Development and characterization of a gill cell line from striped catfish, *Pangasianodon hypophthalmus*, Sauvage (1878)

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Abstract

Cell lines as an in vitro model developed from different target organs of fish find its use in virus susceptibility, cytotoxicity, gene expression studies etc. The striped catfish, *Pangasianodon hypophthalmus* is one of the main species in Asian aquaculture, especially in countries like Thailand, Indonesia, China, India, Bangladesh and Vietnam. The present study reports the development of a permanent cell line from gill of *P. hypophthalmus* designated as PHG and its application in toxicological research. Leibovitz's-15 (L-15) cell culture medium supplemented with 15% fetal bovine serum (FBS) was used for the maintenance of cell line PHG. The morphology of the PHG cell line was fibroblastic in nature. PHG cells grew well at varied temperatures ranging from 24 to 300C with an optimum temperature of 280C. The PHG cell line was characterized using sequence of mitochondrial cytochrome C oxidase subunit I, which authenticated the species of origin of the cell line. The cell line was transfected with pEGFP-C1 plasmid and transfection reporter gene was successfully expressed 48 hr post-transfection with 9% transfection efficiency. The toxicity assessment of two organophosphate pesticides, chlorpyrifos and malathion using PHG cell line revealed that the two organophosphate pesticides were cytotoxic to the cell line at varied concentrations.

Keywords Cell line; Gill; *Pangasianodon hypopthalmus*; Transfection. *Accepted on September 04, 2021*

Introduction

Cell line is one of the widely used tools for carrying out in vitro research in virology, toxicology carcinogenesis and transgenesis. Piscine cell lines have emerged as important model systems in embryology, neurobiology, endocrinology and environmental biology. Further, cell culture systems can also be used to study and identify new biomarkers and also in the drug development process [1]. Several cell lines have been developed from different kind of fishes and a wide range of tissues such as ovary, fin, swim bladder, heart, spleen, liver, eye muscle, vertebrae, brain, and skin. The first permanent fish cell line was originated from the gonad of rainbow trout, *Salmo gairdneri* designated as RTG-2[60]. Recently 551 fish cell lines globally have been made available for the research community.

Pangasianodon hypophthalmus commonly called as striped or sutchi catfish belongs to the class Actinopterygii of order Siluriformes and family *Pangasiidae. P. hypophthalmus* is indigenous to South-East Asian countries namely Thailand and Vietnam. It is a fast growing fish and its potential in terms of production and export has been greatly increasing. It has got its own demand because of its white flesh, low price and flavour. In Indian scenario the production of *P. hypophthalmus* is practised intensively in monoculture as well as in polyculture with Indian Major Carps. Due to the intensive culture practice, channel catfish viral disease and number of bacterial diseases such as bacillary necrosis, Icthyophthiriasis, epitheliocystis have been reported in this species. Hence developing cell line from this species will serve as an in vitro model for studying the pathogenesis of the virus and bacteria.

Piscine cell lines developed from the gill tissue aid in assessing the acute toxicity of aquatic pollutants . Cell line originated from the gill tissues also utilised as valuable, rapid, and cost-effective tool for evaluation of environmental samples [2]. Many piscine cell lines have been used for the assessment of heavy metals, organophosphate pesticides, nanoplastics and simultaneously comparing both in vitro and in vivo systems. The practise of cell lines-based cytotoxicity assessment of aquatic pollutants also replaces the use of animal model. In vitro data generated from the toxicity assessment after exposure of heavy metals have shown significant correlation with in vivo acute cytotoxicity and also it can be correlated with the effect on human physiology and health. This study was aimed to develop cell line from *P. hypophthalmus* which would be an in vitro tool for studying the cytotoxicity of organophosphates pesticide. This paper reports the development and cytotoxicity characterization of PHG cell line from gill tissue of *P. hypophthalmus*.

Materials and Methods

Ethic statement

The live experimental animals were acquired from the ornamental fish farm (Shark point aquarium, Kurla, Mumbai). Since, the present study is on the development of cell line, the study does not need an ethical approval.

Experimental animal

Healthy fingerlings of *P. hypophthalmus* were maintained in the Central Wet Laboratory, Central Institute of Fisheries Education. Fingerlings (15 to 20 g) were transported live to the Fish Genetics and Breeding laboratory, and maintained in sterile, aerated water before explant preparation. Donor fish (P. *hypophthalmus)* was then sacrificed by euthanization using ice-cold water containing 1000 IU/ml penicillin and 1000 µg/ml streptomycin, dipped in 5% Sodium hypochlorite for 5 minutes and wiped with 70% alcohol.

