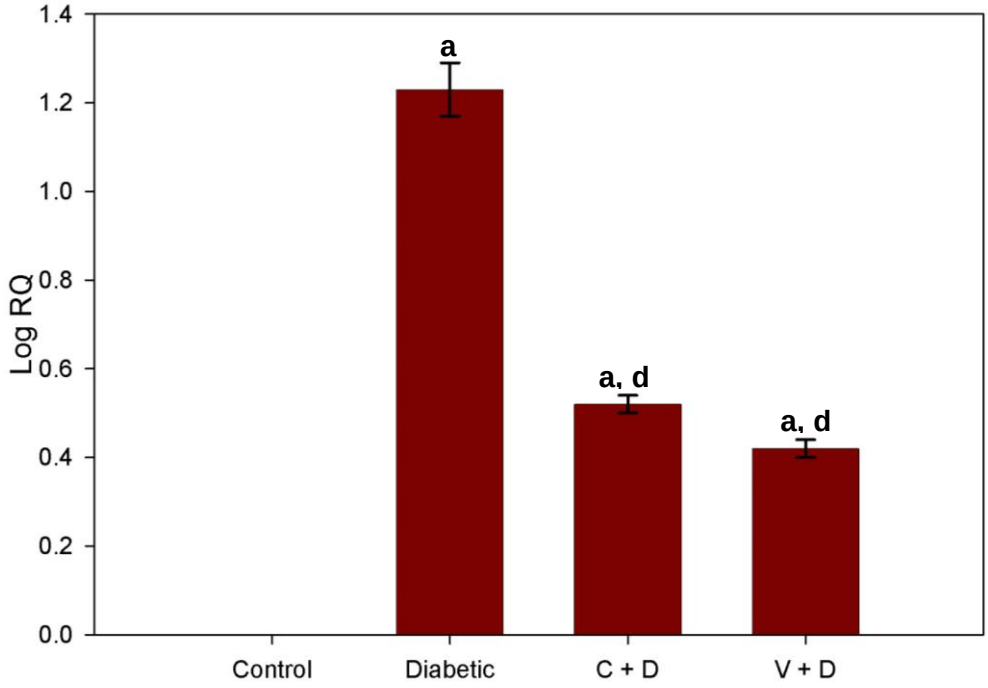


Table-105
Real Time PCR amplification of superoxide dismutases mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.23 ± 0.06 **a**
C + D	0.52 ± 0.02 **a, d**
V + D	0.42 ± 0.02 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-106
Real Time PCR amplification of glutathione peroxidase mRNA in the hippocampus of experimental rats

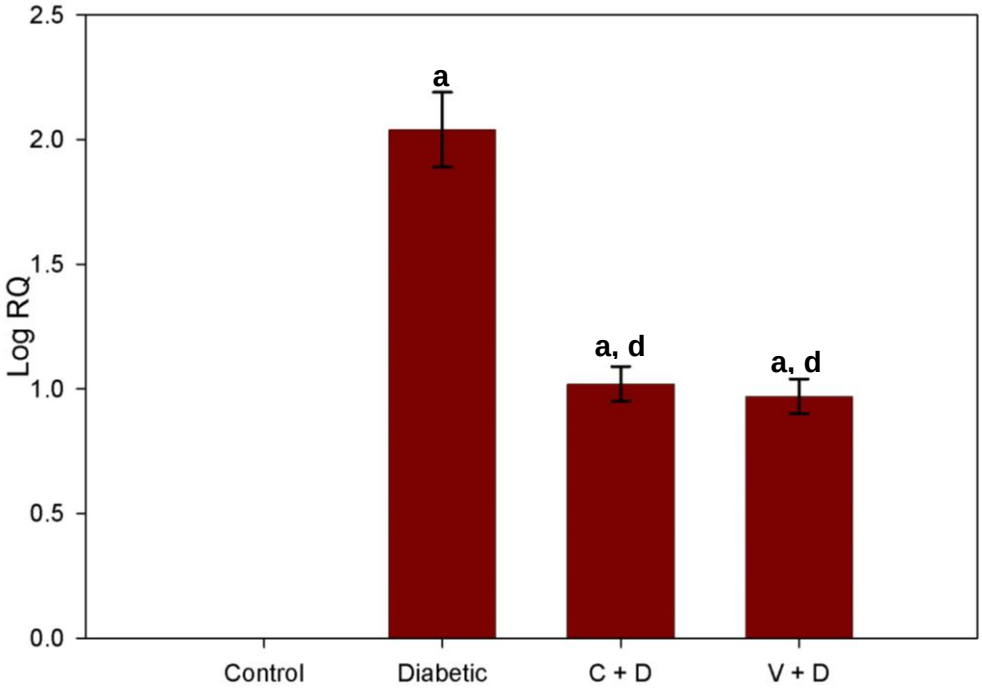


Table-106
Real Time PCR amplification of glutathione peroxidase mRNA in the hippocampus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	2.04 ± 0.15 **a**
C + D	1.02 ± 0.07 **a, d**
V + D	0.97 ± 0.07 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-107
cAMP content in the hippocampus of experimental rats

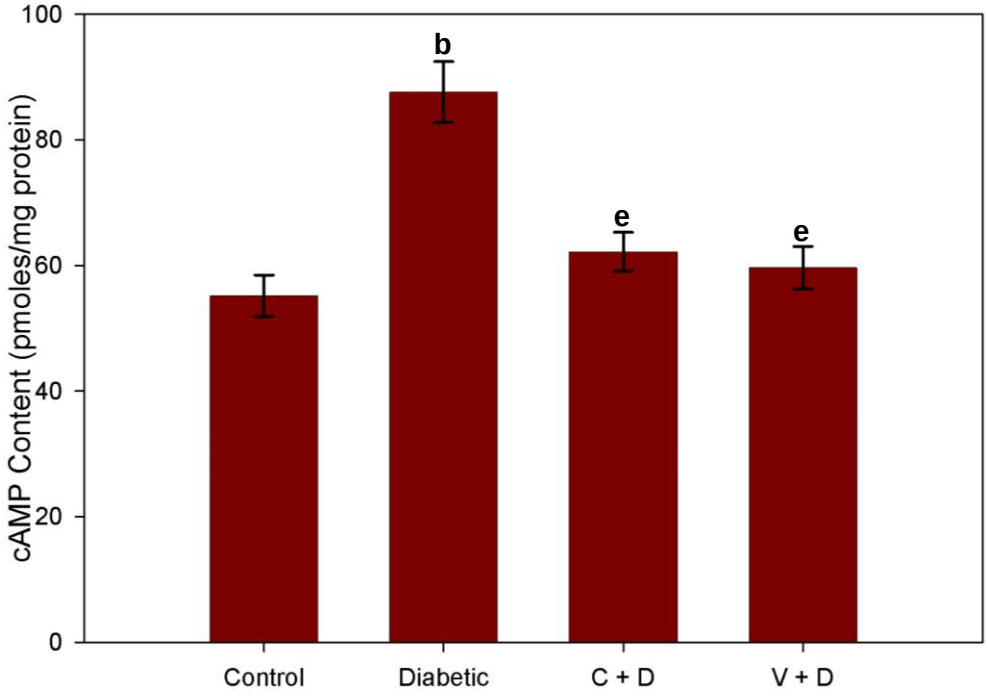


Table-107
cAMP content in the hippocampus of experimental rats

Experimental groups	cAMP content (pmoles/mg protein)
Control	55.16 ± 3.30
Diabetic	87.60 ± 4.86 **b**
C + D	62.18 ± 3.09 **e**
V + D	59.65 ± 3.38 **e**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$ when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-108
cGMP content in the hippocampus of experimental rats

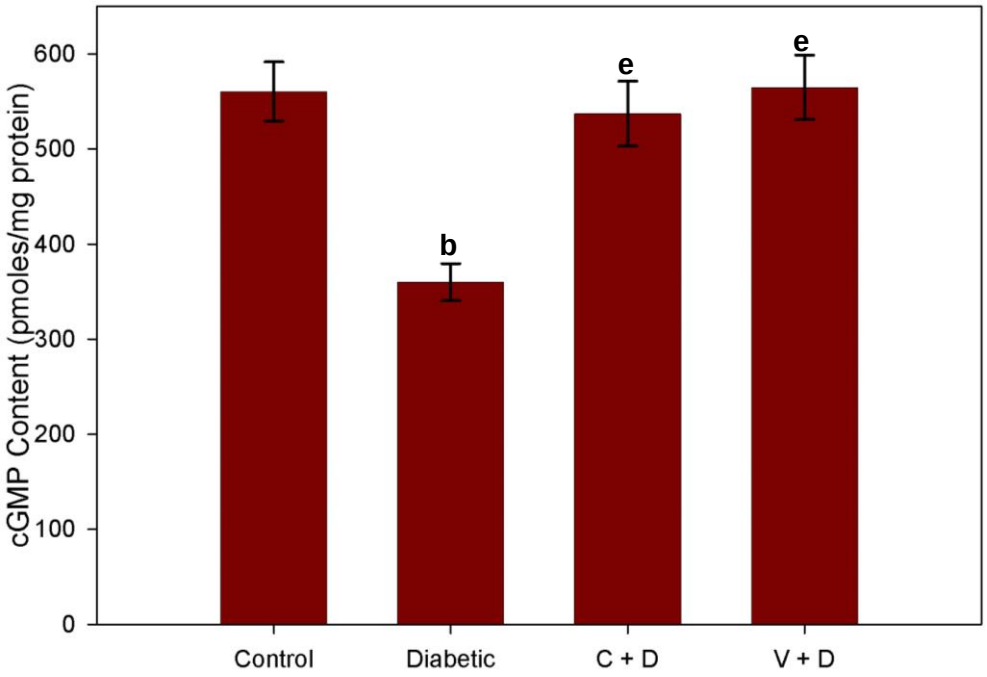


Table-108
cGMP content in the hippocampus of experimental rats

Experimental groups	cGMP content (pmoles/mg protein)
Control	560.43 ± 31.08
Diabetic	359.94 ± 19.50 **b**
C + D	537.19 ± 34.11 **e**
V + D	564.93 ± 33.87 **e**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
b $p<0.01$ when compared to Control. **e** $p<0.01$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-109
IP3 content in the hippocampus of experimental rats

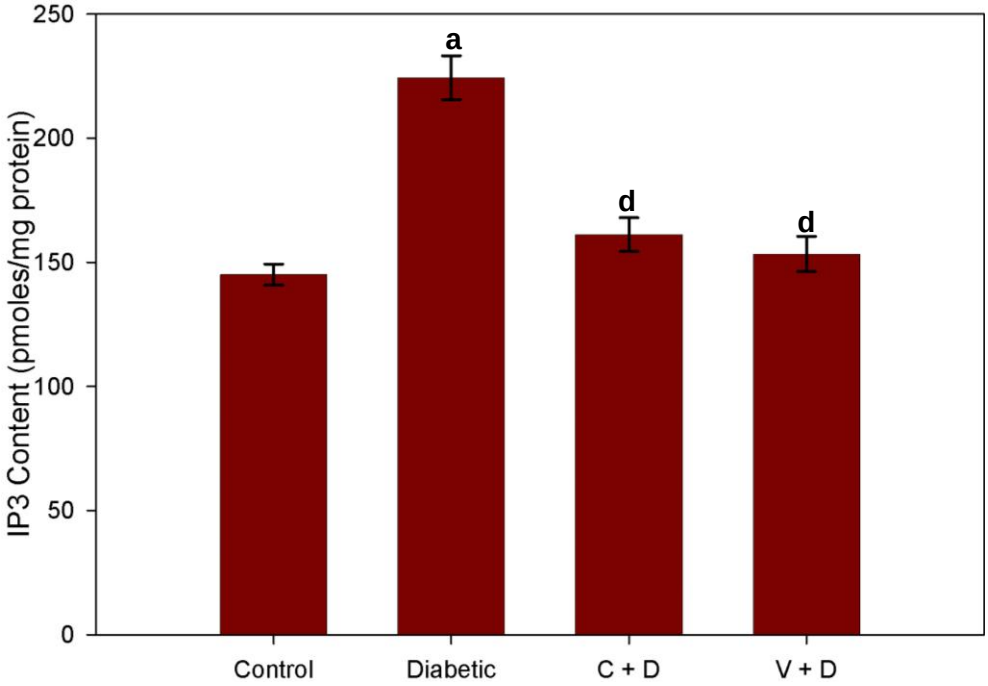


Table-109
IP3 content in the hippocampus of experimental rats

Experimental groups	IP3 content (pmoles/mg protein)
Control	145.01 ± 4.23
Diabetic	224.34 ± 8.83 **a**
C + D	161.15 ± 6.85 **d**
V + D	153.35 ± 7.13 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

HYPOTHALAMUS

Real Time PCR amplification of α2 adrenergic receptor mRNA in the hypothalamus of experimental rats

α2 adrenergic receptor gene expression showed a significant up regulation in the hypothalamus of diabetic ($p<0.001$) and vitamin D_3 pre-treated ($p<0.01$) rats when compared with control. A significant ($p<0.001$) decrease in α2 adrenergic receptor mRNA was observed in pre-treated groups when compared with diabetic group. Further, a significant ($p<0.05$) increase was observed in the V + D when compared with C + D (Figure- 110, Table- 110).

Real Time PCR amplification of β2 adrenergic receptor mRNA in the hypothalamus of experimental rats

Real time PCR gene expression of β2 adrenergic receptor showed a significant ($p<0.001$) up regulation in the hypothalamus of diabetic rats when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) decrease when compared with diabetic group (Figure- 111, Table- 111).

Real Time PCR amplification of muscarinic M1 receptor mRNA in the hypothalamus of experimental rats

Gene expression of muscarinic M1 receptor mRNA showed a significant down regulation in the hypothalamus of diabetic ($p<0.001$) and V + D ($p<0.05$) rats when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) increased the mRNA expression when compared with diabetic group. A significant ($p<0.01$) down regulation was observed in the V + D when compared with C + D (Figure- 112, Table- 112).

Real Time PCR amplification of muscarinic M3 receptor mRNA in the hypothalamus of experimental rats

Gene expression of muscarinic M3 receptor mRNA showed a significant up regulation in the hypothalamus of diabetic ($p<0.001$) and pre-treated ($p<0.01$) rats when compared with control. When compared with diabetic group, pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) decreased the gene expression (Figure- 113, Table- 113).

Real Time PCR amplification of choline acetyltransferase mRNA in the hypothalamus of experimental rats

The gene expression studies showed that choline acetyltransferase mRNA was significantly ($p<0.001$) down regulated in diabetic and pre-treated group when compared with control. In C + D and V + D there was a significant ($p<0.001$) increase in the gene expression of choline acetyltransferase when compared with diabetic group (Figure- 114, Table- 114).

Real Time PCR amplification of acetylcholinesterase mRNA in the hypothalamus of experimental rats

Gene expression of acetylcholinesterase mRNA showed a significant ($p<0.001$) up regulation in the hypothalamus of diabetic, C + D and V + D rats when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) decreased the gene expression when compared with diabetic group (Figure- 115, Table- 115).

Real Time PCR amplification of muscarinic M2 receptor mRNA in the hypothalamus of experimental rats

Muscarinic M2 receptor expression showed a significant up regulation in the hypothalamus of diabetic ($p<0.001$), C + D ($p<0.01$) and V + D ($p<0.001$) rats compared to control. A significant ($p<0.001$) decrease in the muscarinic M2 receptor mRNA was observed in the pre-treated groups when compared with diabetic group. In pre-treated groups, curcumin pre-treated group showed a

significant ($p<0.01$) decrease when compared with vitamin D_3 pre-treated group (Figure- 116, Table- 116).

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the hypothalamus of experimental rats

The gene expression studies showed that α7 nicotinic acetylcholine receptor mRNA was significantly ($p<0.001$) up regulated in diabetic and pre-treated groups when compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) decreased the α7 nicotinic acetylcholine receptor expression when compared with diabetic group (Figure- 117, Table- 117).

Real Time PCR amplification of vitamin D receptor mRNA in the hypothalamus of experimental rats

Real-time PCR gene expression of vitamin D receptor showed a significant ($p<0.001$) down regulation in the hypothalamus of diabetic and V + D rats when compared with control. C + D group showed a significant ($p<0.01$) up regulation when compared with control. Curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) increase in vitamin D receptor mRNA levels when compared with diabetic group. Further, vitamin D receptor expression was significantly ($p<0.001$) up regulated in the C + D group when compared with V + D (Figure- 118, Table- 118).

Real Time PCR amplification of CREB mRNA in the hypothalamus of experimental rats

The gene expression of CREB mRNA showed a significant ($p<0.001$) down regulation in diabetic and pre-treated groups when compared with control. In C + D and V + D there was a significant ($p<0.001$) increase in the CREB expression when compared with diabetic (Figure- 119, Table- 119).

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

Real time PCR gene expression of phospholipase C showed a significant down regulation in the hypothalamus of diabetic ($p<0.001$) and V + D ($p<0.05$) rats when compared with control. Curcumin and vitamin D_3 pre-treatment leads to a significant ($p<0.001$) increase in the phospholipase C expression when compared with diabetic. Further, a significant ($p<0.01$) down regulation was observed in the V + D when compared with C + D (Figure- 120, Table- 120).

Real Time PCR amplification of insulin receptor mRNA in the hypothalamus of experimental rats

The gene expression studies showed that insulin receptor mRNA was significantly ($p<0.001$) down regulated in the diabetic, C + D and V + D group when compared with control. In C + D and V + D there was a significant ($p<0.001$) increase in the insulin receptor mRNA level when compared with diabetic group. Among pre-treated groups, vitamin D_3 pre-treated group showed a significant ($p<0.001$) increase when compared with curcumin pre-treated group (Figure- 121, Table- 121).

Real Time PCR amplification of GLUT 3 mRNA in the hypothalamus of experimental rats

Gene expression of GLUT 3 mRNA showed a significant ($p<0.001$) down regulation in the hypothalamus of diabetic and pre-treated rats when compared with control. When compared with diabetic group, curcumin and vitamin D_3 pre-treated group showed a significant ($p<0.001$) increase in GLUT 3 expression (Figure- 122, Table- 122).

Real Time PCR amplification of superoxide dismutases mRNA in the hypothalamus of experimental rats

Gene expression of superoxide dismutases mRNA showed a significant ($p<0.001$) up regulation in the hypothalamus of diabetic and pre-treated rats when

compared with control. Pre-treatment using curcumin and vitamin D_3 significantly ($p<0.001$) decreased the superoxide dismutases expression when compared with diabetic group (Figure- 123, Table- 123).

Real Time PCR amplification of glutathione peroxidase mRNA in the hypothalamus of experimental rats

Real time PCR amplification of glutathione peroxidase mRNA showed a significant ($p<0.001$) up regulation in the hypothalamus of the diabetic and pre-treated rats when compared with control. When compared with diabetic group, curcumin and vitamin D_3 pre-treated rats showed a significant ($p<0.001$) decrease. Further, a significant ($p<0.05$) increase was observed in V + D when compared with C + D (Figure- 124, Table- 124).

Figure-110

Real Time PCR amplification of α2 adrenergic receptor mRNA in the hypothalamus of experimental rats

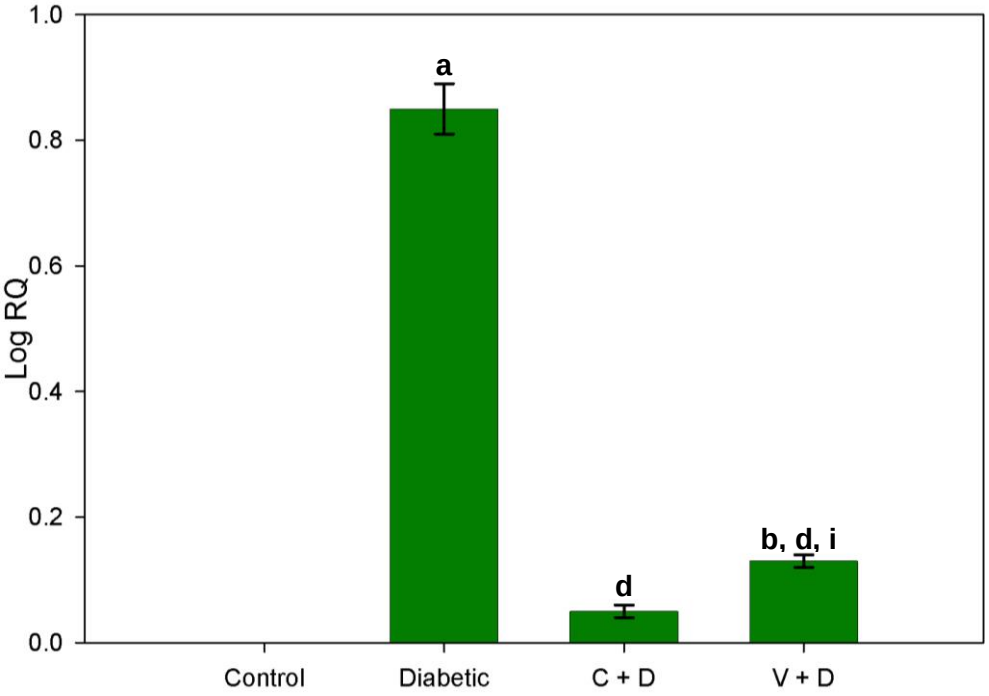


Table-110

Real Time PCR amplification of α2 adrenergic receptor mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.85 ± 0.04 **a**
C + D	0.05 ± 0.01 **d**
V + D	0.13 ± 0.01 **b, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001, **b**p<0.01 when compared to Control. **d**p<0.001 when compared to Diabetic group. **i**p<0.05 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-111

Real Time PCR amplification of β2 adrenergic receptor mRNA in the hypothalamus of experimental rats

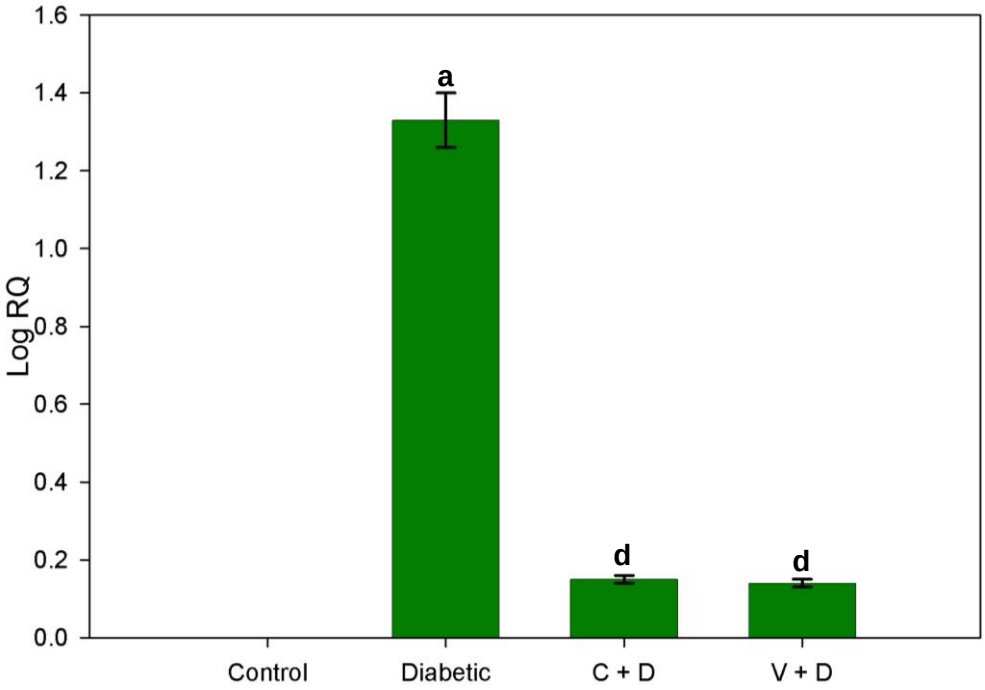


Table-111

Real Time PCR amplification of β2 adrenergic receptor mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.33 ± 0.07 **a**
C + D	0.15 ± 0.01 **d**
V + D	0.14 ± 0.01 **d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-112

Real Time PCR amplification of muscarinic M1 receptor mRNA in the hypothalamus of experimental rats

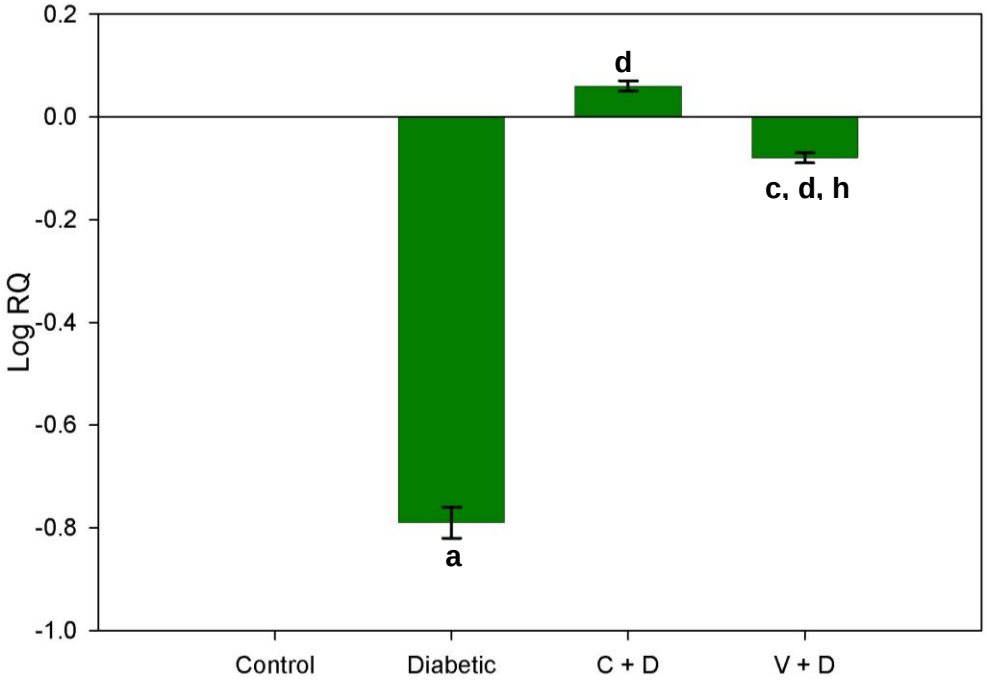


Table-112

Real Time PCR amplification of muscarinic M1 receptor mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.79 ± 0.03 **a**
C + D	0.06 ± 0.01 **d**
V + D	-0.08 ± 0.01 **c, d, h**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001, **c**p<0.05 when compared to Control. **d**p<0.001 when compared to Diabetic group. **h**p<0.01 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-113

Real Time PCR amplification of muscarinic M3 receptor mRNA in the hypothalamus of experimental rats

