aJava

Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Animal and Veterinary Advances 9 (5): 281-291, 2014
ISSN 1683-9919 / DOI: 10.3923/ajava.2014.281.291

Comparative Study on Enzymatic and Explant Method in Establishing Primary Culture from Different Cultivable Cells of Indian Major Carp, *Cirrhinus mrigala*

[1]P.K. Nanda, [2]P. Swain, [2]S.K. Nayak, [2]T. Behera and [3]K. Dhama

[1]Indian Veterinary Research Institute, Eastern Regional Station, Belgachia, Kolkata, 700 037, India
[2]Fish Health Management Division, Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, 751002, India
[3]Division of Pathology, Indian Veterinary Research Institute, Izatnagar, 243 122, Bareilly, Uttar Pradesh, 243122, India

Corresponding Author: P. Swain, Fish Health Management Division, Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar 751002, India

ABSTRACT

Enzymatic method using trypsin to establish primary culture from various cells from heart, liver, kidney, brain, testis, ovary, fin and embryo of Indian Major Carp, *Cirrhinus mrigala* was investigated and compared with that of explant culture method. The dissociation time to obtain cells from individual tissues and the viability of cells for primary culture varied at 0.125 and 0.25% trypsin concentration. Single cell culture of trypsinised cells from testis, ovary and embryo showed good attachment irrespective of trypsin concentration. Cells from liver and heart were found to be sensitive to trypsin only at higher concentration (0.25%); whereas, no attachment/or proliferation was observed from cells of kidney, brain and fin due to sensitivity to trypsin at both the concentrations. In contrast to this method to obtain primary culture, explant culture from most of the tissues showed better attachment, resulting in subsequent growth and proliferation of cells forming monolayer. Overall, the explant culture of most of the tissues of *C. mrigala* was found to be suitable and survived more passages as compared to single cell culture obtained through trypsinisation. For obtaining primary culture from single cells, further investigations are desired to identify tissue specific enzymes, standardization of dose, duration and temperature of enzymatic treatment.

Key words: *Cirrhinus mrigala*, explant culture, Indian major carp, primary culture, trypsin

INTRODUCTION

Over the years and with the advancements in technology, fish cell culture is continuing to expand rapidly with the use of micro carriers and polymeric scaffolds (Nicholson, 1980; Nanda *et al*., 2014). However, like human and animal sciences, short-term primary cultures from different tissues and organs of fish are also necessary because of their applications in many fields of biological research (Hightower and Renfro, 1988; Babich *et al*., 1993; Villena, 2003; Chen *et al*., 2004; Ye *et al*., 2006). To obtain primary cell culture, two basic methods (tissue dissociation and explant) are routinely employed and still in vogue (Bols and Lee 1991). Out of them, tissue disaggregation or dissociation using proteolytic enzymes is one of the common methods to obtain single cell suspensions during culture and/or subsequent sub-culture practices

(Ma and Collodi, 1999; Ganassin *et al.,* 2000; Ossum *et al.,* 2004). Although, many proteolytic enzymes are commercially available, enzymatic disaggregation using trypsin is the most widely used technique in cell culture, as it dissociates adhesive cells from the tissues, substratum etc. and also allows rapid passaging for large scale cell expansion (Cruz *et al.*, 1997; Mitalipova *et al.*, 2005). However, many literatures indicate that trypsinization can affect and change various properties of the cells including modification of adhesive properties; dysregulation of the cell functions; loss of cellular activity; alteration of cell membrane permeability and disruption of the cellular membranes increasing cell deformities etc. (Smets *et al.*, 1979; Pleskach *et al.*, 1993; Collett *et al.*, 2007; Yanase *et al.*, 2007; Huang *et al.*, 2010). Exposure of cells to high concentration of trypsin during culture, or over trypsinization is a common cause of subculture problem as trypsin can damage the cell surface by digesting exposed cell surface proteins (Glade *et al.*, 1996), damage cell membranes and cause lysis (Melican *et al.*, 2005; Sutradhar *et al.*, 2010).

Although, reports are available on the establishment of primary and explant cell culture of fish by many researchers (Sathe *et al.*, 1995; Rao *et al.*, 1997; Joseph *et al.*, 1998; Lakra *et al.*, 2005; Rathore *et al.*, 2007), till date no information is available on time dependent quantitative effect of trypsinization in literature. Further, no systematic attempt in previous studies has been made to discuss thoroughly the possible trypsin sensitivity of different cultivable cells of fish, which is very essential for usual cell culture of any species, since trypsin sensitivity of tissues from different animal species including fish varies (Pumper *et al.*, 1971; Mastan *et al.*, 2007). Looking into the fact that use of trypsin could have a remarkably adverse effect on cell culture practices; this prompted us to investigate the effect of trypsin on attachment, growth, proliferation and survival of different cells from various tissues of Indian Major Carp, mrigal, *Cirrhinus mrigala* and compare their growth characteristics with that of explant culture method.

