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Development of a cell culture system from the ovarian tissue of African catfish (*Clarias gariepinus*)

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Abstract

A growth medium with Leibovitz-15 (L-15) as the base, supplemented with foetal bovine serum (10% v/v), fish muscle extract (10% v/v), prawn muscle extract (10% v/v), lectin (concanavalin A) (0.02 μg ml^{-1}), lipopolysaccharide (0.02 μg ml^{-1}), glucose D (0.2 mg ml^{-1}), ovary extract (0.5% v/v) and prawn haemolymph (0.5%) has been formulated with 354 ± 10 mOsm for the development and maintenance of a cell culture system from the ovarian tissue of African catfish, *Clarias gariepinus*. For its subculturing, a cell dissociation/extracting solution, composed of equal portions of trypsin phosphate versene glucose (TPVG) (containing 0.0125% (w/v) trypsin and 25% (v/v) non-enzymatic cell dissociation solution 1 and 2, has also been developed with which the cell culture can be passaged 15 times after which they cease to multiply and consequently perish. The cell cultures can be maintained for 12–15 days without fluid change between the passages. This is the first report of a cell culture system from the ovarian tissues of African catfish.

Keywords: African catfish; *Clarias gariepinus*; Primary cell culture system; Ovary

1. Introduction

Fish cell lines have become an important tool for biomedical research (Hightower and Renfro, 1988) and more than 61 cell lines have been developed from teleost fishes (Wolf

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and Mann, 1980). Several workers (Bols et al., 1994; Chen et al., 1983; Driever and Rangini, 1993; Eun-Ho et al., 1989; Komura et al., 1987; Lu et al., 1990; Nicholson et al., 1987; Ostrander et al., 1995) claimed that they could passage the primary cell cultures and cell lines developed from fish species such as *Ctenopharyngodon idella* (fin, snout, swimbladder), *Oncorhynchus mykiss* (gills, ovary), *Brachydanio rerio* (embryo), *Umbra limi* (fin), *Oryzias latipes* (fin), *Epinephelus amblycephalus* (kidney), *Lateolabrax japonicus* (liver and heart), and *O. mykiss* (liver) 50–120 times, but no reports were available about their commercial availability. At present, established fish cell lines such as BB (bullhead brown trunk), GF (grunt blue stripped fin), FHM (fat head minnow skin), RTG-2 (rainbow trout gonads) and RTH-149 (rainbow trout hepatoma) are available commercially. But, for any genuine virus isolation, fish cell lines developed from the fish species concerned or related fish species are always advisable (Singh et al., 1995). In that case, the researchers in the tropics are deprived of cell lines of their choice, and that may be the reason for the limited number of reports on viruses from warm water fishes compared with those from cold water ones (Chen et al., 1983). Concomitant with the high culture density and spread of aquaculture, fish diseases especially with viral aetiology have become important, demanding effort in developing cell lines from the tropical cultivable fish species. In India, a cell line from caudal fin of rohu, *Labeo rohita* (Lakra and Bhonde, 1996), heart tissue of major carp (Rao et al., 1997), gill of mrigal *Cirrhinus mrigala* (Sathe et al., 1995), primary cell culture from kidney of *Heteropneustus fossilis* (Singh et al., 1995), and the primary cell culture system from the larvae of *Poecilia reticulata* (Kumar et al., 1998) are the only reports available.

Clarias gariepinus, known as African catfish, is the recent addition to aquaculture in India. The omnivorous fish (Pillai, 1995) breeds in captivity and due to the same reason it becomes a good donor fish for various organs and tissues for cell line development. For any such work, availability of an appropriate medium and subculturing techniques are the limiting factors as these are specific to the type of tissue used and the type of cells developed irrespective of the fish species. The present work was aimed at developing a cell culture system from the ovarian tissue of *C. gariepinus* using a modified medium and a subculturing technique.

