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A cell culture system developed from heart tissue of the greasy grouper, *Epinephelus tauvina* (Forsskal 1775) by enzymatic dissociation

K. S. SOBHANA, K. C. GEORGE, G. VENKAT RAVI, GIJO ITTOOP AND R. PAULRAJ
Marine Biotechnology Division, Central Marine Fisheries Research Institute,
Kochi - 682 018, India
e - mail : sobhana_pradeep@yahoo.co.in

ABSTRACT

Heart tissue samples aseptically excised from healthy juveniles of the grouper, *Epinephelus tauvina* were subjected to enzymatic dissociation using 0.25 % trypsin solution, under aseptic conditions. The resultant cells were suspended in Leibovitz' L-15 medium supplemented with 20 % fetal bovine serum. The cells were subsequently seeded into 25 cm^2 tissue culture flasks at a density of 10^6 ml^{-1} and incubated at 28 ± 2°C. Cells showed spreading and attachment to the culture flasks within 24 h and formed monolayers comprising of epithelioid as well as fibroblast-like cells within 20 days. A confluent monolayer filling the flask surface comprising predominantly fibroblastic cells was formed within 30 to 35 days. The cell monolayer was harvested for passage by trypsinisation, which formed successful monolayers in subsequent subcultures.

Introduction

In recent years, with the rapidly developing mariculture activities world over, outbreak of viral diseases has become a serious issue causing heavy economic losses (Seng and Colorni, 2002). Diseases due to viral etiology, such as the viral nerval necrosis virus (VNN) and iridoviruses have been reported from many parts of Asia with mariculture activities (Chang *et al*., 2001; Lai *et al*., 2003). For development of precise diagnostics and prophylactics of the viral pathogens, establishment of cell lines is an absolute necessity. Fish cell lines also have widespread application in cytogenetics, transgenics, toxicology, as *in vitro* models for studying cellular and physiological processes and also in comparative immunology.

Most of the established fish cell lines were derived from temperate species, such as salmonids and channel cat fish (Nicholson, 1988). However, many new continuous cell cultures are constantly being developed as a result of intensive efforts in several parts of the world, to provide cell cultures from local species utilised in aquaculture (Fernandez *et al.*, 1993 a, b; Chang *et al*., 2001; Lai *et al*., 2003). Since cell cultures derived from the same species or a species closely related to that in which the disease occurs would be the most sensitive for virus isolation, cell lines derived from local species need to be given high priority. The host and tissue specificity of virus underlines the need for developing cell lines from different species in different regions (Cheng *et al.*, 1993).

Published information from India is available on development of three cell lines and two cell culture systems from the marine teleost, *Lates calcarifer* (Sahul Hameed *et al*., 2006; Parameswaran *et al*., 2006 a, b; Lakra *et al*., 2006 a). Few cell lines (Sathe *et al*., 1995, 1997; Lakra *et al*., 2006 b) and primary cell culture systems (Singh *et al*., 1995; Lakra and Bhonde, 1996; Rao *et al*., 1997; Lakra *et al*., 2005) have also been developed from freshwater teleosts.

Groupers (*Epinephelus* spp.) are highly priced and popular seafood fishes among the major farmed fish species in south-east Asia. But the yield from wild stocks is unable to meet the demands and therefore have recently become one of the most important aquaculture and trade commodities in the Asia-Pacific region (Seng and Colorni, 2002). Though India is one of the major producer in Asia in the aquaculture sector, finfish production from mariculture is meagre and still in the experimental stage (Pillai *et al.,* 2002). Marine finfish farming is gaining importance these days since brackishwater farmers are looking for species diversification in the light of severe crisis faced by the shrimp farming sector due to diseases and other problems. A major constraint in grouper hatchery production and farming is outbreak of diseases due to viral etiology. Iridovirus and nodavirus have been identified as the most important pathogens of grouper culture causing major problems at the fry and fingerling stages (Chi *et al*., 1999; Lai *et al*., 2003). In this context, development of grouper cell lines, anticipating problems such as viral disease outbreaks is very important. The present study was

aimed at developing a successful cell culture system from the heart tissue of *E. tauvina* by trypsinisation.