MATERIALS AND METHODS

Fish: Indian Major Carp, mrigal (*C. mrigala*) juveniles (40-50 g) and matured fish (800-1000 g) were brought from nearby farms and maintained in wet laboratory of Fish Health Management Division of Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, India. Fish were acclimatized for 15 days before start of the experiment.

Media and additives: Dulbecco's Modified Eagle's Medium (DMEM), Glutamine (0.3% w/v), non-essential amino acids solution (100×) and 1% antibiotic-antimycotic solution (Sigma, USA) were used throughout the study for culture of different cells.

Serum: Based on our previous work and success in using goat serum in establishing cell culture (Nanda *et al.*, 2009), it was also used in all the cell culture experiments in this study. In brief, blood was aseptically collected from jugular vein of goat (*Capra indica*) and allowed to clot at room temperature. After which, serum was collected by centrifuging at 1500×rpm for 10 min at 4°C. The supernatant obtained was inactivated at 56°C for 30 min and pre-filtered through 0.45 μm filter (Millipore, India) followed by with 0.2 μm filter. The serum, thus obtained, was stored at -20°C until further use.

Collection/isolation of tissues: Different tissues like brain, liver, kidney, heart, fin, testis and ovary were collected from *C. mrigala* by sacrificing the fish following standard ethical protocols of CIFA. Briefly, Fish (juveniles and matured) were first killed by an overdose of the anaesthetic

MS-222 (tricaine methane sulphonate) (Sigma, USA) and then the entire body was swabbed with 70% alcohol. Processing of fish was done aseptically one at a time using sterile instruments under laminar air flow. Testis and ovary were only collected from matured fish whereas fin, liver, kidney, heart and brain were excised in succession from juveniles and immersed immediately in DMEM. Similarly, embryos were collected 2 h after fertilization of eggs at 32 cell stage and washed for several times in medium before processing for cell culture.

Prior to cultivation, the tissues were minced using surgical scalpel blades/a pair of scissors to a size of 1-2 mm^3 and repeatedly washed with DMEM containing 1% antibiotics to remove excess blood cells and cellular debris.

Experimental design: Two sets of experiment were conducted. In one set, sensitivity of different tissues of mrigal was investigated using trypsin at different concentrations (0.125 and 0.25%) in primary culture. Upon reaching confluency (80-90%), subsequent subculture was done using trypsin-versene (0.125%) solution as cell dissociating agent as well. The other set of experiment (explants culture method) was conducted without involving trypsin either during primary cell culture or subsequent subcultures. The latter was carried out using non-enzymatic cell dissociating agent.

Cell culture with trypsin: Enzymatic disaggregation of tissues using trypsin to obtain primary culture was done following the method described by Wolf and Quimby (1976a). Briefly, minced tissues [of equal weight (0.5 g)], were taken in separate erlenmeyer flasks containing 0.125 and 0.25% of trypsin (Source: Porcine pancreas, 1000-1500 BAEE units mg^{-1}, Sigma, USA) in phosphate buffered saline (PBS, pH 7.2). Trypsinisation was done up to 30 min and within that period, the effect of different concentration of trypsin (0.125 and 0.25%) with regard to time on dissociation of cells (complete/partial) of each tissue was recorded. After trypsinisation, goat serum @ 5% (v/v) was then added to stop the trypsin activity. The solution containing dissociated cells and tissue debris were filtered through sterile gauze and then centrifuged at 1500×rpm for 10 min. The supernatant was discarded and the pellet obtained was suspended @1.5×10^5 cells mL^{-1} in 25 cm^2 tissue culture flasks (Nunc, Denmark) containing 5 mL DMEM supplemented with glutamine (0.3% w/v) and non-essential amino acids solution (100×). To this, 10% goat serum and 1% antibiotic-antimycotic solution (Sigma, USA) per mL of medium were also added. The flasks were then incubated at 27±1°C in CO_2 incubator with 5% CO_2 tension. After 1st day, the flasks were observed for attachment of cells and the un-attached cells were removed by discarding the media and adding fresh media to the cultured flasks till confluency (80-90%) attained.

The viability of the each trypsinised cells were done by trypan blue exclusion method. To 20 µL of cell suspension, an equal volume of trypan blue (0.4%) was added. The trypan blue-cell suspension was mixed thoroughly and allowed to stand for 5-15 min before counting the cells in haemocytometer. The percentage (%) of viable number of cells was determined by dividing the viable cells (unstained) with total cells and then multiplying by a factor of 100.