2. Materials and methods

2.1. Preparation of media

Minimum essential medium (MEM) with Earle's salts and without L-glutamine, sodium bicarbonate and antibiotics, Leibovitz medium (L-15) with glutamine and without antibiotics, and the medium M199 with Hank's salt, L-glutamine and without sodium bicarbonate and antibiotics (HI-MEDIA Laboratories, Bombay) were employed as the basal media simultaneously. The media were prepared in glass double distilled water and when MEM was autoclaved at 10 lb for 10 min, the other media were filter sterilized using membrane of 0.22 μm mesh (Sartorius India, Bangalore). The media

were completed, by adding aqueous, 3.5% (w/v) sodium bicarbonate (HIMEDIA Laboratories), antibiotic mixture (0.2 ml/100 ml) containing Benzyl penicillin i.p. (1 000 000 units) and streptomycin sulphate i.p. equivalent to 1 g of streptomycin base dissolved in 5 ml each in sterile double distilled water and mixed together. The procedure described by Schmidt (1969) was followed for the preparation and reconstitution of media.

To arrive at an appropriate composition of the media for the cell culture development, the experiment was undertaken in 19 different phases as summarized in Table 1. In the first phase of the experiment, completed media such as M199, minimum essential medium (MEM) and Leibovitz-15 (L-15) supplemented with foetal bovine serum (FBS) (HI-MEDIA Laboratories) to the final concentration 10% (v/v) was used. Subsequently, during the second phase, to the above media, fish muscle extract (FME) was added to a final concentration of 10% (v/v). To prepare FME, 10 g muscle tissue of *C. gariepinus* was macerated in 100-ml phosphate buffered saline (PBS) (1 ×) and centrifuged at 6000 rpm in order to remove the debris. The supernatant was inactivated in a water bath at 56°C for half an hour. The fluid was again centrifuged to remove the coagulated proteins, filtered through a Seitz filter of 0.45 μm mesh and then filter sterilized by passing it through a membrane of 0.22 μm mesh after which it was stored at 4°C (Singh et al., 1995). Meanwhile, in the third phase, to the completed media with FBS, prawn muscle extract (PME) derived from *Penaeus monodon* was incorporated to a final concentration of 10% (v/v). The procedure adapted for preparing PME was analogous to the one used for fish muscle extract, but without the step involved for inactivation. Following this, as the fourth phase, to the media completed with FBS both FME and PME were added to a final concentration of 10% (v/v) each. During the fifth phase, in addition to FBS, FME and PME, lectin (L 9132, from *Phaseolus vulgaris*, red kidney bean, Sigma), represented as lectin-1 was supplemented to a final concentration of 0.02 μg ml^{-1} dissolved in PBS. Subsequently lectin (L 5275 concanavalin A, from *Canavalia ensiformia* (Jack bean Type IV S, Sigma) represented as lectin-2 was incorporated to a final concentration of 0.02 μg ml^{-1} which formed the sixth phase of the experiment. Later, lectin-1 and lectin-2 were replaced (seventh phase) with lipopolysaccharides, (L 2654, Sigma) to a final concentration of 0.02 μg ml^{-1}. During the eighth phase, the above growth factors were replaced by glucose-D, in the ninth phase by sucrose, and in the tenth phase by trehalose to a final concentration of 0.2 mg ml^{-1} and in the eleventh phase by insulin to a final concentration 0.02 μg ml^{-1}. In the twelfth phase, the above-mentioned basic media with FBS, FME and PME were supplemented with ovary extract (OE) to a final concentration of 10% (v/v). For preparing ovary extract (OE) 10 g ovary collected aseptically from *C. gariepinus* was macerated in 100 ml PBS (1 ×) and centrifuged at 6000 rpm for 15 min passed through a seitz filter of 0.45 μm mesh, again filtered through a membrane of 0.22 μm mesh and stored at 4°C. In the next phase (thirteenth), the media with FBS, PME and FME were supplemented with 0.5% (v/v) prawn shell extract (PSE). For preparing PSE 10 g fresh shells from *P. monodon* were repeatedly washed with PBS, macerated with the help of glass wool in 100 ml PBS, centrifuged at 6000 rpm for 20 min at 4°C and passed through a Seitz filter of 0.45 μm mesh and finally passed through a membrane of 0.22 μm mesh and kept at 4°C. Subsequently, in the fourteenth phase, the prawn shell extract was substituted with