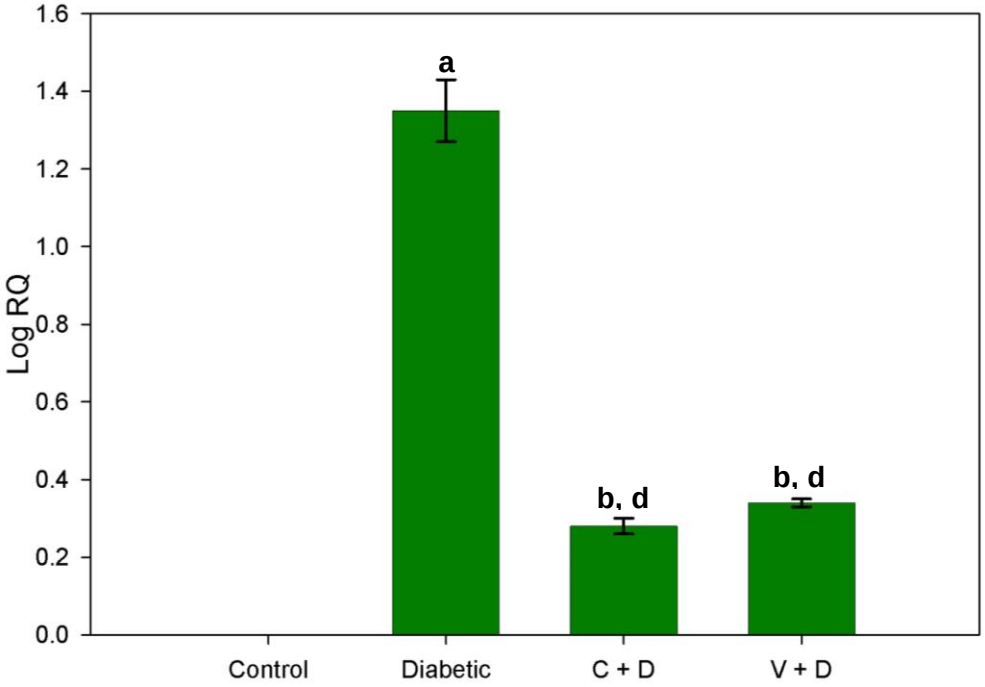


Table-113

Real Time PCR amplification of muscarinic M3 receptor mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.35 ± 0.08 **a**
C + D	0.28 ± 0.02 **b, d**
V + D	0.34 ± 0.01 **b, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a$p<0.001$, **b**$p<0.01$ when compared to Control. **d**$p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-114

Real Time PCR amplification of choline acetyltransferase mRNA in the hypothalamus of experimental rats

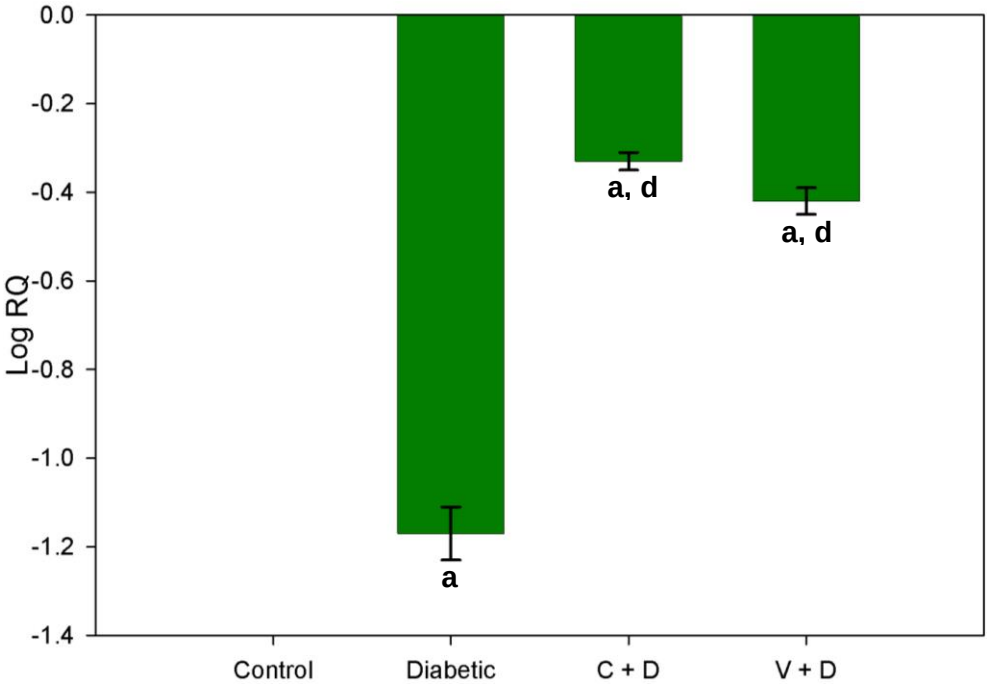


Table-114

Real Time PCR amplification of choline acetyltransferase mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.17 ± 0.06 **a**
C + D	-0.33 ± 0.02 **a, d**
V + D	-0.42 ± 0.03 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-115

Real Time PCR amplification of acetylcholinesterase mRNA in the hypothalamus of experimental rats

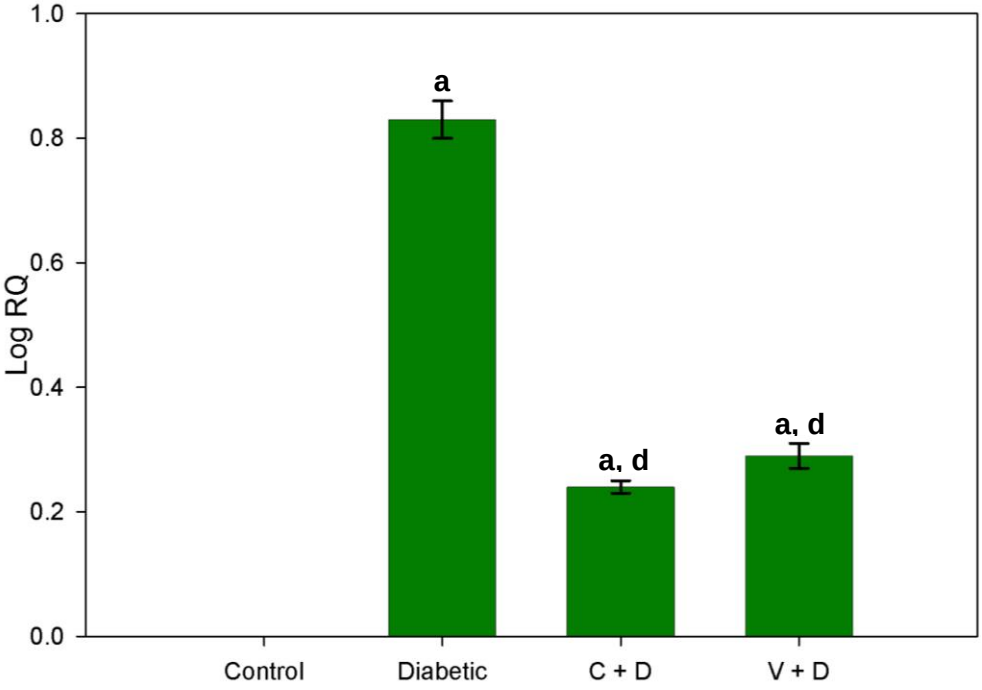


Table-115

Real Time PCR amplification of acetylcholinesterase mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.83 ± 0.03 **a**
C + D	0.24 ± 0.01 **a, d**
V + D	0.29 ± 0.02 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a$p<0.001$ when compared to Control. **d**$p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-116

Real Time PCR amplification of muscarinic M2 receptor mRNA in the hypothalamus of experimental rats

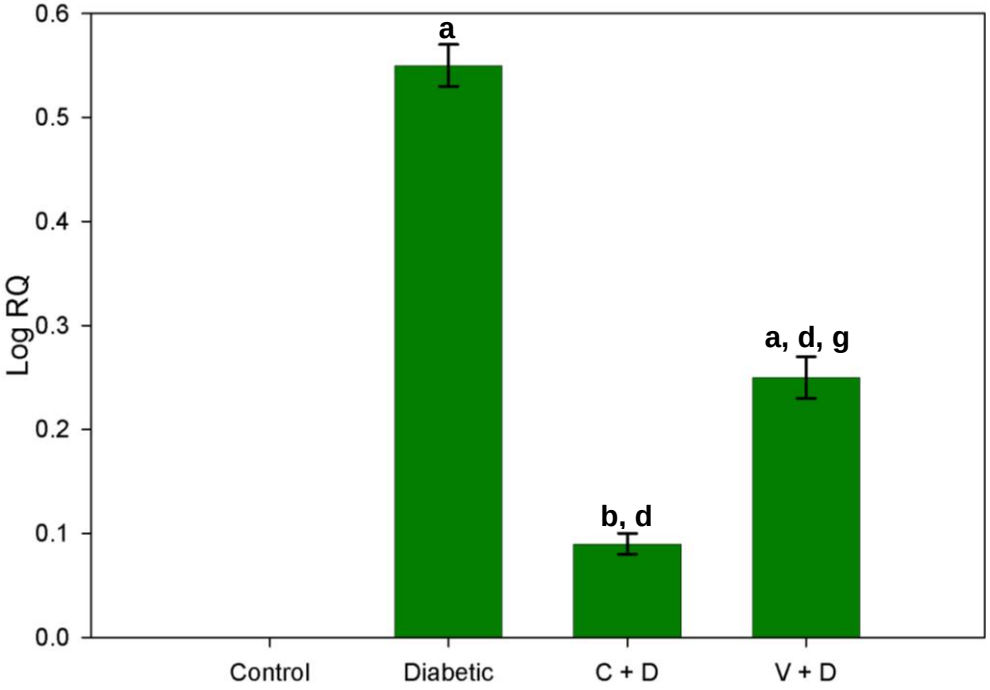


Table-116

Real Time PCR amplification of muscarinic M2 receptor mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.55 ± 0.02 **a**
C + D	0.09 ± 0.01 **b, d**
V + D	0.25 ± 0.02 **a, d, g**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001, **b**p<0.01 when compared to Control. **d**p<0.001 when compared to Diabetic group. **g**p<0.01 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-117

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the hypothalamus of experimental rats

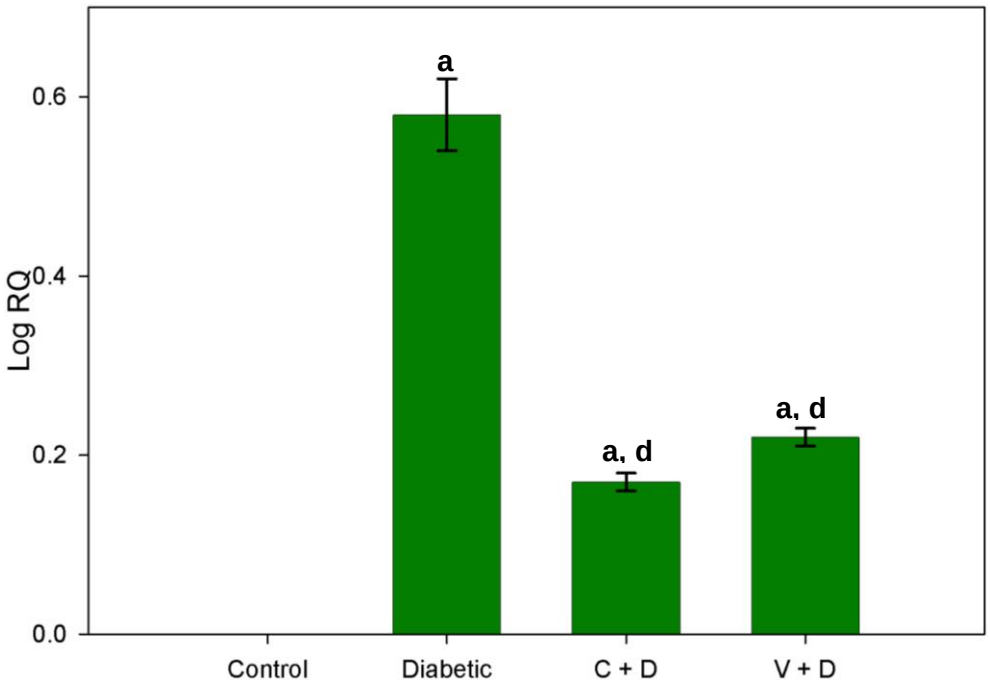


Table-117

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.58 ± 0.04 **a**
C + D	0.17 ± 0.01 **a, d**
V + D	0.22 ± 0.01 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a $p<0.001$ when compared to Control. **d** $p<0.001$ when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-118

Real Time PCR amplification of vitamin D receptor mRNA in the hypothalamus of experimental rats

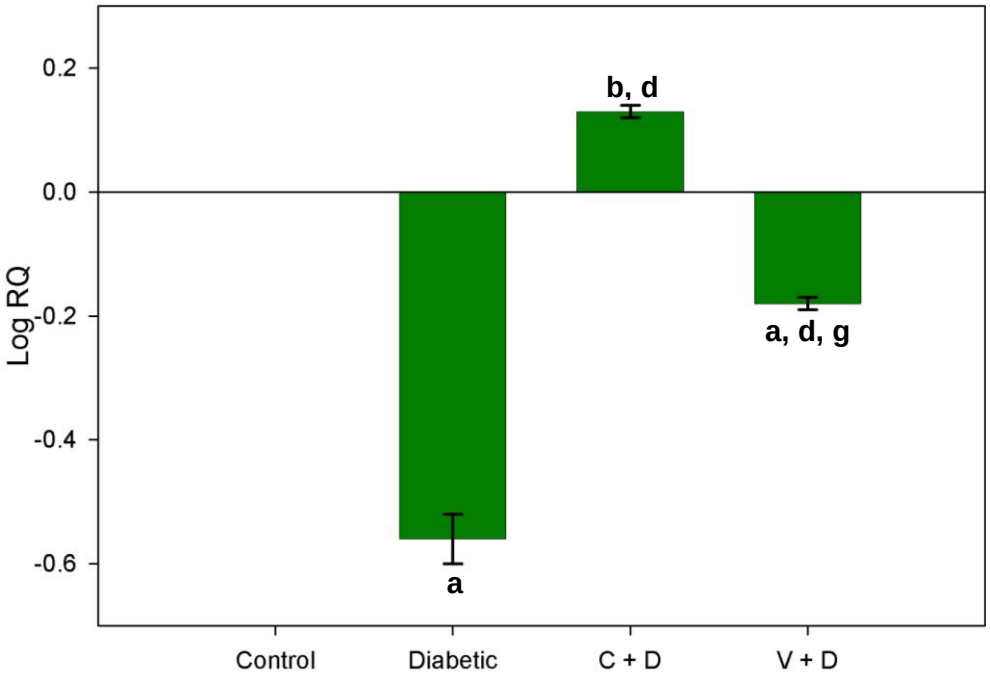


Table-118

Real Time PCR amplification of vitamin D receptor mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.56 ± 0.04 **a**
C + D	0.13 ± 0.01 **b, d**
V + D	-0.18 ± 0.01 **a, d, g**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001, **b**p<0.01 when compared to Control. **d**p<0.001 when compared to Diabetic group. **g**p<0.001 when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-119

Real Time PCR amplification of CREB mRNA in the hypothalamus of experimental rats

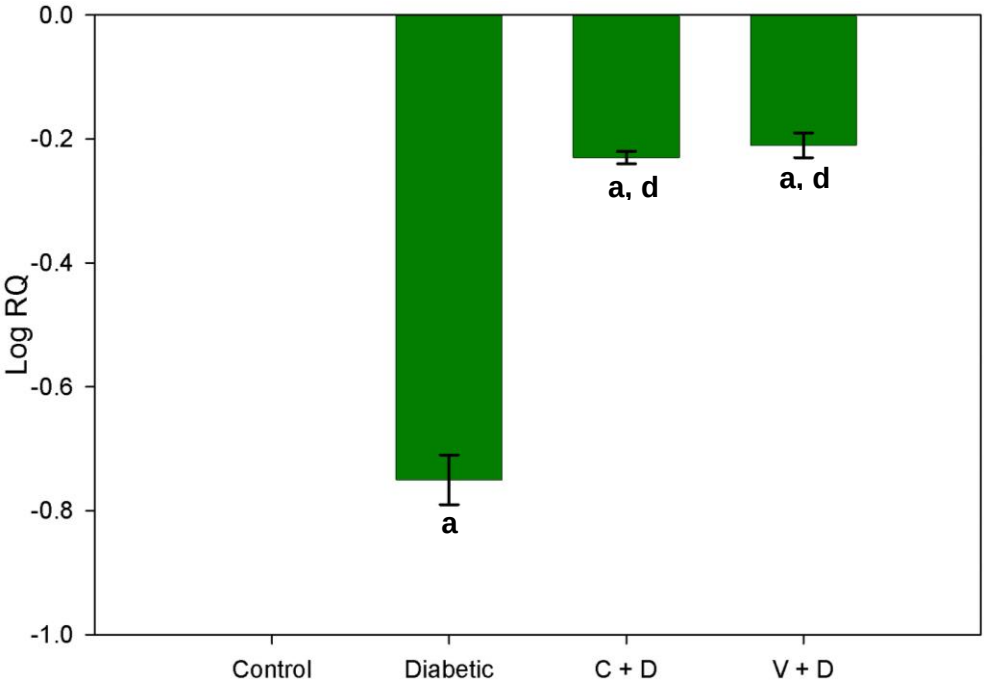


Table-119

Real Time PCR amplification of CREB mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.75 ± 0.04 **a**
C + D	-0.23 ± 0.01 **a, d**
V + D	-0.21 ± 0.02 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-120

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

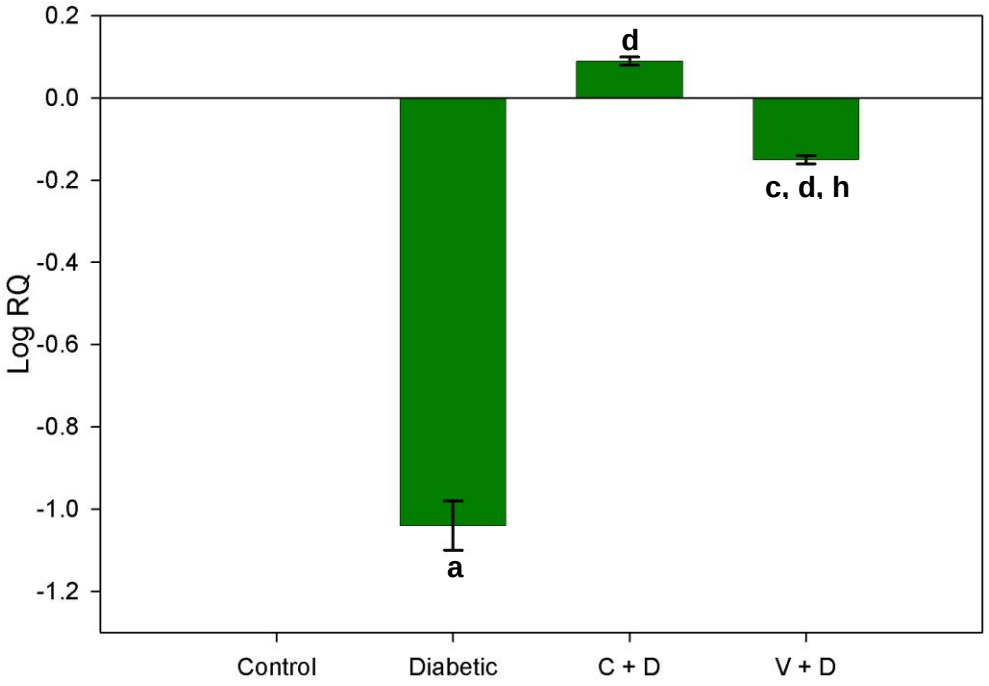


Table-120

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

Experimental groups	Log RQ
Control	0
Diabetic	-1.04 ± 0.06 **a**
C + D	0.09 ± 0.01 **d**
V + D	-0.15 ± 0.01 **c, d, h**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**$p<0.001$, **c**$p<0.05$ when compared to Control. **d**$p<0.001$ when compared to Diabetic group. **h**$p<0.01$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-121

Real Time PCR amplification of insulin receptor mRNA in the hypothalamus of experimental rats

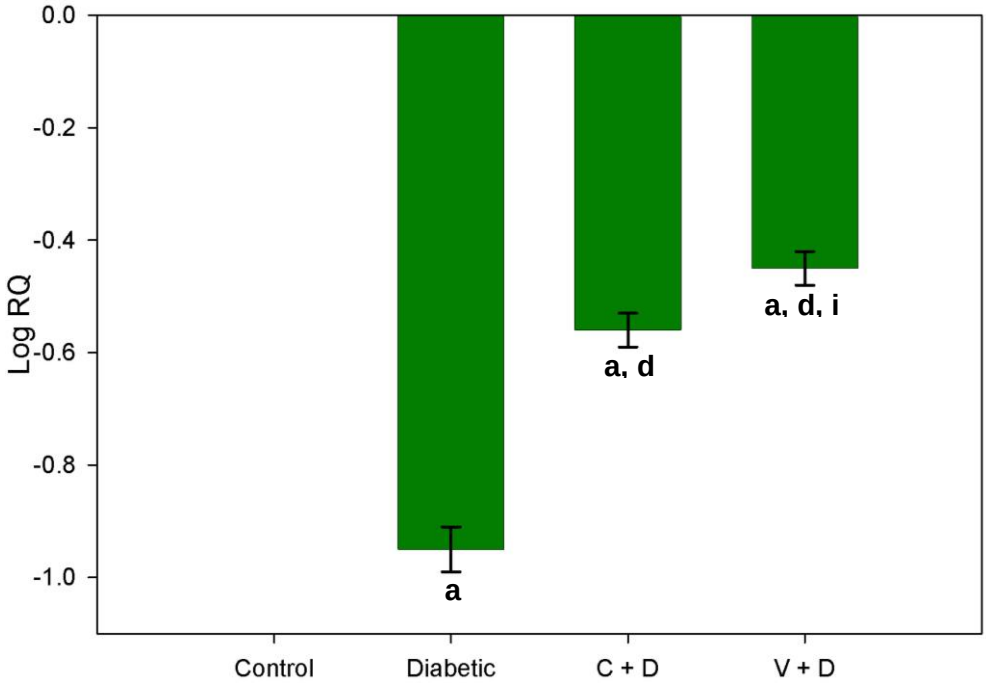


Table-121

Real Time PCR amplification of insulin receptor mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.95 ± 0.04 **a**
C + D	-0.56 ± 0.03 **a, d**
V + D	-0.45 ± 0.03 **a, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats. **a**p<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group. **i**p<0.05 when compared to C + D group.

C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-122

Real Time PCR amplification of GLUT 3 mRNA in the hypothalamus of experimental rats

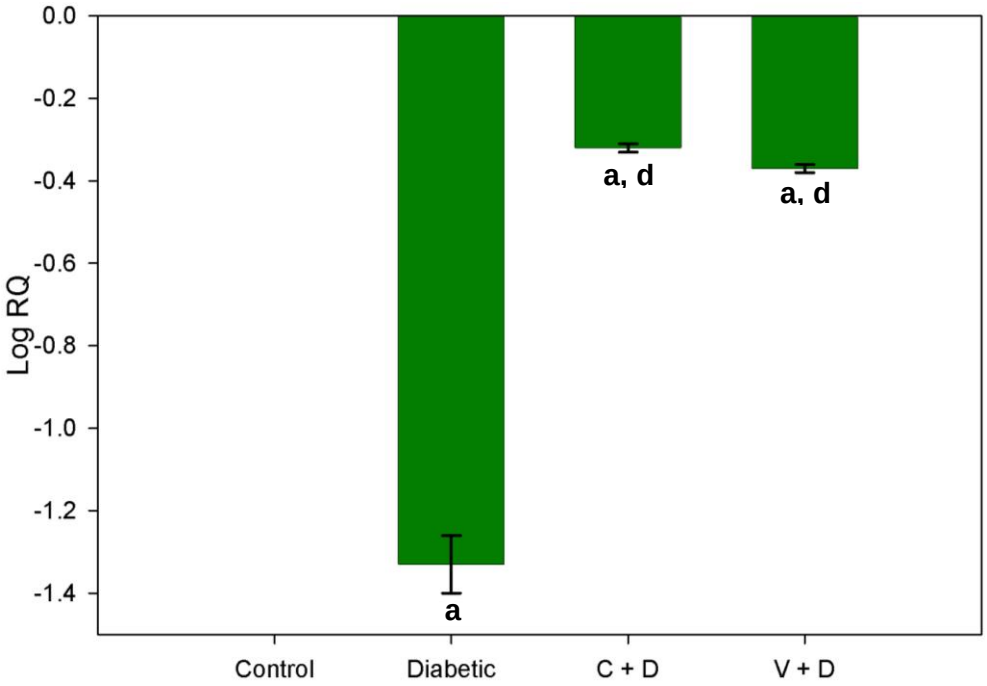


Table-122

Real Time PCR amplification of GLUT 3 mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.33 ± 0.07 **a**
C + D	-0.32 ± 0.01 **a, d**
V + D	-0.37 ± 0.01 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-123

Real Time PCR amplification of superoxide dismutases mRNA in the hypothalamus of experimental rats

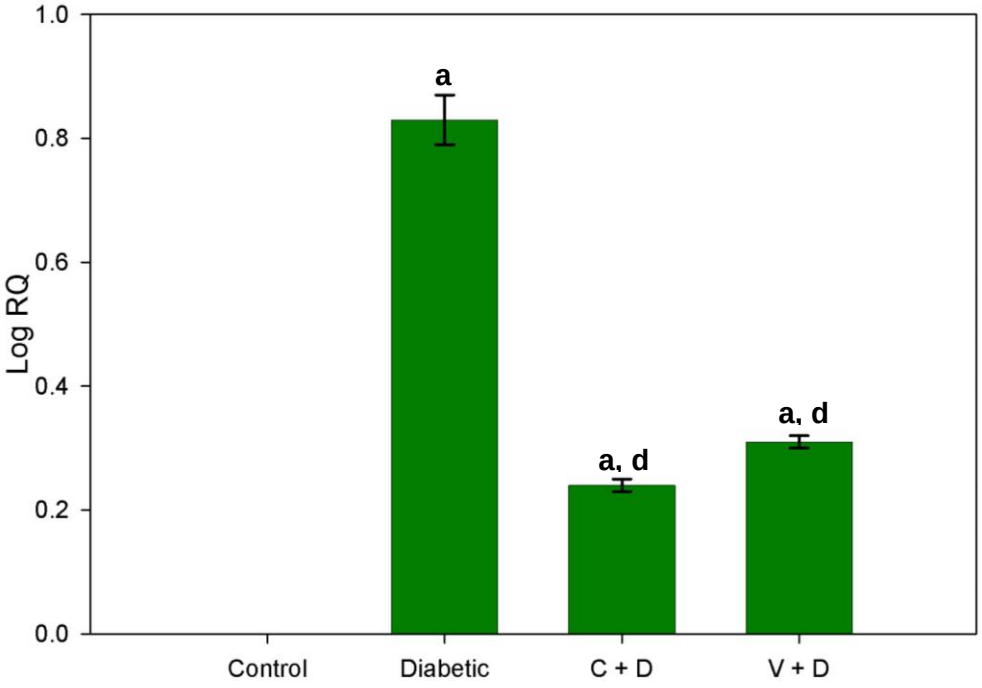


Table-123

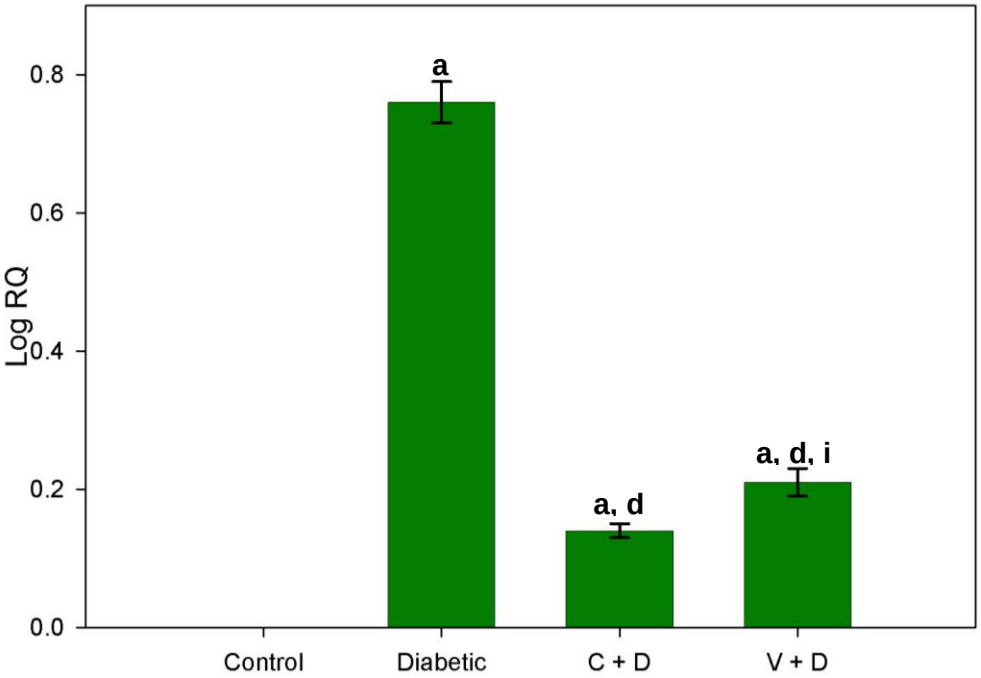
Real Time PCR amplification of superoxide dismutases mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.83 ± 0.04 **a**
C + D	0.24 ± 0.01 **a, d**
V + D	0.31 ± 0.01 **a, d**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
ap<0.001 when compared to Control. **d**p<0.001 when compared to Diabetic group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Figure-124

Real Time PCR amplification of glutathione peroxidase mRNA



in the hypothalamus of experimental rats

Table-124

Real Time PCR amplification of glutathione peroxidase mRNA in the hypothalamus of experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.76 ± 0.03 **a**
C + D	0.14 ± 0.01 **a, d**
V + D	0.21 ± 0.02 **a, d, i**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 4-6 rats.
a$p<0.001$ when compared to Control. **d**$p<0.001$ when compared to Diabetic group. **i**$p<0.05$ when compared to C + D group.
C+D- Curcumin pre-treated diabetic rats and V+D- Vitamin D_3 pre-treated diabetic rats.

