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DEVELOPMENT OF CELL CULTURE SYSTEM FROM SELECTED TISSUES OF *PANGAIUS HYPOPTHALMUS*

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Abstract

Cell line provides a valuable biological tool for carrying out investigations into physiology, virology, toxicology, carcinogenesis, and transgenesis. The operation by which cells are grown under the control conditions, generally outside their natural habitat, is called as cell culture. Cells are isolated from the animals and plants cells, these isolated cells are allowed to grow and culture in artificial media. The cell lines from different tissues of different species are valuable for studying species-specific responses to viral infection at the cellular level. Cell culture systems were developed from different tissues of P.Hypopthalmus Namely Caudal Fin, heart, swim bladder, gills and eye tissues. A total of seven explants were prepared, but only two were used to develop cell culture. Cell culture systems developed from fin and heart were successfully subcultured to establish cell lines designated as PHCF respectively. Morphological evaluation under the inverted microscope revealed that all the explants prepared from fin explants were found to be adequately attached after 18-24 hours of explant preparation. The fish cell line is dominant in vitro tool for studying epidemiology, molecular carcinogenesis, toxicology and functional genomics in fish.

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INTRODUCTION:

In recent year cell lines became an essential tool for the study of epidemiology, molecular carcinogenesis, toxicology, and functional genome in fish. It is also crucial for the isolation and identification of the fish virus [1]. Cell culture technique was started as tissue culture with the development of "hanging drop technique" by Ross Harrison, in which a small piece of tissue placed in a drop of the medium, and cells migrated from the stuff into the surrounding environment [2]. The operation by which cells are grown under the control conditions, generally outside their natural habitat, is called as cell culture. In recent year cell culture have marked a significant change in the research [3]. Cells are isolated from the animals and plants cells, these isolated cells are allowed to grow and culture in artificial media. The cell can be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or it can be derived from the cell line or cell strain. The stage of the culture, after that the cell are isolated from a tissue and reproduced under the appropriate condition are called as a primary culture. At this stage, the cell is used to subcultured (i.e., passage) by transferring them into a new vessel on the fresh growth medium to provide more place for continuous growth. After the first passage, the primary culture is referred to the cell line or subclone. As cell lines derived from primary culture, have a limited lifespan. Normal cells divide only a limited number of times before they lose their ability to increase, these cell lines are called as finite but when cell lines receive transformation and acquire the ability to divide indefinitely, known as a continuous cell line [4].

Pangasius hypopthalmus (Sauvage, 1878) belong to the Pangasiidae family, also known as *Pangasius sutuchii* (Flower, 1937) [5]. They are highly migratory riverine fish species and omnivorous in nature. They eat algae, higher plants, insects, while mature fish use to eat fruits, crustoceans and fish [6].

MATERIALS AND METHODS:

Fry and fingerlings of *P.hypophthalmus* collected from the Narmada River, Hoshangabad, Madhya Pradesh, India. They were maintained at a tank of ICAR-CIFE, Mumbai. Fingerlings (15-20g) were transported live to the laboratory and kept in sterile, aerated water containing 1000 KJ/ml penicillin and 1000 µg/ml streptomycin for 24 hours at room temperature before experimentation.

Explant Preparation:

Explant were prepared by using the standard method of Goswami et al. 2012 [7].

Preparation of Donor Fish:

As contamination is the major problem in tissue culture experiments, adequate care was taken to minimize the possible routes of contamination. The donor fish was starved for a one or two hours to reduce the possibility of gross contamination from feces and regurgitated feed. During this period, fish was allowed to swim in well aerated autoclaved water for reducing the microbial load adhered on to the skin and gills. Donor fish (*P. hypophthalmus*) was then sacrificed by euthanizing in ice for 10-15 min.

Decontamination:

External decontamination is necessary before dissection. The decontaminating solutions for this purpose included chlorine solution (500 ppm available chlorine), 70% ethanol, iodophore solution (0.5 w/v iodine). For external organs like gills, skin or fin intended for culture, strong disinfectants were avoided because they damage the tissue too. Instead, an antibiotic solution prepared in a balanced salt solution was used. The commonly used antibiotics in the present experiment were penicillin (400 IU/ml) and streptomycin (400 ng/ml) with an anti-fungal amphotericin B (10 ag/ml). The tissue of interest was aseptically picked up and washed three to five times with the antibiotic solution.