chitosan (chit) prepared according to Madhavan and Ramachandran Nair (1974). In the fifteenth phase, the chitosan was substituted with 0.5% (v/v) clam haemolymph (CHL). To collect haemolymph, the cell surface of (*Villoritta villoritta*) was washed thoroughly with autoclaved seawater, and shells were opened by inserting a scalpel blade, and the haemolymph collected from adductor muscle using tuberculin syringe aseptically. An equal quantity of PBS was added to the haemolymph, centrifuged thrice at 10 000 rpm for 10 min and passed through a membrane filter of 0.22 μm mesh and stored at 4°C. In the sixteenth phase, CHL 0.5% (v/v) was replaced with fish skin extract (FSE) prepared from *C. gariepinus* following the procedures used for preparing FME. In the seventeenth phase, the FSE was replaced with 50% diluted prawn haemolymph (PHL) to a final concentration of 0.5% (v/v). Haemolymph was collected aseptically from juvenile *Penaeus indicus* using capillary tube from a sinus situated beneath the rostral spine. To prevent clotting, 0.015% (w/v) aqueous L (−) cystine (Loba Chemicals, Bombay) was used for rinsing capillary and eppendorf tubes. An equal quantity of PBS was added to the haemolymph and centrifuged at 10 000 rpm at 4°C for 10 min and passed through a membrane filter of 0.22 μm mesh and stored at 4°C. In the seventeenth phase, in addition to FBS, FME and PME a cocktail of growth factors was added, containing (OE) to an end concentration of 0.5% (v/v) glucose D (0.2 mg ml^{-1}), LPS (0.02 μg ml^{-1}), and lectin-2 (0.02 μg ml^{-1}). In the last phase (nineteenth), in addition to the above additives, (eighteenth phase) PHL, to a final concentration of 0.5% (v/v), was also added. Osmolarity of this preparation was determined using FISKE 110 osmometer (USA).

2.2. Fish and tissue explants

Female *C. gariepinus* weighing 20–30 g each were obtained from M/s Rosen Fisheries, Trichur, Kerala and starved in an aquarium for 3 days with frequent water exchange to facilitate the emptying of the fish intestine. The fishes were sacrificed by plunging them in crushed ice for 10–15 min. Their surface was disinfected by immersing them in sodium hypochlorite diluted in distilled water to a final concentration of 600 ± 25 ppm chlorine for 15 min. The dead fishes were washed 5 times with autoclaved tap water at room temperature (28 ± 0.5°C), immersed in 70% (v/v) ethanol for 10 min, washed again with sterile distilled water, and swabbed with 70% ethanol (v/v). The ventral side of the body was cut open aseptically, the ovarian tissue was removed and placed in cold phosphate buffered saline (PBS) containing 0.2% glucose (w/v) and 0.2 ml of the earlier described antibiotic mixture per 100 ml, after which it was washed three times with the same fluid. The tissue was minced into 20 pieces of 1 mm^3 in growth medium using a surgical scalpel blade placed on a rubber cork in laminar flow cabinet. Before seeding, the tissue culture flasks (25 mm^2, Borosil India, Bombay), the growth surface was conditioned with 0.5 ml foetal bovine serum (FBS) for 24 h at room temperature (28 ± 2°C). The tissue pieces were then spread over the growth surface uniformly using a wide mouthed Pasteur pipette and allowed to stick onto the surface for 8–10 h without adding the growth medium. Subsequently, the growth media were gently added through the side without dislodging the explant, the flasks were stoppered with rubber corks, and incubated at 26 ± 2°C. Daily observations

were made for the percentage of attachment, proliferation of cells and monolayer formation under an inverted phase contrast microscope (Zeiss Axiovert 25 CFL, Germany). The incubation continued until sufficient cells were present for subculturing.