Discussion

Diabetes mellitus is a chronic metabolic disease of multifactorial origin, caused by poor lifestyle choices and genetic predisposition. The increased incidence of this medical catastrophe of worldwide dimensions, is attributed to the adoption of modern life style. Sedentary life style, unhealthy diet, lack of exercise, obesity and stress are the major life style factors that contribute to the development of diabetes. Lifestyle modification pivoting on healthy diet, weight reduction, nutritional intervention and increased physical activity can prevent or delay the onset of diabetes. Out of all these factors, nutritional management holds tremendous potential to meet the challenge of diabetes management.

ANTI-DIABETOGENIC EFFECT OF CURCUMIN AND VITAMIN D_3

The present study substantiates the role of curcumin and vitamin D_3 pre-treatment in the prevention of diabetogenesis in multiple low dose streptozotocin (MLD-STZ) induced diabetic rat model. Wistar rats injected with MLD-STZ developed diabetes mellitus, characterized by hypoinsulinemia, hyperglycaemia and decreased body weight. In the curcumin and vitamin D_3 pre-treated rats, only a prediabetes condition developed after fourteen days of MLD-STZ injection. Prediabetes is a transition stage between normoglycaemia and uncontrolled hyperglycaemia characterized by fasting blood glucose levels between 100 to 125 mg/dL (Garber *et al*., 2008). On the fourteenth day after MLD-STZ administration, the pre-treated rats maintained fasting blood glucose level just above the normal, but not high enough to be diagnosed as diabetes. Prediabetes condition can either revert back to normoglycaemia or progress to a diabetic state. Annually, up to around 10% of prediabetic individuals regain normal glucose homeostasis (Tabák *et al*., 2012). The time taken for the progression of prediabetes to diabetes vary based on sex, age, physical activity, body mass index, food habits, ethnicity and geographical characteristics. Population based studies reveal that approximately 5%–10% of prediabetes cases progress to diabetes,

annually (Souza *et al*., 2012). Drugs like metformin, acarbose, orlistat and rosiglitazone have been shown to be effective in preventing the progression of prediabetes (Nathan *et al*., 2007). Several clinical trials and observational studies have demonstrated the ability of lifestyle changes and drug-based interventions to reduce the risk of diabetes by 55% to 80% (Forouhi *et al*., 2007).

To understand the pattern of glycemic changes in experimental rats, random blood glucose levels were quantified from the first day of streptozotocin injection to the fourteenth day. After MLD-STZ administration, a progressive increase in the blood glucose levels were observed in diabetic and pre-treated rats till the seventh day. But the diabetic group showed an exponential increase in the blood glucose levels after seventh day and it got stabilized at hyperglycaemic levels after eleventh day. This was the typical pattern of circulating glucose observed after MLD-STZ injection to untreated rodents (Holstad & Sandler, 1999). In case of curcumin and vitamin D_3 pre-treated rats, the glycemic level tend to fall progressively from the seventh to the twelfth day. Subsequently, the blood glucose levels were maintained in the prediabetic range. Genetic, environmental and lifestyle factors lead to the reversal or maintenance of this prediabetes condition or its progress to diabetes. (Tabák *et al*., 2012).

Circulating insulin levels of experimental rats on fourteenth day after the beginning of MLD-STZ, were in concordance with the blood glucose levels. Hyperglycaemia of the diabetic group is associated with significant hypoinsulinemia. Pre-treated rats in the intermediate hyperglycaemic state showed a corresponding reduction in circulating insulin levels. The pattern of blood glucose levels and increased circulating insulin concentration in the pre-treated group validate the activation of beta cell compensatory response. Natural physiological circumstances like insulin resistance, pregnancy, obesity and aging can significantly increase the insulin demand and induce beta cell compensatory response for the optimal control of glucose homeostasis (Matveyenko *et al*., 2008). This also indicates increased insulin secretion by beta cell mass expansion in response to the transient hyperglycaemia of the pre-treated group. Pancreatic

beta cell mass is the product of size and number of beta cells. Postnatal beta cell mass expansion is mediated by hypertrophy and mitotic cell division. The number of beta cells in pancreas is regulated by the balance between cell proliferation and apoptosis.

In our study, curcumin and vitamin D_3 pre-treatment for 60 days significantly increased the body weight of experimental rats. Earlier studies have reported the increase in lean body mass of rodents upon curcumin supplementation (Weisberg *et al*., 2008). But curcumin is also reported to have anti-angiogenic activity, necessary for the growth of adipose tissue (Alappat & Awad, 2010). It modulates adipocyte metabolism by stimulation of fatty acid degradation and inhibition of lipid synthesis and storage. Previous clinical, observational and experimental studies have suggested the association of vitamin D_3 nutritional status with changes in total body weight (Bell *et al*., 1985; Jungert *et al*., 2012; Wamberg *et al*., 2013). The major physiological function of vitamin D_3 is to maintain serum calcium and phosphorus levels to aid bone mineralization. Vitamin D_3 induced increased deposition of calcium hydroxyappatite in the bone matrix will increase the bone density and mass. So the increased body weight after vitamin D_3 supplementation is partially due to increased bone mineralization (Holick, 2004). Action of vitamin D_3 through vitamin D receptor suppresses adipogenesis by inhibition of the parathyroid hormone (McCarty & Thomas, 2003). Further, vitamin D_3 deficiency is associated with an increased risk of lipid accumulation and obesity. So vitamin D_3 and curcumin induced body weight increase is unlikely to cause obesity or hyperlipidemia. Hence the body weight increase after curcumin and vitamin D_3 supplementation can be considered as a good sign of overall health improvement.

Blood glucose and insulin levels of diabetic rats clearly emphasize the lack of beta cell compensatory response. This is due to the inability of diabetic group to expand functional beta cell mass according to the increased metabolic demand (Butler *et al*., 2003). Streptozotocin mediated beta cell damage disturb the metabolic balance between beta cell birth and death in response to the insulin

demand. Beta cell proliferation and size expansion will no longer be able to compensate the increased insulin demand (Meier *et al*., 2005). The resultant hyperglycaemia induce further beta cell death due to glucotoxicity mediated free radical production and apoptosis. This mechanism of beta cell dysfunction and death is similar to human diabetogenesis induced by pregnancy, obesity and insulin resistance (Ritzel *et al*., 2006).

Curcumin and vitamin D_3 pre-treated rats activate beta cell compensatory response against streptozotocin induced loss of insulin producing cells. Beta cells respond to transient hyperglycaemia by increasing cell size and number in order to maintain adequate population of functional beta cells in the pancreas. These compensatory changes in beta cell mass help to retain sufficient pancreatic insulin output (Sorenson & Brelje, 1997). Pre-treatment induced increase in beta cell regeneration potential partially compensates for streptozotocin mediated beta cell dysfunction and apoptosis. Increased beta cell proliferation and neogenesis has also been observed to compensate apoptosis in Zucker diabetic fatty rats (Pick *et al*., 1998). Likewise, during pregnancy, a 3 to 4 fold increase in beta cell mass occur in rodents by hypertrophy and proliferation (Rieck *et al*., 2009). So essentially, the pre-treatments help to activate the natural compensatory mechanism regardless of the presence of beta cell toxin, streptozotocin.

Curcumin and vitamin D_3 action on pancreatic beta cells are mediated through both genomic and non-genomic mechanisms. The non-genomic actions are mediated by its antioxidant and anti-inflammatory properties, regulation of protein expression and cellular metabolism. Antioxidant property of curcumin and vitamin D_3 help to reduce streptozotocin induced oxidative stress and protect beta cells from free radical toxicity. Beta cell damage also occurs due to the inflammatory response of the immune system against streptozotocin mediated tissue damage. Thus, the anti-inflammatory property of curcumin and vitamin D_3 support the maintenance of a functional beta cell population in pancreas (Luca *et al*., 2000; Menon & Sudheer, 2007).

Role of curcumin

Active constituents of plants have received considerable attention in the treatment and prevention of diabetes and associated complications. Various studies have demonstrated several health benefits of oral administration of curcumin (Ammon & Wahl, 1991). Anti-hyperglycaemic and anti-hyperlipidemic property of curcumin is well documented in rodent models of diabetes, induced by alloxan and streptozotocin (Zhang *et al*., 2013). Studies in experimental diabetes have revealed several possible mechanisms of action of curcumin on pancreatic beta cells. They also have a role in maintaining pancreatic insulin output and glucose homeostasis after MLD-STZ treatment, as observed in the present study. Persistent hyperglycaemia during diabetes produce glucotoxicity to beta cells by autoxidation of glucose, activation of polyol pathway and increased glycation of proteins. Curcumin treatment prevents oxidative stress in the pancreas of diabetic rats by reduction of reactive oxygen free radicals, lipid peroxidation and thiobarbituric acid reactive substances. Curcumin exerts its antioxidant effect through both direct and indirect mechanisms. Curcumin mechanistically interferes with calcium regulation and protein kinases to inhibit the generation of reactive oxygen species (Balasubramanyam *et al*., 2003). Further, it prevents reactive oxygen species induced apoptosis by inhibiting caspase activation and blocking cytochrome c release (Chan *et al*., 2005). Curcumin administration significantly reduces circulatory lipid peroxidative markers and inhibit TBARS and protein carbonyls (Mahesh *et al*., 2005). Balamurugan *et al*., (2009) reported that curcumin protects transplanted pancreatic beta cells from oxidative stress by the induction of endogenous antioxidant defence system in beta cell. Curcumin also protects beta cells from the toxicity of diabetes associated pancreatic amyloid deposits (Sparks *et al*., 2012).

Pancreatic inflammation is one of the main contributors to beta cell dysfunction and death. Curcumin reduces inflammatory response against beta cells by decreasing lymphocyte infiltration in pancreatic islets and normalizing the key cytokine levels. Curcumin has the capacity to limit the release of pro-

inflammatory factors from immune cells by modulating the membrane fluidity (Margina *et al*., 2013). Anti-inflammatory property of curcumin suppresses the activation and lymphokine secretion of lymphocytes and macrophages by modulating its CD markers. It also induces epigenetic changes in the monocytes by activating histone deacetylases and inhibiting histone acetyltransferase (Pham & Lee, 2012). Best *et al*., (2007) associated the hypoglycaemic actions of curcumin to its ability to normalize electrophysiological activities of beta cells. This electrical activity is essential to maintain the neuronal excitability of islet cells. This implicates the significance of sympathetic and parasympathetic nerve innervations to beta cells in the maintenance of normoglycaemia. Systemic action of curcumin also helps to stimulate glucose utilization and reduce diabetes associated complications.

Role of vitamin D_3

The extant relationship between diabetes and vitamin D_3 was demonstrated a long time back. The increased prevalence of vitamin D deficiency and current epidemics of diabetes mellitus can be correlated. In case of humans, only a small amount (~30%) of vitamin D is obtained from diet, as only a few foods contain it naturally. As a result, overall vitamin D requirements are covered from sunlight-induced photochemical conversion of 7-dehydrocholesterol (Holick, 2004). Industrialization has reduced exposure to sunlight, thus increasing our dependence on dietary sources of Vitamin D. National Health and Nutrition Examination Survey (NHANES) statistics demonstrate that more than 90% of the pigmented population of the United States (Blacks, Hispanics and Asians) are sufferers of vitamin D insufficiency (25-hydroxyVitamin D<30 ng/mL), with nearly three fourths of the white population also being vitamin D insufficient (Adams & Hewison, 2010). Several studies have described an intriguing inverse correlation between sunshine exposure, vitamin D_3 nutritional status and the pathogenesis of diabetes (Takiishi *et al*., 2010).

Based on various observational studies, the dietary intake of 2,000 IU of vitamin D during the first year of life diminishes the risk of developing type 1 diabetes (Seshadria *et al*., 2011). Vitamin D deficiency causes bone-deforming disease, rickets during development; while in adults, it causes osteomalacia and disturbed muscle metabolism owing to the impairment in calcium balance. These effects have also been observed in patients with type 2 diabetes; they exhibit abnormalities in mineral and vitamin D metabolism eventually resulting in osteopenia (Inaba *et al*., 1999). The increased prevalence of diabetes in obesity can be associated to hypovitaminosis D. Obesity promotes chronic vitamin D deficiency due to its efficient sequestration in fat stores, where it is not bioavailable (Holick, 2007). Evidence from various experimental studies also supports the correlation between vitamin D status and diabetes. In non-obese diabetes type 1 mice, vitamin D_3 administration reduced the incidence of diabetes to 80%. Similarly in vitamin D supplemented children, the risk of developing childhood-onset diabetes is reduced to 33% when compared with non-supplemented children. Our previous studies have demonstrated the use of vitamin D as a therapeutic agent for diabetes. In diabetic Wistar rats, vitamin D_3 treatment significantly ameliorated hypoinsulinemia and hyperglycaemia (Kumar *et al*., 2011). Ismail and Namala (2000) observed impaired glucose tolerance and altered insulin sensitivity in diet-induced Vitamin D deficient rats. Thus, the deficiency in vitamin D increases the susceptibility to glucose intolerance, alters insulin secretion and hence lead to the development of diabetes.

Several lines of evidence emphasize that vitamin D_3 is a potential risk modifier for diabetes mellitus. Vitamin D is thought to have both genomic and non-genomic effects on various mechanisms related to the pathophysiology of diabetes. Lee *et al*., (1994) reported that genomic action of vitamin D_3 in pancreatic beta cells is mediated through vitamin D receptor. 1-alpha-hydroxylase enzyme expressed by beta cells help to convert biologically inactive 25-hydroxyvitamin D to the active form 1,25-dihydroxyvitamin D. So beta cells respond to both circulating 1,25-dihydroxyvitamin D and 25-hydroxyvitamin D.

Vitamin D_3 directly increases insulin secretion and insulin sensitivity from beta cells by the stimulation of expression of different growth promoting receptors and transcription factors (van Etten & Mathieu, 2005; Pittas *et al*., 2007). Its role in maintaining circulating calcium homeostasis indirectly supports insulin secretion by regulating the calcium flux through beta cell membrane.

As the aetiology of diabetogenesis is intricate and multifaceted, anti-diabetogenic property of vitamin D_3 will be multidimensional. According to the present literature, there are four possible mechanisms by which vitamin D_3 can control diabetes. Firstly, the immunomodulatory action of vitamin D (Mathieu *et al*., 2005) helps to prevent the beta cell death. Secondly, circulating vitamin D binds to the vitamin D receptor and enhances insulin receptor expression; thereby improving insulin sensitivity (Calle *et al*., 2008). Thirdly, vitamin D facilitates calcium absorption in the intestine. Vitamin D and calcium act synergistically to reduce the risk of diabetes (Pittas *et al*., 2007). Lastly, vitamin D itself is essential for glucose induced insulin release and the maintenance of glucose tolerance (Ismail & Namala, 2000). Vitamin D_3 helps to prevent pancreatic beta cell dysfunction, inhibits inflammation and autoimmune destruction of pancreatic beta cells, improves insulin action and regulates calcium homeostasis. These effects of vitamin D_3 contribute to the prevention of diabetogenesis after MLD-STZ administration to the pre-treated group.

PANCREATIC BETA CELL COMPENSATORY RESPONSE

Since reduced beta cell mass is a hallmark of all types of diabetes, it is worthwhile to study the pancreatic regeneration associated with the anti-diabetogenesis effect of curcumin and vitamin D_3 pre-treatment. Beta cell regeneration occurs *via* three mechanisms-proliferation of existing beta cells, neogenesis from precursor cells and trans-differentiation of other cell types (Wang *et al*., 2008). Dor *et al*., (2004) performed genetic lineage tracing studies to determine the type of differentiation that contributes to beta cell compensatory response. They concluded that terminally differentiated pancreatic beta cell

proliferation is the main source of new beta cells. Out of the various methods available for measuring cell proliferation potential, direct measurement of DNA synthesis is the most reliable one (Rubini *et al*., 1964). So, we used tritiated thymidine incorporation studies to quantify DNA synthesis in pancreatic beta cells isolated from experimental rats. Our data showed that, beta cells isolated from diabetic rats have a significantly decreased tritiated thymidine incorporation capacity. Hence, the amount of DNA synthesis in diabetic group is significantly reduced when compared to control. This reduced regenerative capacity is a result of toxicity of streptozotocin in beta cells (Rossini *et al*., 1977). Streptozotocin directly hinders the natural ability of pancreatic beta cells to proliferate in culture medium. In diabetic group, persistent hyperglycaemia induced glucotoxicity and lipotoxicity also limits proliferative potential of beta cells. Similar to human diabetogenesis process, the decreased proliferative capacity of pancreatic beta cells has an important role in the pathophysiology of MLD-STZ induced diabetic rat models (Del Prato, 2009).

Curcumin and vitamin D_3 supplemented rats showed a significant increase in the DNA synthesis when compared to control and diabetic group. This indicates stimulated proliferation of beta cells upon pre-treatment with curcumin and vitamin D_3. Pancreatic beta cells are known to have the ability to proliferate in response to metabolic demands to maintain or increase beta cell mass. In normal rat pancreas, 2–3% of beta cells replicate every 24 hours, thus accounting for slow turnover during adult life. Pregnancy promotes up to a 10-fold increase in beta cell division and insulin resistance induces a 30-fold amplification of beta cell mass (Banerjee *et al*., 2005). The significant increase in cell proliferation in the pre-treated group of rats when compared with diabetic group is due to the protective role of curcumin and vitamin D_3 against streptozotocin. Intrinsic antioxidant capacity of beta cells are considerably low due to naturally decreased expression levels of major antioxidant enzymes like superoxide dismutases, glutathione peroxidase and catalase (Tiedge *et al*., 1998). Hence, the beta cells are particularly vulnerable to oxidative stress, which hinder the proliferative capacity of cells. The

intrinsic antioxidant capacity of curcumin and vitamin D_3 induces antioxidant defence in beta cells, thereby protecting them from free radical damage (Luca *et al*., 2000; Menon & Sudheer, 2007). This also counters the inhibitory action of streptozotocin on cell proliferation.

The significant increase in beta cell proliferation potential compared to control indicates the activation of beta cell compensatory response in pre-treated rats. Curcumin is a regulator of cell cycle progression particularly in S and G2/M phase. Curcumin treatment was reported to improve stem cell potency and activate cell proliferation in precursor cells (Kim *et al*., 2011). Similar effect of curcumin was also observed in hippocampal neural progenitor cell and mesenchymal stem cell differentiation (Gu *et al*., 2012; Kim *et al*., 2008). Activation of beta cell proliferation in vitamin D_3 pre-treated rats are mediated by vitamin D receptors. Vitamin D receptor response element mediated activation and inhibition of cell cycle regulators control cell cycle progression and cell proliferation (Samuel & Sitrin, 2008). Vitamin D is already known to have a major role in regulating cell proliferation in cardiac myocytes, smooth muscle cells, chondrocytes and several cultured cell lines (Binderup & Bramm, 1988; Schwartz *et al*., 1989; O'Connell *et al*., 1997).

Beta cell compensatory response to hyperglycaemia also includes beta cell size expansion and increased insulin secretion. Substantial increase in protein synthesis is essential to increase growth and secretory activities of cells. So, assessment of protein synthesis is a valuable parameter for evaluating the growth and protein secretion of isolated pancreatic beta cells. Leucine is an essential amino acid present in most eukaryotic proteins. Incorporation of radiolabelled leucine into cultured cells is used as an index of protein synthesis (Freshney *et al*., 1975; Ignotz & Massagué, 1986). Our data showed that leucine incorporation was significantly low in beta cells isolated from the diabetic group of rats. Streptozotocin treatment selectively damages the synthetic activity of beta cells and consequent reduction in tritiated leucine incorporation. Further, streptozotocin induced diabetes is associated with a reduced protein synthesis in cytoplasm and

mitochondria of various tissues including pancreas (Martin *et al*., 1995). Significant reduction in protein synthesis in the beta cells of diabetic rats validate the reduced capacity of growth and insulin secretion in response to increased metabolic load.

The significant increase in leucine incorporation in the curcumin and vitamin D_3 pre-treated rats, compared to the control is due to their protective effect against streptozotocin. This shows increased protein synthesis, secretory activity and size expansion of beta cells of pre-treated rats. Up regulation of protein biosynthesis in beta cells of pre-treated rats facilitates the synthetic activity of cell, which mainly comprises of insulin release (Molleson *et al*., 1973). Intensification of thymidine and leucine incorporation in pre-treated group indicates the accelerated growth and proliferation of beta cells, which can account for the significant increase in insulin secretion.

Proliferating cells have hypomethylated DNA and this DNA can incorporate more methyl groups than DNA from non-proliferating cells (Cravo *et al*., 1996). To confirm the increased beta cell proliferation *in vivo*, tritiated methyl group incorporation studies were carried out in the beta cells of experimental rats. DNA from the beta cells of diabetic group incorporated significantly higher concentration of tritiated methyl group than the control. This indicates reduced methylation of beta cell DNA. Streptozotocin is a DNA alkylating agent, it directly methylates purine bases and disturbs the normal methylation pattern of genome (Pegg *et al*., 1985; Murata *et al*., 1999). In response to increased DNA methylation, beta cells activate the enzyme DNA alkyltransferase that remove methyl group form DNA (Pegg *et al*., 1985). Further, streptozotocin reduces DNA methylation by stimulating unscheduled DNA synthesis in cell (Tyson & Mirsalis, 1985). The reduced methylation in beta cell DNA of diabetic group without any marked increase in DNA replication, protein synthesis and insulin release could be due to the genotoxicity of streptozotocin rather than increased beta cell proliferation. Beta cells isolated form curcumin and vitamin D_3 pre-treated rats showed a significant increase in methyl group incorporation capacity when

compared with both control and diabetic groups. This might be due to the production of unmethylated DNA strands during rapid S phase DNA replication. Active demethylation of DNA by DNA glycosylase and AID/Apobec family of deaminases also reduce methylation of DNA (Zhu, 2009). Hypomethylation of DNA is a major epigenetic marker of proliferating cells. This epigenetic change in curcumin and vitamin D_3 pre-treated rats are associated with modulation of some important genes associated with cell survival and cell cycle progression (Ehrlich, 2009).

OXIDATIVE STRESS IN PANCREATIC BETA CELLS

Pancreatic beta cells are extremely sensitive towards oxidative stress due to the weak antioxidant defence system present. Redox imbalance due to the overproduction of free radicals contribute to beta cell dysfunction, necrosis and/or apoptosis. All types of diabetogenesis are accompanied by beta cell functional impairment and mass reduction due to oxidative stress (Kajimoto & Kaneto, 2004). Oxidative stress can be measured accurately by quantifying lipid peroxidation. One of the end products of lipid peroxidation of polyunsaturated fatty acids is malondialdehyde (MDA). Variations in the free radical levels in the cells are directly reflected in the MDA levels. It is a commonly accepted biomarker for the assessment of oxidative stress and antioxidant status (Gaweł *et al*., 2004).

