Indian J. Fish., **48**(2) : 183-188, Apr.-Jun., 2001

Studies on a cell culture derived from hepatopancreas of tiger prawn *Penaeus monodon*

B. MURALI MANOHAR, K.V. VIVEK AND K. KUMANAN

Department of Pathology, Madras Veterinary College, Chennai - 600 007, India.

ABSTRACT

Hepatopancreatocytes from hepatopancreas of penaeid shrimp, monodon were cultured in artificial medium Leibovitz-15, supplemented with glucose and sodium chloride in vitro. Two different approaches for culturing of these cells were followed. i) Culturing, enzymatically disrupted cells and ii) Explant culture of small tissue fragments. The second approach of explant cultures was observed to be highly adva- ntageous as a monolayer was observed within 4-6 days of culture. On the whole, cells could be maintained for as long as 45 days with a maximum of 5 passages. On subculturing, generally one subpopulation of cells with high proliferative capacity was observed to be predominant. Morphological studies lead to the conclusion that these cells were embryonic cells from the tubular epithelium of hepatopancreas.

Introduction

The past decade has been witness to a mammoth leap in the aquaculture industry. India's contribution to this global trend has been comparable with China, Taiwan, Japan and other south east Asian countries, the other key players in the industry. The commonly sought after shrimp species from India are *Penaeus monodon* and *Penaeus indicus*. However recent studies confirm the wide spread occurence of viral diseases in shrimp. Monodon Baculo Virus (MBV), Reolike Yellow Head Virus (YHV), Systemic Ectodermal-Mesodermal Baculovirus (SEMBV), Infectious Hypodermal Hematopoietic Necrosis Virus (IHHNV) infections are known to cause irreparable and severe loss to the industry.

This has prompted researchers to carry research on the development of cell cultures as the first step towards resolving this crisis. Cell cultures once developed would facilitate to carry out virus isolation and to develop diagnostic tools. It would also pave the way to develop models to study the shrimp metabolism and thereby design better feed for cultured shrimp.

Attempts in the past to culture crustacean tissue have lead to partial success only. Although primary cultures were established, till date no subcultures have been available. Chen *et al.* (1986) were the first to successfully culture cells from gonad of *P.monodon* being subcultured for three passages. An attempt is made to culture hepatopancreatocytes of *Penaeus monodon* and the results are

described.

Materials and methods

Wild shrimp were procured from a local supplier and transported in bags containing aerated sterile seawater and maintained in glass tanks in sterlie seawater for 2-3 hours before sacrificing them. Animals were anesthetised by maintaining in ice for 3-5 minutes (cold shock). Surface sterilization was done using 2% tincture iodine.

Two different approaches to culturing hepatopancreatocytes were attempted. i) Culturing cells dissociated enzymatically and ii) Explant culture of tissue fragments.

Culturing cells dissociated enzymatically : The organ was carefully dissected out and incubated in standing buffer containing sodium chloride-14 mM, Potassium chloride -0.6mM, HEPES - 0.996mM, and EDTA-0.52mM for 30 minutes. Further incubation in collagenase buffer containing NaCl, KCl, HEPES, $CaCI_2$, BSA, collagenase type IV at room temperature for 20 minutes was done. Tissue was thoroughly minced and filtered to get a cell suspension. Suspension was spun at 2000 rpm for 10 minutes in a highspeed centrifuge and the pellet formed was washed in plain Leibovitz - 15 medium by centrifugation at 2000 rpm. This was repeated three times. After final washing, the pellet was resuspended into growth medium (L-15 medium,15% FCS, 100U/ml Penicillin, 100ug/ml Streptomycin, lg/l dextrose and 5 g/l sodium chloride) and the entire contents were emptied into culture dishes (Falcon 35X10mm). These dishes were maintained at 27°C in a water-jacketed CO_2 incubator with 5% CO_2. The percentage viability of cells was calculated by the 'Dye Exclusion' method (0.4% trypan blue in saline) before seeding cells (= 85% viability was used). The viability was checked periodically and also prior to subculturing.

Subculturing was done by collecting the spend medium, containing cells in suspension, recentrifuging at 1500rpm and seeding the cells with fresh growth medium. A primary culture was maintained for 10-12 days after which subculturing was carried out at a 5 day frequency.

Explant culture of tissue fragments : Small tissue fragments from hepatopancreas were aseptically dissected out. The organ was incubated in standing buffer for 20 minutes. Fragments were thoroughly washed in standing buffer and kept in culture dishes containing growth medium. These dishes were kept in water-jacketed CO_2 incubator at 27°C and 5% CO_2

Subculturing was carried out after 12 days of primary culture. Since cell attachment was observed, subculturing was done by gentle flushing of the culture dish with fresh medium, recentrifuging cells at 1500 rpm and then seeding them into fresh growth medium. The cells in suspension were subcultured.