2.3. Subculturing

To develop an appropriate cell dislodgement solution and a protocol for its effective application, the following series of experiments were conducted in 12 phases. First, a trypsin phosphate versene glucose (TPVG) solution, containing (in g l^{-1}) NaCl, 8; KCl, 0.2; Na_2HPO_4, 1.15; KH_2PO_4, 0.2 (SLR Bombay); disodium versenate (EDTA) (Difco), 0.2; glucose, 0.5 (HI-MEDIA Laboratories); trypsin (Sigma) 1.0 and phenol red 0.01, was made. In the subsequent three phases, the above TPVG solution was diluted with phosphate buffered saline (PBS) to obtain final concentrations of 0.1, 0.05 and 0.025 mg l^{-1} trypsin. In the fifth and sixth phases of the experiment, non-enzymatic cell dissociation solution-1 (NES 1) (C.5789, Lot 12540258, Sigma) prepared in Hank's balanced salts solution (HBSS) without calcium and magnesium, Sigma) and non-enzymatic cell dissociation solution-2 (NES 2) (C.5914 Lot 125H 0010, Sigma) prepared in HBSS without calcium and magnesium were employed. In the subsequent two phases, the NESs were diluted to obtain 50% strength using PBS as described before. As the ninth and tenth phases, the above enzymatic and non-enzymatic solutions, such as diluted TPVG solution containing 0.025% trypsin and NES 1 (50%) as one lot and TPVG containing 0.025% trypsin and NES 2 (50%) as the other were mixed at 1:1 ratio and used. In all subculturing ways, complete medium was decanted off, the cells were washed gently thrice with PBS without calcium and magnesium, and rinsed twice with the cell extraction solution, after which the supernatant was decanted every time. The bottles were observed for rounding of cells, and once rounding was observed, the reaction was stopped by adding 0.5-ml foetal bovine serum (FBS). Subsequently, the growth medium was added and the flask was swung around gently to dislocate/extract the cells. The cell suspensions were transferred to fresh sterile tissue culture 25 mm^2 flasks. In all instances, the percentage of extracted cells and the subsequent level of attachment of cells in new flasks were estimated by microscopic observations. In addition to this, cells were also extracted with Pasteur pipettes and also by scraping using cell scrapers with fresh growth medium at 26 ± 0.5°C.

3. Results

The response of the ovarian tissue of African catfish (*C. gariepinus*) to the different media is summarized in Table 1. Among the three basic media tested Leibovitz-15 (L-15), when supplemented with foetal bovine serum (FBS) and fish muscle extract (FME) supported higher percentage attachment of explants leading to cell proliferation than minimum essential medium (MEM) and M199 with the same supplements. When this medium was further enriched with prawn muscle extract (PME), lectin-2 or lipopolysaccharides (LPS), the tissue attachment and percentage confluency increased. Final addition of lectin-1 did not make considerable changes. If in place of the above

Table 1
Attachment of explants and subsequent growth after passaging ovarian tissue of *C. gariepinus* in relation to varying combinations of media, growth factors and mitogens