Materials and methods

Experimental fish

Healthy juveniles of the grouper (*E. tauvina*) having average weight 60 ± 10 g, collected from the coastal waters of Cochin were used for developing primary cell culture. Fishes were acclimatised for a period of two weeks on a diet of marine shrimp/fish meat, in circular fibre glass tanks having *in situ* biological filtration system and holding 300 l of well aerated and dechlorinated sea water of 30-32 ‰ salinity. The fishes were subsequently transferred to rectangular perspex tanks (90 cm X 60 cm X 45 cm) holding 50 l of well aerated and dechlorinated sea water (30 ‰) in the pathology laboratory of CMFRI.

Tissue culture medium

The tissue culture medium, Leibovitz' L -15 (GIBCO) was used for cell culture in the present study. The medium was reconstituted in Milli Q synthesis grade water with added NaCl (0.07 M). The pH of the medium was adjusted to 7.2 ± 0.2. After adjusting the pH, medium was filter sterilised (0.2 µ), dispensed in sterile screw cap bottles and stored at 4°C until use. For preparation of the growth medium, sterile Fetal Bovine Serum (PAN Biotech, Germany) was used (20 % v/v) after inactivation at 56^0C for 30 min in a water bath. Antibiotics such as penicillin and streptomycin (Sigma, USA) (100 IU ml^{-1} and 100 ìg ml^{-1} respectively) and the fungizone, amphotericin B (GIBCO) (0.25 ìg ml^{-1}) were used in the preparation of growth medium.

Preparation of fish and tissue collection

Before dissecting out the tissues for primary culture, the fishes were starved for two days and maintained overnight in sterile, aerated seawater containing 1000 IU ml^{-1} penicillin and 1000 ìg ml^{-1} streptomycin. Prior to sacrifice, the fishes were tranquilised by plunging in iced water for 5 min, then disinfected in sodium hypochlorite (500 ppm available chlorine) for 5 min, washed in sterile sea water and swabbed with 70 % ethyl alcohol. The heart tissue was aseptically excised from the fishes and collected in sterile vials containing phosphate buffered saline (PBS, pH 7.2) having 500 IU ml^{-1} penicillin, 500 ìg ml^{-1} streptomycin and 1.25 ìg ml^{-1} amphotericin B. Subsequently, the tissues were washed thrice in the same medium prior to trypsinisation.

Primary culture

The tissue samples were minced thoroughly with scissors aseptically and transferred to sterile glass beakers containing 0.25 % trypsin solution (0.25 % trypsin and 0.2 % ethylenediaminetetraacetic acid, EDTA) in PBS. The contents were gently agitated with a magnetic stirrer at room temperature for 30 min. After settling of the larger undigested tissue pieces, the supernatant was transferred into an equal volume of complete medium (L-15) containing 20% FBS, 100 IU ml^{-1} penicillin, 100 ì g ml^{-1} streptomycin and 0.25 ìg ml^{-1} amphotercin B and mixed well to inhibit trypsin activity. The resultant cells were centrifuged at 200 *g* for 10 min and the pellet resuspended in fresh complete medium (L-15, pH 7.2 + 0.2). The cells were seeded into 25 cm^2 tissue culture flasks at a density of 10^6 ml^{-1} and incubated at 28 ± 2°C.

Subculture and maintenance

When confluent monolayers were formed in primary culture, cells were carefully dislodged from the flask surface by treatment with 1 - 2 ml of 0.25 % trypsin (0.25 % trypsin and 0.2 % EDTA in PBS). Two milliliter of fresh medium (L-15 containing 20 % FBS, 100 IU ml^{-1} penicillin, 100 ì g ml^{-1} streptomycin and 0.25 ìg ml^{-1} amphotercin B) was then added to neutralise the action of trypsin. The detached cells were harvested in 5 ml of fresh growth medium (L-15 with 20 % FBS), then split into two portions and transferred to new culture flasks. The flasks were then incubated at 28 ± 2°C and observed for growth, cell attachment and formation of monolayer using an inverted microscope (Nikon TS 100).