In our experiment, the pancreatic beta cells isolated from the diabetic group showed a significant increase in MDA concentration when compared with control. This can be due to the direct or indirect action of streptozotocin. Streptozotocin increases lipid peroxidation in the beta cells by stimulating the production of free radicals like nitrogen oxide and reactive oxygen species. Streptozotocin treatment is also known to inhibit the antioxidant enzymes, superoxide dismutases and glutathione peroxidase and reduce the level of non-enzymatic antioxidants like glutathione, vitamin C and vitamin E (Naresh *et al*., 2013). This leads to oxidative stress induced partial loss of beta cell function and

result in hyperglycaemia. The high blood glucose levels further increase reactive oxygen species accumulation in the beta cells (Newsholme *et al*., 2007). The main sources of hyperglycaemia induced free radicals are increased oxidation in mitochondrial electron transport chain, activation of protein kinase C and increase in intracellular calcium ion levels (Newsholme *et al*., 2009). Increased production of free radicals stimulates peroxidation of polyunsaturated fatty acids in the membrane, DNA damage and oxidation of proteins. This, in turn contributes to the development of glucotoxicity and lipotoxicity in beta cells (Robertson *et al*., 2004). Hence hyperglycaemia associated oxidative stress combined with streptozotocin toxicity contribute to free radical production and beta cell dysfunction, apoptosis and necrosis.

Lipid peroxidation associated MDA levels were significantly decreased in the pancreatic beta cells of curcumin and vitamin D_3 pre-treated rats when compared to the diabetic group. Curcumin is a strong antioxidant and has the ability to protect bio-membranes from free radical mediated lipid peroxidation. It effectively scavenges reactive oxygen and nitrogen radicals by its phenolic and central methylenic hydrogen functional groups (Menon & Sudheer, 2007). Curcumin also strengthens the antioxidant defence system by inducing transcriptional level changes in the cell through haem oxygenase-1. α, β-unsaturated carbonyl groups in the curcumin activate the translocation of Nrf2 protein into the nucleus. Nrf2 protein binds to the antioxidant-responsive element present in the promoter region of several proteins involved in antioxidant stress response (Balogun *et al*., 2003; Jeong *et al*., 2009).

Vitamin D is a lipophilic membrane antioxidant that accumulates in bio-membranes to effectively inhibit the peroxidation of polyunsaturated fatty acids. Secosteroid ring structure of vitamin D_3 mediates its interactions with saturated, monounsaturated and polyunsaturated residues of membrane phospholipids to decrease membrane fluidity and lipid peroxidation (Wiseman, 1993). It is also reported to have a role in strengthening the existing free radical scavenging system by inducing the expression of superoxide dismutases, glutathione peroxidase and

thioredoxin reductase in various tissues *via* the activation of vitamin D receptor (Noyan *et al.,* 2005). Vitamin D supplementation is also known to modulate the nitric oxide generating enzyme, nitric oxide synthase (Garcion *et al*., 1997). Like curcumin and many other plant derived polyphenols, vitamin D activates Nrf2-antioxidant-responsive element pathway against the oxidative stress (Balogun *et al*., 2003; Bobilev *et al*., 2011).

Results of radiolabeled precursor incorporation studies, together with the analysis of lipid peroxidation and circulating insulin levels of experimental rats suggest that MLD-STZ cause a substantial reduction in the functional pancreatic beta cell population. As the beta cells are inherently prone to oxidative stress, streptozotocin induced free radical production and DNA damage lead to permanent dysfunction and death of beta cells. These rats are also unable to mount an effective beta cell compensatory response against the resultant hyperglycaemia. Curcumin and vitamin D_3 pre-treatments protect beta cells against streptozotocin toxicity by their endogenous antioxidant properties and by their ability to promote the antioxidant defence system of cell. They initiate effective beta cell compensatory response by accelerating beta cell growth, insulin secretion and proliferation in response to increased metabolic demands. The change in the pattern of blood glucose levels in pre-treated rats show that hyperglycaemia induced activation of beta cells could compensate the streptozotocin toxicity.

Curcumin and vitamin D_3 stimulated insulin synthesis and secretion by activation of protein biosynthesis and direct modulation of beta cell mass expansion appears to be regulated by multiple factors. The mechanism of pre-treatment induced increased beta cell regeneration involves the active signalling through many critical cell proliferation and differentiation pathways. Curcumin and vitamin D_3 also increase functional pancreatic beta cell population by providing resistance against streptozotocin induced apoptosis and necrosis. Delineating the molecular mechanisms that promote beta cell compensatory response, maintain pancreatic insulin output and protect cells against death is

important to elucidate the anti-diabetogenesis property of curcumin and vitamin D_3.

PANCREATIC BETA CELL REGENERATION

Pancreatic beta cell compensatory response is mediated by the activation of several intercellular pathways controlling cell growth, proliferation and differentiation. Several transcription factors and intracellular signalling molecules are involved in the process. Keller *et al*., (2008) identified a module of 217 genes that are involved in the regulation of beta cell replication. Further, beta cell mass and insulin secretion are controlled by sympathetic and parasympathetic nerve innervations to pancreas. The mechanism underlying the anti-diabetogenesis effect of curcumin and vitamin D_3 can be elucidated only by studying the molecular level changes in pancreas and brain. This was investigated by further studies on the gene expression of key regeneration markers: Akt, NeuroD1, Pax, Pdx-1, insulin like growth factor-1, NF-κB and cyclin D2.

Akt, also known as protein kinase B, has a major role in insulin dependent glucose uptake and regulation of beta cell hypertrophy, proliferation and cell survival. Gene expression studies and confocal analysis of Akt showed a significant down regulation in diabetic group. This suppression of Akt activity in beta cells activates cytokine induced apoptosis and inhibits insulin secretion (Chen *et al*., 2006). It also alters beta cell response to increased free radical levels through PI3K/Akt pathway and contributes to the accumulation of reactive oxygen and nitrogen species in beta cells (Størling *et al*., 2005). This stimulates progressive deterioration of beta cell function and activates apoptotic cell death. Decreased expression of Akt also impairs insulin and insulin like growth factor signalling pathway for beta cell survival and mass expansion. Studies on Akt dead mutants of the beta cells have revealed the critical role of Akt for maintaining pancreatic insulin output. Pancreas of these transgenic animals shows severe alterations in beta cell architecture, total beta cell mass and insulin synthesizing capacity (Bernal-Mizrachi *et al*., 2004).

When compared to both control and diabetic, Akt mRNA and protein expressions were significantly up regulated in curcumin and vitamin D_3 pre-treated rats. Increased Akt expression is known to regulate many processes including metabolism, proliferation, cell survival, growth and angiogenesis in many tissues including pancreas. It also promotes beta cell compensatory response against hyperglycaemia (Srinivasan *et al*., 2002). The over expression of Akt in the beta cells of mice significantly increase beta cell mass by increased beta cell proliferation and size expansion. It increases beta cell regeneration through stimulation of cell cycle progression by the activation of cyclin D1, cyclin D2 and cyclin-dependent kinase (Fatrai *et al*., 2006). Glucose- mediated PI3-kinase/Akt beta cell survival pathway becomes more active due to the over expression of Akt (Withers *et al*., 1998). Bernal-Mizrachi *et al*., (2001) observed that significant increase in the Akt gene expression in pancreas is associated with beta cell survival and differentiation. Activated Akt directly phosphorylates MAP3K5 and GSK3, which mediate cell survival and proliferation signals respectively. Akt induced phosphorylation reduces oxidative stress mediated stimulation of MAP3K5 kinase activity and prevents apoptosis. Akt has a regulatory role in gene transcription and activity of NF-κB and CREB. Phosphorylated CREB in turn induces the transcription of pro-survival genes such as BCL2 and MCL1 (Hers *et al*., 2011). Zhang and Zanello (2008) showed that vitamin D_3 acts through vitamin D receptor to up regulate Akt to increase cell survival and supresses apoptotic cell death. Taken together, a significant up regulation of Akt expression in curcumin and vitamin D_3 pre-treated group stimulate an increase in pancreatic cell proliferation potential and provide resistance to apoptotic cell death. This helps in the maintenance of functional beta cell population after MLD-STZ injection.

Formation of new beta cells in pancreas, by the differentiation of progenitor cells was studied by monitoring the gene expression of transcription factors like NeuroD1, Pax and Pdx-1. NeuroD1 is a key regulator of pancreatic islet maturation and insulin hormone gene transcription. Its gene expression was significantly down regulated in diabetic rats when compared to control. This

decrease in NeuroD1 expression is correlated with the inability of diabetic rats to maintain a mature beta cell population and insulin production. Further, NeuroD1 controls insulin gene transcription by binding to the E-box of the insulin promoter and interacts with the p300 co-activator in pancreatic beta cells (Atouf *et al*., 1997). So decreased NeuroD1 expression directly supresses insulin biosynthesis at the transcriptional level. Further, it was shown that mutation induced decrease in promoter binding of NeuroD1 causes diabetes in humans (Malecki *et al*., 1999). Experiments using targeted disruption of the NeuroD1 gene induces a striking reduction in beta cells mass with impaired beta cell maturation. Homozygous NeuroD1-null mice die shortly after birth due to neonatal diabetes that result from developmental and functional defects of the pancreatic endocrine cells (Naya *et al*., 1997). Pre-treated rats showed a significant up regulation in NeuroD1 expression when compared to control and diabetic groups. This helps in increasing insulin gene transcription and secretion from pancreas. Increased expression also helps to augment the glucose sensitivity of beta cells. Hyperglycaemia induced nuclear translocation of NeuroD1 modulate the expression of different ion channels and regulators involved in glucose mediated insulin release (Andrali *et al*., 2008). Further, increased NeuroD1 expression is a marker for beta differentiation from progenitor cells. Recombinant expression of NeuroD1 genes in hepatocytes transforms it into insulin producing cells expressing most of the beta cell specific markers (Kojima *et al*., 2003). Among the pre-treated groups, vitamin D_3 induced significant increase in NeuroD1 expression when compared to curcumin. This up regulation might be due to the genomic action of vitamin D_3 *via* vitamin D receptor.

During development, Pax gene expression assign early pancreatic epithelium proliferation, branching and subsequent differentiation. It is a critical transcription factor for normal formation of all islet cell types and is involved in the differentiation of beta cells of pancreas (Frost *et al*., 2008). Significantly increased Pax expression in the pancreas denote increased beta cell regeneration. In diabetic rats, increased Pax expression was not able to compensate for the

streptozotocin induced loss of beta cells. In curcumin and vitamin D_3 pre-treated rats, Pax expression was significantly up regulated when compared to both control and diabetic. This indicates increased beta cell neogenesis from pancreatic stem cells and precursor cells. Sosa-Pineda *et al*., (1997) demonstrated the importance of Pax in differentiation of the beta cell lineage. Homozygous Pax mutant mice did not have any insulin producing beta cells in the pancreas. After the completion of development, Pax expression is significantly low in adult pancreas. Beta cell compensatory response induced beta neogenesis activates Pax expression. It helps in beta cell mass expansion by regulating beta cell neogenesis, differentiation, stem cell maintenance and apoptosis. In pre-treated rats, increased Pax expression activates insulin gene transcription and maintains insulin producing beta-cell populations by controlling maturation and differentiation of pancreatic precursor cells (Fowden & Hill, 2001; Frost *et al*., 2008). The significant increase in Pax expression in the vitamin D pre-treated rats compared to that of curcumin pre-treated rats might be due to vitamin D receptor mediated signalling through Ets proteins (Tolón *et al*., 2000; Drané *et al*., 2004).

Pancreatic stem cell marker Pdx-1 is a transcription factor that has a critical role in the development of endocrine pancreas and beta cell regeneration. We observed a significant down regulation of Pdx-1 gene in the pancreas of diabetic rats. The decreased expression of Pdx-1 attained using Tet-On system in transgenic mouse resulted in severe beta-cell dysfunction (Lottmann *et al*., 2001). Streptozotocin induced oxidative stress plays an important role in down regulation of Pdx-1 gene through the activation of c-Jun N-terminal kinase (JNK) pathway. This suppression is also reported to decrease the gene expression of insulin gene (Kaneto & Matsuoka, 2013). Pdx-1deficient mice lack a pancreas and die shortly after birth, indicating the absolute requirement for Pdx-1 during pancreatic development. The significant decrease in Pdx-1 expression of diabetic rats impairs beta cell function, abrogates beta cell compensatory response and glucose-stimulated insulin secretion due to the metabolic impairment (Brissova *et al*., 2002, 2005). Curcumin and Vitamin D_3 pre-treatment before the administration of

streptozotocin significantly up regulates the expression of Pdx-1 in pancreas. This Pdx-1 up regulation in adult rats appears to be a prerequisite for the differentiation of precursor cells into insulin producing beta cells. Induced overexpression of Pdx1 result in the formation of the endocrine lineages together with a significant up regulation of insulin and other pancreas-related genes (Kubo *et al*., 2011). Pdx-1 also regulates the transcription of several genes like glucokinase, insulin and glucose transporter-2, involved in glucose sensing and insulin production. Increased expression of Pdx-1 together with NeuroD1, helps to stimulate glucose mediated insulin gene transcription. Collectively these data show that decreased expression of pancreatic transcription factors like NeuroD1, Pax and Pdx-1 expression was not contributing significantly to beta cell differentiation in the diabetic group. Significant increase of NeuroD1, Pax and Pdx-1 levels in curcumin and vitamin D_3 pre-treated rats promote beta cell mass expansion by influencing several cell differentiation pathways and increase the insulin production from existing beta cells by the transcriptional activation of its promoter. It also contributes to the regulation of several cell differentiation pathways that promote the survival of beta cells of the pancreas.

Insulin like growth factor-1 belongs to a family of peptide growth factors that stimulate cell growth, proliferation and differentiation. Diabetic, C + D and V + D groups showed a significant increase in the expression of insulin like growth factor-1 when compared to control group. This increase can be considered as a protective response against increased oxidative stress in beta cells. Oxidative stress induced by hydrogen peroxide is known to increase the expression of insulin like growth factor-1 to specifically activate NF-κB mediated cell survival pathway (Heck *et al*., 1999). Pre-treated rats showed a further increase in insulin like growth factor-1 expression, compared to diabetic rats. This over expression plays an important role in pancreatic regeneration by autocrine and paracrine mechanisms to stimulate DNA synthesis and act as beta cell differentiation activator (Smith *et al*., 1988). The crucial event in insulin like growth factor-1 induced signalling is the activation of the Akt pathway through phosphorylation of

insulin like growth factor-1 receptor kinase that mediates its anti-apoptotic effect (Dickson *et al*., 2001). Increased insulin like growth factor-1 expression together with increase in Akt promotes beta cell proliferation, development and survival through IRS-2 signalling pathway. It is also reported to prevent apoptotic cell death by activating a number of cell survival signal transduction pathways (Párrizas *et al*., 1997). Transgenic mice over expressing insulin like growth factor-1 in beta cells are protected from streptozotocin induced beta cell apoptosis (George *et al*., 2002).

Transcription factor NF-κB has a major role in cell growth, survival, apoptosis and cellular stress responses. In pancreatic beta cells, its overexpression is known to cause cytokine-induced beta cell death (Baker *et al*., 2001). NF-κB expression was significantly up regulated in diabetic rats when compared to control and pre-treated rats. This over expression of NF-κB initiates beta cell apoptotic cell signalling cascade (Cnop *et al*., 2005). In the case of curcumin and vitamin D_3 pre-treated rats, NF-κB expression was significantly reduced when compared to diabetic group. Reduced NF-κB levels increase the resistance of pancreatic beta cells against cytokine and TNF-α induced extrinsic apoptosis (Ortis *et al*., 2008). Eldor and colleagues previously showed that specific inhibition of NF-κB in beta cells of pancreas prevents apoptotic cell death caused by the MLD-STZ (Giannoukakis *et al*., 2000; Eldor *et al*., 2006).

Genetic studies have demonstrated that cyclin D2 is a key D-type cyclin in beta cell proliferation and mass homeostasis. Thus, significant decrease in cyclin D2 mRNA in the pancreas of diabetic rats will hinder the beta cell replication. Maintenance of cyclin D2 mRNA levels is important for beta cell mass expansion in response to hyperglycaemia in postnatal life (Georgia & Bhushan, 2004). Hence, streptozotocin induced reduction in cyclin D2 expression to levels less than that of control will hinder beta cell expansion. Curcumin and vitamin D_3 pre-treatment significantly increased cyclin D2 expression compared to both diabetic and control groups. This helps the quiescent G0 phase cells to enter cell cycle for proliferation (Salpeter *et al*., 2011). Transient overexpression of cyclin D2 in the

pancreas initiated a robust beta cell proliferation and prevented streptozotocin induced diabetes (Chen *et al*., 2012). Cyclin D2 is also known to induce beta cell replication in response to insulin resistance and the presence of extracellular mitogens (Stamateris *et al*., 2013). Increased cyclin D2 expression can also help to protect beta cells from apoptotic cell death (He *et al*., 2009). In pancreatic beta cells, the major regulator of cyclin D2 expression is glucose (Salpeter *et al*., 2011). So, in curcumin and vitamin D_3 pre-treated rats, MLD-STZ induced hyperglycaemia trigger beta cell proliferation by cyclin D2 mediated cell cycle progression.

APOPTOSIS IN PANCREAS

Diabetes occurs when beta cell mass is reduced below a critical level, due to the insufficient regenerative capacity of pancreatic cells to replace dying beta cells. Excessive apoptotic death of pancreatic beta cells has been considered as a hallmark of diabetogenesis. MLD-STZ induced diabetes resemble human diabetogenesis in the irreversible loss of beta cells due to apoptosis (O'Brien *et al*., 1996). Compounds capable of inhibiting beta cell apoptosis can be used to delay or prevent the development of diabetes (Mabley *et al*., 2001; Bhakkiyalakshmi *et al*., 2014). Beta cell toxins and hyperglycaemia activate a number of apoptotic cell signalling pathways and cause deleterious effects. In the present study, we analysed apoptosis in pancreas by studying the expression of markers like Bax, caspase 3, caspase 8 and TNF-α.

MLD-STZ administration induced glucotoxicity activates multiple pathways of apoptosis. Hyperglycaemia associated pancreatic beta cell death *via* intrinsic apoptosis pathway is revealed through the increased gene expression of pro-apoptotic gene, Bax in the diabetic and pre-treated groups. The elevated level of Bax mRNA is closely followed by an increase of Bax protein levels. This increase in Bax concentration leads to its translocation to mitochondrial membrane and mitochondrial transmembrane pore formation, which lead to the release of cytochrome c from mitochondria. Cytochrome c binds tightly to Apaf-1 in the

cytosol and forms an apoptosome, which induce irreversible cell apoptosis (Rossé *et al*., 1998; Gallaher *et al*., 2001). A significant decrease in Bax gene expression in the pancreas of curcumin and vitamin D_3 pre-treated rats compared to the control is due to its direct and indirect effects. Pre-treatment directly reduces streptozotocin toxicity and beta cell dysfunction to inhibit Bax mediated apoptosis. As increased blood glucose is the major trigger of Bax expression, anti-hyperglycaemic effect of curcumin and vitamin D_3 indirectly helps to reduce Bax mRNA levels (Leinninger *et al*., 2006).

Activation of cysteine-aspartyl specific proteases called caspases, have a critical role in mediating pancreatic beta cell apoptosis. Significantly increased expression of caspase 3 and caspase 8 in the pancreas of diabetic rats indicate streptozotocin stimulated apoptosis of beta cells. Increased expression of caspase 3 indicates activation of intrinsic and extrinsic apoptotic pathways. This lead to the proteolytic processing of many inactive pro-apoptotic zymogens and induce selective morphological changes during apoptosis (Hoshi *et al*., 1998). Caspase 3 also contribute to the autoimmune destruction of beta cells by stimulating the antigen cross-presentation for initiating T-cell priming (Hakem *et al*., 1998; Liadis *et al*., 2005). Confocal and Real Time PCR amplification studies were used to confirm the significant decrease in caspase 3 expression in curcumin and vitamin D_3 pre-treated rats when compared with diabetic. Decreased Bax expression is reported to protect pancreatic beta cells from MLD-STZ induced diabetes in caspase 3 knockout mice (Liadis *et al*., 2005). Decreased Bax and increased insulin like growth factor 1 expression prevent beta cell apoptosis in the pre-treated group by suppressing caspase 3 expression (Cregan *et al*., 1999; Fukaya & Yamaguchi, 2005). Caspase 8 is an upstream protease in the extrinsic pathway of apoptosis. Increased expression of caspase 8 in diabetic rats induces apoptosis by initiating mitochondrial death pathway with release of cytochrome c and activation of downstream effector caspases. Curcumin and vitamin D_3 pre-treated rats showed a significant down regulation of caspase 8 when compared with diabetic group. Liadis *et al*., (2007) showed that caspase 8 is one of the main

mediators of diabetogenesis in the presence of apoptotic stimuli. But it also has a key role in the maintenance of beta cell mass and insulin secretion in response to varying metabolic demands. Further, decrease in caspase 8 expression has been shown to increase protection against streptozotocin induced beta cell death and development of diabetes. In the pre-treated rats, suppression of caspase 8 expression influences cell signalling through insulin receptors, insulin like growth factor receptor, Pdx-1 and CREB (Tuttle *et al*., 2001).

TNF-α is a key regulator of pro-inflammatory cytokines and leukocyte adhesion molecules that cause unwanted effects in chronic metabolic and inflammatory diseases, including diabetes. Our study showed that TNF-α gene expression was significantly up regulated in the pancreas of diabetic rats when compared to that of control. Mueller *et al*., (1995) observed increased expression of TNF-α mRNA in the beta cells during diabetogenesis. This increase in the expression causes pathogenic destruction of pancreatic cells due to its direct role in mediating apoptosis. The sequential steps of TNF-α induced death receptor pathway ultimately lead to the activation of initiator caspases like caspase 8 and the effector caspase, caspase 3 (Nakagawa & Yamaguchi, 2005). Anti-diabetogenesis effect of curcumin and vitamin D_3 pre-treatment is associated with significant reduction in TNF-α mRNA levels compared to the diabetic group. Decreased TNF-α levels helps to reduce apoptotic beta cell death and subsequent diabetogenesis. Yang *et al*., (1994) demonstrated that the inhibition of TNF-α using anti-TNF monoclonal antibody prevented the development of diabetes in non-obese diabetic mice. Decreased TNF-α expression in the pre-treated rats decreases the susceptibility to diabetes by suppressing the cell surface expression of ICAM-1 and Fas (Wachlin *et al*., 2003).

Taken together, these data confirm that diabetic rats have a significant up regulation of apoptotic markers, Bax, caspase 3, caspase 8 and TNF-α in pancreatic beta cells when compared to that of control. Significant reduction in the expression of these markers in curcumin and vitamin D_3 pre-treated rats indicate the effect of pre-treatment in preventing or delaying beta cell death after

streptozotocin injection. Collectively, these data demonstrate that curcumin and vitamin D_3 pre-treatment facilitate the rats to maintain a functional and sufficiently large beta cell mass after MLD-STZ injection to ensure sufficient pancreatic insulin output. Expression studies of genetic markers have revealed that this beta cell mass expansion is due to replication of the existing mature beta cells and differentiation of precursor cells and reduced apoptosis of the existing beta cells.

FUNCTIONAL REGULATION OF ADRENERGIC AND MUSCARINIC RECEPTOR SUBTYPES IN PANCREAS

Pancreatic insulin output and beta cell compensatory response is regulated by nutritional status, hormonal levels and by neuronal signalling (Mobbs *et al*., 2005). Nutrient sensing and energy balance is regulated by the brain to maintain systemic glucose homeostasis. Autonomous nervous system is the prime controller of biphasic release of insulin and beta cell mass balance according to metabolic demand (N'Guyen *et al*., 1994; Kiba, 2004). This neuronal signalling could be accountable for curcumin and vitamin D_3 mediated pancreatic beta cell hyperplasia. Autonomous neuronal innervations to pancreas include the parasympathetic vagus nerve and sympathetic splanchnic nerve. Adrenergic sympathetic neurons release noradrenaline and cholinergic parasympathetic neurons release acetyl choline to regulate the beta cells of pancreas (Campfield & Smith, 1983; Tarussio *et al*., 2014). Pancreatic beta cells resemble neurons in electrical excitability, depolarization induced secretion and expression of various neurotransmitter receptors (Liu *et al*., 2010). Hence, activation of neurotransmitter receptors present on the beta cells directly control insulin synthesis, storage, secretion and beta cell mass. Stimulation of this neuronal pathway is critical for an effective beta cell compensatory response. In this context, it is essential to study adrenergic and muscarinic receptor subtype functional regulation in the pathogenesis of diabetes. Studying the impact of curcumin and vitamin D_3 pre-treatment on pancreatic adrenergic and muscarinic receptor system will reveal

neuronal action of these compounds in stimulating beta cell compensatory response.

In our study, total adrenergic receptor number in diabetic rat pancreas showed a significant increase when compared to control. When compared to diabetic rats, curcumin and vitamin D_3 pre-treated group showed a significant decrease in total receptor number. The K_d, which is inversely proportional to receptor affinity, did not show any significant changes in experimental rats. Detailed analysis of adrenergic receptor subtypes showed differential functional regulation of α and β adrenergic receptors in pancreas. Out of the different subtypes of α adrenergic receptors, α2 is the predominantly expressed one in pancreas. Radio receptor assay, Real-Time PCR analysis and confocal studies confirmed the significant increase of α2 adrenergic receptors in the pancreas of diabetic rats. Binding affinities of this receptor didn't show any significant change when compared to control. Increased expression of α2 adrenergic receptor mediate norepinephrine induced inhibition of insulin release by sympathetic nerve stimulation. In diabetic rats, streptozotocin-induced high blood glucose levels stimulate the increased expression of α2 adrenergic receptor in pancreas (Angel & Langer, 1988; Angel *et al*., 1990). Activation of α2 subtype of adrenergic receptors regulate different intracellular pathways to modulate beta cell function through coupling to different G proteins (Docherty, 1998). Up regulated α2 adrenergic receptor levels in the pancreas inhibit insulin release from pancreas *via* three major pathways. Firstly, activated α2 adrenergic receptors inhibit the activity of the enzyme, adenylyl cyclase and reduce intracellular cAMP levels. cAMP potentiates hyperglycaemia induced insulin secretion by activating protein kinase A and Epac (Peterhoff *et al*., 2003; Seino *et al*., 2009). Thus, significant increase of α2 adrenergic receptors in the pancreas contribute to the impairment of glucose-stimulated insulin secretion. Secondly, α2 adrenergic receptors prevent calcium mediated exocytosis of insulin vesicles by potassium channel mediated hyperpolarization (Nilsson *et al*., 1989). Finally, suppression of calcium channels by α2 adrenergic receptors that act *via* G proteins decrease calcium mediated

insulin secretion. Increased potassium channel activity and suppression of calcium current deteriorates beta cell membrane depolarization induced insulin secretion (Rajan *et al*., 1990). So, increased expression of α2 adrenergic receptor in diabetic rats reduce insulin granule docking, impair glucose stimulated insulin release and increase the risk of diabetes (Rosengren *et al*., 2010).

