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Primary cell culture from fin explants of *Labeo rohita* (Ham.)

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ABSTRACT

The study reports a successful development of primary cell culture from fin explants of *labeo rohita* and subsequent subculture up to eight passages. Explant tissues of fin were seeded and incubated at 30°C in DMEM supplemented with 10% FCS. On day 5 of seeding, the differentiation of the cells was visible and a complete monolayer was obtained on day 20. A cell count of 2 X 10^6 cells was sufficient to produce monolayer in 5 days on 8^{th} passage. These encouraging results are indicative of a high probability of developing a cell line from the fin tissue of *L. rohita.*

Introduction

Fish cell lines have both fundamental and practical importance The most widely employed application of fish cell culture is the isolation of fish viruses from infected fish which is highly dependent on the availability of susceptible cell lines. Fish cell lines are also useful as in-vitro model for studying the replication and genetics of viruses, effects of anti-viral drugs and production of experimental vaccines. Fish cell lines can also be used for studying biological process such as determining karyotypes, chromosomal abnormalities, chromosomal polymorphism and other related aspects of fish cytogenetics. In addition, fish cell lines find increasing applications in fish toxicology, carcinogenesis, evaluation of immune responses etc.

The commercialization of trout and other salmonid fish culture in North America, Europe and Japan has led to development of number of salmonid cell lines (Wolf and Quimby, 1962) for various purposes. On the other hand, only a few cell lines have been developed from warm water species. However, with the intensification of aquaculture in the Asia-Pacific region, several disease problems have cropped up. Consequently, there has been a rapid increase in efforts to develop cell lines from species such as carp, grouper, tilapia, eel, etc. These cell lines are being used for isolation and characterization of previously unknown viruses of this region.

In India, there has been quite a progress towards the development of cell lines from Indian major carps and other

warm water fishes (Singh *et al.*, 1995, Lakra and Bhonde, 1996; Rao *et al.*, 1997; Kumar *et al.*, 1998; Prassanna *et al.*, 2000; KAnar *et al.*, 2001, Rathore *et al.*, 2001). A few cell lines MG-3 (Sathe *et al.*, 1995) and RG-I and RG-2 (Sathe *et al.*, 1997) derived from gills of *Cirrhinus mrigala* and *Labeo rohita,* respectively have been established and characterized. Most recently, new cell lines have been developed from *Lates calcarifer* (Lakra *et al.*, 2005a; Hameed *et al.*, 2006) and *Tor putitora* (Lakra *et al.*, 2005b). A perusal of literature reveals that most of the earlier efforts focused on the development of complete monolayer formation and factors influencing the attachment of explants and growth of cells However, one of the most important aspects in the development of cell line is the continuous subculture of the monolayer with a high split ratio. In the present study, successful development of a monolayer from fin explants of *Labeo rohita* and subsequent subculture up to eight passages is reported.

Materials and methods

L. rohita fingerlings (2-3 g) were externally disinfected with 70% ethanol and washed wtth sterile phosphate buffer saline (PBS). The tail fin was aseptically cut and transferred to a petri-plate containing PBS plus an antibiotic-antimycotic solution (peni-cillin G sodium-200 I U, streptomycin sulfate-200 ug and amphotericin B -50 ug per ml PBS) and rinsed three times. The fin tissue was cut in to small pieces of 1-2mm and again rinsed three times with PBS. The tissue explants (15-20 no) were carefully seeded onto the surface of a 40 mm fresh petri-dish (Nunc) and allowed to attach for one hour before the addition of 50 ul of foetal calf serum (PCS) on each explant. Care was taken not to dislodge the explants from their sites of attachment. The petri-dish was placed in a CO2 incubator at 30°C with 5% CO_2 tension. After 24 hours (day 1), 100 ul of Dulbecco's Minimum Essential Medium (DMEM) containing 10% PCS was carefully added to each explant and further incubated. The addition of DMEM was done every 24 hours so as to immerse the tissue explants completely in approx 4ml medium. Thereafter, 50% of the medium was replaced with fresh DMEM every 5 day, till the formation of a complete monolayer.

Results

Monolayer formation

On day 1, large number of cells emerged around the periphery of the explant. These cells aided in providing support to the explants for attachment The migrating cells were almost oval in shape with very little differentiation in structure. By day 5, the differentiation of the cells was visible (Fig. I) As the culture progressed, the migration of the cells from the explants continued and on 14^{th} day, the multiplication of the

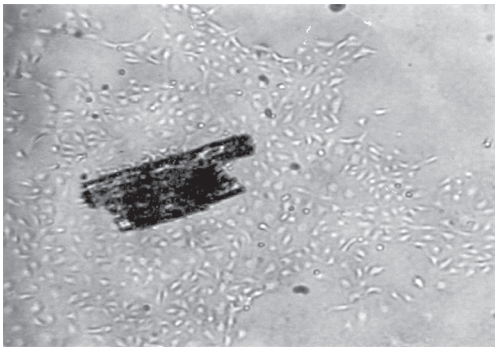

Fig. 1. Fin explant showing widespread cell growth on day 5, (40X).

fibroblasts was observed in the marginal areas (Fig. 2). Microscopically, the cells were predominantly fibroblastic with a few epithelial like cells (Fig. 3). The widespread cell growth continued till it

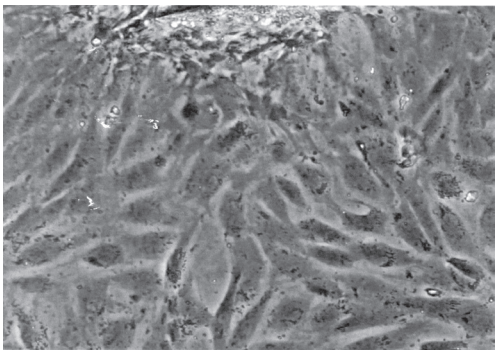

Fig. 2. Fin explant showing fibroblast like cell growth on day 14, (200X).