Sub-culture of cells: Subculture of the cells was carried following the procedure described by Wolf and Quimby (1976b). After attaining confluency (80-90%), the cells obtained from different tissues were treated with 1 mL of trypsin-versene (0.125%) solution for up to 60 sec until the cells began to round up and lifting of the cell monolayer occurred. The treated cells were harvested and

the percentage viability of cells was calculated. A seeding density of 1.5×10^5 live cells^{-1} mL of medium, as determined by a haemocytometer, was maintained for each subculture (passage) of cells from different tissues following the same procedure, till their growth was poor.

Cell culture without trypsin

Primary cell culture following explant method: In order to compare the effect of trypsin, primary cell culture by using explants under semi-dried conditions was done as per our earlier study (Nanda *et al*., 2009). Briefly, 0.5 mL of goat serum was pipetted out and spread uniformly in 25 cm^2 tissue culture flasks and kept at room temperature for overnight. Approximately, 30 No. of tissue explants (1-2 mm^3 size) were distributed uniformly in the culture flasks and kept in semi drying condition for 3 h at room temperature. After incubation, the flasks were fed with 5 mL of DMEM along with supplements and antibiotic-antimycotic solution as mentioned in previous method.

Sub-culture of cells: Subculture of the cells derived from explants was carried out using non-enzymatic tissue dissociation solution (Sigma, USA). The non-enzymatic tissue dissociation solution was first treated with the monolayers (80-90%) of cells for 60 sec. After which, washing and harvesting of cells were done in a similar manner like trypsin treated subculture.

Parameters studied: During both primary and sub-culture of both trypsinised and un-trypsinised cells, different parameters like attachment of cells to substratum, proliferation, growth and time taken to attain the confluency (formation of monolayer) by cells were observed routinely under phase contrast microscope. The survivability and number of passages for individual cells of different mrigal tissues in trypsin treated and untreated groups were recorded. The morphological growth characteristics of cells were studied by removing the medium followed by ethanol fixation and stained with Giemsa and observed under phase-contrast microscope (Nikon, Japan) (Freshney, 2005). After air drying the flasks, photographs were taken to know the morphological characteristic of the cells obtained from different tissues of *C. mrigala*.

RESULTS

Dissociation time and attachment of trypsin treated cells: Table 1 shows the dissociation time, condition of tissue, attachment efficiency of trypsinised cells from tissues of *C. mrigala*. The dissociation of cells from different tissues of *C. mrigala* was found to vary with regard to time and concentration. Tissues like liver and kidney were found to be dissociated completely within 20 and 21 min when treated with 0.125% trypsin, but the time reduced to 16 and 18 min when treated with 0.25% trypsin, respectively. Similarly, embryos were found to dissociate completely within 20 and 16 min with 0.125 and 0.25% trypsin, respectively. On the other hand, heart, ovary and fin tissues dissociated partly after 30 min with 0.25% trypsin treatment. On contrary, within the same exposure time, tissues of brain and testis partly dissociated with 0.125% trypsin but dissociated completely at 0.25% concentration.

Trypsinisation of cells at different concentration also affected the attachment efficiency of cells to the culture flasks during primary culture. Although cells derived from testis, ovary and embryo exhibited good attachment irrespective of trypsin concentration, cells from heart and liver failed to

Table 1: Effect of trypsinisation on culture viability of tissues of *Cirrhinus mrigala*

Tissue	Trypsin concentration (%)	Dissociation time (min)	Tissue condition	Attachment of cells to culture flasks
Heart	0.125	30	Partly dissociated	Attached
	0.250	30	Partly dissociated	Poor attachment
Liver	0.125	20	Completely dissociated	Attached
	0.250	16	Completely dissociated	Poor attachment
Kidney	0.125	21	Completely dissociated	Poorly attached
	0.250	18	Completely dissociated	No attachment
Brain	0.125	30	Partly dissociated	Poor attachment
	0.250	30	Completely dissociated	No attachment
Testis	0.125	30	Partly dissociated	Good attachment
	0.250	30	Completely dissociated	Good attachment
Ovary	0.125	30	Partly dissociated	Good attachment
	0.250	30	Partly dissociated	Good attachment
Fin	0.125	30	Undissociated	No attachment
	0.250	30	Partly dissociated	No attachment
Embryo	0.125	20	Completely dissociated	Good attachment
	0.250	16	Completely dissociated	Good attachment

attach when trypsinised with 0.25% trypsin but fairly attached at lower trypsin treatment i.e., 0.125%. On the other hand, trypsinised (0.125%) cells from dissociated tissues of kidney and brain exhibited poor to no attachment at higher trypsin (0.25%) concentration. Effect of trypsin concentration was found on subsequent growth and formation of monolayer during primary culture of cells. Although cells derived from heart, liver, testis, ovary and embryo showed good growth characteristics resulting in proliferation of cells and formation of monolayer (confluency); cells from kidney, brain and fin either failed to attach or grow and proliferate, thus ruling out the possibility of subculture (Table 1).