Pre-treatment with curcumin and vitamin D_3 before streptozotocin administration significantly decreased the α2 adrenergic receptor binding affinity, mRNA and protein expressions, when compared to the diabetic group. This decline in α2 adrenergic receptor affinity and expression helps to reduce inhibition over insulin release through the activation of adenylyl cyclase, decrease of potassium channel activity and increase of calcium current. α2 adrenergic receptor knockout mice have improved glucose tolerance and increased insulin secretion (Savontaus *et al*., 2008). Administration of α2-adrenoceptor specific antagonist to diabetic rats and humans showed similar results (Berlin *et al*., 1994; Abdel-Zaher *et al*., 2001). Hence, curcumin and vitamin D_3 pre-treatment induced reduction in the α2-adrenoceptor has a major role in the maintenance of pancreatic insulin output and glucose tolerance.

In contrast to α2-adrenoceptors, pancreatic β2 adrenergic receptors showed a significant decrease in receptor binding, mRNA levels and protein expression when compared to the control group. Kaneto *et al*., (1975) demonstrated that β2 subtype is the major β adrenergic receptor that mediates adrenergic control of pancreas. Decreased activation and expression of β2 adrenergic receptor is reported to induce G1/S arrest of cell cycle by inhibition of extracellular signal-regulated kinase (ERK), Akt pathway and NF-κB activation (Chambard *et al*., 2007). Curcumin and vitamin D_3 pre-treatment significantly increased the overall expression of β2 adrenergic receptor when compared to diabetic group. Increased β2 adrenergic receptor activates cell cycle progression and beta cell proliferation by activating adenylyl cyclase and protein kinase A (Zhang *et al*., 2011). Activated protein kinase A promote pancreatic regeneration by transcriptional activation of several key cell proliferation factors. cAMP

influences expression of genes carrying cAMP response element *via* CREB (Zhang *et al*., 2010). β2 adrenergic receptor action through ERK and Akt pathways inhibit apoptotic factors like Bax and caspase-3 and activate anti-apoptotic proteins like Bad , Bcl-2, Bcl-XL and Mcl-1 (Herr & Debatin, 2001). Ras activation mediated by β2 adrenergic receptor stimulates several key signalling proteins including Raf, MEK, ERK, PI3K, Akt, PTEN and NF-κB, to support beta cell regeneration and to prevent apoptotic cell death (McCubrey *et al*., 2007; van der Weyden & Adam, 2007). Variations in receptor affinity among experimental rats might be due to the reversible phosphorylation of β2 adrenergic receptor by protein kinases. Insulin and insulin like growth factor 1 induced phosphorylation of cytosolic domain of β2 adrenergic receptors promote receptor sequestration. Mitogen-activated protein kinase (MAP kinase) pathway is activated by phosphorylated β2 adrenergic receptors (Karoor & Malbon, 1996). Hence, increased expression of β2 adrenergic receptor in the pancreas of pre-treated rats helps to activate beta cell compensatory response by inducing beta cell proliferation.

Parasympathetic nervous system modulates pancreatic insulin output and beta cell mass through acetylcholine, released by the vagal efferent fibres. The parasympathetic stimulation of pancreas is particularly important in the maintenance of blood glucose homeostasis by the activation of beta cell compensatory response to counter insulin resistance and obesity (Rohner-Jeanrenaud & Jeanrenaud, 1985). The principle mediators of acetylcholine signalling in pancreas are metabotropic muscarinic M1 and M3 receptors (Iismaa *et al*., 2000; Niebergall-Roth & Singer, 2003). Total muscarinic acetylcholine receptor number was significantly reduced in diabetic rat pancreas without any marked change in binding affinity. Radio receptor assay, Real Time PCR analysis and confocal microscopy confirmed a significant reduction in pancreatic muscarinic M1 and M3 receptors of diabetic rats. Muscarinic M1 receptors in the pancreas has a major role in the glucose and autonomous nervous system induced insulin secretion (Renuka *et al*., 2006). Significantly decreased M1receptors in the

pancreas of diabetic rats reduce the sensitivity of pancreatic beta cells to vagal stimulation. Pre-treatment using curcumin and vitamin D_3 significantly increased muscarinic M1 mRNA and protein levels when compared to diabetic group. It aids the neuronal regulation of pancreatic hormone release in response to varying metabolic demands. Increased expression of M1 receptor induces multiple cellular responses by the stimulation of tyrosine kinase activity (Huang *et al*., 1993). Muscarinic M1 receptors induce cell proliferation by the epidermal growth factor independent transactivation of epidermal growth factor receptor (Tsai *et al*., 1997). Increased expression and activation of M1 receptor increase the secretory activities of cells by promoting tyrosine kinase-dependent regulation of potassium channels (Huang *et al*., 1993). So, the increased expression of muscarinic M1 receptor stimulates pancreatic beta cell proliferation and insulin secretion in response to increased vagal stimulation.

Out of the different neurotransmitter receptors present in pancreas, muscarinic M3 acetylcholine receptors are the predominant subtype. In different muscarinic receptor subtypes, M3 plays a crucial role in acetylcholine mediated pancreatic endocrine regulation (Duttaroy *et al*., 2004). Diabetes associated decrease in muscarinic M3 receptors in the pancreas has a major impact on neuronally regulated beta cell mass expansion and insulin release. This reduces pancreatic insulin output by reducing the intracellular calcium levels and by inhibiting the calcium induced exocytosis of insulin vesicles from beta cells (Gilon & Henquin, 2001). Yamada *et al*., (2001) developed muscarinic M3 receptor deficient rats and reported that these rats showed hypophagia, decreased body weight and hypoinsulinemia. Hence, reduced muscarinic M3 receptor expression in our diabetic group induces an impairment of insulin release.

Curcumin and vitamin D_3 pre-treated rats showed a significant up regulation of muscarinic M3 receptors after MLD-STZ administration. Increased expression of M3 has been reported to stimulate pancreatic insulin secretion and increase glucose tolerance. Overexpression of M3 also provides resistance against diet induced glucose intolerance and hyperglycaemia in mice (Gautam *et al*.,

2006). Recently Nakajim *et al*., (2013) demonstrated that drug induced activation of muscarinic M3 receptors significantly improves beta cell function and protect rodents from diabetes and glucose intolerance induced by diet or streptozotocin. Binding of muscarinic M3 receptors by acetylcholine induces insulin secretion by the immediate activation of Gq-type G proteins (Boschero *et al*., 1995). Sustained muscarinic M3 receptor mediated insulin release is stimulated by the increased expression in pre-treated rats. Phosphorylation and arrestin-dependent coupling of muscarinic M3 receptor to protein kinase D1 is the main mediator of sustained G protein independent activation of insulin release (Kong *et al*., 2010). Curcumin and vitamin D_3 pre-treatment induced activation of M3 stimulate insulin release in a glucose dependent manner. Increased muscarinic receptors also mediate hyperglycaemia induced beta cell mass expansion by the stimulation of beta cell regeneration by the activation of MAPK pathway, calcium signalling and protein kinase C (Ma *et al*., 2000). High levels of M3 receptor expression are essential to initiate acetylcholine mediated beta cell proliferation (Brown *et al*., 1997). Up regulated M3 receptor also activate several cell proliferation pathways by the transactivation of growth hormone signalling cascade (Felder, 1995; Ukegawa *et al*., 2003). So collectively, pre-treatment induced up regulation of muscarinic receptor subtypes in the pancreas increase the sensitivity of beta cells to neuronal signalling, stimulate glucose induced insulin secretion and promote beta cell compensatory response.

The parasympathetic cholinergic innervations to pancreas originate from the intrapancreatic ganglia (Miller, 1981). These cholinergic fibres densely innervate the islets of Langerhans than the exocrine pancreas (Godfrey & Matschinsky, 1975). Parasympathetic neurons regulate insulin secretion and beta cell compensatory response by the release of the neurotransmitter acetylcholine. Two essential enzymes of the cholinergic system: choline acetyltransferase and acetylcholinesterase catalyse the synthesis and hydrolysis of acetylcholine, respectively. The ratio of choline acetyltransferase to acetylcholinesterase determine acetylcholine releasing potential (Fonnum, 1969). In our experiment,

choline acetyltransferase mRNA expression in diabetic rats was significantly reduced when compared to control. This indicates reduced cholinergic neuron number and cholinergic tone in the pancreas of diabetic rats. Diabetes associated impairment in cholinergic nerve impulse transmission was reported in central and peripheral nervous system (Gilon & Henquin, 2001; Kumar *et al*., 2011). The decreased cholinergic activity in the pancreas of diabetic group might be due to streptozotocin induced degeneration of intra-insular cholinergic neurons and reduction in beta cell mass (Luiten *et al*., 1986). The increase in acetylcholinesterase expression in diabetic rats was not statistically significant. Streptozotocin injection after pre-treatment with curcumin and vitamin D_3 significantly increased the expression of choline acetyltransferase when compared to both control and diabetic groups. The expression of acetylcholine degrading acetylcholinesterase was significantly down regulated in pre-treated rats when compared to diabetic. These changes help to increase the production of acetylcholine and stimulate cholinergic tone in pancreas (Fonnum, 1969). Increased expression of muscarinic acetylcholine receptors along with increased acetylcholine secretion potential might be due to improved parasympathetic stimulation of pancreas to increase insulin production in response to hyperglycaemia. Action of curcumin and vitamin D_3 in modulating neuronal stimulation from different brain regions to pancreas play a key role in stimulating cholinergic neurons in pre-treated rats (Rossi *et al*., 2005; Balakrishnan *et al*., 2009).

Apart from the major acetylcholine receptors like muscarinic M1 and M3, pancreas has also been reported to express muscarinic M2 receptors in low concentrations. Muscarinic M2 receptor present in pancreas is distinct from the M2 receptor of heart (Henquin & Nenquin, 1988). Unlike M1 and M3, muscarinic M2 activation leads to the G protein mediated inhibition of adenylyl cyclase (Migeon *et al*., 1995). Present study reports significantly increased muscarinic M2 receptor gene expression in the MLD-STZ induced diabetic rat pancreas. This exerts an inhibitory effect on glucose induced insulin secretion. Increased M2

expression also counteract M1 and M3 mediated stimulation of beta cell insulin release. Curcumin and vitamin D_3 prevented the up regulation of M2 receptor and maintained its expression to near control even after streptozotocin injection. This reduces insulin secretion inhibitory signalling by acetylcholine.

α7 NICOTINIC ACETYLCHOLINE RECEPTOR

Pancreatic α7 nicotinic acetylcholine receptor is a mediator of cholinergic signalling. The significant increase in α7 nicotinic acetylcholine receptors in diabetic and pre-treated rats might be due to its critical role in modulating inflammatory response. Streptozotocin induced beta cell damage induces an inflammatory response in the pancreas. The parasympathetic signals to this inflammation is mediated through the release of acetylcholine and activation of α7 nicotinic acetylcholine receptors (van Westerloo *et al.*, 2006). It stimulates the production of several pro-inflammatory cytokines to inhibit inflammatory damages to the tissue. Activation of α7 nicotinic acetylcholine receptors stimulate the nicotinic anti-inflammatory response *via* Jak/STAT and NF-κB pathways (de Jonge & Ulloa, 2007). It also suppresses TNF-α release to inhibit the death of existing pancreatic cells (Parrish *et al*., 2008). A significant decrease in the mRNA content was observed in curcumin and vitamin D_3 pre-treated rats when compared to diabetic group. This might be due to the direct protection of pancreatic beta cells from streptozotocin induced damage by curcumin and vitamin D_3. The antioxidant and anti-inflammatory properties of these compounds help to reduce pancreatic inflammation and thereby reduce nicotinic anti-inflammatory response.

VITAMIN D RECEPTOR

The anti-diabetic actions of vitamin D_3 are mediated not only through the regulation of plasma calcium levels that regulate insulin synthesis and secretion, but also *via* a direct action on pancreatic beta cell homeostasis. A number of potential mechanisms have been postulated to explain reduced pancreatic beta cell death in vitamin D_3 pre-treated rats. The major pathway of vitamin D action

involves its binding to the cytosolic/nuclear vitamin D receptor, a transcriptional activator (Palomer *et al*., 2008). Vitamin D receptor present in pancreatic beta cells is known to regulate insulin release and beta cell mass regulation. 25-hydroxyvitamin D_3-1α-hydroxylase expressed by beta cell catalyse the local conversion of biologically inert 25-hydroxyvitamin D to its active form 1,25-dihydroxyvitamin D_3 (Bland *et al*., 2004). In our experiment, diabetic, curcumin pre-treated and vitamin D_3 pre-treated rats showed a significant increase in vitamin D receptor expression. This could be due to the activation of vitamin D receptors in response to streptozotocin induced hyperglycaemia. Vitamin D_3 bound vitamin D receptor can directly modify insulin secretion from pancreatic beta cells by altering insulin gene transcription (Ortlepp *et al*., 2003). Increased vitamin D receptor expression increases insulin sensitivity of beta cells by stimulating the expression of insulin receptors and glucose transporters (Maestro *et al*., 2003).

On comparing control, diabetic and curcumin pre-treated groups, pre-treatment with vitamin D_3 was found to significantly increase vitamin D receptor expression at both mRNA and protein levels. This indicates the active signalling *via* vitamin D receptors in the pancreas. Increased expression of vitamin D receptors increases insulin storage in beta cells. It also enhances insulin synthesis in pancreatic beta cells by stimulating the insulin gene transcription and by enhancing the stability of insulin mRNA (Zeitz *et al*., 2003). Vitamin D_3 mediated regulation of calcium homeostasis helps pancreatic beta cells in glucose induced depolarization stimulated insulin release. The significant increase in vitamin D receptor gene expression in vitamin D_3 pre-treated rats might be due to vitamin D receptor autoregulation. Activated vitamin D receptor bound to enhancers located in the promoter region of vitamin D receptor gene and stimulate its own transcription (Zella *et al*., 2007). Increased vitamin D receptor number in the pancreas also has immunomodulatory functions, that have been reported to prevent the onset of diabetes by inhibiting cytokine mediated beta cell apoptosis and necrosis (Mathieu *et al*., 2005). During the periods of pregnancy and insulin

resistance, increased vitamin D receptor aid the initiation of beta cell compensatory response against increased insulin demand (Chiu *et al*., 2004). Inability of vitamin D receptors to stimulate gene expression due to mutations is associated with an increased risk of diabetes (Palomer *et al*., 2008). Further, vitamin D_3 action through vitamin D receptors is reported to promote cell regeneration in various tissues (Stratos *et al*., 2013). It also plays an important role in maintaining functional stem cell population to sustain the regenerative capacity of the tissue (Cianferotti *et al*., 2007). Active signalling through vitamin D receptors is also essential for the proper development of various organs. In the present study, pre-treatment with vitamin D_3 increased pancreatic beta cell mass expansion in response to increased insulin demand. Gene expression studies showed a significant up regulation in key cell proliferation, differentiation and anti-apoptotic factors. Vitamin D receptors directly or indirectly regulate the gene expression of most of these factors. This points out that vitamin D receptor mediated cell signalling is involved in regulation of pancreatic beta cell survival, regeneration, proliferation and differentiation.

CREB AND PHOSPHOLIPASE C

Cell signalling through G protein coupled receptors and tyrosine kinase receptors control pancreatic beta cell survival, proliferation and insulin secretion by the activation of key intracellular transcription factors. Among these factors, CREB and phospholipase C have a crucial role in maintaining sufficient pancreatic insulin output to maintain glucose homeostasis. Our streptozotocin induced diabetic rats showed a significant down regulation of CREB gene expression in pancreas. This reduces growth factor induced cAMP signal transduction *via* CREB. CREB on activation stimulates several cAMP response element containing proteins including those regulating glucose sensing, insulin gene expression, insulin secretion, beta cell survival and mass expansion (Dalle *et al*., 2011). Jhala *et al*., (2003) reported that, deficiency in functional CREB expression leads to beta cell apoptosis and subsequent development of diabetes.

The diabetes associated down regulation of CREB expression is due to increased cytokine mediated inhibition of adenyl cyclase (Jambal *et al*., 2003). Curcumin and vitamin D_3 pre-treatment significantly increases the expression of CREB when compared to both control and diabetic groups. This increases survival and proliferation of beta cells after the injection of streptozotocin. Increased cAMP levels accompanied by increased CREB expression activate insulin receptor substrate 2. Activated insulin receptor substrate 2 mediates insulin like growth factor 1, gastric inhibitory polypeptide and glucagon-like peptide-1 cell signalling (Jhala *et al*., 2003). Glucose responsive insulin secretion and beta cell proliferation is mediated by insulin receptor and extracellular signal–regulated kinase induced CREB activation (Costes *et al*., 2006). It stimulates Pdx-1 gene expression to increase beta cell survival, regeneration and insulin secretion. It also helps to inhibit pancreatic beta cell apoptosis by increasing the expression of anti-apoptotic factors (Jambal *et al*., 2003).

Insulinotropic signalling that includes sympathetic and parasympathetic neuronal stimulation is mediated through the activation of phospholipase C in beta cells. Activated phospholipase C produce two important second messengers critical for insulin release. Inositol trisphosphate (IP3) induces an increase in intracellular Ca^{2+} levels and diacylylglycerol (DAG) enable exocytosis of insulin granules *via* protein kinase C (De Camilli *et al*., 1996). Diabetic rats in our experiment showed a significant decrease in phospholipase C expression. Sustained hyperglycaemia in these diabetic rats induce a deficiency in phospholipase C expression and led to the impairment of postprandial insulin secretion (Zawalich *et al*., 2006). Pre-treatment with curcumin and vitamin D_3 resulted in a significant increase in phospholipase C expression when compared to both control and diabetic rats. This indicates stimulation of insulin release in response to various insulinotropic signals including hyperglycaemia, during the prediabetic condition. Increased parasympathetic activation of muscarinic M1 and M3 receptors in pancreas induce insulin secretion by activating phospholipase C (Thore *et al*., 2005).

INSULIN RECEPTOR

Insulin receptor plays an important role in normal beta cell function by mediating autocrine positive feedback for insulin synthesis and secretion (Aspinwall *et al*., 1999). Diabetic group showed a significant down regulation of insulin receptor expression in the pancreas. This impairs insulin receptor mediated positive feedback system and leads to an alteration in glucose induced insulin secretion (Jackerott *et al*., 2001). Reduced insulin receptor expression also leads to the development of beta cell insulin resistance. Progressive pancreatic insulin resistance results in glucose unresponsiveness (Kulkarni *et al*., 1999). Curcumin and vitamin D_3 pre-treated rats showed significant up regulation of insulin receptor mRNA when compared to both control and diabetic rats. This stimulates the positive feedback system to increase insulin synthesis and secretion. Activated insulin receptors also activate glucose utilization by beta cells and stimulate various pathways leading to beta cell survival and proliferation (Leibiger *et al*., 2008).

GLUT 2

GLUT 2 is low affinity glucose transporter that plays an important role in glucose sensing and glucose stimulated insulin secretion. In concordance with previous reports, MLD-STZ induced diabetic rats in our experiments showed a significant down regulation of GLUT 2 mRNA expression (Ferrer *et al*., 1995; Wang & Gleichmann, 1998). This leads to impaired glucose sensing by pancreatic beta cells and subsequent development of glucose unresponsiveness. Deficiency in functional GLUT 2 expression is associated with the development of hyperglycaemia and hypoinsulinemia (Guillam *et al*., 1997). Pre-treatment with both curcumin and vitamin D_3 were able to significantly increase GLUT 2 mRNA levels compared to control and diabetic rats. This helps to maintain normoglycaemia after MLD-STZ administration by promoting normal functioning

and development of beta cells. Pre-treatment induced increased expression of Pdx-1 that binds to TAAT motif in GLUT 2 gene to enhance its gene expression (Waeber *et al*., 1996). Prediabetic condition present in curcumin and vitamin D_3 pre-treated rats stimulates the expression of GLUT 2 in pancreas to enhance glucose sensitivity and increase pancreatic insulin output.

ANTIOXIDANT ENZYMES

Antioxidant enzymes, superoxide dismutases and glutathione peroxidase has an important role in maintaining physiological levels of free radicals by hastening the dismutation of free radicals and elimination of organic peroxides and hydro-peroxides. In our study, the gene expression of superoxide dismutases and glutathione peroxidase was significantly increased in all MLD-STZ treated rats. This could be a cellular response against the streptozotocin induced increased oxidative stress in pancreas. Pancreatic beta cells are considered as a susceptible target for reactive oxygen species because it contains low levels of free radical scavengers (Szkudelski *et al*., 2001). So increased free radical levels in beta cells lead to apoptosis and necrosis of a part of insulin producing cells, which subsequently lead to the development of hyperglycaemia. The persistent hyperglycaemia associated glucotoxicity and lipotoxicity also increase the reactive oxygen species production and oxidative injury. Accumulation of reactive oxygen species in the cells leads to the activation of apoptotic mediators and inhibition of anti-apoptotic factors (O'Brien *et al*., 1996). In diabetic rats, increased superoxide dismutases and glutathione peroxidase expression was not able to protect beta cell from free radical induced beta cell dysfunction and mass reduction. Hence, pancreatic insulin output becomes insufficient to compensate for the initial hyperglycaemia induced by streptozotocin. Persistent hyperglycaemia inactivates antioxidant enzymes by glycation and further increase oxidative damage to beta cells. Rats that received curcumin and vitamin D_3 pre-treatment before MLD-STZ administration showed a significant increase in the gene expression of superoxide dismutases and glutathione peroxidase when compared to both control and

diabetic. Curcumin and vitamin D_3 is reported to increase the intracellular antioxidant defence system by increasing the expression of key enzymes *via* the activation of Nrf2- antioxidant-responsive element pathway (Balogun *et al*., 2003; Bobilev *et al*., 2011). To an extent, this protects beta cells from streptozotocin and hyperglycaemia induced oxidative stress in pancreas (Wang *et al*., 1994; Dai *et al*., 2003). This alleviates the imbalance between the generation of reactive oxygen species and activity of scavenging enzymes. Increase in the expression of key antioxidant enzymes protect beta cells from free radical induced cellular dysfunction and prevent the activation of apoptotic pathways (Selvam & Anuradha, 1990).

SECOND MESSENGERS

Second messengers like cAMP, cGMP and IP3 play a critical role in signal transduction pathways, downstream of receptors for many hormones and neurotransmitters that regulate insulin secretion and beta cell mass expansion. We observed significant decrease in these second messengers in the pancreas of diabetic rats, when compared to control. cAMP mediated signal transduction regulates glucose stimulated insulin release, proliferation, differentiation and apoptosis in pancreatic beta cells (Holz, 2004). Decreased cAMP content in the pancreas of diabetic rats leads to reduced insulin secretion in response to various insulinotropic stimuli. Reduction in cAMP levels also activates cellular apoptosis by removing the CREB mediated inhibition on apoptotic factors. Curcumin and vitamin D_3 pre-treatment was able to maintain a near control level of cAMP content. This helps to retain the sensitivity of beta cells towards various secretagogue stimuli. cAMP mediated short term effects on insulin secretion are mediated by increased cytoplasmic Ca^{2+} concentration and long term effects by transcriptional mechanisms that involve the cAMP-responsive element pathway (Holz *et al*., 2008). Further, cAMP dependent PKA-mediated phosphorylation of CREB stimulates islet-cell function, protect beta-cells against apoptosis and *via* Akt, it promotes islet-cell survival (Hussain *et al*., 2006). Second messenger

cGMP regulates various cellular functions through the activation of a specific cGMP-dependent protein kinase, protein kinase G (Pilz & Broderick, 2005). Significant decrease in cGMP content of diabetic rat pancreas induces a disturbance in intracellular Ca^{2+} homeostasis and alters glucose stimulated insulin release (Ishikawa *et al*., 2003). Curcumin and vitamin D_3 pre-treatment retained the cGMP at near control levels. This helps beta cells to respond to increased plasma glucose levels by activating protein kinase G mediated cell signalling to potentiate glucose stimulated insulin secretion, promote beta cell differentiation and prevent beta cell apoptosis (McCarty, 2006). IP3 is a second messenger that induces glucose-stimulated insulin secretion through the release of Ca^{2+} from endoplasmic reticulum. Pancreatic beta cell dysfunction and impaired insulin secretion is associated with a significant reduction in the pancreatic IP3 content of diabetic rats (Zawalich *et al*., 1995). Significant reduction in muscarinic receptor and phospholipase C expression also contributes to the decline in IP3 levels in pancreas. This leads to decreased calcium mobilization from endoplasmic reticulum in response to glucose and other secretagogue stimuli. Impairment in pancreatic calcium dynamics reduces insulin transcription and granule docking (Somesh *et al*., 2013). Curcumin and vitamin D_3 pre-treated rats were able to maintain a near control level of IP3 when compared to diabetic rats. This has a positive effect on insulin synthesis and secretion in response to hyperglycaemia and neuronal stimulation. Significant increase in IP3 levels of pre-treated rats also induces an increase in cAMP levels to aid glucose mediated oscillation in insulin secretion (Dyachok *et al*., 2008).

Collectively these data confirm that pre-treatment with curcumin and vitamin D_3 increases the pancreatic insulin output in response to increased insulin demand through the functional regulation of neurotransmitter receptors. Pre-treated rats increase beta cell glucose response by up regulating the expression of insulinotropic receptors like muscarinic M1, muscarinic M3 and β adrenergic receptors and down regulation of inhibitory signals like α2 adrenergic and muscarinic M2 receptors. Increased cholinergic signalling in pancreas will helps

to initiate beta cell compensatory response by inducing beta cell growth and proliferation. In pre-treated rats, expression of intracellular signalling and glucose sensing molecules increase in response to increased metabolic demands induced by streptozotocin. The enzymatic antioxidant defence system in pancreas gets strengthened by curcumin and vitamin D_3 pre-treatment.

NEURONAL REGULATION OF PANCREAS

The sympathetic and parasympathetic autonomic nerve fibres innervating the pancreas control the functional regulation of various pancreatic neurotransmitter receptor subtypes to induce beta cell compensatory response. These neuronal innervations originate mainly from brain stem, hippocampus and hypothalamus. Hence, the study of adrenergic and muscarinic receptor functional regulation in these brain areas are important to confirm the role of neuronal stimulation in insulin release and beta cell mass expansion. Behavioural studies were done in the experimental rats to evaluate the effect of curcumin and vitamin D_3 pre-treatment on motor and cognitive functions of rats. Diabetic rats showed an impaired performance in Y maze, rotarod and grid walk test. This clearly indicates the deficit of cognitive functions, exploratory behaviour, learning, memory and motor coordination. Curcumin and vitamin D_3 pre-treated rats attained normal scores in these behavioural tests. This indicates the ameliorating effect of pre-treatment on brain stem, hippocampus and hypothalamus (McEwen & Sapolsky, 1995). Previous studies have reported the ability of curcumin and vitamin D_3 administration to prevent diabetes associated behavioural alterations (Kuhad & Chopra, 2007; Kumar *et al*., 2011). Further, adrenergic and cholinergic neurons in different brain regions have been reported to play a key role in the modulation of behaviour (Kow *et al*., 1992).