Primary culture

Aseptically dissected gill samples were washed with PBS (phosphate buffer saline) supplemented with 500 IU/ml penicillin, 500 µg/ml Amphotericin and 2.5 mg/ml Fungizone (Hyclone, USA). The tissues were minced into small pieces; explants of 1mm3 sizes were prepared and washed thrice with PBS containing antibiotics (Hyclone, USA).Then the minced explants were seeded into 25 cm2 cell culture flasks (Tarsons, India). Adherence of explants was accomplished by the addition of 200 µl of fetal bovine serum (FBS) (Gibco, USA) and incubated at 28C overnight and after that Leibovitz-15 medium (Hi-Media, India) supplemented with 20% FBS.

Subculture of monolayers

Primary cultures upon reaching 70-90% confluency, the cells were harvested using TPVG solution (0.1% trypsin) (Thermo scientific) and 0.2% ethylenediaminetetraacetic acid (EDTA). The concentration of the FBS for primary cultures was initially 20% and then reduced to 15% as the culture progress. The adherence of explants, proliferation of cells from the adhered explants, and contamination of flasks were observed regularly using an inverted microscope (Nikon, Japan).

Growth studies

The optimum temperature for the growth of the PHG cells were determined by incubating at varying temperature of 20, 24, 28 and 32 C over seven days at seeding concentration of 1x105 cells in 25 cm2 culture flasks. Two flasks from different temperatures at which they were incubated, were trypsinized and cell counting was performed using a haemocytometer on an alternate day. Similar procedures were performed for the effects of varied concentrations of FBS (10, 15, and 20 %) on cell growth at 28 C over seven days.

Species authentication by PCR amplification

Genomic DNA from the PHG cell line and the gill tissue of P. hypophthalmus were isolated following [35] with minor modifications. The detached cells were suspended with 2 ml PBS and then transferred to a centrifuge tube and centrifuged at 8000 rpm for 8 minutes. For lysis of the cells, 500 µl of TEN buffer, 50 µl of 10% SDS and five µl of proteinase K were added, homogenised and incubated at 55 C for 1 hour. After cell lysis, the digested samples were deproteinized by successive Phenol: chloroform-isoamyl alcohol and DNA was recovered by an ethanol wash. Eventually, the DNA pellets recovered from the ethanol wash were air dried and suspended in TE buffer and stored at -20°C for further use.

Mitochondrial COI region (Cytochrome C oxidase sub unit I) was amplified using universal pair of primersFish F25'TCGACTAATCATAAAGATATCGGCAC3' and FishR2 5'

ACTTCAGGGTGACCGAAGAATCAGAA 3. The mitochondrial gene cytochrome C oxidase subunit I (COI) was amplified in a 12.5 µl reaction volume with 1.25 µl of 10X Taq polymerase buffer, 0.25 µl of each dNTP (0.05 mM), 0.5µl of each primer (0.01 mM), 0.125 µl of Taq polymerase and 0.5µl of genomic DNA (50-100 ng /µl). The thermal cycler Agilent, Sure cycler, 8800, USA was used for PCR amplification. Thermal regime and the cycle parameters involved an initial denaturation step at 94 C for 3 min followed by 35 cycles of denaturation at 94 C for 30 sec, annealing at 54 C for 30 sec, an extension at 72 C for 1 min, with a final extension of 72 ºC for 7 min. Amplified products were analyzed in 1.0% agarose gel containing ethidium bromide and visualized with a UV transilluminator and the amplified products after purification were subjected to sequencing by the external sequencing facility (Xcelris, Labs, Ltd). The obtained forward and reverse DNA sequences were aligned and compared with the standard sequences of P. hypophthalmus voucher specimen in Genbank, NCBI using Basic Local Alignment Search Tool (BLAST).