Attachment, proliferation and growth of trypsin un-treated cells: Explant method of tissue culture showed firm attachment of most of the tissues like heart, liver, ovary, testis and embryo resulting in subsequent growth and proliferation (Table 2). Cell proliferation began within 2-6 days from tissues like liver, ovary, heart, testis and embryo while brain explants took 12 days to proliferate. Likewise, the monolayer formation took least time (6-8) days in liver while it was prolonged up to 28 days in case of brain.

Morphological growth characteristics and survivability of trypsin treated and un-treated cells: The morphological growth characteristics, proliferation, duration to attain confluency (monolayer formation) and survivability (passage) of different cells of *C. mrigala* obtained from trypsin treated and trypsin un-treated (explants) methods is shown in Table 2. Proliferating cells cultured from different tissues of mrigal exhibited different morphological growth characteristics. Heart cells of *C. mrigala* showed epithelioid (epithelial-like) growth characteristic (Fig. 1) while typical glial-like cells were observed in case of brain cell culture (Fig. 2). Proliferation of cells from other cultured tissues including liver exhibited fibroblast-like growth pattern throughout the culture period (Fig. 3). Upon sub culture, cells from most of the tissues of *C. mrigala* survived more passages in trypsin un-treated i.e., explant culture method. Liver, kidney and heart cells survived up to 10th, 9th and 7th passages, respectively by explant method as compared to 2nd

Table 2: Morphological growth characteristics and survivability (passage) of cells of *Cirrhinus mrigala* obtained from trypsin treated and trypsin un-treated (explant) methods

Tissue	Trypsin treated	Trypsin un-treated (explant) method
Heart	Epithelioid but poor growth and proliferation, monolayer 16 days, survived up to 2nd passage at 0.125% trypsin concentration	Epithelioid on sub culture, spindle shaped fibroblasts predominated proliferation from 3rd day, monolayer 10-12 days, survived up to 7th passage
Liver	Fibroblast-like but poor growth and proliferation, monolayer 10-12 days, survived up to 2nd passage at 0.125% trypsin concentration	Fibroblast-like proliferation from 2nd day, monolayer 6-8 days, survived up to 10th passage
Kidney	Poor attachment, no growth and proliferation, no subculture (passage)	Fibroblast-like proliferation from 7th day, monolayer 16-18 days, survived up to 9th passage
Brain	Poor attachment, no growth, proliferation and subsequent subculture (passage)	Glial-like cells, proliferation from 12th day, monolayer 28 days, survived up to 3rd passage
Testis	Fibroblast-like proliferation from 3rd day, monolayer 6-8 days, Survived up to 4th passage (at 0.125%) and 3rd passage at 0.25% trypsin concentration	Fibroblast-like proliferation from 3rd day, monolayer 7-8 days, survived up to 5th passage
Ovary	Fibroblast-like; proliferation 3-4 days; monolayer 8-9 days, survived up to 3rd passage at both trypsin concentration	Fibroblast-like; proliferation 3-4 days; monolayer 7-9 days, survived up to 4th passage
Fin	No attachment, subsequent proliferation and growth, No subculture (passage)	Fibroblast-like proliferation from 7th day, monolayer 20-22 days, survived up to 3rd passage
Embryo	Fibroblast-like proliferation from 3-4 days, monolayer 6-8 days, Survived up to 3rd passage at both trypsin concentration	Fibroblast-like, proliferation from 3rd day, monolayer 6-8 days, survived up to 4th passage

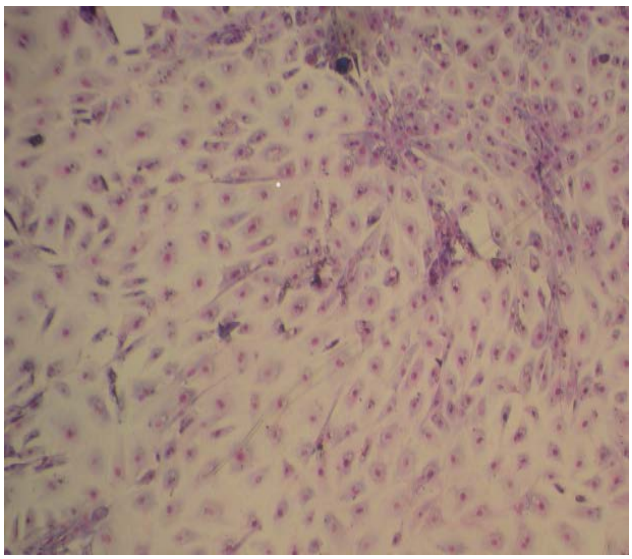

Fig. 1: Typical epithelioid (epithelial-like) growth characteristics of heart explant culture of *Cirrhinus mrigala* (Giemsa stained, ×100)