Phase number	Medium	Supplements	Percent attachment of the explants	Time taken for attachment and beginning of cell proliferation in hours	Growth and monolayer formation @
1	M199	10% FBS	30	72	+
	MEM	10% FBS	30	72	+
	L-15	10% FBS	35	72	+
2	M199	10% FBS + 10% FME	30	72	+
	MEM	10% FBS + 10% FME	30	72	+
	L-15	10% FBS + 10% FME	40	72	+ +
3	M199	10% FBS + 10% PME	30	72	+
	MEM	10% FBS + 10% PME	35	72	+
	L-15	10% FBS + 10% PME	40	48	+ +
4	M199	10% FBS + 10% FME + 10% PME	40	72	+
	MEM	10% FBS + 10% FME + 10% PME	44	72	+
	L-15	10% FBS + 10% FME + 10% PME	65	48	+ + +
5	M199	10% FBS + 10% FME + 10% PME + Lec1*	40	72	+
	MEM	10% FBS + 10% FME + 10% PME + Lec1*	45	72	+
	L-15	10% FBS + 10% FME + 10% PME + Lec1*	65	48	+ + +
6	M199	10% FBS + 10% FME + 10% PME + Lec2*	40	72	+
	MEM	10% FBS + 10% FME + 10% PME + Lec2*	45	72	+
	L-15	10% FBS + 10% FME + 10% PME + Lec2*	75	48	+ + + +
7	M199	10% FBS + 10% FME + 10% PME + LPS*	43	72	+
	MEM	10% FBS + 10% FME + 10% PME + LPS*	45	72	+ +
	L-15	10% FBS + 10% FME + 10% PME + LPS*	70	48	+ + + +
8	M199	10% FBS + 10% FME + 10% PME + Glu•	45	72	+ +
	MEM	10% FBS + 10% FME + 10% PME + Glu•	48	72	+ +
	L-15	10% FBS + 10% FME + 10% PME + Glu•	80	36	+ + + +
9	M199	10% FBS + 10% FME + 10% PME + Suc•	48	72	+ +
	MEM	10% FBS + 10% FME + 10% PME + Suc•	45	72	+ +
	L-15	10% FBS + 10% FME + 10% PME + Suc•	70	48	+ + +
10	M199	10% FBS + 10% FME + 10% PME + Tre•	35	72	+ +
	MEM	10% FBS + 10% FME + 10% PME + Tre•	40	72	+ +
	L-15	10% FBS + 10% FME + 10% PME + Tre•	65	48	+ +
11	M199	10% FBS + 10% FME + 10% PME + insulin*	30	72	+
	MEM	10% FBS + 10% FME + 10% PME + insulin*	30	72	+
	L-15	10% FBS + 10% FME + 10% PME + insulin*	20	72	+
12	M199	10% FBS + 10% FME + 10% PME + 0.5% OE	70	72	+ + +
	MEM	10% FBS + 10% FME + 10% PME + 0.5% OE	65	72	+ + +
	L-15	10% FBS + 10% FME + 10% PME + 0.5% OE	90	24	+ + + + +
13	M199	10% FBS + 10% FME + 10% PME + 0.5% PSE	10	72	+
	MEM	10% FBS + 10% FME + 10% PME + 0.5% PSE	10	72	+
	L-15	10% FBS + 10% FME + 10% PME + 0.5% PSE	30	48	+ +
14	M199	10% FBS + 10% FME + 10% PME + 0.5% Chit	10	72	+
	MEM	10% FBS + 10% FME + 10% PME + 0.5% Chit	12	72	+
	L-15	10% FBS + 10% FME + 10% PME + 0.5% Chit	30	48	+ +

Table 1 (*continued*)

Phase number	Medium	Supplements	Percent attachment of the explants	Time taken for attachment and beginning of cell proliferation in hours	Growth and monolayer formation @
15	M199	10% FBS + 10% FME + 10% PME + 0.5% CHL	25	72	+
	MEM	10% FBS + 10% FME + 10% PME + 0.5% CHL	30	72	+
	L-15	10% FBS + 10% FME + 10% PME + 0.5% CHL	25	72	+
16	M199	10% FBS + 10% FME + 10% PME + 0.5% FSE	60	48	+
	MEM	10% FBS + 10% FME + 10% PME + 0.5% FSE	45	72	+
	L-15	10% FBS + 10% FME + 10% PME + 0.5% FSE	70	48	+
17	M199	10% FBS + 10% FME + 10% PME + 0.5% PHL	60	48	+ +
	MEM	10% FBS + 10% FME + 10% PME + 0.5% PHL	65	48	+ +
	L-15	10% FBS + 10% FME + 10% PME + 0.5% PHL	70	48	+ + +
18	M199	10% FBS + 10% FME + 10% PME + 0.5% OE + Lec2 * + LPS * + Glu•	45	72	+ + +
	MEM	10% FBS + 10% FME + 10% PME + 0.5% OE + Lec2 * + LPS * + Glu•	40	72	+ + +
	L-15	10% FBS + 10% FME + 10% PME + 0.5% OE + Lec2 * + LPS * + Glu•	95	24	+ + + + + +
19	M199	10% FBS + 10% FME + 10% PME + 0.5% OE + Lec2 * + LPS * + Glu• + 0.5% PHL	60	72	+ + +
	MEM	10% FBS + 10% FME + 10% PME + 0.5% OE + Lec2 * + LPS * + Glu• + 0.5% PHL	50	72	+ + +
	L-15	10% FBS + 10% FME + 10% PME + 0.5% OE + Lec2 * + LPS * + Glu• + 0.5% PHL	96	24	+ + + + + + +