Transfection

70-90% confluent cells at 10th passage were trypsinized from Flask and then seeded at a density of 1 x 105 in a six well plate individually at 28 C normal atmospheric incubator. The culture medium was aspirated from each well, and the cells were gently rinsed twice with PBS and supplemented with 400 µl of fresh L-15 medium devoid of serum and antibiotics. The plasmid DNA pEGFP-C1 with a concentration of 200 ng was suspended in 100 µl of Opti- MEM and 0.5µl of plus reagent and the mixture was incubated for 5 minutes at room temperature. The Opti-MEM and plus reagent mixture containing plasmid DNA was further added with transfecting reagent Lipofectamine LTX and incubated for 30 min at room temperature. The mixture was added dropwise to each well containing cells. Then the cells were incubated overnight at 28ºC in the BOD incubator and tested for transgene expression. Then the old medium was changed with fresh medium after 4-6 hours. The cells were examined under a fluorescence microscope for expression of green fluorescence signals. Transfection efficiency was determined by the percentage of the fluorescence protein-positive cells to the number of viable cells 24~48 h after the start of transfection [3].

Pesticide Exposure

84 wells of a 96-well microplate (Nunc) were seeded with 100 µl of cell suspension containing 1.5 X 105 cells/ml. This seeding density was found to be optimal to achieve the desired confluency (following 48 h in culture) for the cell lines. Instead of complete L-15 medium containing FBS, test chemicals were

prepared in L-15/ex solution to reduce the effects of metal binding to serum proteins. Cells were exposed to chemicals in a range of concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 mg/l) based on preliminary results obtained for different range of concentrations on the cell line. 12 wells containing cells with medium only were used as negative control in 96 well plates. All the pesticides were tested in six replicates per microplate. Chemical exposure was conducted for 24 h on PHG cell line. After this incubation period, morphological damage was evaluated in comparison to the negative controls. The test medium was aspirated off; cell monolayers were washed with phosphate buffered saline (PBS) and prepared for the cytotoxicity assays.

Cytotoxicity Assays

Cytotoxicity of pesticides was determined by MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) reduction and neutral red uptake assay. The cytotoxicity assays used in this study were performed in three independent replicates with six replicates for each pesticide concentration.

MTT Reduction Assay

The MTT reduction assay is used for determination of cell viability by measuring the reduction of the yellow colour water soluble Tetrazolium dye 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to formazan crystals by mitochondrial lactate dehydrogenase produced by live cells. These formazan crystals, exhibits purple color upon dissolution. Following 24 h exposure to the selected pesticides, the media was aspirated off and fresh L-15/ex solution was added to each well for 4 h. 10µL of MTT solution (5 mg/ml) was then added in each well and the plates were incubated for 4 h at 28 °C. Following incubation, the MTT containing medium was aspirated off from the microtiter plate and the intracellular formazan crystals were extracted and solubilized in solubilization buffer. The plates were gently shaken for 10 min and the absorbance was recorded with the help of ELISA reader (SunriseTM, Tecan) at 570 nm wavelength.

Neutral Red (NR) Uptake Assay

Viable cells accumulate NR (3-amino-7-dimethylamino-2-methylphenzine hydrochloride) in lysosomes. Following 24 h exposure to the selected pesticides, media was aspirated off and the cells were incubated with 100 µl of 33 µg/ml of neutral red solution prepared in L-15/ex solution at 28oC for 2 h. The cells were then washed with NR fixative solution (0.5 % (v/v) formaldehyde and 1 % (w/v) CaCl2 in deionized distilled water). 100 µl/well of NR extraction solution (1 % (v/v), acetic acid and 50 % (v/v) ethanol in deionized distilled water) was then added to solubilize the lysosomal neutral red. The plates were gently shaken for 10 minutes and the absorbance was recorded with the help of ELISA reader (SunriseTM, Tecan) at 530 nm wavelength.

Statistical Analysis

Experiments were performed in triplicates with six replicates for each exposure concentration. Data was analyzed with Graph Pad Prism 5. The individual data points of the concentration–response cytotoxicity charts are presented as the arithmetic mean percent inhibition relative to the control ± standard deviation (S.D.). Statistical analysis of data was carried out using oneway ANOVA ($p \leq 0.05$) followed by Dunnett's multiple comparison test ($p \leq 0.05$).