Dissection:

Eye, fin, heart, gill and swim bladder tissues were taken out aseptically and washed with PBS containing 500 IU/ml penicillin, 500 ng/ml streptomycin and 2.5 mg/ml Fungizone. The stuff was minced into small pieces; explants of 1mm3 sizes were prepared and washed thrice with PBS containing antibiotics. These explants were then seeded into 25 cm2 cell culture flasks. Addition of 0.5 ml of FBS accomplished the adherence of explants, these flasks were then incubated at 28 °C and allowed to attach to the surface of the vial overnight. After 18-22 hours, the growth medium, L-15 (Leibovitz) supplemented with 20% FBS was added gently. The medium was changed after five days.

Subculture and Maintenance:

The flasks were observed daily for attachment of explants, spreading and proliferation of cells, morphological details using an inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The dispersed cells adhere to the culture substrate and start to reproduce. Dead cells cannot secrete substrate that helps in attachment to the flask and hence floats. They were removed during the subsequent medium exchange. The optimum pH and incubation temperature maintained were 7.4 and 28 °C respectively for a culture of fish cells.

Upon reaching 90%-95% confluency, the cells were trypsinized using TPVG solution (0.1%trypsin, 0.2% ethylenediaminetetraacetic acid (EDTA), and 2% glucose in 1 x PBS).

At the time of the 1st subculture, the cells were carefully detached from the flask surface using TPVG solution (0.1% Trypsin, 0.2% EDTA and 0.2% glucose in PBS IX) without dislodging the explants. The detached cells were harvested in 5mL of growth medium and transferred to fresh flasks. The explants were maintained further to collect new migrating cells. When the confluent monolayer had formed in the primary culture, the old medium was removed, and cells were dislodged by treatment with TPVG solution twice for 30 seconds each. The detached cells were resuspended in 5mL of fresh growth medium (L-15 plus 20% FBS) and seeded in 25 cm2 plastic culture flasks.

RESULTS AND DISCUSSION:

Primary Culture:

Primary cultures were established by using explants technique. Explants were prepared from different tissues such as heart, gill, eye, swim bladder and caudal fin tissues. However, explants prepared with caudal fin were successfully subcultured up to 9th passage. Primary cultures were established from fin, heart, eye, swim bladder and gills of *P.Hypopthalmus* by explant technique. The explant technique has many advantages over the trypsinization method regarding speed, ease, and maintenance of cell interactions and the avoidance of enzymatic digestion which can damage the cell surface (Avella et al., 1994) [8]. Number of continuous cell lines has been developed by using the explant procedure (Lakra and Bhonde 1996; Akinoto et al., 2000; Kumar et al., 2001; parameshwaran et al., 2006b; 2006c; Ahmed et al., 2009 a,b; Goswami et al., 2012) [9, 10, 11, 12, 13, 14, 15, 7].

Cell culture systems were developed from different tissues of *P.Hypopthalmus* Namely Caudal Fin, heart, swim bladder, gills and eye tissues. Cell culture systems developed from fin and heart were successfully subcultured to establish cell lines designated as PHCF respectively. The fish cell line is a powerful in vitro tool for studying epidemiology, molecular carcinogenesis, toxicology and functional genomics in fish (Hightower and Renfro, 1988; Bahich and Borenfreund, 1991; Wise et al., 2002) [16, 17, 18]. Since the development of first fish cell line (RTG-2 from gonad tissue of rainbow trout, numbers of cell lines have been established from different organs of different fish species. Since some Pathogenic viruses are known to be organ or tissue-specific, the establishment of cell lines from different organs and tissues of a host species will be valuable for studying species-specific responses to viral infection at the cellular level.

Cell Culture System from Caudal Fin Explant:

A total of seven explants were prepared, but only two were used to develop cell culture. Morphological evaluation under the inverted microscope revealed that all the explants prepared from fin explants were found to be adequately attached after 18-24 hours of explant preparation. The radiation of cells started after 3-4 days of explant preparation from fin and a confluent monolayer around the explants was observed within 7-10 Days. Majority of the cells proliferating from fin explant were reasonably heterogeneous i.e., consist of both epithelial and fibroblastic cells (Fig. 1 & 2). Cells radiating from fin, eye, and gill were reasonably heterogeneous i.e., consisting of both epithelial and fibroblastic cells. Varied nature of cells during early passages has been reported by many researchers during early cell cultures (Lakra et al., 2010a; Parameswaran et al., 2006b) [19, 13]. Fibroblast cells predominated aver epitheloid cells during subculturing of TTCF cells. Predomination of fibroblastic calls over epithelioid cells in cell cultures from fish has been reported (Bejar et al., 1997; Lai et al., 2003, Lakra et al., 2006a, Goswami et al., 2012) [20, 21, 22, 7]. In case of eye tissue, during initial subculture from eye explants, the trypsinized cells attached to flasks in clumps and cells were of both epithelial and fibroblastic morphology. After initial passages, the epithelial population of cells predominated over fibroblastic cells. Ahmed et al., (2008) [23] also reported the presence of both epithelial-like cells and fibroblast-like cells during primary culture and predominance of epithelial-like cells over fibroblastic cells in subsequent subcultures from eye cells of Catla catla, the similar morphological change was also observed in the cells eye of *P.Hypopthalmus*.