passage through trypsination. However, testis, ovary and embryo showed comparable survivability (passages) in both the methods.

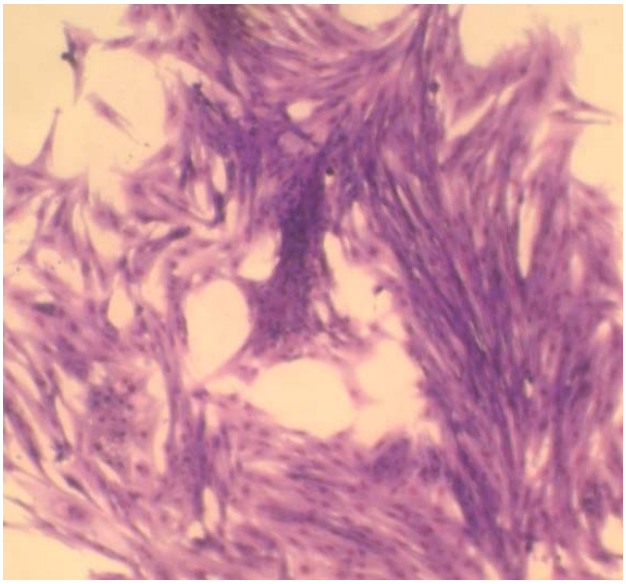

Fig. 2: Typical glial-like growth characteristics of brain explant culture of *Cirrhinus mrigala* (Giemsa stained, ×200)

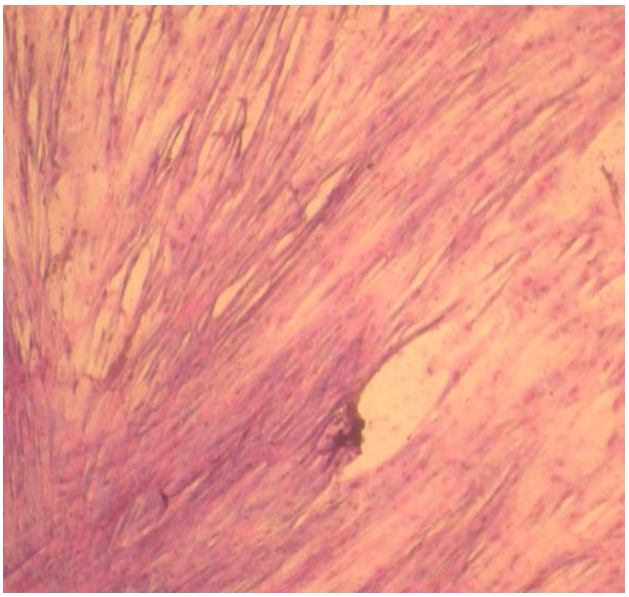

Fig. 3: Typical fibroblast-like growth characteristics of liver explant culture of *Cirrhinus mrigala* (Giemsa stained, ×100)

DISCUSSION

Cell culture involves the process by which cells derived from various types of tissues are grown under artificial *in vitro* conditions. To obtain primary culture, either of the two basic methods are employed i.e., enzymatic dissociation of tissues or explant culture method (Bols and Lee 1991). In case of enzymatic method, separation or disaggregation of cells from tissues is critical to initiate primary culture. Therefore, to separate and harvest cells for culture and subsequent subculture (passage), different types of cell dispersing agents (chemical, enzymatic) are employed. Amongst these, tissue dissociation using proteolytic enzyme such as trypsin is mostly used to obtain primary culture (Noga, 1980; Bols and Lee, 1994; Sathe *et al*., 1995; Swain *et al*., 2014), as it dissociates adhesive cells from the tissues, substratum etc. and also allows rapid passaging for large scale cell expansion (Cruz *et al*., 1997; Mitalipova *et al*., 2005). Although trypsinisation is a fast and reliable method to detach cells from either static or carrier surfaces (Cruz *et al*., 1997), several researchers have obtained mixed results with enzymatic dissociation to initiate primary cultures, as sensitivity of tissues to trypsin from different animal species including fish varies (Pumper *et al*., 1971; Mastan *et al*., 2007).