Results and discussion

Dissociation of tissue using trypsin-EDTA (0.25 % trypsin and 0.2 % EDTA) yielded dispersed cells and the resuspended pellet consisted of individual cells as well as cell clumps (Fig.1). Within 24 h of seeding, initiation of

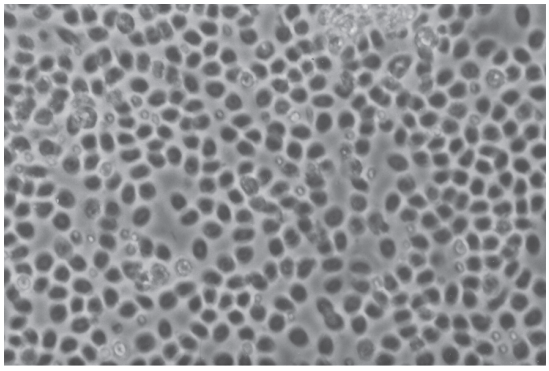

Fig. 1. Phase contrast photomicrograph of dissociated cells from heart tissue of *E. tauvina* after trypsinisation (X 200)

spreading and attachment of the cells to the surface of the culture flasks was noticed (Fig. 2). Several spindle shaped spreading and attaching cells were noticed within 6 days post-seeding (Fig. 3). Patches of cell monolayers were

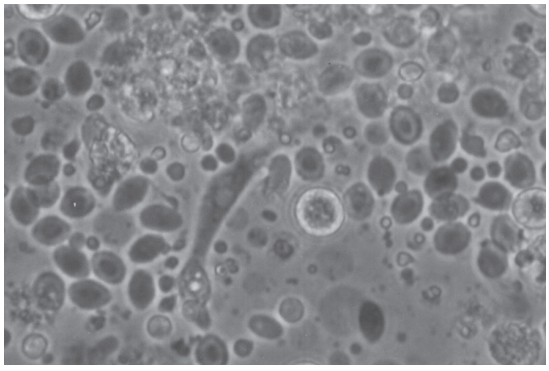

Fig. 2. Initiation of spreading and attachment of cells from trypsinised heart tissue of *E. tauvina* at 24 h post-seeding (X 400)

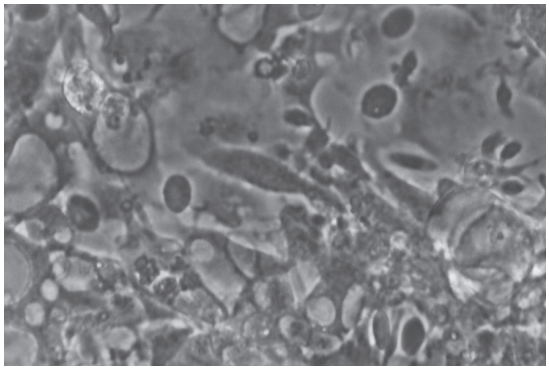

Fig. 3. Spreading and attaching cells from trypsinised heart tissue of *E. tauvina* on 6 days post-seeding (dps) (X 400)

formed within 20 days consisting of both epithelioid and fibroblast-like cells (Fig. 4). However, as the culture progressed fibroblast-like cells predominated and a confluent monolayer filling the surface of the culture flask was formed within 30-35 days (Fig. 5 and 6). After obtaining a complete monolayer, cells were passaged to new flasks by trypsinisation. The trypsinised cells exhibited

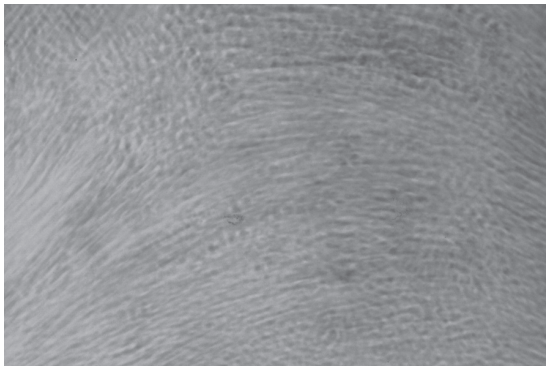

Fig. 4. Cell monolayer formed from trypsinised heart tissue of *E. tauvina* consisting of both epithelioid and fibroblast-like cells on 20 dps (X 40)