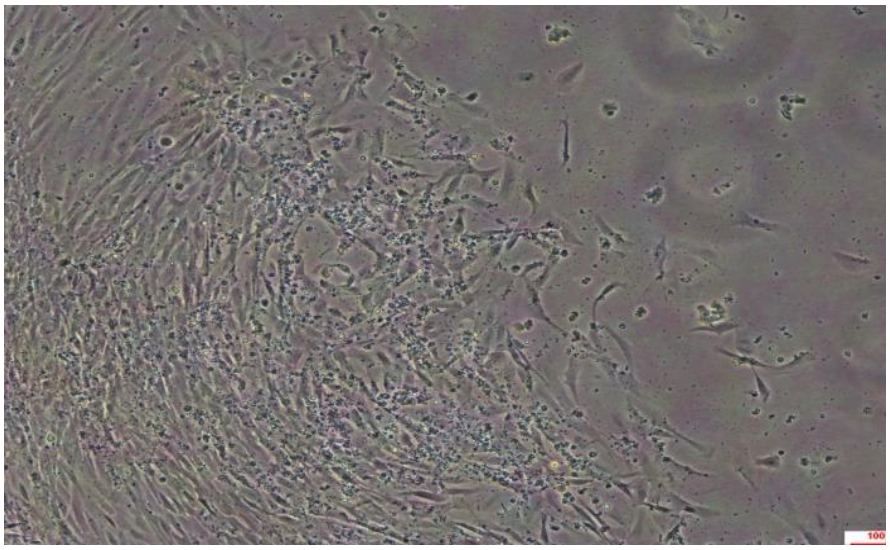

Fig 1: The Radiation Of Cells Started After Four days of Explant Preparation From Caudal fin (at 100X)

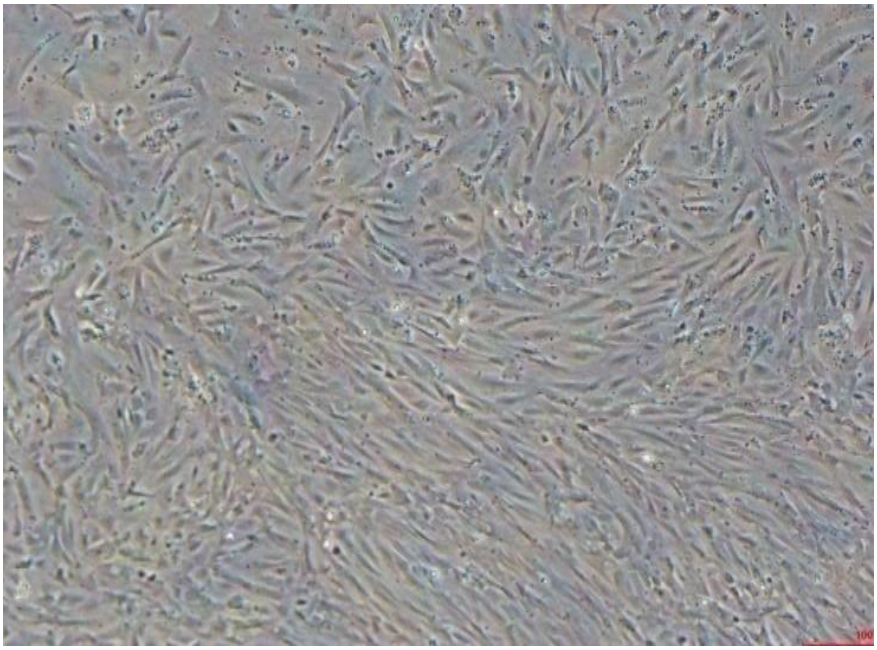

Fig 2: A Confluent Monolayer Covered the Surface Flask the Explants was Observed within Fifteen days which of the cells were ready for subculture (at 100X).

Morphological Observation:
Fibroblast

Subculture and Maintainance:
After 5th passage, fibroblastic cells dominated over epithelial cells resulting in a homogeneous population of a fibroblastic cell. During the first ten subcultures, a blend of 50% each of new and old medium was used as an interval of 5-7 days. In subsequent subcultures, the cell was subcultured in L-15 with 20% FBS at 5-7 days of range. The consistent subculturing of fin cells resulted in the development of a cell line designated as PHCF (*P.Hypopthalmus* Caudal Fin).