In this study, attempts were made to obtain single cell from tissues like fin, heart, liver, kidney, brain, testis, ovary and embryo of *C. mrigala* for primary culture and study various parameters like dissociation time, attachment efficiency, monolayer formation etc. using trypsin at two different concentrations (0.125 and 0.25%) and compared with explant culture method. The dissociation time among the tissues of *C. mrigala* to obtain single cell for primary culture varied greatly, when treated with 0.125 and 0.25% trypsin concentration. Tissues like liver and embryo were dissociated completely within 16 and 20 min at 0.25 and 0.125% trypsin treatment, respectively while ovary, testis and fin were found to be partially dissociated even after 30 min with 0.25% trypsin treatment. The variation in dissociation time to obtain single cell may be related to types of tissue, species, age and efficacy of enzymes to act on tissues at different concentrations as observed by Wolf and Quimby (1969).

Further, enzymatic treatment can weaken the cell membranes and decrease their ability to attach to the substrate and trypsinisation under standard conditions can be highly cytotoxic (McKeehan, 1977; Smets *et al*., 1979; Pleskach *et al*., 1993). This was observed in case of cells from tissues of brain, fin and kidney of mrigal as these cells were sensitive at both the concentrations of trypsin and failed to attach and proliferate. On contrary to enzymatic dissociation method to obtain primary culture, explant culture method in semi-dried conditions showed firm attachment of most of the tissues like heart, liver, ovary, testis and embryo resulting in subsequent growth and proliferation. Cell proliferation began within 2-6 days from tissues like liver, ovary, heart, testis and embryo while explant culture of brain tissues took 12 days to proliferate. Likewise, the monolayer formation took least time (6-8) days in liver while it was prolonged up to 28 days in case of brain. In fish, the predominance of fibroblast-like cells over epithelioid (epithelial-like) cells has been reported in a number of tissues in both primary and explants cultures (Singh *et al*., 1995; Lakra and Bhonde, 1996; Lai *et al*., 2003) and similar types of observations were noticed in both types of cell culture methods in this study.

The usual trypsin concentrations for detaching cells range from 0.05 to 0.5% (Brown *et al*., 2007). Although, tissues like heart and liver showed sensitivity at 0.25% trypsin concentration, but upon trypsinisation at lower concentration (0.125%), they attained confluency and survived up to 4th passage. On the other hand, ovary, testis and embryo cells were not

sensitive to trypsin at both concentrations and exhibited better attachment, growth and proliferation like that of explant culture method which indicates that the effect of trypsin could be tissue specific. Highest passage was recorded for liver (10th), kidney (9th) and heart (7th) explants, as compared to others but afterwards their growth was poor. Normally, *in vitro* growth of cell depends on nature of the cell, their ability to attach to the substratum and nutrient requirements (Part and Bergstrom, 1995).

In enzymatic method, the dissociation time to obtain single cell to initiate primary culture depends upon the nature of proteolytic enzyme, its efficacy or potency at different dose or concentrations, temperature and above all, the nature of tissue or organ. Furthermore, growth and proliferation of cells during culture and subsequent subculture may also vary depending on the cell type, age of monolayer, cell density, serum concentration etc. (Melican *et al*., 2005). The difference in passage number and survival of cells obtained from different tissues and organs of *C. mrigal* using enzymatic method during this investigation could be related to some of these factors.

CONCLUSION

The findings of the study indicate that enzymatic dissociation of tissue using trypsin not only affected the attachment but also their subsequent growth, proliferation and survival of cells obtained from different tissues of *C. mrigala*. On the contrary, explants culture method was found to be better for obtaining primary culture as far as attachment, growth, proliferation and subsequent subculture (passage) is concerned. To obtain primary culture from single cells; investigations are required to identify tissue specific enzymes along with standardization of dose, duration and temperature of enzymatic treatment.

REFERENCES

Babich, H., M.R. Palace and A. Stern, 1993. Oxidative stress in fish cells: *In vitro* studies. Arch. Environ. Contam. Toxicol., 24: 173-178.

Bols, N.C. and L.E.J. Lee, 1991. Technology and uses of cell cultures from the tissues and organs of bony fish. Cytotechnology, 6: 163-187.

Bols, N.C. and L.E.J. Lee, 1994. Cell Lines: Availability, Propagation and Isolation. In: Biochemistry and Molecular Biology of Fishes, Hochachka, P.W. and T.P. Mommsen (Eds.). Vol. 3, Elsevier Science, The Netherlands, pp: 145-149.