BRAIN STEM

Adrenergic receptor subtypes mRNA and protein expression were significantly reduced in the brain stem of diabetic rats when compared to control.

Previous studies have reported increased epinephrine and norepinephrine levels in brain stem during hyperglycaemia (Jones *et al*., 1990; Gupta *et al*., 1992). Observed decrease in α2 and β2 adrenergic receptors in the brain stem might be a compensatory response to increased ligand levels. This type of stress adaptation responses mediated by the adrenergic receptors have been reported in various stress models (Torda *et al*., 1981; Stone & Platt, 1982). This lead to the development of diabetes associated neuronal complications like thermoregulatory deficit, decreased spontaneous motor activity and higher levels of spontaneous pain threshold (Chu *et al*., 1986). Decreased adrenergic receptor expression in the brain stem is known to stimulate pancreas to increase insulin production and beta cell proliferation in response to hyperglycaemia (Das *et al*., 2006).

When compared to diabetic rats B_{max}, mRNA and protein expression of α2 and β adrenergic receptors were significantly increased in curcumin and vitamin D_3 pre-treated rats. This helps to retain a near control presynaptic modulation of central noradrenergic function and postsynaptic nerve impulse transmission (Scheinin *et al*., 1994). α2 adrenergic receptors are the major receptors in the brain stem that signal epinephrine and norepinephrine induced inhibition of cAMP production. Reduction in the expression of α2 adrenergic receptors in brain stem impairs blood pressure regulation (MacMillan *et al*., 1996). Diabetes associated reduction in the adrenergic receptors of brain stem increases food intake (polyphagia) through the impairment of glucagon-like peptide 1 producing preproglucagon neurons (Hisadome *et al*., 2011). Increased adrenergic receptor number in the pre-treated group normalize the food intake by maintaining cholecystokinin induced adrenergic receptor activation (Baptista *et al*., 2005). This helps to maintain serotonin metabolism in the brain by regulating tryptophan accumulation. Diabetes associated alterations in serotonergic systems also control appetite (Svensson *et al*., 1975).

Parasympathetic control of pancreatic beta cell insulin secretion and beta cell regeneration is regulated through the vagus nerve. We observed a significant reduction in muscarinic M1 and M3 subtypes mRNA and protein expression in the

brain stem of diabetic rats when compared to control. Dorsal motor nucleus and nucleus tractus solitarius of dorsal vagal complex present in the brain stem receive neuronal inputs from many other regions of brain stem (Spanswick *et al*., 2000). Hyperglycaemia induced stimulation of dorsal vagal complex by different areas of brain stimulates vagal signalling. Diabetes associated decreased muscarinic M1 and M3 receptors reduce cholinergic vagal signalling to increase insulin synthesis, secretion and beta cell mass expansion (Balfour *et al*., 2006). Decreased vagal stimulation, together with reduced muscarinic receptor expression in the pancreas of diabetic rats reduce the effect of parasympathetic stimulation. Curcumin and vitamin D_3 pre-treatment significantly increased the muscarinic M1 and M3 receptor expression towards near control, when compared to diabetic rats. This indicates normalization of cholinergic neuronal signalling in brain stem and dorsal vagal complex. Parasympathetic hyperglycaemia induced stimulation of vagus nerve promotes insulin secretion (Wu *et al*., 2004; Balakrishnan *et al*., 2009). Physiological functions of cholinergic signalling also include stimulation of beta cell compensatory response by the stimulation of beta cell proliferation in response to persistent hyperglycaemia (Lausier *et al*., 2010).

Gene expression of enzymes involved in acetylcholine metabolism, choline acetyltransferase and acetylcholinesterase were significantly increased in the brain stem of diabetic rats. Increase in acetylcholine synthesis due to the increased expression of choline acetyltransferase is partially compensated by the simultaneous increase in hydrolysing enzyme, acetylcholinesterase. The imbalance in acetylcholine metabolism in the brain stem leads to diabetes associated impairment of cardiorespiratory control and central chemosensitivity (Mallard *et al*., 1999). Curcumin and vitamin D_3 pre-treated rats showed a significant increase in choline acetyltransferase and a decrease in acetylcholinesterase, when compared to diabetic group. Increased synthesis and reduced hydrolysis leads to increased cholinergic neuronal signalling in brain stem. This induces sufficient parasympathetic stimulation of vagus nerve to improve pancreatic insulin output.

The expression of another muscarinic receptor subtype, M2 was significantly down regulated in the brain stem of diabetic and pre-treated rats, when compared to control. But when compared to diabetic, muscarinic M2 receptor expression was up regulated in pre-treated rats. The reduced expression of muscarinic M2 receptor prevents the hyperpolarisation of cholinergic neurons in the brain stem and increases the release of endogenous acetylcholine (Baghdoyan *et al*., 1998; Shabani *et al*., 2010). Decreased muscarinic M2 autoreceptors aid the increased cholinergic stimulation of pancreas *via* dorsal vagal complex in response to hyperglycaemia. Ionotropic α7 nicotinic acetylcholine receptor expression was significantly increased in diabetic rats, when compared to control. Shi *et al*., (1993) reported that the increased nicotinic acetylcholine receptor expression is associated with a decrease in acetylcholine function. α7 nicotinic acetylcholine receptors are expressed by cholinergic, serotonergic and noradrenergic neurons in the brain stem (Bitner *et al*., 2002). Pre-treated group showed a significant increase in α7 nicotinic acetylcholine receptor expression when compared to control and a significant decrease when compared to the diabetic group. This can be considered as a response to prediabetes associated hyperglycaemia. Increased α7 nicotinic acetylcholine receptor number in the dorsal vagal complex of brain stem promotes fast synaptic coupling (Zaninetti *et al*., 1999).

MLD-STZ induced diabetes is associated with significant alterations in the expression of key intracellular signal mediators like vitamin D receptor, CREB and phospholipase C. Serum levels of vitamin D_3 are significantly reduced in diabetic subjects (Ishida *et al*., 1985). Significant increase in the vitamin D receptors in the brain stem could be a compensatory response to the decreased availability of vitamin D_3. Pre-treatment induced down regulation of vitamin D receptor when compared to diabetic rats. This helps to normalize glucose transport and cholinergic receptor function in brain stem (Kumar *et al*., 2011). CREB and phospholipase C mediate neurotransmitter induced nerve impulse transmission and hormonal signalling in brain. Decreased expression of CREB during diabetes,

inhibits the neuronal NO synthase and cause the modifications in somatosensory functions (Ma *et al*., 2008). Pre-treatment induced up regulation of CREB expression helps to induce transcriptional changes in brain stem to produce plasticity changes in neurocircuitry. These changes modify the feeding behaviour by regulating the meal size and inter-meal intervals (Berthoud *et al*., 2006). Decreased expression of phospholipase C could reduce the signal transduction through several important neuronal signalling pathways. It reduces the sensitivity of cholinergic and adrenergic neuronal signal transduction pathways. It also reduces the insulin sensitivity of brain by reducing the insulin mediated intracellular signal transduction (Häring *et al*., 1991).

The gene expression of insulin receptor and glucose transporter, GLUT 3 was significantly increased in response to streptozotocin induced diabetes. Increased insulin receptor expression leads to alterations in energy balance through impaired regulation of food intake, gastric tone and endocrine control (Hjelland *et al*., 2005). Insulin controls energy homeostasis in brain stem by modulating the presynaptic and postsynaptic neuronal receptor expression, particularly in dorsal motor nucleus of the vagus nerve (Blake & Smith, 2012). Increased insulin receptor expression can also help to increase glucose utilization by activating Erk1/2 signalling pathway in the brain stem (Filippi *et al*., 2014). Curcumin and vitamin D_3 treatment significantly down regulated insulin receptor expression when compared to diabetic rats. The insulin receptor expression in diabetic and pre-treated rats showed an inverse relationship with circulating insulin levels. Neuron specific GLUT 3 is another important part of brain glucose sensing machinery, whose expression was significantly up regulated after streptozotocin administration. Increase in GLUT 3 might be due to increased insulin receptor expression and/or due to decreased insulin sensitivity (Reagan *et al*., 1999). Diabetic group showed significant increase in GLUT 3 mRNA when compared to curcumin and vitamin D_3 pre-treated rats. GLUT 3 mediated increased glucose import into nerve cells produce glucotoxicity and increased

oxidative free radical production in brain stem. This impairs glucose utilisation and sensitivity of brain stem (Das *et al*., 2009).

The expression of antioxidant enzymes, superoxide dismutases and glutathione peroxidase was significantly increased in response to the magnitude of hyperglycaemia. This increase in antioxidant enzyme expression is necessary to protect nerve cells from increased oxidative stress. Decreased expression of superoxide dismutases and glutathione peroxidase in pre-treated rats compared to diabetic group might be due to reduced glycemic levels, decreased GLUT 3 expression and the endogenous antioxidant properties of curcumin and vitamin D_3 (Wiseman, 1993; Kuo *et al*., 1996). Signal transduction at the level of second messengers was also observed to be impaired in the brain stem of our diabetic rats. The cyclin mononucleotide second messenger level was significantly increased in the brain stem while the IP3 levels were significantly reduced during diabetes. Alterations in the second messengers indicate impairment of nerve impulse transmission and alterations in cell signalling pathways in the diabetic rats (Mørk *et al*., 1992). These alterations in the brain stem negatively affect neuronal glucose sensing and energy homeostasis. Parasympathetic stimulation of beta mass expansion and regeneration in response to increased insulin demand will also be impaired. Curcumin and vitamin D_3 mediated normalization of adrenergic and muscarinic receptors are partly responsible for the near normal levels of cAMP, cGMP and IP3 in the brain stem of pre-treated rats (Tonnaer *et al*., 1991; Banks *et al*., 1993). This promotes hyperglycaemia induced parasympathetic cholinergic stimulation of pancreatic beta cells by protecting brain stem neuronal signalling pathways.

HIPPOCAMPUS

Our studies in the hippocampus of experimental rats showed a significant increase in the major adrenergic receptor subtypes expression in diabetic rats. Diabetes associated hyperglycaemia induces significant increase in brain catecholamine levels in the hippocampus (Bitar *et al*., 1986). Increased receptor

number, together with increased epinephrine and norepinephrine stimulate neuronal signalling through α and β adrenergic receptors. Together with our behavioural studies, it shows a significant functional impairment in the hippocampal adrenergic signalling. α and β adrenergic receptors in the hippocampal neurons are opposite in their physiological actions. Activation of α2 adrenergic receptors inhibit presynaptic neurons to reduce excitatory postsynaptic potential and β adrenergic activation stimulates receptor mediated increase in postsynaptic excitability (Woodward *et al*., 1979; Scanziani *et al*., 1993). Hence, functional regulation of adrenergic receptor subtypes in hippocampus determines the postsynaptic responsiveness of neurons. In diabetic rats, functional alterations of adrenergic receptor cause various behavioural and physiological complications. Alterations in the expression of presynaptic and postsynaptic α adrenergic receptors of the hippocampus lead to the development of amnesia (Stuchlik *et al*., 2008). Impaired β adrenergic receptor function alters MAP kinase mediated long term potentiation (Winder *et al*., 1999). Increased β adrenergic receptor expression also increases the proliferation of hippocampal glial cells and cause impairment of learning, memory and spatial navigation (Kalaria *et al*., 1989). When compared to diabetic rats, curcumin and vitamin D_3 pre-treated rats showed a significant decrease in mRNA and protein expression of α and β adrenergic receptors in the hippocampus. Together with the increased insulin levels in the pre-treated rats, hippocampal adrenergic receptors activate the endogenous noradrenergic activity to regulate long feeding behaviour, learning and memory (Zhao *et al*., 1999). Further, pre-treatment induced functional improvement in the hippocampal adrenergic system stimulates brain stem to initiate parasympathetic beta cell compensatory response through multi-synaptic relays (Castle *et al*., 2005).

Cholinergic neurons in the hippocampus play an important role in regulation of cognition and extrapyramidal motor activity. Muscarinic M1 and M3 receptors showed differential expression in the hippocampus of diabetic rats. Muscarinic M1 expression was significantly decreased and the muscarinic M3 expression was significantly increased in hippocampus of MLD-STZ induced

diabetic rats. Radio receptor assay, Real Time PCR and confocal microscopic analysis confirmed this differential expression at mRNA and protein levels. Diabetes induced alterations in the hippocampal acetylcholine metabolism could be responsible for muscarinic M1 and M3 receptor alterations (Ragozzino *et al*., 1998). We found increased gene expression of choline acetyltransferase and acetylcholinesterase in the hippocampus of diabetic group. This disturbance in acetylcholine metabolism induces functional regulation of hippocampal muscarinic receptors according to their binding affinities. Decreased expression of low affinity M1 receptors reduces the neuronal excitability at high acetylcholine levels (Auerbach & Segal, 1996). Gireesh *et al*., (2008) suggest that alteration of muscarinic M1 receptors in the hippocampus is due to the impairment of neuronal insulin signalling. The most important function of muscarinic M1 receptors in the hippocampus is regulation of locomotor activity (Miyakawa *et al*., 2001). Altered muscarinic M1 mediated signalling also disturbs the γ oscillation mediated cognitive functions and hippocampal information processing (Fisahn *et al*., 2002). Muscarinic M3 receptors are high affinity receptors that respond to high levels of acetylcholine and have a major role in fear conditioning, learning and memory. Increased expression of muscarinic M3 receptor is reported to stimulate cholinergic depression in hippocampus (Auerbach & Segal, 1996). Like muscarinic M3 receptors, high affinity muscarinic M2 receptor expression and ionotropic α7 nicotinic acetylcholine receptor expression was significantly increased in diabetic rats, when compared to control. Both these receptors are present in the hippocampal interneurons presynaptically and posynaptically to modulate cholinergic nerve impulse transmission (Hájos *et al*., 1998; Freedman *et al*., 2000). Taken together, these data suggest that there is a significant impairment of the hippocampal cholinergic system, responsible for the cognitive and locomotor deficits in behavioural studies. Curcumin and vitamin D_3 pre-treatment induced increase in muscarinic M1 receptor expression and decrease in muscarinic M2, M3 and α7 nicotinic receptor expression, compared to the diabetic group helps to improve cognitive functions and locomotor activity. It helps to sustain normal levels of hippocampal neurogenesis and synaptic plasticity.

Vitamin D receptor expression was significantly increased in the hippocampus of all streptozotocin injected rats. Pre-treated rats showed a significant increase in vitamin D receptor expression when compared to diabetic rats. Persistent hyperglycaemia induces an increase in the expression of vitamin D receptor in the hippocampus (Ji *et al*., 2014). Increased expression of vitamin D receptor could modulate the expression of several genes involved in the hippocampal neurogenesis and memory consolidation. Among the pre-treated rats, a more significant increase was observed in vitamin D_3 pre-treated rats. It might be due to the auto-stimulatory effect of vitamin D receptor on increasing its own transcription. Significant alterations in the hippocampal intracellular pathways are corroborated by significant decrease in CREB and phospholipase C expression. Decreased expression of CREB in the hippocampus leads to an impairment of long-term facilitation of synaptic strength, long-term memory and long-term potentiation (Dash *et al*., 1990; Bourtchuladze *et al*., 1994). Pre-treatment induced up regulation of CREB expression helps to maintain the memory process intact. Increased CREB expression also reduces the chance of development of depression and many other diabetes associated cognitive dysfunctions (Chen *et al*., 2001). Altered expression of phospholipase C in streptozotocin induced diabetic rats have been reported to impair calcium dependent long-term potentiation (Chabot *et al*., 1997). Curcumin and vitamin D_3 pre-treatment induced increase in the phospholipase C mRNA levels in the hippocampus, normalizes adrenergic, muscarinic and insulin mediated cell signalling.

Like brain stem, gene expression of insulin receptor and GLUT 3 in the hippocampus was significantly up regulated in diabetic group, when compared to control. Impaired insulin receptor mediated signalling and neuronal glucose transport leads to the dysfunction of hippocampal neurons. Impaired GLUT 3 and insulin receptor expression along with decreased circulating insulin levels reduces hippocampal neuronal glucose utilization and increases oxidative free radical production (Reagan *et al*., 2000). The gene expression of superoxide dismutases and glutathione peroxidase showed an increase in line with the glycemic levels of

diabetic and pre-treated rats. This increase in antioxidant enzyme expression helps to protect cells from hyperglycaemia induced glucotoxicity (Stranahan *et al*., 2008). Further, second messenger content in hippocampus was measured to assess intracellular signalling. cAMP and IP3 content was significantly increased and cGMP levels were significantly reduced in diabetic rats, when compared to control. These impairments indicate hyperglycaemia induced changes in many cell signalling pathways including that of norepinephrine, acetylcholine and insulin. This, in turn contribute to diabetes associated reduction in hippocampal function. Hence, pre-treatment with curcumin and vitamin D_3 before MLD-STZ administration helps to protect hippocampal cell signalling by its direct action and protects pancreatic beta cells.

HYPOTHALAMUS

Adrenergic receptor functional regulation in the hypothalamus is responsible for maintaining energy homeostasis by controlling food intake. mRNA expression of both α2 adrenergic and β2 adrenergic receptors were significantly increased in the hypothalamus of diabetic rats, when compared to control. Curcumin and vitamin D_3 pre-treated rats showed a significant down regulation of both α2 and β2 adrenergic receptors, when compared to diabetic rats. Impaired glycemic levels have been reported to increase norepinephrine activity through hypothalamic adrenergic receptor subtypes (Szepietowska *et al*., 2011). Increased adrenergic receptor expression stimulates glucose recovery. Glucose sensing neurons in the brain stem communicate with the hypothalamic glucose sensors through catecholaminergic projections to integrate the response to impaired blood glucose homeostasis (Watts & Donovan, 2010). Increased expression of adrenergic receptor subtypes weakens this neuronal regulatory network and promotes the development of metabolic syndromes in diabetic rats (Boundy & Cincotta, 2000). It also leads to diabetes associated changes in the feeding behaviour due to increased expression of α2 adrenergic receptors in the hypothalamic satiety centre and β2 adrenergic receptors in the feeding centre

(Leibowitz, 1970; King, 2006). Impaired adrenergic receptor expression also cause alterations in corticotrophin releasing hormone secretion, sympathoadrenal actions and cardiovascular regulation (Boudier *et al*., 1974; Kiss & Aguilera, 1992).

Choline acetyltransferase gene expression was significantly decreased and acetylcholinesterase was increased in the hypothalamus of diabetic rats. Hence, diabetes associated reduction in the acetylcholine content of the hypothalamus is due to reduced synthesis of acetylcholine, together with the increased hydrolysis (Malouf & Birks, 2004; Sakr, 2013). Curcumin and vitamin D_3 induced reduction in oxidative stress and glycation end product levels helps to prevent the drop in hypothalamic acetylcholine levels (Irie *et al*., 2008). As observed in hypothalamus, a similar pattern of expression was observed in hypothalamic muscarinic M1, M2 and M3 receptors. Diabetes induced a significant decrease in low affinity muscarinic M1 receptors and a significant increase in high affinity M2 and M3 subtypes. α7 nicotinic acetylcholine receptor expression was also found to be increased significantly in the diabetic group. These changes are induced by impaired cholinergic metabolism and lead to the development of cholinergic symptoms including sweating, hunger and paresthesias *via* the alterations in sympathetic postganglionic neurons (Towler *et al*., 1993). Curcumin and vitamin D_3 induced significant reversal of muscarinic and nicotinic receptor subtypes and therefore helps to protect hypothalamic glucose sensing from hyperglycaemia induced stress. They help to maintain glucose homeostasis by modulating feeding behaviour, adiposity and pancreatic hormone release (Ahrén, 2000; Schwartz *et al*., 2000).

Gene expressions of vitamin D receptor, CREB, phospholipase C, insulin receptor and GLUT 3 were significantly down regulated in the hypothalamus of diabetic rats, when compared to control. Significant up regulation of these receptors were observed in curcumin and vitamin D_3 pre-treated rats, when compared to diabetic. Decreased vitamin D receptor reduces the biological action of vitamin D_3 in hippocampus and stimulates winter response by modulating

glucose and lipid metabolism. Increased hypothalamic vitamin D_3 sensitivity helps to intensify vitamin D_3 mediated cell signalling to inhibit fat accumulation and development of obesity in pre-treated rats (Cooper *et al*., 1997; Foss, 2009). CREB and phospholipase C are the downstream targets of many important cell signalling pathways. Alterations in the glucocorticoid levels of diabetic rats might be due to the impairment of CREB mediated cell signalling pathways in hypothalamus (Légrádi *et al*., 1997). Curcumin and vitamin D_3 mediated down regulation of CREB when compared to diabetic rats. This helps in the proper regulation of sympathetic and parasympathetic autonomic nerves to stimulate anorectic and energy wasting responses (Sarkar *et al*., 2002). Diabetes associated impairment of several different nutritive metabolic pathways reduces the expression of phospholipase C and cause subsequent impairment of intracellular Ca^{2+} release (Muroya *et al*., 2004).

In the central nervous system, hypothalamus is the primary target of insulin. Decreased insulin receptor expression in diabetic rats alters energy homeostasis by affecting hypothalamic neuropeptide release (Leibowitz & Wortley, 2004). Reduced expression of insulin receptors and insufficient circulating insulin levels directly induce hyperglycaemia by affecting hypothalamus mediated energy homeostasis and fuel metabolism (Obici *et al*., 2002). This also induces diabetes associated metabolic syndrome like insulin resistance, glucose intolerance, dyslipidemia and leptin resistance (Kubota *et al*., 2004). Prediabetic condition present in the curcumin and vitamin D_3 pre-treated rats also cause a significant increase in their gene expression, according to the glycemic levels. Intact insulin signalling in the pre-treated group stimulates insulin induced hypothalamic homeostatic signals (Polonsky *et al*., 1988). In diabetic rats, decreased insulin response of hypothalamus causes the down regulation of GLUT 3 expression. This impairs hypothalamic glucose sensing ability and lead to impairments in systemic dysregulation of energy metabolism. Curcumin and vitamin D_3 pre-treated rats maintain the expression of insulin receptor and GLUT 3 to elicit a normal sympathoadrenal response against hyperglycaemia (Diggs-

Andrews *et al*., 2010). Decreased GLUT 3 expression also stimulates neuronal damage by reducing glucose transport into the cell. The observed increase in the superoxide dismutases and glutathione peroxidase expression might be a counter regulatory response towards increased oxidative stress. Direct and indirect antioxidant and anti-hyperglycaemic actions of curcumin and vitamin D_3 reduces the oxidative free radical load. It accounts for the significant decrease in antioxidant enzyme gene transcription in hypothalamus.

Taken together, these data confirm that curcumin and vitamin D_3 pre-treatment helps to maintain functional adrenergic and muscarinic receptor functions in brain stem, hippocampus and hypothalamus after the injection of MLD-STZ. Pre-treatment induced ameliorations in the intracellular cell signalling molecules, glucose sensing machinery, antioxidant system and second messengers in brain stem, hippocampus and hypothalamus helps to retain the neuronal response to hyperglycaemia. This, in turn, helps to activate an autonomic neuronal signalling to pancreas in response to MLD-STZ induced increased metabolic demand of insulin. In pancreas, curcumin and vitamin D_3 induced a significant increase in the expression of β adrenergic, muscarinic M1 and M3 receptors to increase the insulin secretion and beta cell mass expansion in response to hyperglycaemia. Decreased expression of α2 adrenergic receptors in the pancreas helps to reduce epinephrine and norepinephrine mediated inhibition of cell insulin release. Pre-treatment induced enhancements in intracellular cell signalling system increases the responsiveness of pancreas to autonomic stimulations to activate beta cell compensatory response. Hence, the anti-diabetogenesis effect of curcumin and vitamin D_3 is due to its action on pancreas, brain stem, hippocampus and hypothalamus to stimulate insulin secretion, beta cell mass expansion and reduce apoptosis. Thus, curcumin and vitamin D_3 administration has been shown to reduce functional beta cell mass deterioration associated with pathophysiology of all types of diabetes. In this backdrop, it has immense value in better management of incidence of diabetes and has great therapeutic significance as well.

Summary

1. The anti-diabetogenesis property of curcumin and vitamin D_3 pre-treatment was assessed using MLD-STZ induced diabetic Wistar rat models.
2. Curcumin and vitamin D_3 pre-treated rats developed only a prediabetic condition after MLD-STZ administration, with a significant increase in circulating insulin levels when compared to the diabetic group of rats. Diabetic rats showed a significant reduction in body weight when compared to control. Body weight of the pre-treated group was significantly more than that of the diabetic rats.
3. Behavioural studies like Y maze test, rotarod test and grid walk test were conducted to evaluate exploratory behaviour, learning and memory, motor learning and sensory motor coordination. MLD-STZ induced was associated with a significant reduction in cognitive and motor functions. Pre-treatment with curcumin and vitamin D_3 was able to prevent behavioural deficits, associated with MLD-STZ administration.
4. DNA and protein synthesis in the pancreatic beta cells isolated from experimental rats were determined using [^{3}H] thymidine and [^{3}H] leucine incorporation studies. There was a significant reduction in DNA and protein biosynthesis in beta cells isolated from diabetic rats when compared to control. Curcumin and vitamin D_3 pre-treated rats showed a significant increase in the DNA and protein synthesis when compared to both control and diabetic group.
5. Beta cell proliferation studies based on tritiated methyl group incorporation to DNA, showed a significant increase in incorporation in diabetic and pre-treated rats when compared to control. Methyl group incorporation was more prominent in curcumin and vitamin D_3 pre-treated rats when compared to the diabetic group.