The cells radiating from heart and swim bladder explants were round and homogenous with fibroblast-like morphology from the inception. Rougee et al., (2007) [24] also described that the morphology of GFSB cells obtained from swim bladder of Carassius auratus, remained consistently fibroblastic from their initial inception to the subsequent passage, while Lakra et al., (2010a) and Lai et al., (2003) [19, 21] described epithelial-like morphology of cells derived from *Labeo rohita* and *Epinephelus awoara* swim bladder respectively. In the process of subsequent passaging, the cells of fin and heart are successfully subcultured upto 68 and 53 passages which emerge as cell lines. These cell

lines were designated as TTCF for fin cell line and TTH for heart cell line.

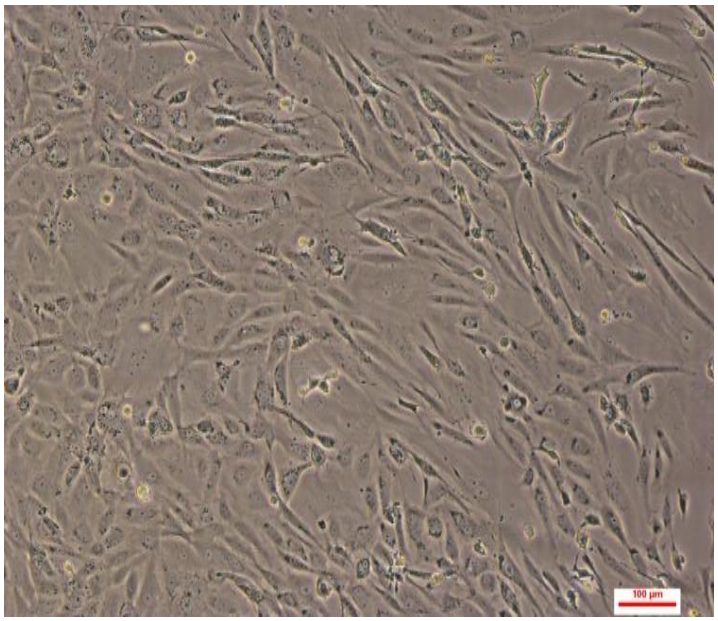

Fig 3: Subcultured 5th Cells look like Epithelial and Fibroblast (at 100X)

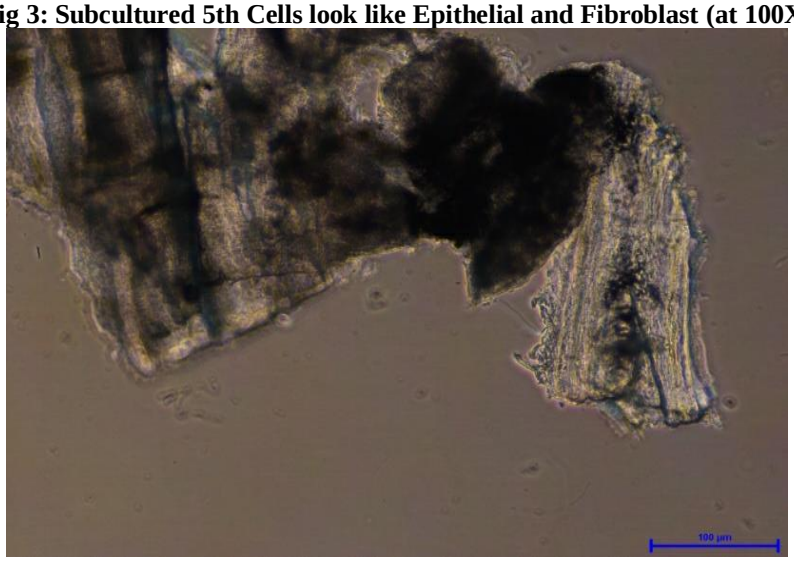

Fig 4: Passaged 10th Cells look like Fibroblast (at 100X)

CONCLUSION:

Nowadays year cell culture finds much application in the different area of research, including cancer research, gene therapy, tissue or organ replacement, etc. cell culture, in particular, those derived from fish, have been successfully employed as a biological alternative to the use of the whole animal. The increasing use and importance of fish cell lines suggest that cell culturists should be encouraged to place these lines with the international cell repositories like ATCC, EACC or another appropriate repository to provide a dependable, high-quality source of cells for the benefits of all. To further ensure the use of authenticated cell lines, full cell line documentation, including the source and passage numbers used during experiments and should be considered as standard research reagents and given the same care and quality control measures that surround the use of kits, enzymes, and other laboratory products commercially obtained.

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