Brown, M.A., C.S. Wallace, C.C. Anamelechi, E. Clermont, W.M. Reichert and G.A. Truskey, 2007. The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces. Biomaterials, 28: 3928-3935.

Chen, S.L., G.C. Ren, Z.X. Sha and C.Y. Shi, 2004. Establishment of a continuous embryonic cell line from Japanese flounder *Paralichthys olivaceus* for virus isolation. Dis. Aquat. Org., 60: 241-246.

Collett, J., A. Crawford, P.V. Hatton, M. Geoghegan and S. Rimmer, 2007. Thermally responsive polymeric hydrogel brushes: Synthesis, physical properties and use for the culture of chondrocytes. J. Royal Soc. Interface, 4: 117-126.

Cruz, H.J., E.M. Dias, J.L. Moreira and M.J.T. Carrondo, 1997. Cell-dislodging methods under serum-free conditions. Applied Microbiol. Biotechnol., 47: 482-488.

Freshney, R.I., 2005. Culture of Animal Cells: A Manual of Basic Techniques. 5th Edn., Wiley-Liss, New York, USA., pp: 387-389.

Ganassin, R.C., K. Schirmer and N.C. Bols, 2000. Methods for the use of Fish Cell and Tissue Cultures as Model Systems in Basic and Toxicology Research. In: The Laboratory Fish, Ostrander, G.K. (Ed.). Academic Press, San Diego, CA., ISBN: 9780125296502, pp: 631-651.

Glade, C.P., B.A. Seegers, E.F. Meulen, C.A. van Hooijdonk, P.E. van Erp and P.C. van de Kerkhof, 1996. Multiparameter flow cytometric characterization of epidermal cell suspensions prepared from normal and hyperproliferative human skin using an optimized thermolysin-trypsin protocol. Arch. Dermatol. Res., 288: 203-210.

Hightower, L.E. and J.L. Renfro, 1988. Recent applications of fish cell culture to biomedical research. J. Exp. Zool., 248: 290-302.

Huang, H.L., H.W. Hsing, T.C. Lai, Y.W. Chen and T.R. Lee *et al*., 2010. Trypsin-induced proteome alteration during cell subculture in mammalian cells. J. Biomed. Sci., Vol. 17. 10.1186/1423-0127-17-36

Joseph, M.A., R.K. Sushmitha, C.V. Mohan and K.M. Shankar, 1998. Evaluation of tissues of Indian major carps for development of cell lines by explant method. Curr. Sci., 75: 1403-1406.

Lai, Y.S., J.A.C. John, C.H. Lin, I.C. Guo, S.C. Chen, K. Fang and C.Y. Chang, 2003. Establishment of cell lines from a tropical grouper, *Epinephelus awoara* (Temminck and Schlegel) and their susceptibility to grouper irido-and nodaviruses. J. Fish Dis., 26: 31-42.

Lakra, W.S. and R.R. Bhonde, 1996. Development of a primary cell culture from the caudal fin of an Indian major carp, *Labeo rohita* (Ham.). Asian Fish. Sci., 9: 149-152.

Lakra, W.S., M.R. Behera, N. Sivakumar, M. Goswami and R.R. Bhonde, 2005. Development of cell culture from liver and kidney of Indian major carp, *Labeo rohita* (Hamilton). Indian J. Fish., 52: 373-376.

Ma, C. and P. Collodi, 1999. Preparation of primary cell cultures from lamprey. Methods Cell Sci., 21: 39-46.

Mastan, S., S. Goswami, V. Kakaria and T. Qureshi, 2007. Development of primary cell culture from heart explants of common carp, *Cyprinus carpio* (Linn). J. Cell Tissue Res., 7: 1053-1056.

McKeehan, W.L., 1977. The effect of temperature during trypsin treatment on viability and multiplication potential of single normal human and chicken fibroblasts. Cell Biol. Int. Rep., 1: 335-343.

Melican, D., R. Butler, N. Hawkins, L.H. Chen and E. Hayden *et al*., 2005. Effect of serum concentration, method of trypsinization and fusion/activation utilizing transfected fetal cells to generate transgenic dairy goats by somatic cell nuclear transfer. Theriogenology, 63: 1549-1563.

Mitalipova, M.M., R.R. Rao, D.M. Hoyer, J.A. Johnson and L.F. Meisner *et al*., 2005. Preserving the genetic integrity of human embryonic stem cells. Nat. Biotechnol., 23: 19-20.

Nanda, P.K., P. Swain, S.K. Nayak, S. Dash, P. Routray, S.K. Swain and B.C. Patra, 2009. Goat serum as an alternative to establish cell culture from Indian major carp, *Cirrhinus mrigala*. *In vitro* Cell. Dev. Biol. Anim., 45: 148-151.