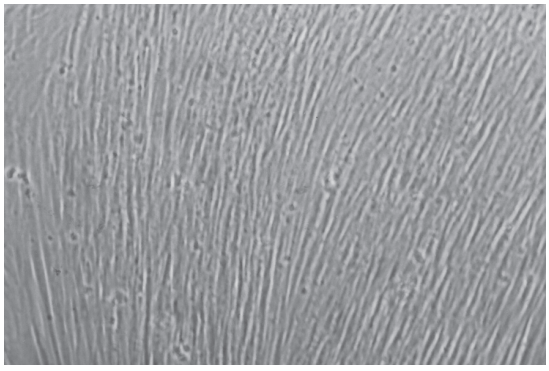

Fig. 5. Confluent monolayer formed from trypsinised heart tissue of *E. tauvina* comprising predominantly fibroblast-like cells on 31 dps (X 100)

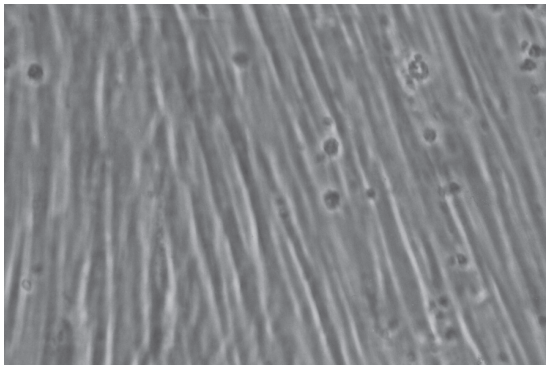

Fig. 6. Confluent monolayer formed from trypsinised heart tissue of *E. tauvina* comprising predominantly fibroblast-like cells on 31 dps (X 200)

good attachment to the flask surface (Fig. 7) and formed successful monolayers in subsequent subcultures (Fig. 8).

Several researchers have achieved successful results with trypsin dissociation, to initiate primary cultures. Enzymatic dissociation is one of the conventional methods

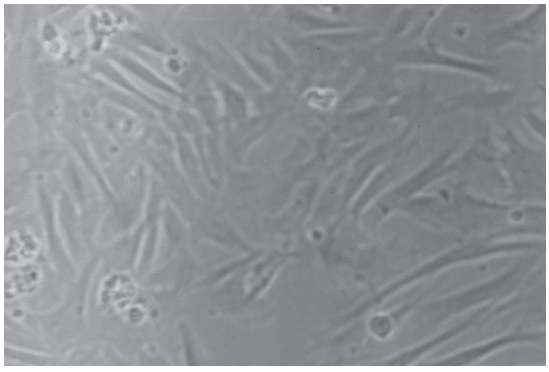

Fig. 7. Attachment and formation of monolayer by the subcultured heart cells in the first passage (X 200)

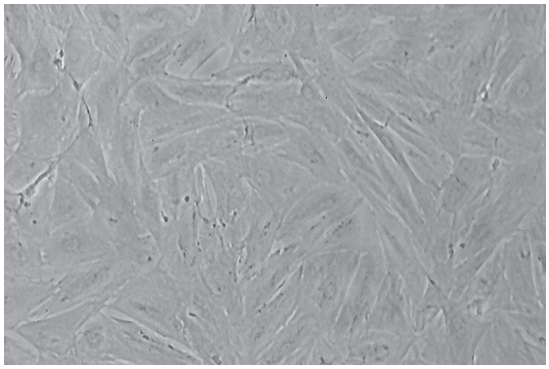

Fig. 8. Confluent monolayer formed by the subcultured heart cells at passage 6 (X 200)

for obtaining cell cultures. Grutzner (1958) made the first attempt for trypsinisation of fish tissue from the tench, *Tinca tinca*, yielding cultivable cells which grew in monolayer and successfully subcultured. Wolf *et al*. (1960), using extended trypsinisation at 4-6°C, cultured cells of six freshwater teleosts. The cells were subcultured by mechanical dispersion as well as with trypsin or disodium versenate. Subsequently, preparation of monolayer cell cultures from enzymatically disaggregated fish tissues has been reported by a number of other workers. Trypsinised gonad tissue of *Clarias batrachus* (Noga and Hartmann, 1981) and *Ictalurus nebulosus* (Alguacil *et al*., 1991) have shown good attachment and successful primary cell cultures were developed. The degree of attachment of trypsin dissociated embryonic tissue of *Poecilia reticulata* and *Betta splendens* was considerably good and spindle-shaped cells were formed within 48-72 h (Singh *et al*., 1995). Gill of rohu, *Labeo rohita* was subjected to trypsinisation with encouraging results (Sathe *et al*., 1997). A tropical marine fish cell line (SF) was established from fry of *Lates calcarifer* by trypsinisation (Chang *et al*., 2001).