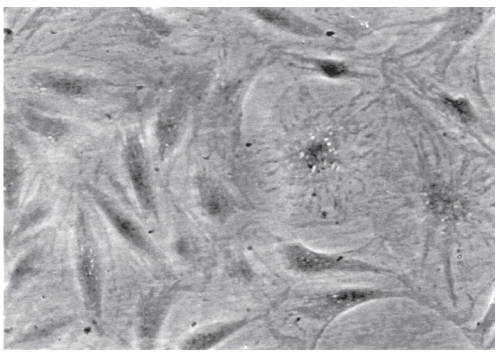

Fig. 3. Cell population showing fibroblast and few epithelial cells, (200X).

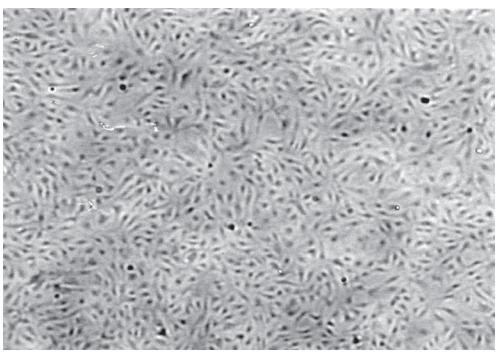

Fig. 4. Complete monolayer formation on day 20, (40X).

extended to the periphery of the neighbouring explant. A complete monolayer was observed on day 20 (Fig 4).

Subculture and propagation of cells

A confluent monolayer on the surface of the petridish was subcultured by treatment with one ml of trypsin solution (0.25% trypsin and 02% EDTA in PBS) after one wash with 5 ml PBS. The trypsin solution was allowed to act for one minute and then withdrawn. The cells were dislodged by vigorous shaking of petridish. Once the detachment of cells was confirmed under inverted microscope, 5ml of DMEM was added to the petridish to arrest the activity of trypsin. The cell suspension was pipetted to make a single cell suspension after washing with PBS. The cells were seeded to a 25 cm^2 flask (Nunc) with vented lid and incubated at 30^0C. Within 24 hours, attachment of nearly 80% of cells was visible. On day 4 of subculture, 50% of medium was replaced with fresh DMEM. A confluent monolayer formation was observed in 7 days (I^{s1} passage). Similar procedure was followed for 2^{nd}, 3^{rd} and 4^{th} passages. By the end of this period, the confluent monolayer formation was found developing within 5 days, indicating faster multiplication of cells and higher cell count. In the subsequent passages, the subcultured cells were seeded into two new flasks at a split ratio of 1:2. A cell count of 2 X $I0^6$ cells was sufficient to produce monolayer in 5 days on 8^{th} passage.

Discussion

Since viral infections of tropical fishes are increasingly reported, a cell culture from *L rohita* may greatly facilitate initial investigations on viral etiology, besides their potential applications in genetics, toxicology and transgenics. There have been a few attempts to develop cell culture from caudal fin and gills of *L. rohita* (Lakra and Bhonde, 1996; Sathe *et al.,* 1997). The advantage of caudal fin is that it has a high regenerating capacity and can be used as good model for development of

cell lines. Moreover, non-lethal sampling of the fish can be undertaken with fin tissue, without sacrificing the fish. In the early stages of the primary culture, the cell population mainly consisted of fibroblast like cells and epithelial cells which were round in structure. As the culture progressed the large round cells declined in number due to limited multiplication. In contrast, the population of the fibroblast cells increased rapidly and formed monolayer. It has been earlier reported (Freshney, 1994) that serum factors derived from platelets have strong mitogenic effect on fibroblasts and have a propensity to inhibit epithelial proliferation, thereby causing fibroblasts to predominate in subcultures The risk of microbial contamination is one of the major hurdles in primary cell cultures from fin tissues. This can be avoided by the use of healthy fish and judicious use of antibiotics in the culture medium. However, higher doses can also lead to slow or no growth from the explants. Attachment of explants to plastic substrate is another important aspect in development of primary cultures. Optimum attachment of explants was achieved between temperature ranges of 28-30°C, while at 37°C, low attachment was observed. Therefore, use of low temperature CO_2 incubator would greatly enhance the chances of development of primary cultures from tropical fishes. Fibronectin treatment of the plastic surface or coating of the cell surface with positively charged polymer, such as poly-D-lysine are also known to improve cell attachment and favoring replication of cells Coating of the plate surface with FCS, containing fibronectin, helped in better attachment of the explants.

The results indicate that fibroblasts derived from caudal fin of *L. rohita* have a high multiplication potential and can be successfully subcultured. Therefore, there is high probability to develop an established cell line from fin tissue of *L. rohita*.

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