FBS: foetal bovine serum; FME: fish muscle extract; PME: prawn muscle extract; Lec1: lectin1; Lec2: lectin2; LPS: lipopolysaccharides; Glu: glucose D; Suc: sucrose; Tre: trehalose; OE: ovary extract; PSE: prawn shell extract; Chit: chitosan; CHL: clam haemolymph; PHL: prawn haemolymph; FSE: fish skin extract; +: 10% confluency; + +: 20% confluency; + + +: 30% confluency; + + + +: 40% confluency; + + + + +: 50% confluency; + + + + + +: 60% confluency; + + + + + + + +: 80% confluency; *(0.02 μg ml^{-1}); •(0.2 mg ml^{-1}).

mitogens, glucose D or sucrose were added, an increased explants attachment and proliferation were observed. However, addition of trehalose, insulin, and prawn shell extract (PSE), chitosan (Chit), clam haemolymph (CHL) or fish skin extract (FSE), in its place decreased the attachment of explants and cell proliferation. The additions of ovary

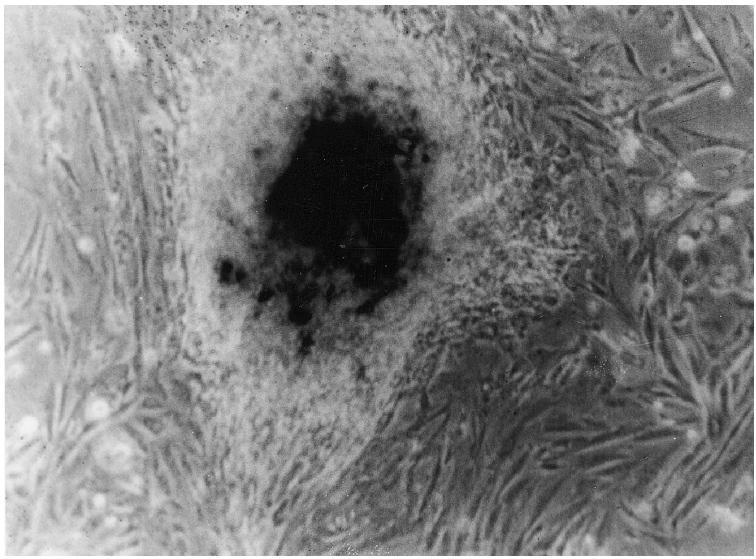

Fig. 1. Attachment of an explant of ovarian tissue from *C. gariepinus* with the formation of monolayer (220×).

extract (OE) in place of other growth factors resulted in strong attachment of the explants and relative good growth of cells. Addition of prawn haemolymph (PHL) in the place of above growth factors yielded equally good explants attachment and cell

Table 2
Application of different cell extraction solutions and techniques in subculturing the primary cell culture systems developed from the ovarian tissue of *C. gariepinus*

Solution number	Dissociation solution	Extracted cells in percent	Attached cells in percent in new bottles
1	TPVG (0.2% trypsin (w/v))	100	0
2	TPVG (0.1% trypsin (w/v))	100	5
3	TPVG (0.05% trypsin (w/v))	70	10
4	TPVG (0.025% trypsin (w/v))	65	45
5	NES 1	50	50
6	NES 2	60	45
7	NES 1 (50%)	55	65
8	NES 2 (50%)	60	65
9	Swinging flask and suck off cells	30	10
10	With cell scraper	70	20
11	TPVG (0.025% trypsin(w/v)) + NES 1 50% + NES 2 50%	80	85
12	TPVG (0.0125% trypsin(w/v)) + NES 1 25% + NES 2 25%	95	90

NES 1: non-enzymatic solution 1; NES 2: non-enzymatic solution 2; TPVG: trypsin phosphate versene glucose.