Periodically both the culture types were observed under an inverted microscope and photographed. Sterile coverslip preparations were also made by placing coverslips into culture dishes before seeding cells into them, to study cell morphology. These coverslips were stained with crystal violet stain (0.1% crystal violet in 0.25% formal saline for 30 seconds) and the different subpopulations of hepatopancreatocytes were identified.The effect of extra cellular matrix proteins namely collagen (40ug/ml, 60ug/ml, 80ug/ml and 100ug/ml) and fibronectin (0.5mg/ml) at various concentrations were also studied. Culture dishes were precoated with the desired concentration of the protein and BSA (100ug/ml) and were incubated at room temperature prior to seeding of cells.

Results

Ten primary and four secondary cultures were established. Since the objective of the study was to establish a primary culture, a greater number of primary cultures were maintained. The primary cultures were used to standardise culture conditions, media requirements, optimal serum supplementation levels and a staining technique for morphological studies. The changes in cellular morphology with passaging were studied and cellular longevity *in virto* estimated.

The optimum temperature ideal for maintaining shrimp tissues was observed to be in the range of 22-27°C. The pH of the medium was optimal at 7.0 to 7.2. However the osmolality of the medium was not checked for specific levels. *Penaeus monodon* are highly euryhaline marine organisms and hence are efficient osmoregulators (Cheng and Liao, 1986). The cells were observed to adapt to different osmolality levels *in vitro*. 15% serum supplementation (FCS) was observed to be ideal for shrimp tissues in culture. This level supplies most of the cellular requirements needed for cellular growth and differentiation. 5% and 20% serum supplementation failed to produce the same kind of cellular proliferation. The use of double strength medium to culture these cells gave better results and greater longevity for cells. Supplementation of sugars - dextrose (1g/1) and salts - sodium chloride (5g/1) in 2xL-15 medium (double strength) improved medium profile ensuring better cell growth.

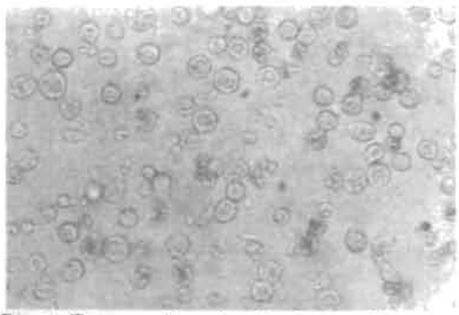

Fig. 1. Enzyme dissociated culture of hepatopancreas (Day4) showing cells in suspension with two major cell types: Phase-contrast photomicrograph, 200X.

In the first approach, cells were observed to be highly spherical in appearance. These cells remained in suspension and were observed to divide. Maximum division of cells was observed with in a period of 6 to 8 days. Generally two types of cells were observed to dominate. One group of cells having a large nucleus and small cytoplasm and the other with a lipid rich cytoplasm (Fig.1). Small cells were occasionally visible. No morphological changes with passaging were observed as cells remained spherical.

In the second approach, two types of cells described in normal shrimp histology were observed. The smallest of cells were seen to proliferate and attach to the substrate to form a diffuse monolayer (Fig.2). Large cells with a granular cytoplasm and occasionally binucleate were also observed (Fig.3). After 2-4days postculture, cell migration from explant fragments was observed. Some of these cellular aggregates were observed to

Fig. 2. Explant culture of hepatopancreas (Day5) showing a diffuse monolayer of small cells : Phase-contrast photomicrograph, 120X.

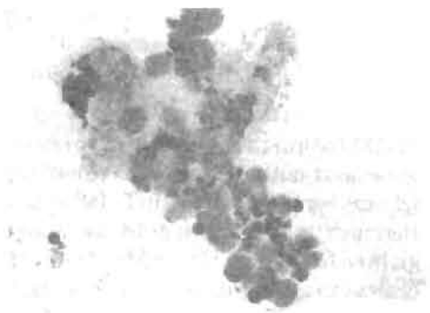

Fig. 3. Explant culture of hepatopancreas showing a cell culture from the explant with two cell types, small cells and large cells with granular cytoplasm within a scaffolding membrane 0.1% crystal violet in 0.25% formal saline, 650X.

mutiply. With progress, cellular migration was observed to increase. Some intact tubules were also seen to migrate into the medium. These tubules could be maintained for as long as 50 days. These tubules showed a group of small undifferentianed cells at the blunt end and a highly pleomorphic population of cells to the distal end of the tubule. The pleomorphic population showed different cell types (Fig. 4).