Nanda, P.K., P. Swain, S.K. Nayak, S.S. Mishra, P. Jayasankar and S.K. Sahoo, 2014. Use of polymeric scaffold for *In vitro* growth of fibroblast-like cells of Indian major carp, *Cirrhinus mrigala*. Adv. Anim. Vet. Sci., 2: 177-182.

Nicholson, B.L., 1980. Growth of fish cell lines on microcarriers. Applied Environ. Microbiol., 39: 394-397.

Noga, E.J., 1980. Establishment of primary cell cultures using short-term trypsinization of organ fragments. J. Tissue Culture Methods, 6: 55-56.

Ossum, C.G., E.K. Hoffmann, M.M. Vijayan, S.E. Holt and N.C. Bols, 2004. Characterization of a novel fibroblast-like cell line from rainbow trout and responses to sublethal anoxia. J. Fish Biol., 64: 1103-1116.

Part, P. and F. Bergstrom, 1995. Primary Cultures of Teleost Branchial Epithelial Cells in Cellular and Molecular Approaches to Fish Ionic Regulation. In: Fish Physiology, Wood, C.M. and T.J. Shuttleworth (Eds.). Academic Press, New York, USA., pp: 207-227.

Pleskach, V.A., I.V. Kozhukharova, I.V. Artsybasheva, T.A. Goilo, M.V. Tarunina and N.A. Filatova, 1993. [The cultivation features, growth characteristics and oncogenicity of serum-free populations of transformed fibroblasts]. Tsitologiia, 36: 806-815 (In Russian).

Pumper, R.W., P. Fagan and W.G. Taylor, 1971. Trypsin sensitivity of mammalian cells grown in a serum-free medium. *In Vitro*, 6: 266-268.

Rao, K.S., M.A. Joseph, K.M. Shanker and C.V. Mohan, 1997. Primary cell culture from explants of heart tissue of Indian major carps. Curr. Sci., 73: 374-376.

Rathore, G., G. Kumar, T.R. Swaminathan, N. Sood, V. Singh, R. Abidi and W.S. Lakra, 2007. Primary cell culture from fin explants of *Labeo rohita* (Ham.). Indian J. Fish., 541: 93-97.

Sathe, P.S., D.T. Mourya, A. Basu, S.S. Gogate and K. Banerjee, 1995. Establishment and characterization of a new fish cell line, MG-3, from gills of mrigal, *Cirrhinus mrigala*. Indian J. Exp. Biol., 33: 589-594.

Singh, I.S.B., P. Rosamma, M. Raveendranath and J. Shanmugam, 1995. Development of primary cell cultures from kidney of freshwater fish *Heteropneustes fossilis*. Indian J. Exp. Biol., 33: 595-599.

Smets, L.A., C. Homburg and H. van Rooy, 1979. Selective effects of trypsinization on established and tumour-derived mouse 3T3 cells. Cell Biol. Int. Rep., 3: 107-111.

Sutradhar, B.C., J. Park, G. Hong, S.H. Choi and G. Kim, 2010. Effects of trypsinization on viability of equine chondrocytes in cell culture. Pak. Vet. J., 30: 232-238.

Swain, P., P.K. Nanda, S.K. Nayak and S.S. Mishra, 2014. Basic techniques and limitations in establishing cell culture: A mini review. Adv. Anim. Vet. Sci., 2: 1-10.

Villena, A.J., 2003. Applications and needs of fish and shellfish cell culture for disease control in aquaculture. Rev. Fish Biol. Fish., 13: 111-140.

Wolf, K. and M.C. Quimby, 1969. Fish Cell and Tissue Culture. In: Fish Physiology, Hoar, W.S. and D.J. Randall (Eds.). Academic Press, New York, pp: 253-306.

Wolf, K. and M.C. Quimby, 1976a. Primary monolayer culture of fish cells initiated from trypsinized tissues. Tissue Cult. Assoc., 2: 453-456.

Wolf, K. and M.C. Quimby, 1976b. Procedures for subculturing fish cells and propagating fish cell lines. Methods Cell Sci., 2: 471-474.

Yanase, Y., H. Suzuki, T. Tsutsui, I. Uechi, T. Hiragun, S. Mihara and M. Hide, 2007. Living cell positioning on the surface of gold film for SPR analysis. Biosensors Bioelectron., 23: 562-567.

Ye, H.Q., S.L. Chen, Z.X. Sha and M.Y. Xu, 2006. Development and characterization of cell lines from heart, liver, spleen and head kidney of sea perch *Lateolabrax japonicus*. J. Fish Biol., 69: 115-126.