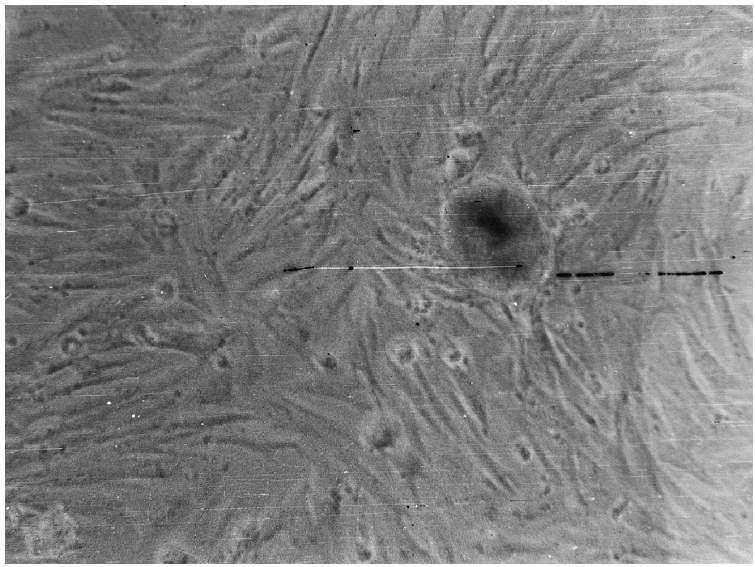

Fig. 2. Monolayer formation of the cell culture system developed from ovarian tissue of *C. gariepinus* in the second passage (225×).

proliferation. Subsequently, when FBS, FME, PME, OE, lectin-2, LPS and glucose D were added in the form of a cocktail to L-15, better results as rapid attachment and formation of a monolayer with 60% confluence (Fig. 1) could be obtained. Later, the

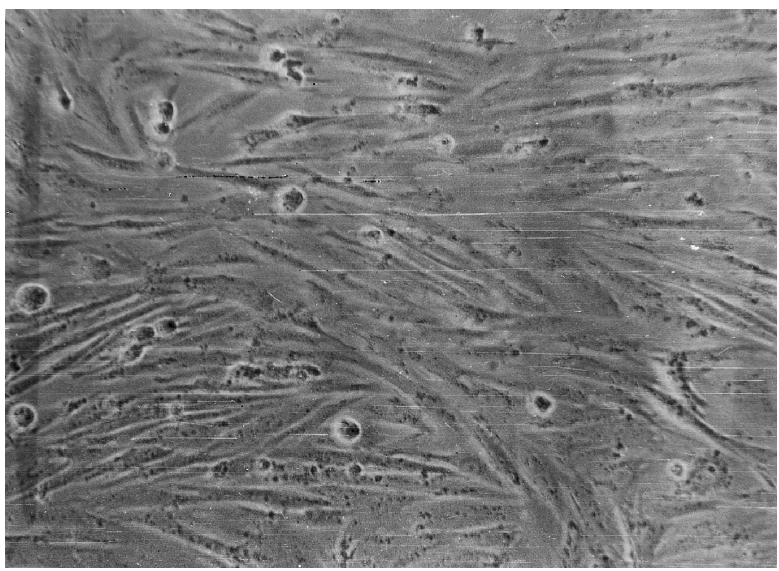

Fig. 3. Monolayer formation of the cell culture system developed from ovarian tissue of *C. gariepinus* in the fourth passage (225×).

above results could further be enhanced by incorporating PHL also to above cocktail by which 80% confluence in the monolayer formation could be achieved. Its osmolarity was found to be 354 ± 10 mOsm. On the basis of this L-15 of phase 19 was used for all further experiments undertaken to develop an appropriate subculturing technique.

The results on the subculturing techniques are presented in Table 2 . Among all the types and combinations of cell extraction solution used, the one which was prepared with equal quantities of trypsin phosphate versene glucose (TPVG containing 0.0125% trypsin), non-enzymatic solution 1 (NES 1) (25% v/v) and non-enzymatic solution 2 (NES 2) 25% (v/v) was found to be best as it resulted in 95% extraction of the cells and 90% subsequent attachment in the new flasks. By using this solution, the cell culture could be passaged 15 times (Figs. 2 and 3) after which they stopped dividing, got loosened and subsequently died off. Between every passage the cell cultures could be maintained for 12–15 days without fluid change. The fingerlings of *C. gariepinus* grow sufficiently large with in 3 months, enough for the removal and utilization of ovary for the cell culture development. From one such fish, more than 50 bottles of primary cell cultures and double the number of diploid cell cultures can be developed for biomedical applications.