Fig. 4. Explant culture of hepatopancreas showing intact tubule with a pleomorphic cellular population with smaller cells towards the blunt end of the tubules and larger cells with granular cytoplasm towards the distal end : 0.1% crystal violet in 0.25% formal saline, 150X.

The use of precoated culture dishes in the first approach however did not contribute to cell attachment and cellular longevity. At a collagen concentration of 100ug/ml and fibronectin concentration of 0.5 mg/ml, cells were observed to attach to the substrate. They were however immediately lost or cellular ghosts were observed. Precoating plates for the second approach did not improve cellular attachment and hence no further trails were made. The cells were observed to be spherical and the same morphology was observed in the subcultures.

Discussion

The loss of some hepatopancreatocytes during enzymatic dissociation in enzyme dissociated cell cultures best explained the cellular diversity observed with explant cultures. Enzyme treatment of tissues are known to weaken cell membranes (Toullec, 1999) and cause cellular damage. The damage mostly occurs in case of highly fragilc cells like the secretory cells. In the first approach, cells with granular cytoplasm were sparsely observed. The existing cells in culture showed highly damaged membrance. These secretory cells with granular cytoplasm are depots of digestive proenzymes or zymogens that get activated on membrane lysis. In the normal physiology of the animal, these cells function in a similar manner and are trigerred by a natural signal. Their release can further damage other cells bringing about a near total cellular damage, as their own secretions are powerful digesters. This vindicates our observation made in the first approach. Moreover residual enzyme activity in culture reduces cellular life. As reported, enzymatically disrupted cells were observed to die 20 days in primary cultures in some of the cultures maintained. Cellular ghosts were observed in these cultures. Enzymatic dissociation of cells is also known to decrease the ability of cells to attach to the substrate (Toullec, 1999).

This explains our observations made with the first approach-cells remaining in suspension.

The explant method was observed to be a highly advantageous approach except for problems of contamination that were encountered. This could be overcome by thorough washing of the tissue fragment with perfusion buffer containing antibiotics. Shrimp tissues harbour several natural endoparasites that find their way into the culture. The percentage of cell attachment was observed to tbe considerably high in this approach. The larger cells with granular cytoplasm were identified as the secretory cells or the B cells. The smaller cells observed to attach and proliferate faster are the undifferentiated embryonic cells (Liping *et al.*, 1990). This property can be attributed to the outer membrane of the tubule (Toullec, 1999). Since the intact tubular membrane comes into the culture, it provides a natural surface for cellular attachment. The localised nature of this monolayer could also be due to the envelope that prevents cellular migration. However isolated subculturing of these cells did not give the desired result. Extensive cell death was observed in some of these cultures. As a primary culture, these cells along with the other cells in suspension could be maintained for a maximum of 45 days. The mitotic stages of some of these cells were also observed. The nature of cellular co-existence comes to light, since these cells function in tandem in the organ. Hence co-culturing E-cells (embryonic cells) with the other subpopulations can provide better cell longeivity. The other cells observed include the binucleated and secretory cells with the typical peripherally displaced nucleii or the B-cell (Blasenzellen cells). The intact tubule described earlier lends credence to the fact that the E-cell stock give rise to the other cell types namely B-cell, R-cell and the F-cell that have specific functional role in the normal physiology of the animal (Bell and Lightner, 1988).

Studies to date have also revealed that the various approaches to hepatopancreas culture can give different results and that a primary culture can be established. However the successful subculture of cells and their further characterisation to develop cell lines is still in its infancy. For this to be accomplished, fabricating a medium exclusively for shrimp tissue culture becomes highly essential. The use of 2XL-15 nedium partially helps in overcoming the problem. It largely caters to the needs of the shrimp tissue in terms of the high sugar, inorganic ion, methionine, cystine and glutamine requirements. It has 10 times more aminoacids and 100 times more vitamins in comparison with M-199 (Toullec, 1999). Identifying ideal supplements that confers to shrimp tissue needs which can replace FCS is equally important. However studies on Y-organ cells from the crab *Carcinus maenas* with FCS supplementation have shown an increased secretory activity. (Toullec and Dauphin, 1994). FCS is a good supplement for shrimp tissue as well. Novo serum supplements that substitute for the internal shrimp fluids and provide the necessary growth factors needed for the cells will enable better cellular differentiation. Culturing organs from juvenile shrimp has been shown to give better results as a large number of potent cells becomes available. The use of cells from undifferentiated tissue (Toullec *et al.*, 1996) and cell transfected with a tumor gene have given encouraging results (Tapay *et al.*, 1995) and constitutes the future in shrimp tissue culture.

Acknowledgements

The authors wish to thank the Department of Biotechnology, Ministry of Science And Technology, Government of India for the financial assistance provided