6. Oxidative stress and antioxidant status was measured by quantifying malondialdehyde content. A significant increase was observed in the beta cells of diabetic and pre-treated rats when compared to control. Pre-treatment with curcumin and vitamin D_3 significantly decreased malondialdehyde levels, when compared to the diabetic group.
7. Beta cell compensatory response to hyperglycemia was studied by analysing the gene expression of regeneration markers like Akt, NeuroD1, Pax, Pdx-1, insulin like growth factor-1, NF-κB and cyclin D2. Real Time PCR and immunohistochemical studies confirmed significant down regulation of Akt expression in the pancreas of diabetic rats when compared to control. Curcumin and vitamin D_3 pre-treatment induced a significant up regulation of Akt expression when compared to both control and diabetic groups.
8. MLD-STZ induced diabetes was associated with significant down regulation in the expression of NeuroD1, Pdx-1 and cyclin D2 in the pancreas when compared to control. When compared to both control and diabetic groups, pre-treatment using curcumin and vitamin D_3 significantly up regulated the expression of these markers. Pax expression was significantly increased in the pancreas of diabetic and pre-treated rats when compared to control. Pre-treated rats showed a prominent up regulation in Pax expression when compared with diabetic group. Amongst the pre-treated rats, vitamin D_3 pre-treated rats showed a prominent up regulation of NeuroD1 and Pax mRNA expression when compared to the curcumin pre-treated group.
9. The gene expression of insulin like growth factor-1 and NF-κB in the pancreas of diabetic and pre-treated rats was significantly up regulated when compared to the control. More prominent up regulation of insulin like growth factor-1 was observed in curcumin and vitamin D_3 pre-treated rats when compared to the diabetic group. NF-κB mRNA levels were

significantly decreased in the pre-treated groups when compared to the diabetic group.

10. The gene expression of apoptotic markers like Bax, caspase 3, caspase 8 and TNF-α were significantly up regulated in the pancreas of diabetic and pre-treated groups when compared to control. The expression of all the apoptotic markers was significantly down regulated in the pre-treated group, when compared with diabetic. Immunohistochemical studies using confocal microscope with specific antibodies of caspase 3 confirmed a significantly increased expression in diabetic group. Curcumin and vitamin D_3 pre-treated rats showed a significant decrease in caspase 3 protein expression in pancreas when compared to diabetic group.
11. Adrenergic and muscarinic receptors ligand binding parameters were measured by Scatchard analysis. Total adrenergic receptor number was significantly increased in the pancreas of diabetic rats when compared to control. Curcumin and vitamin D_3 pre-treatment significantly decreased the total adrenergic receptor number. There was a significant decrease in total muscarinic receptor number in the diabetic rat pancreas when compared with control. Pre-treated rats showed a significant increase in total muscarinic receptor number.
12. Scatchard analysis, Real Time PCR and confocal microscopic studies confirmed a significant increase in α2 adrenergic receptor expression in the pancreas of diabetic rats. Pre-treatment with curcumin and vitamin D_3 significantly decreased α2 adrenergic receptor expression when compared to the diabetic group. In the diabetic group, mRNA and protein expression of β adrenergic receptors were decreased significantly when compared to control. Curcumin and vitamin D_3 pre-treatment significantly increased the β adrenergic receptors expression.
13. There was a significant reduction in receptor binding, mRNA expression and protein level expression of muscarinic M1 and M3 subtypes in the pancreas of diabetic rats, when compared to control. Curcumin and

vitamin D_3 pre-treatments were significantly increased the muscarinic M1 and M3 expression when compared with the diabetic. Choline acetyltransferase and acetylcholine esterase expression control the synthesis and hydrolysis of acetylcholine. Choline acetyltransferase gene expression was significantly decreased in diabetic rat pancreas when compared to control. The curcumin and vitamin D_3 pre-treated rats showed a significant increase in the mRNA expression of choline acetyltransferase and a significant decrease in acetylcholine esterase when compared with both control and diabetic rats. The high affinity muscarinic M2 and α7 nicotinic acetylcholine receptors expressions were significantly increased in the pancreas of diabetic rats. Pre-treatments could significantly decreased their expression when compared to the diabetic group.

14. The signalling mediator for vitamin D_3 is the vitamin D receptor. Its mRNA expression was significantly increased in all MLD-STZ treated rats when compared to control. A prominent up regulation in the vitamin D receptor mRNA and protein expression was observed in vitamin D_3 pre-treated rats when compared to all the other groups. CREB and phospholipase C have a crucial role in maintaining sufficient pancreatic insulin output. In diabetic rat pancreas, there was a significant down regulation of CREB and phospholipase C mRNA expression when compared to control. When compared with both control and diabetic rats, a significant up regulation of CREB and phospholipase C was observed in curcumin and vitamin D_3 pre-treated rats. MLD-STZ induced diabetes was associated with a significant down regulation of beta cell insulin sensing components like insulin receptor and GLUT 2. Pre-treatment with curcumin and vitamin D_3 significantly up regulated the insulin receptor and GLUT 2 expression when compared to both control and diabetic rats.
15. Antioxidant enzymes like superoxide dismutases and glutathione peroxidase gene expression was significantly increased in diabetic and

pre-treated groups. Compared to control and diabetic rats, a prominent up regulation was observed in curcumin and vitamin D_3 pre-treated rats. cAMP, cGMP and IP3 are critical second messengers in many signal transduction pathways. In diabetic rat pancreas, there was a significant reduction in the levels of these second messengers when compared to control. Curcumin and vitamin D_3 pre-treatments were able to maintain a near control levels of cAMP, cGMP and IP3 when compared to the diabetic group.

16. B_{max} and K_d of total adrenergic and α2 and β receptor subtypes were significantly decreased in the brain stem of diabetic rats when compared to control. When compared to diabetic rats, curcumin and vitamin D_3 pre-treatment significantly increases their binding parameters in brain stem. mRNA and protein expression of α2 and β2 adrenergic receptors in the brain stem also showed a significant down regulation in diabetic rats when compared to control. Pre-treatment with curcumin and vitamin D_3 were able to significantly increase their gene expression when compared to the diabetic group. Hippocampal total, α2 and β adrenergic receptors B_{max} and K_d was significantly increased in diabetic rats when compared to control. Pre-treatment with curcumin and vitamin D_3 significantly decreased B_{max} and K_d of adrenergic receptors. Similarly, Real Time PCR and confocal studies revealed a significant increase in the expression of α2 and β2 adrenergic receptors in hippocampus of diabetic rats when compared to control. Curcumin and vitamin D_3 pre-treatments significantly down regulated the expression of α2 and β2 adrenergic receptors. In hypothalamus, α2 and β2 adrenergic receptor gene expression was significantly up regulated in diabetic rats when compared to control. Pre-treatment with curcumin and vitamin D_3 significantly down regulated the gene expression.
17. Total muscarinic and muscarinic M1 receptor number was significantly decreased in the brain stem and hippocampus of experiment rats when

compared to control. When compared to the diabetic group, total muscarinic and muscarinic M1 receptor number was significantly increased in the pre-treated groups. mRNA and protein expression of muscarinic M1 receptor showed a significant down regulation in the brain stem and hippocampus when compared to control. Curcumin and vitamin D_3 pre-treatments were able to significantly up regulate M1 expression. Muscarinic M3 receptor B_{max}, mRNA level and protein expression was significantly decreased in brain stem and increased in hippocampus when compared with control. M3 expression was significantly up regulated in brain stem and down regulated in the hippocampus of pre-treated group when compared to diabetic. In hypothalamus, mRNA expression of M1 receptor was down regulated and M3 receptor was up regulated when compared to control. Pre-treated rats showed a significant up regulation of M1 receptor and down regulation of M3 receptor when compared to the diabetic group.

18. Choline acetyltransferase expression was significantly up regulated in brain stem and hippocampus and down regulated in hypothalamus of diabetic and pre-treated rats when compared with control. In the pre-treated rats, its expression was significantly up regulated in brain stem and hypothalamus and down regulated in hippocampus when compared to the diabetic group. Acetylcholine esterase expression was significantly up regulated in brain stem, hippocampus and hypothalamus of diabetic rats when compared to control. A significant down regulation was observed in brain stem, hippocampus and hypothalamus of pre-treated group when compared to diabetic group.
19. Muscarinic M2 receptor expression was significantly up regulated in hippocampus and hypothalamus, but down regulated in the brain stem of diabetic and pre-treated rats, when compared to control. When compared to the diabetic group, muscarinic M2 receptor expression was significantly up regulated in brain stem and down regulated in the hippocampus and

hypothalamus of pre-treated group. α7 nicotinic acetylcholine expression was significantly up regulated in the brain stem, hippocampus and hypothalamus of diabetic and pre-treated rats, when compared to control. Pre-treated rats showed a significant down regulation of α7 nicotinic receptor when compared to diabetic group.

20. In diabetic rats, vitamin D receptor expression was significantly up regulated in brain stem and hippocampus, but down regulated in hypothalamus when compared to control. Its expression was significantly up regulated in hippocampus and hypothalamus, but down regulated in brain stem of the pre-treated group when compared to diabetic rats. In diabetic rats, CREB and phospholipase C expression was significantly down regulated in brain stem, hippocampus and hypothalamus, when compared to control. Pre-treated groups showed a significant increase in the mRNA levels of CREB and phospholipase C when compared to diabetic. Insulin receptor and GLUT 3 expressions were significantly up regulated in the brain stem and hippocampus and down regulated in hypothalamus of diabetic and pre-treated rats when compared to control. Pre-treated rats exhibited a significant up regulation of insulin receptor and GLUT 3 in hypothalamus and down regulation in brain stem and hippocampus when compared to diabetic rats.
21. Superoxide dismutase and glutathione peroxidase gene expression was significantly up regulated in brain stem, hippocampus and hypothalamus of diabetic and pre-treated rats when compared to control. The down regulation was more pronounced in the pre-treated rats, when compared to the diabetic.
22. cAMP content in the brain stem and hippocampus was significantly increased in diabetic rats when compared to control. cGMP content was significantly increased in brain stem and decreased in hippocampus of diabetic rats. Diabetic rats also showed a significant decrease in IP3 in brain stem and a significant increase in hippocampus when compared to

control. Pre-treatment with curcumin and vitamin D_3 retained a near control level of cAMP, cGMP and IP3 levels in the brain stem and hippocampus when compared with diabetic group.

Collectively, the present study showed that the functional regulation of adrenergic and muscarinic receptor subtypes in pancreas, brain stem, hippocampus and hypothalamus play a key role in pathogenesis of diabetes and associated complications in MLD-STZ induced diabetic rat model. The anti-diabetogenesis property of curcumin and vitamin D_3 is due to the increased insulin secretion from existing beta cells together with increased beta cell mass expansion and reduced apoptosis. This beta cell compensatory response against hyperglycemia is induced by adrenergic and muscarinic receptor subtype functional regulation in the pancreas. Pre-treatments also improve pancreatic intracellular signalling, glucose sensitivity, antioxidant defence system and second messenger levels to sustain considerable insulin release even after MLD-STZ administration. Curcumin and vitamin D_3 pre-treatment helps to maintain adrenergic and muscarinic receptor function, intracellular signalling, insulin sensitivity, antioxidant defence and second messenger levels in brain stem, hippocampus and hypothalamus to elicit a proper stimulation of beta cells in response to impaired glucose homeostasis. Our results confirmed the anti-diabetogenesis property of curcumin and vitamin D_3 pre-treatment. In curcumin and vitamin D_3 pre-treated rats, the adrenergic and muscarinic receptor subtypes functional regulation in the pancreas, brain stem, hippocampus and hypothalamus are the major stimulators for beta cell compensatory response against increased metabolic demand.

Conclusion

Administration of MLD-STZ to curcumin and vitamin D_3 pre-treated rats induced only an incidental prediabetic condition. Curcumin and vitamin D_3 pre-treated groups injected with MLD-STZ exhibited improved circulating insulin levels and behavioural responses when compared to MLD-STZ induced diabetic group. Activation of beta cell compensatory response induces an increase in pancreatic insulin output and beta cell mass expansion in the pre-treated group. Cell signalling proteins that regulate pancreatic beta cell survival, insulin release, proliferation and differentiation showed a significant increase in curcumin and vitamin D_3 pre-treated rats. Marked decline in α2 adrenergic receptor function in pancreas helps to relent sympathetic inhibition of insulin release. Neuronal stimulation of hyperglycemia induced beta cell compensatory response is mediated by escalated signalling through β adrenergic, muscarinic M1 and M3 receptors. Pre-treatment mediated functional regulation of adrenergic and cholinergic receptors, key cell signalling proteins and second messengers improves pancreatic glucose sensing, insulin gene expression, insulin secretion, cell survival and beta cell mass expansion in pancreas. Curcumin and vitamin D_3 pre-treatment induced modulation of adrenergic and cholinergic signalling in brain stem, hippocampus and hypothalamus promotes insulin secretion, beta cell compensatory response, insulin sensitivity and energy balance to resist diabetogenesis. Pre-treatment improved second messenger levels and the gene expression of intracellular signalling molecules in brain stem, hippocampus and hypothalamus, to retain a functional neuronal response to hyperglycemia. Curcumin and vitamin D_3 protect pancreas and brain regions from oxidative stress by their indigenous antioxidant properties and by their ability to stimulate cellular free radical defence system. The present study demonstrates the role of adrenergic and muscarinic receptor subtypes functional regulation in curcumin and vitamin D_3 mediated anti-diabetogenesis. This will have immense clinical significance in developing effective strategies to delay or prevent the onset of diabetes.

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1. **George N**, Kumar TP, Antony S, Jayanarayanan S, Paulose CS. (2012). Effect of vitamin D3 in reducing metabolic and oxidative stress in the liver of streptozotocin-induced diabetic rats. Br J Nutr., 108:1410-1418. [PMID:22221397]

2. Kumar PT, Antony S, Nandhu MS, Sadanandan J, **Naijil G**, Paulose CS. (2011). Vitamin D3 restores altered cholinergic and insulin receptor expression in the cerebral cortex and muscarinic M3 receptor expression in pancreatic islets of streptozotocin induced diabetic rats. J Nutr Biochem., 22:418-425 [PMID:20655720].

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13. Kumar PT, **George N**, Antony S, Paulose CS. (2013). Curcumin restores diabetes induced neurochemical changes in the brain stem of Wistar rats. Eur J Pharmacol., 702:323-231 [PMID:23380686].

BOOK PUBLISHED

1. **Naijil George**, Ajayan MS, Paulose CS. (2012). Muscarinic Receptor Subtypes Functional Regulation in the Pancreas of Multiple Low Dose Streptozotocin Induced Diabetic Rats. In 'Current Scenario in Biotechnology'. Edited by Ponmurugan *et al*., Blooms Bury, London, Pages 3-8.

ABSTRACTS PRESENTED

1. **Naijil George**, Peeyush KT, Jayanarayanan S, Paulose CS. Vitamin D_3 induced reversal of metabolic and oxidative stress in the liver of streptozotocin diabetic rats. In 'National Conference on Innovations in Biotechnology'. Organized by the SRM University, Chennai. October 2010.
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3. **Naijil George**, Peeyush Kumar T, Sherin Antony, Paulose CS. Cholinergic, Insulin Receptor and GLUT3 Functional Regulation in Brain Stem of Streptozotocin Induced Diabetic Rats: Effect of Curcumin Supplementation. In 'Neurocon 2011', Organized by the IPGME&R, Kolkata, West Bengal. January 2011.
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8. **Naijil George**, Anju T., Paulose CS. Induced pluripotent stem cell technology and ethics. In 'International Symposium on Genetics and Neuroscience'. Organized by the Institute of Science and Religion, Aluva. February 2014.

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Effect of vitamin D_3 in reducing metabolic and oxidative stress in the liver of streptozotocin-induced diabetic rats

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Abstract

Diabetes mellitus is a growing health problem worldwide and is associated with severe liver complications. The aim of the present study is to analyse the status of metabolic and free-radical-scavenging enzymes and second messengers in the liver of streptozotocin (STZ)-induced diabetic rats, and to determine the hepatoprotective role of vitamin D_3. All studies were performed using the liver of adult male Wistar rats. Gene expression studies were carried out using real-time PCR with specific probes. Second messenger levels were determined using ^{3}H-labelled Biotrak assay kits, and glucose uptake assay with D-[^{14}C]glucose. The present results show that there was a decrease in hepatic glucose uptake, malate dehydrogenase activity, glycogen content, inositol triphosphate (IP_3) and cyclic GMP levels, and superoxide dismutase, glutathione peroxidase, phospholipase C, cyclic AMP-responsive element-binding protein, vitamin D receptor (VDR) and insulin receptor (INSR) gene expression in the diabetic rats when compared with the controls (all $P<0{\cdot}05$), while cyclic AMP levels and GLUT2 expression were increased ($P<0{\cdot}05$). Treatment of the diabetic rats with vitamin D_3 and insulin reversed the altered parameters to near control values. In conclusion, the data suggest a novel role of vitamin D_3 in restoring impaired liver metabolism in STZ-induced diabetic rats by regulating glucose uptake, storage and metabolism. We demonstrated that the restoring effect of vitamin D_3 is mediated through VDR modulation, thereby improving signal transduction and controlling free radicals in the liver of diabetic rats. These data suggest a potential role for vitamin D_3 in the treatment of diabetes-associated hepatic complications.

Key words: Vitamin D: Diabetes: Liver: Oxidative stress: Metabolism: Insulin

Diabetes-associated complications are major causes of morbidity and mortality. The liver plays a unique role in glucose homeostasis. Because of its anatomical location, it is ideally suited to control the systemic supply of absorbed nutrients, and it is one of only two organs that both consume and produce substantial amounts of glucose. The central role of the liver in the maintenance of blood glucose levels is assured by complex regulation by metabolic substrates, insulin and other hormones[1]. Diabetes-associated hyperglycaemia and hypoinsulinaemia lead to the impairment of hepatic glucose and lipid metabolism. Virtually, the entire spectrum of liver diseases is seen in patients with diabetes, including liver cirrhosis, a significant cause of death in diabetic patients, even more relevant than CVD[2]. Interest in the development of novel antidiabetic agents has been fuelled by the intense complications due to therapeutic treatment of diabetes and associated liver failure[2,3]. An increased prevalence of diabetes has been observed in vitamin D-deficient individuals[4], and our previous study has shown that vitamin D_3 treatment restores blood glucose homeostasis in streptozotocin (STZ)-induced diabetic rats[5]. Binding of vitamin D_3 promotes the vitamin D receptor (VDR) to form a heterodimer with the retinoid X receptor and transactivates vitamin D-responsive elements present in target genes[6]. VDR mRNA and protein have previously been detected and studied in several rat tissues including liver[7,8].

Diabetes-associated liver complications are widely studied using STZ-induced diabetic rats[9]. Net glucose uptake and its metabolism in hepatocytes depend on intracellular metabolic status, which is determined by metabolic enzymes, second messengers and transcription factors. Evidence for the role of free radicals and oxidative stress in diabetes-associated complications has been established. Implication of oxidative stress in the pathogenesis of diabetes mellitus is

Abbreviations: cAMP, cyclic AMP; cGMP, cyclic GMP; CT, threshold cycle; INSR, insulin receptor; IP_3, inositol triphosphate; LPO, lipid peroxidation; MDH, malate dehydrogenase; STZ, streptozotocin; VDR, vitamin D receptor.

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Vitamin D_3 restores altered cholinergic and insulin receptor expression in the cerebral cortex and muscarinic M3 receptor expression in pancreatic islets of streptozotocin induced diabetic rats

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Abstract

Nutritional therapy is a challenging but necessary dimension in the management of diabetes and neurodegenerative changes associated with it. The study evaluates the effect of vitamin D_3 in preventing the altered function of cholinergic, insulin receptors and GLUT3 in the cerebral cortex of diabetic rats. Muscarinic M3 acetylcholine receptors in pancreas control insulin secretion. Vitamin D_3 treatment in M3 receptor regulation in the pancreatic islets was also studied. Radioreceptor binding assays and gene expression was done in the cerebral cortex of male Wistar rats. Immunocytochemistry of muscarinic M3 receptor was studied in the pancreatic islets using specific antibodies. Y-maze was used to evaluate the exploratory and spatial memory. Diabetes induced a decrease in muscarinic M1, insulin and vitamin D receptor expression and an increase in muscarinic M3, α7 nicotinic acetylcholine receptor, acetylcholine esterase and GLUT3 expression. Vitamin D_3 and insulin treatment reversed diabetes-induced alterations to near control. Diabetic rats showed a decreased Y-maze performance while vitamin D_3 supplementation improved the behavioural deficit. In conclusion, vitamin D_3 shows a potential therapeutic effect in normalizing diabetes-induced alterations in cholinergic, insulin and vitamin D receptor and maintains a normal glucose transport and utilisation in the cortex. In addition vitamin D_3 modulated muscarinic M3 receptors activity in pancreas and plays a pivotal role in controlling insulin secretion. Hence our findings proved, vitamin D_3 supplementation as a potential nutritional therapy in ameliorating diabetes mediated cortical dysfunctions and suggest an interaction between vitamin D_3 and muscarinic M3 receptors in regulating insulin secretion from pancreas.

Keywords: Diabetes; Cerebral cortex; Insulin; Vitamin D_3; Cholinergic receptor pancreas and muscarinic M3

1. Introduction

Diabetes mellitus is a common metabolic disorder characterized by hyperglycemia due to an absolute or relative insulin deficiency. Diabetes mellitus is known to be associated with neurological complications in both peripheral nervous system and the central nervous system (CNS) [1]. Brain cells are particularly vulnerable to oxidative stress. Controlling blood glucose is essential for avoiding long-term complications of diabetes like learning and memory deficit.

Vitamin D_3 is either synthesised in the epidermis from 7-dehydrocholesterol by the absorption of ultraviolet light, or obtained from the diet in a limited number of foods such as eggs, fish oils and fortified milk. The biological actions of vitamin D_3 are mediated through binding to the vitamin D receptor (VDR), a member of the nuclear steroid hormone receptor family. An increased prevalence of diabetes has been described in vitamin D-deficient individuals [2]. Insulin synthesis and secretion has been shown to be impaired in β cells in vitamin D-deficient animals. It was also of interest to determine whether changes in the expression of the muscarinic M3 receptors using vitamin D_3 supplementation in pancreas might account for the increased synthesis and secretion of insulin. Immunohistochemistry showed the presence of VDR in human pituitary gland [3], suggesting a possible role of vitamin D in regulation of the brain endocrine system. It is of particular importance that VDR and catalytic enzymes are colocalized in the brain, supporting an autocrine/paracrine function for vitamin D. These findings support a functional role for vitamin D in the human brain [4].

Diabetes is also found to be associated with changes in somatic sensations which involve the cerebellum, cerebral cortex and thalamus. The cholinergic innervation of the cerebral cortex has been extensively investigated because of its role in arousal, learning and memory [5]. Alterations in glucose utilization are known to occur in the important regions of brain connected with learning and memory [6]. The brain glucose uptake is ultimately dependent on facilitative glucose transporters, the modulation of brain glucose transporters intrinsic activity. GLUT3 is the main neuronal glucose transporter [7] abundant in the brain.

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At the Cutting Edge

Role of curcumin in the prevention of cholinergic mediated cortical dysfunctions in streptozotocin-induced diabetic rats

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ABSTRACT

Diabetes exacerbates neuronal injury mediated through neurotransmitters deregulation in cerebral cortex. Our study analyzed the neuroprotective effect of curcumin to prevent cortical dysfunction associated with diabetes. Our study revealed decreased gene expression of muscarinic M1, insulin receptor, SOD, choline acetyl transferase and increased gene expression of muscarinic M3, α7-nicotinic acetylcholine receptor, acetylcholine esterase and GLUT3 in cerebral cortex of diabetic rats. Curcumin and insulin treatment reversed this altered parameters to near control. Immunohistochemistry studies of muscarinic M1 and M3 confirmed the gene expression at protein level. Decreased novel arm entry of diabetic rats in Y-maze test, improved in treatment group. These results suggest that cholinergic dysfunction, impaired glucose transport and oxidative stress contributes to learning and memory deficits in diabetes and further suggest that antioxidant curcumin has potential therapeutic role in preventing and/or delaying the diabetic complications associated with brain.

Contents

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ORIGINAL PAPER

Decreased GABA Receptor Binding in the Cerebral Cortex of Insulin Induced Hypoglycemic and Streptozotocin Induced Diabetic Rats

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Abstract Hypoglycemia is the major problem to blood glucose homeostasis in treatment of diabetes and is associated with severe irreversible consequences including seizures, coma and death. GABAergic inhibitory function in the cerebral cortex plays an important role in controlling the excitability and responsiveness of cortical neurons. Present study analysed effects of insulin induced hypoglycemia and streptozotocin induced diabetes on the cortical GABA receptor binding, $GABA_{A\acute{z}1}$, $GABA_B$ receptor subtype expression, GAD and GLUT3 expression. Diabetic rats showed decreased [^{3}H] GABA binding in the cerebral cortex compared to control while hypoglycemia exacerbated the decrease. GABA receptor subunits; $GABA_{A\acute{z}1}$, $GABA_B$ and GAD expression significantly decreased in diabetic rats whereas hypoglycemia significanly decreased the expression compared to diabetic. GLUT3 expression significantly up regulated during both hypo and hyperglycemia. Our results showed that hypoglycemia and hyperglycemia decreased GABAergic neuroprotective function in the cerebral cortex, which account for the increased vulnerability of cerebral cortex to subsequent neuronal damage during hypo/hyperglycemia.

Keywords Diabetes · Hypoglycemia · GABA receptor · GLUT3

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Abbreviation

GABA	Gamma-aminobutyric acid
GAD	Glutamate decarboxylase
STZ	Streptozotocin

Introduction

Hypoglycemia is a relatively common episode primarily affecting diabetic patients receiving treatment with insulin or other hypoglycemic drugs. A major concern of diabetic patients is that repeated episodes of hypoglycemia result in neuronal loss because of impaired glucose supply and utilization. Recurrent hypo/hyperglycemia reduces the supply of glucose to the brain which will have deleterious effect to the functioning of brain cells.

The central nervous system (CNS) neurotransmitters play an important role in the regulation of glucose homeostasis. Neurotransmitters have been reported to show significant alterations during hyperglycemia resulting in altered functions causing neuronal degeneration [1]. Hypoglycemia has been shown to produce neuronal damage and death, primarily in cortex [2]. As neuronal glucose uptake depends on the extracellular concentration of glucose, cellular damage ensues after persistent episodes of hyperglycemia [3]. Symptoms of hypoglycemia result from the actions of hormones and neurotransmitters in the process of restoring blood glucose levels. Neuronal damage caused by energy deprivation is due to imbalance between excitatory and inhibitory neurotransmission [4, 5]. Limited glucose availability aggravates hypoglycemia-induced brain damage [6]. Various animal models reportedly show seizures when rendered hypoglycemic. Previous studies from our laboratory reported enhanced glutamate toxicity

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Research report

Hypoglycemia induced behavioural deficit and decreased GABA receptor, CREB expression in the cerebellum of streptozoticin induced diabetic rats

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ABSTRACT

Intensive glycemic control during diabetes is associated with an increased incidence of hypoglycemia, which is the major barrier in blood glucose homeostasis during diabetes therapy. The CNS neurotransmitters play an important role in the regulation of glucose homeostasis. In the present study, we showed the effects of hypoglycemia in diabetic and non- diabetic rats on motor functions and alterations of GABA receptor and CREB expression in the cerebellum. Cerebellar dysfunction is associated with seizure generation, motor deficits and memory impairment. Scatchard analysis of [^{3}H]GABA binding in the cerebellum of diabetic hypoglycemic and control hypoglycemic rats showed significant ($P < 0.01$) decrease in B_{max} and K_d compared to diabetic and control rats. Real-time PCR amplification of GABA receptor subunit $GABA_{A\alpha1}$ and GAD showed significant ($P < 0.001$) down-regulation in the cerebellum of hypoglycemic rats compared to diabetic and control rats. Confocal imaging study confirmed the decreased GABA receptors in hypoglycemic rats. CREB mRNA expression was down-regulated during recurrent hypoglycemia. Both diabetic and non-diabetic hypoglycemic rats showed impaired performance in grid walk test compared to diabetic and control. Impaired GABA receptor and CREB expression along with motor function deficit were more prominent in hypoglycemic rats than hyperglycemic which showed that hypoglycemia is causing more neuronal damage at molecular level. These molecular changes observed during hypo/hyperglycemia contribute to motor and learning deficits which has clinical significance in diabetes treatment.