The L-15 medium supplemented with 0.07 M NaCl and 20 % FBS supported growth of the cells from heart tissue of *E. tauvina* in the present study. Several researchers have studied the suitability of various mammalian and insect cell culture media and supplements required for growth of fish cells. The medium L-15 has been successfully used to support the growth of fish cells (Lakra *et al*., 2006 a, b; Sahul Hameed *et al*., 2006). The Leibovitz medium was designed to maintain pH in the physiological range under normal atmosphere without added CO_2. The suitability of L-15 in supporting fish cell lines has been studied and confirmed by Fernandez *et al*. (1993 a) using 28 fish cell lines. Among the various media tested [Leibovitz' L-15, Medium 199 (M-199) and Eagle's minimum essential medium (Eagle's MEM)], L-15 was found to be the most suitable for the attachment and proliferation of cells (Lakra *et al.*, 2005). Faster growth and better proliferation was noticed in cells cultured with L-15 medium at pH 7.4. The use of serum at levels above 10 % has been recommended for primary cultures and for the initial passages, whereas an optimal concentration of 5 % is enough in later stages (Sahul Hameed *et al.*, 2006; Parameswaran *et al.*, 2006 a, b).

Cells from the heart tissue of *E. tauvina* grew well in L-15 with additional NaCl which is needed for marine fish cells (Clem *et al*., 1961; Fernandez *et al*., 1993 a). Clem *et al*. (1961) were the first to establish monolayer cell cultures from marine teleosts and obtained best results in commercial medium modified with 0.07 M NaCl. The JSKG cell line established from gonads of Japanese striped knife jaw, *Oplegnathus fasciatus* and PAS cell line from skin of purplish amberjack, *Seriola dumerili* were initiated at a higher NaCl concentration of 0.206 M, but gradually adapted to a low NaCl concentration of 0.116 M after several subcultures (Fernandez *et al*., 1993 b). However, Chang *et al*. (2001) successfully established SF cell line from Asian sea bass, *L. calcarifer* without using increased NaCl concentrations in the cell culture medium. Sahul Hameed *et al*. (2006) also concluded that for the establishment of SISK cell line from sea bass kidney, additional NaCl was not needed.

In the present study with grouper heart cells, initially epithelial cells and fibroblast-like cells coexisted. However, as the culture progressed fibroblast-like cells were predominant. Chi *et al*. (1999) reported the presence of both epithelial cells and fibroblast-like cells in the primary culture of grouper fin cells. However, they reported that in subsequent subcultures, fibroblast-like cells proliferated more rapidly than the epithelial cells and ultimately predominated. Many serum factors derived from platelets have a strong mitogenic effect on fibroblasts and also tend to inhibit epithelial proliferation, subsequently causing fibroblasts to overgrow in subcultures (Freshney, 1994). Usually a predomination of fibroblastic cells over epithelioid cells in cell cultures from fish has been reported (Chi *et al*., 1999; Lai *et al*., 2003). The primary culture developed from heart explants of Indian major carps comprised mainly of fibroblast-like cells (Rao *et al*., 1997). Similarly the GH cell line developed from trypsinised heart tissue of the tropical grouper, *Epinephelus awoara* was also composed of fibroblast-like cells (Lai *et al*., 2003).

The results of the present study have clearly demonstrated good growth and formation of confluent monolayer of cells from trypsinised heart tissue of *E. tauvina*, which has been successfully subcultured. Besides, heart tissue of groupers appear to be ideal for cell culture, as it is easy to collect aseptically compared to other visceral organs. Hence there is scope and prospect for development of cell line from heart tissue of grouper.

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