4. Discussion

Leibovitz-15 (L-15) was found to be the most suitable medium of the three basic one minimum essential medium (MEM), M199 and L-15, each supplemented with foetal bovine serum (FBS) and fish muscle extract (FME) in terms of its efficacy to support rapid explant attachment and cell proliferation. Suitability of L-15 in supporting fish cell lines compared to that of other media has been documented by Fernandez et al. (1993) when they compared the growth responses of 28 fish cell lines in different media at various temperatures and sodium chloride concentrations. The ovarian tissue of *C. gariepinus* in the present study was found to require tissue derived growth factors present in fish muscle extract (FME), ovary extract (OE) and prawn haemolymph (PHL) for attachment and proliferation. Similar observations were made by Kumar et al. (1998) when they studied the development of a primary cell culture system from the embryonic tissue of *P. reticulata*. Glucose and sucrose were found to be growth enhancers, comparable to lipopolysacharides (LPS) and lectin. However, lectins (concanavalin A) and LPS as mitogens contribute more during subsequent passages of cells to a rapid multiplication, whereby they increase the possibilities of in vitro transformation and establishment. They, therefore, cannot be compared to glucose and sucrose. Yet as energy suppliers, glucose and sucrose have their own importance in the growth medium used. According to Alava and Pascual (1987), glucose, sucrose and trehalose can be used as the energy sources in cell culture systems. By studying in vitro subculture systems from lymphoid organ of *P. monodon*, Hsu et al. (1995) observed that by supplementing the growth medium with glucose, 80% of the cells attached themselves and with trehalose and sucrose an attachment of 50% was seen. In contrast to this, primary cultures of epithelial cells from rainbow trout gills were not found to respond to any of the growth factors employed (Part et al., 1993). This precisely indicates that there

is no fixed rule as far as growth factors are concerned and the required growth factors have to be investigated for every fish species studied, and even for every organ used for the cell culture development. Accordingly, L-15 supplemented with FBS (10% v/v), FME (10% v/v), prawn muscle extract (PME) (10% v/v), OE (0.5% v/v), PHL (0.5%), lectin-2 (concanavalin A) (0.02 $\mu g\ ml^{-1}$), LPS (0.02 $\mu g\ ml^{-1}$), and glucose D (0.2 $mg\ ml^{-1}$) is recommended as the growth medium for the initiation and maintenance of a primary cell culture system from the ovarian tissue of *C. gariepinus*. After the fifteenth passage, though they stop multiplication and subsequently perish, there is every possibility for in vitro transformation by artificial means.

Trypsin (0.2% w/v) present in trypsin phosphate versene glucose (TPVG) was found to be toxic to the primary cell culture system developed. By diluting TPVG to a final concentration of 0.025% trypsin, the survival and attachment potential of extracted cells could be enhanced. Almost the same performance could be observed, when non-enzymatic solution (NES) 1 and 2 were used at 50% v/v dilution. However, the best results were obtained with a cocktail made of equal proportions of TPVG (containing 0.0125% trypsin (w/v)), 25% v/v, NES 1 and 2 were employed. The addition of 0.5 ml foetal bovine serum (FBS) to the cell culture after treating with the cell extracting solution improved the viability of cells. As a matter of fact, the cocktail has to be prepared fresh just before use by mixing the three solutions and should not be maintained for prolonged use.

In summary, in this study, a growth medium for the development and maintenance of a cell culture system from the ovarian tissue of *C. gariepinus* has been developed. Moreover, for its subculturing, a cell extraction solution has been developed with which it could be passaged 15 times, after which it ceased multiplication and perished. Between the passages, the cell cultures could be maintained for 12–15 days without fluid change. More work has to be carried out on its in vitro transformation and establishment. This is the first report of a cell culture system from the ovarian tissue of *C. gariepinus*. By using this technique, it should now be possible to develop primary and diploid cell cultures, because the fish can be bred in captivity and fingerlings raised under laboratory conditions.

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