1. Introduction

Intensive insulin therapy decreases symptoms associated with chronic hyperglycemia, but increases the accompanying risk for episodes of severe hypoglycemia as reported by The Diabetes Control and Complications Trial Research Group, 1993 [14]. Hypoglycemia is the limiting factor in the management of diabetes which leads to brain damage and long-term cognitive impairment [2]. Prolonged insulin-induced hypoglycemia causes widespread loss of neurons and permanent brain damage with irreversible coma [3].

GABA is a predominant neurotransmitter, which is synaptically released, mediates fast inhibitory synaptic transmission and regulates excitatory activity of neurons [40]. GABA acts at one of two types of receptor $GABA_A$ which controls chloride entry into the cell and $GABA_B$, which increases potassium conductance, decreases calcium entry, and inhibits the presynaptic release of other neurotransmitters. The cerebellum is a region of the brain that plays an important role in the integration of sensory perception, memory consolidation, coordination, and motor control. To coordinate motor control, there are many neural pathways linking the cerebellum with the cerebral motor cortex and the spinocerebellar tract [47]. GABA receptor subunits expressed in the granule cells of the cerebellum are a part of the brain responsible for coordination and motor learning. Cerebellar cortex regulates different types of motor response. GABA receptors play critical roles in neuronal excitability and modulation of synaptic neurotransmission [39]. The $GABA_A$ receptor mediates fast synaptic inhibition in the brain and its dysfunction is implicated in several forms of degenerative disorders. The $GABA_{A\alpha1}$ subunit is the most abundant α subunit variant in the brain. At least half of the $GABA_A$Rs are believed to contain the $\alpha1$ subunit that is highly expressed throughout most brain regions [19]. CREB is a transcription factor that has been implicated in the activation of protein synthesis required for long-term memory and seizure formation [28]. CREB is a stimulus-inducible transcriptional activator controlled by phosphorylation that up-regulates endogenous $GABA_{A\alpha1}$ transcription. The differential regulation of $GABA_A$ receptor subtypes represents a major facet of homeostatic synaptic plasticity and contributes to the excitation/inhibition (E/I) balance under physiological conditions and upon pathological challenges [9]. Glutamate decarboxylase (GAD) is an enzyme that catalyzes the decarboxylation of glutamate to GABA. GAD is the rate limiting

Abbreviation: GABA, gamma-aminobutyric acid; GAD, glutamate decarboxylase; STZ, streptozotocin; CREB, cyclic AMP response element binding protein.

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RESEARCH Open Access

Curcumin modulates dopaminergic receptor, CREB and phospholipase c gene expression in the cerebral cortex and cerebellum of streptozotocin induced diabetic rats

T Peeyush Kumar, Sherin Antony, G Gireesh, Naijil George and CS Paulose*

Abstract

Curcumin, an active principle component in rhizome of *Curcuma longa*, has proved its merit for diabetes through its anti-oxidative and anti-inflammatory properties. This study aims at evaluating the effect of curcumin in modulating the altered dopaminergic receptors, CREB and phospholipase C in the cerebral cortex and cerebellum of STZ induced diabetic rats. Radioreceptor binding assays and gene expression was done in the cerebral cortex and cerebellum of male Wistar rats using specific ligands and probes. Total dopaminergic receptor binding parameter, B_{max} showed an increase in cerebral cortex and decrease in the cerebellum of diabetic rats. Gene expression studies using real time PCR showed an increased expression of dopamine D1 and D2 receptor in the cerebral cortex of diabetic rats. In cerebellum dopamine D1 receptor was down regulated and D2 receptor showed an up regulation. Transcription factor CREB and phospholipase C showed a significant down regulation in cerebral cortex and cerebellum of diabetic rats. We report that curcumin supplementation reduces diabetes induced alteration of dopamine D1, D2 receptors, transcription factor CREB and phospholipase C to near control. Our results indicate that curcumin has a potential to regulate diabetes induced malfunctions of dopaminergic signalling, CREB and Phospholipase C expression in cerebral cortex and cerebellum and thereby improving the cognitive and emotional functions associated with these regions. Furthermore, in line with these studies an interaction between curcumin and dopaminergic receptors, CREB and phospholipase C is suggested, which attenuates the cortical and cerebellar dysfunction in diabetes. These results suggest that curcumin holds promise as an agent to prevent or treat CNS complications in diabetes.

Introduction

Diabetes mellitus is a heterogeneous disease characterized by chronic hyperglycaemia and requires long-term management. Chronic changes in the antecedent level of glycaemia induce alterations in brain glucose metabolism in rodents [1,2]. Chronic hyperglycemia in diabetes can lead to various complications, affecting the CNS [3]. A continuous systemic supply of glucose is essential for normal cerebral metabolism [4].

Controlling blood sugar is essential for avoiding long-term complications of diabetes like learning and memory. Although mechanisms leading to cortical and cerebellar dysfunction associated with diabetic complications are not completely understood, brain cells are particularly vulnerable to oxidative stress [5]. Oxidative stress, leading to an increased production of reactive oxygen species, as well as lipid peroxidation is increased in diabetes [6-8]. Hyperglycemia causes the autoxidation of glucose, glycation of proteins, and the activation of polyol metabolism [9]. These changes accelerate the generation of reactive oxygen species to increase oxidative modifications of lipids, DNA, and proteins in various tissues. Oxidative stress is believed to play an important role in the development of complications in diabetes associated neuronal disorders [9]. Greater understanding of CNS (CNS) involvement could lead to new strategies to prevent or reverse the damage caused by diabetes mellitus.

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Mini-Review

Opioid System Functional Regulation in Neurological Disease Management

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There is increasing evidence to suggest a role for the opioid system in the control of pathophysiology of neurological disorders (Alzheimer's, Parkinson's, and Huntington's diseases, spinal cord injury, epilepsy, hypoxia, and autism). Resuscitation of the altered expression of the opioid system in various neurological disorders is of therapeutic importance. Such treatment may be beneficial in ameliorating the clinical symptoms of the disorder. This Mini-Review provides a brief update on opioid system regulation in neurological disorders and focuses on the opioids' pharmacological importance. © 2010 Wiley-Liss, Inc.

Key words: opioid receptors; neurological disorders; resuscitation

Opiates such as morphine and related compounds have been of therapeutic interest for medical scientists and laymen alike for a long time. The demonstration of pharmacologically relevant opiate receptor binding sites in brain and gut (Pert and Snyder, 1973) and subsequent isolation and characterization of opioid peptides paved the way for renewed interest in opiate research. The usual pharmacokinetic parameters (half-life, clearance, volume of distribution) of opioids have been known for some time. However, their metabolism has, until recently, been poorly understood.

The opioid receptors (ORs) are now known to be distributed widely in the central nervous system (CNS) and in peripheral sensory and autonomic nerves. Activation of ORs by endogenous and exogenous ligands results in a multitude of physiological functions and behaviors. Research has been conducted on a wide array of molecular–biochemical effects, along with neurochemical localization studies of endogenous opioids and their receptors; i.e., attempts have been made to clarify the role of OR-mediated signalling mechanisms in pain and analgesia; stress and social status; tolerance and dependence; learning and memory; eating and drinking; alcohol and drugs of abuse; CNS development and endocrinology; mental illness and mood; seizures and neurological disorders; electrical activity and neurophysiology; general activity and locomotion; gastrointestinal, renal, and hepatic functions; cardiovascular responses; respiration and thermoregulation; and immunological responses (Williams et al., 2001).

OPIOID RECEPTORS

There are ORs within the CNS as well as throughout the peripheral tissues. These receptors are normally stimulated by endogenous peptides (endorphins, enkephalins, and dynorphins) produced in response to noxious stimulation (Trescot et al., 2008). A natural corollary of the heterogenecity of the opioid peptides is the presence of mutilple classes of opioid receptors. Pharmacological classification of ORs by use of isolated tissue preparations and radioligand studies indicates that there are primarily four types of opioid receptors, μ, κ, δ, and σ (Traynor, 1994). μ-Receptors are responsible for supraspinal analgesia, respiratory depression, euphoria, sedation, decreased gastrointestinal motility, and physical dependence. Subtypes include μ_1 and μ_2, with μ_1 related to analgesia, euphoria, and serenity and μ_2 related to respiratory depression, pruritus, prolactin release, dependence, anorexia, and sedation (Baumann and Rabii, 1990; Vonhof and Sirén, 1991; Trescot et al., 2008). These receptors are also called OP3 or morphine opioid receptors (MOR). κ-Receptors are found in the limbic and other diencephalic areas, brainstem, and spinal cord and are responsible for spinal analgesia, sedation, dyspnea, dependence, dysphoria, and respiratory depression. These are also known as OP2 or κ-opioid receptors (KOR). δ-Receptors are located largely in the brain, and their effects have not been well studied. They may be responsible for psychomimetic and dysphoric effects. They are also called OP1 and δ-opioid receptors (DOR). σ-Receptors are responsible for psychomimetic effects, dysphoria, and stress-induced depres-

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Neuropharmacology and Analgesia

The effects of abnormalities of glucose homeostasis on the expression and binding of muscarinic receptors in cerebral cortex of rats

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ABSTRACT

Glucose homeostasis in humans is an important factor for the functioning of nervous system. Both hypo and hyperglycemia contributes to neuronal functional deficit. In the present study, effect of insulin induced hypoglycemia and streptozotocin induced diabetes on muscarinic receptor binding, cholinergic enzymes; AChE, ChAT expression and GLUT3 in the cerebral cortex of experimental rats were analysed. Total muscarinic, muscarinic M_1 receptor showed a significant decrease and muscarinic M_3 receptor subtype showed a significant increased binding in the cerebral cortex of hypoglycemic rats compared to diabetic and control. Real-Time PCR analysis of muscarinic M_1, M_3 receptor subtypes confirmed the receptor binding studies. Immunohistochemistry of muscarinic M_1, M_3 receptors using specific antibodies were also carried out. AChE and GLUT3 expression up regulated and ChAT expression down regulated in hypoglycemic rats compared to diabetic and control rats. Our results showed that hypo/hyperglycemia caused impaired glucose transport in neuronal cells as shown by altered expression of GLUT3. Increased AChE and decreased ChAT expression is suggested to alter cortical acetylcholine metabolism in experimental rats along with altered muscarinic receptor binding in hypo/hyperglycemic rats, impair cholinergic transmission, which subsequently lead to cholinergic dysfunction thereby causing learning and memory deficits. We observed a prominent cholinergic functional disturbance in hypoglycemic condition than in hyperglycemia. Hypoglycemia exacerbated the neurochemical changes in cerebral cortex induced by hyperglycemia. These findings have implications for both therapy and identification of causes contributing to neuronal dysfunction in diabetes.

1. Introduction

Hypoglycemia is a major problem to blood glucose homeostasis in treatment of diabetes and is associated with severe irreversible consequences including seizures, coma and death. Hypoglycemic coma and brain injury are potential complications of insulin therapy (Suh et al., 2005) which impose alterations upon both the central (CNS) and peripheral (PNS) nervous systems thereby leading to brain damage and long-term cognitive impairment. Pathological studies in humans and animals indicate hypoglycemia-induced neuronal death occurs preferentially in the hippocampus, superficial layers of the cortex and striatum (Auer and Siesjö, 1993). Severe hypoglycemia triggers a cascade of events in vulnerable neurons that culminate in neuronal death even after glucose normalization. Due to the extensive neuronal loss, one of the neurological sequela associated with hypoglycemia is cognitive decline. According to clinical studies, significant learning and memory deficits correlate with the frequency of hypoglycemia not only in patients with type 1 diabetes, but also in the relatively younger group among the population with type 2 diabetes (Dey et al., 1997).

Acetylcholine in the cerebral cortex serves as a neuromodulator rather than as a classical neurotransmitter. (Krnjevic, 2004). Several studies have shown that changes in the concentration of acetylcholine in the hippocampus and cortex correlate with learning and cognitive function (Chang and Gold, 2003; Fadda et al., 1996; Hironaka et al., 2001; Ragozzino et al., 1996). Any damage in neuronal glucose metabolism and its control cause disturbances in memory function (Hoyer, 2003). The cholinergic system is implicated in functional, behavioural and pathological states including cognitive function by the interaction of acetylcholine with muscarinic receptors (Alkondon et al., 2000). Acetylcholine regulate the activities of central and peripheral nervous system functions through interactions with muscarinic receptors and is released at autonomic synapses modulating a variety of central circuits that support arousal, attention, reward, learning and memory (Dani, 2001; Wess, 2004; Winkler et al., 1995) in the brain. Muscarinic M_1 receptors are abundantly expressed in all major forebrain areas including cerebral cortex, hippocampus and striatum, where they constitute 40–50% of the total muscarinic receptors (Levey, 1993). The duration of action of acetylcholine at the

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Research Paper

Muscarinic M_1, M_3 receptor modulation in the corpus striatum of streptozotocin induced diabetic rats as a function of age

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Abstract

Objectives In this study we have investigated muscarinic M_1, M_3 receptor kinetics and the functional role of IP3 and cGMP in the corpus striatum of both young and old diabetic and insulin-treated diabetic rats.

Methods Radioreceptor binding assays was done in the corpus striatum using specific antagonists QNB and DAMP. IP3 and cGMP assay using [3H]IP3 and [3H]cGMP Biotrak assay system kits.

Key findings M_1 receptor increased and M_3 receptor decreased in control old rats when compared with young control rats. In young diabetic groups M_1 receptor increased and M_3 receptor decreased. Old diabetic groups showed reversed M_1 and M_3 receptors compared with their controls. IP3 and cGMP content increased in old control rats compared with young control rats. IP3 content increased in young diabetic rats and decreased in old diabetic rats. cGMP content was increased significantly in both young and old diabetic groups. Insulin treatment reversed these altered parameters near to control.

Conclusions Our studies showed that M_1 and M_3 receptors, IP3 and cGMP were functionally regulated during diabetes as function of age, which will have immense clinical significance.

Keywords ageing; corpus striatum; diabetic rats; muscarinic receptors; second messengers

Introduction

Muscarinic acetylcholine (ACh) receptors are members of a large family of plasma membrane receptors that transduce the intracellular signals via coupling to guanine nucleotide binding regulatory proteins and subserve numerous vital functions in both the brain and the autonomic nervous system.[1] These receptors are widely distributed throughout the body, predominantly expressed within the parasympathetic nervous system and exert both excitatory and inhibitory control over central and peripheral tissues. ACh has complex and clinically important actions in the striatum that are mediated predominantly by muscarinic receptors. Cholinergic terminals within the striatum contain presynaptic muscarinic receptors that inhibit neurotransmitter release. Various anatomical, electrophysiological, and pathological observations provide evidence that ACh plays a major role in the control of striatal function and in the regulation of motor control.[2] Striatal ACh is released from a population of large cholinergic interneurons that establish complex synaptic contacts with dopamine terminals, originating from the substantia nigra, and with several striatal neuronal populations. The structures within the basal ganglia observed to express muscarinic receptor mRNAs are the striatum, substantia nigra, pars compacta and subthalamic nucleus. The cholinergic innervation of the striatum is intrinsic and originates from a small number (<3%) of large neurons.[3] Streptozotocin (STZ)-induced rats were used as an experimental model for diabetes since they provide a relevant example of endogenous hyperglycaemia.[4] STZ is a toxic glucose analogue that preferentially accumulates in pancreatic beta cells via the GLUT2 glucose transporter and is used as a prominent diabetogenic chemical in diabetes research.[5]

Changes in muscarinic acetylcholine receptors (mAChRs) have been implicated in the pathophysiology of many major diseases of the CNS. Neurochemical studies have revealed age-related changes in neurotransmitter enzyme activity and receptor binding.[6] M_1

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Molecular and Cellular Pharmacology

β_2-Adrenoceptor and insulin receptor expression in the skeletal muscle of streptozotocin induced diabetic rats: Antagonism by vitamin D_3 and curcumin

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ABSTRACT

Diabetes mellitus is a heterogeneous disease and nutritional therapy forms a necessary dimension for its long-term management. Traditional medicinal plants and vitamins are the potentially useful natural products for diabetes control. Diabetes causes atrophy and wasting of skeletal muscles resulting in major peripheral damage. The current study was designed to investigate the therapeutic effect of vitamin D_3 and curcumin treatment on β_2-adrenoceptors, transcription factor CREB, insulin receptors, protein kinase B (Akt) and malate dehydrogenase activity in the skeletal muscle of diabetic rats. Radioreceptor binding assay was done for β_2-adrenoceptors using specific ligand, [^{3}H] propranolol and gene expression studies of β_2-adrenoceptors, transcription factor CREB, insulin receptors and Akt were also done using specific probes. The results of the β_2-adrenoceptor assay showed significant increase in binding parameters, receptor number (B_{max}) and equilibrium dissociation constant (K_d) in the diabetic group in comparison to control. Similarly, an up regulation of β_2-adrenoceptor and CREB gene expression was observed in the diabetic group whereas the insulin receptor expression was down regulated which signifies the increased glycogenolysis, gluconeogenesis and decreased glycogenesis in the muscles. Expression of Akt was found to be up regulated in the diabetic group. Malate dehydrogenase activity was significantly decreased in both cytosolic and mitochondrial fractions of the diabetic group. All these molecular aspects were reversed to near control with vitamin D_3 and curcumin treatment. Our results suggest the rising potential of both vitamin D_3 and curcumin in the management of peripheral complications associated with diabetes.

1. Introduction

Diabetes mellitus is a group of metabolic diseases characterized by high blood glucose levels, resulting from defects in insulin secretion or action, or both. It was first identified as a disease associated with "sweet urine" and "excessive muscle loss" in the ancient world and has currently become the most common endocrine disorder in man, affecting over 170 million people world-wide and, potentially, over 365 million by the year 2030 (Wild et al., 2004). Wasting of muscles, muscle weakness and atrophy are important symptoms characterized in diabetes. As skeletal muscle is the predominant site of insulin-mediated glucose uptake, insulin resistance plays the major role in the pathogenesis of the disease.

Approaches to the prevention and control of hyperglycemia are central to the management of diabetes (Herman and Crofford, 1997). Vitamin D_3 is either synthesized in the epidermis from 7-dehydrocholesterol by the absorption of ultraviolet light or obtained from diet. The biological actions of vitamin D_3 are mediated through binding to vitamin D receptor. An increased prevalence of diabetes has been described in vitamin D_3-deficient individuals. Insulin synthesis and secretion has been shown to be impaired in β-cells in vitamin D_3-deficient animals (Chiu et al., 2004).

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], a yellow pigment from *Curcuma longa*, is a major component of turmeric and exhibits powerful anti-oxidant, anti-diabetic, anti-inflammatory and anti-cancer properties (Commandeur and Vermeulen, 1996; Surh et al., 2001).

The adrenoceptors are cell surface receptors that belong to the large family of metabotropic G-protein coupled receptors. They are targets of catecholamines — epinephrine and norepinephrine and mediate functions such as vasodilation, glycogenolysis, and gluconeogenesis in skeletal muscles (Rang and Maureen Dale, 2003).

The cAMP response element binding protein is a nuclear protein that modulates the transcription of genes with cAMP responsive elements in their promoters (CREs) and epinephrine binding to adrenoceptor causes their activation. In liver and skeletal muscles, several enzymes involved in gluconeogenesis are encoded by genes that contain nearby CREs.

The insulin receptor is a transmembrane receptor, which belongs to the large class of tyrosine kinase receptors and is activated by insulin (Ward and Lawrence, 2009). The activated receptor phosphorylates a

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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-κB) 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 β-(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 β1 suppression of EGF induced DNA synthesis. Thus the activation of GABA receptors, trigger DNA synthesis,

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ORIGINAL ARTICLE

Neonatal hypoxic insult-mediated cholinergic disturbances in the brain stem: effect of glucose, oxygen and epinephrine resuscitation

T. R. Anju · G. Naijil · J. Shilpa · T. Roshni · C. S. Paulose

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Abstract Molecular processes regulating cholinergic functions play an important role in the control of respiration under neonatal hypoxia. The present study evaluates neonatal hypoxic insult-mediated cholinergic alterations and the protective role of glucose, oxygen and epinephrine resuscitation. The changes in total muscarinic, muscarinic M1, M2, M3 receptors and the enzymes involved in acetylcholine metabolism—cholineacetyl transferase and acetylcholine easterase in the brain stem were analyzed. Hypoxic stress decreased total muscarinic receptors along with a reduction in muscarinic M1, M2 and M3 receptor genes in the brain stem. The reduction in acetylcholine metabolism is indicated by the down regulated cholineacetyl transferase and up regulated acetylcholine easterase expression. These cholinergic disturbances in the brain stem were reversed by glucose resuscitation to hypoxic neonates. The adverse effects of immediate oxygenation and epinephrine administration were also reported. This has immense clinical significance in establishing a proper resuscitation for the management of neonatal hypoxia.

Keywords Muscarinic · Cholineacetyl transferase · Acetylcholine easterase · Epinephrine · Hypoxia

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Introduction

Respiration is a highly integrated process that involves a complex network of interplay between the brain, brain stem, spinal cord, cranial and spinal nerves, diaphragm, intercostal muscles, laryngeal and pharyngeal structures, lungs and the vasculature. It also involves diverse sets of neurotransmitters, neuromodulators, receptors, second messengers and transcription factors. The impulse which drives the ventilatory system begins in the brainstem. Postnatal development of respiratory control system is related to structural and functional changes at peripheral and central brain stem components of respiratory control [10, 13, 15]. Hypoxia occurring before or shortly after birth is a major cause of life-threatening injury and lifelong disability [16, 41]. Hypoxia results in multi-organ failure and structural/functional damage especially devastating to the cardiovascular, renal, gastrointestinal and central nervous systems [44, 48]. Oxygen deprivation alters electrical transmission in the brain and generates free radicals, which may mediate neuronal death. Hypoxic brain injury is very complex and has different neuropathological manifestations depending on the maturity of the newborn. Thus, understanding the diagnosis, pathogenesis, resuscitation and treatment of those infants suffering hypoxic brain injury is paramount to reduce disability, improving survival and enhancing quality of life.

The neurotransmitter acetylcholine acting through muscarinic receptors is involved in many aspects of respiratory neuromodulation [27], notably central chemosensitivity in brainstem structures [2, 8]. Cholinergic systems originating in the pons [17] also control respiration in the sleep–wake states by affecting respiratory neurons and motoneurons [4, 5]. Cholinergic control also occurs through direct action on rhythm-generating neurons in the

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Neuropharmacology and analgesia

Curcumin restores diabetes induced neurochemical changes in the brain stem of Wistar rats

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ABSTRACT

Diabetes mellitus, when poorly controlled, leads to debilitating central nervous system (CNS) complications including cognitive deficits, somatosensory and motor dysfunction. The present study investigated curcumin's potential in modulating diabetes induced neurochemical changes in brainstem. Expression analysis of cholinergic, insulin receptor and GLUT-3 in the brainstem of streptozotocin (STZ) induced diabetic rats were studied. Radioreceptor binding assays, gene expression studies and immunohistochemical analysis were done in the brainstem of male Wistar rats. Our result showed that B_{max} of total muscarinic and muscarinic M_3 receptors were increased and muscarinic M_1 receptor was decreased in diabetic rats compared to control. mRNA level of muscarinic M_3, α_7-nicotinic acetylcholine, insulin receptors, acetylcholine esterase, choline acetyltransferase and GLUT-3 significantly increased and M_1 receptor decreased in the brainstem of diabetic rats. Curcumin and insulin treatment restored the alterations and maintained all parameters to near control. The results show that diabetes is associated with significant reduction in brainstem function coupled with altered cholinergic, insulin receptor and GLUT-3 gene expression. The present study indicates beneficial effect of curcumin in diabetic rats by regulating the cholinergic, insulin receptor and GLUT-3 in the brainstem similar to the responses obtained with insulin therapy.

1. Introduction

Diabetes mellitus is a major global health problem currently affecting more than 180 million people worldwide. In recent years it has become evident that diabetes causes significant CNS complications, resulting in important functional impairments (Mijnhout et al., 2006). Prolonged exposure to chronic hyperglycemia in diabetes leads to various complications, affecting the neurological, cardiovascular, renal, visual and auditory systems (Brownlee, 2001). Curcumin is a natural plant polyphenolic compound with powerful anti-oxidant, anti-diabetic, anti-inflammatory and anti-cancer properties and have a long tradition in folk medicine (Kumar et al. 2010a, 2010b).

Brainstem along with hypothalamus serves as the key center of the central nervous system regulating the body homeostasis. Brainstem reticular formation has been considered to play an important role in generating behavioral states as well as in the modulation of pain sensation (Zambreanu et al., 2005) and the reticular functions originate from interacting neuronal groups in the brainstem, including cholinergic, adrenergic and serotoninergic neurons (Steriade, 1996). In addition to glucose, insulin secretion from the endocrine pancreatic b cells is stimulated by several neuropeptide hormones and neurotransmitters, among which acetylcholine; the muscarinic cholinergic receptor agonist plays a prominent role (Ahren, 2000; Gilon and Henquin, 2001). Alterations in glucose transport and utilization are known to occur in the important regions of brain connected with learning and memory (McNay et al., 2000; Krebs and Parent, 2005). Insulin and insulin receptors in the brain suggest their functional involvement in brain cognition phenomena such as learning and memory. Insulin has been shown to exert a memory-enhancing action on both humans and experimental animals (Park et al., 2000; Kern et al., 2001). Also the brain glucose uptake is dependent on facilitative glucose transporters such as GLUT-3. The expression, regulation and activity of glucose transporters play an essential role in neuronal homeostasis, since glucose represents the primary energy source for the brain (Zhao et al., 2010).

Curcumin, the active compound in turmeric, because of its antioxidant and anti-inflammatory properties, has been demonstrated in the prevention and treatment of neurodegenerative disorders such as Alzheimer's disease and multiple sclerosis (Cole et al., 2007). Previous study showed that curcumin and

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