Results

In the current study, primary cultures from the gill tissues were developed by using explant outgrowth technique. Many continuous cell lines have been developed by using explant technique because adherence of cells to the culture flask for formation of monolayer can be achieved greatly by the explant technique. Primary cultures derived from the gill explants revealed that all the explants prepared from gill were attached properly after 18-24 hrs of explant preparation. The radiation of cells started after 72 hrs of explant preparation and a confluent monolayer around the explants was observed within 7-10 Days In the initial stages, the cells radiating from the primary cultures of PHG consisted of a heterogeneous population i.e., cells comprising of two morphology, epithelial and fibroblast.. After fifth subculture fibroblast- like cells gradually dominated. The consistent subculture of gill cells resulted in the development of a permanent cell line designated as PHG (Figure.1)

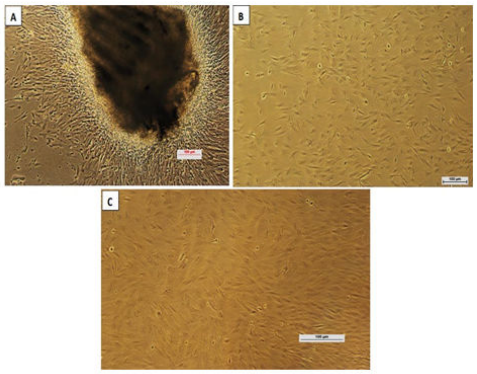

Figure.1 *a) Phase contrast photomicrographs of PHG explant (100X);b) Phase contrast photomicrographs of PHG cells derived from the gill of P. hypophthalmus at passage 5 (X100);c) Phase contrast photomicrographs of PHG cells derived from the gill of P. hypophthalmus at passage 10 (X100).*

In the present study the optimum temperature for the growth of the PHG cells was found to be 28ºC. The cells were capable of proliferating in temperature ranging from 24ºC to 32ºC. Growth of the cells at optimum temperature of 28ºC. The growth of the cells increased as the concentration of the FBS increased from 10 % to 20%. Cells grew reasonably well in the concentration of FBS at 20 % (Figure. 2), in order to meet the cost of serum and optimum growth, the PHG cell line was maintained at 15 % .

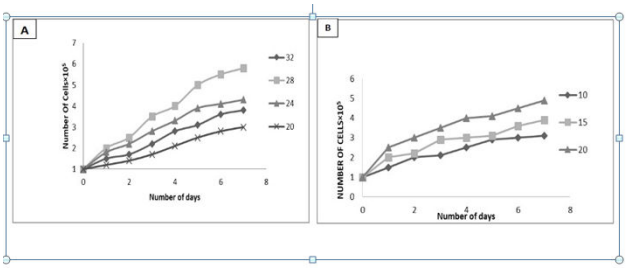

Figure.2 *a). Effect of growth of PHG cell line at different temperatures (ºC); b) Effect of growth of PHG cell line at different concentrations of FBS (percentage).*

The amplification of the mitochondrial region cytochrome C oxidase subunit I (COI) gene from the PHG cell line yielded amplicon size of 655 bp. Sequence alignment of COI gene derived from the PHG cells revealed 99% maximum similarity with the known mitochondrial sequence of P. hypophthalmus voucher specimen and the gene sequences were submitted to GenBank database (Accession number MK116534). PHG cell line at tenth passage was successfully transfected with pEGFP-C1 plasmid using Lipofectamine and plus reagent (Invitrogen). The expression of the pEGFP in the PHG was detected 18 hrs post transfection . The estimated transfection efficiency was 9% this indicated that developed cell line PHG would be useful for in vitro genetic manipulation and transgenic studies. In the present study MTT assay and NR uptake assay were used to assess cytotoxicity of the two organophosphate pesticides, chlorpyrifos and malathion. Morphological appearance of PHG cells was studied and discernible changes were observed between the treated and control cells at the dose of pesticides used for the study. The cell density was reduced as cell damage leading to death and destruction of the exposed monolayer was observed as compared to the control. The cells shrinked in shape and finally dead spherical cells were found floating in the media. Light microscopic inspection in the present study demonstrated a good agreement with the IC50 values obtained with the MTT and NR for the PHG cell line. Twenty-four hours (24 h) exposure of the fish cell line to varying concentrations of two pesticides produced a dose-dependent reduction in the fraction of viable cells. The cytotoxicity effect was found to be statistically significant ($p \leq 0.05$) for the range of pesticide concentration. The dose response curve for each pesticide for the two different cytotoxicity assays are given [4].

The cytotoxicity of pesticides was comparatively analysed on the basis of the calculated IC50 values obtained from MTT and NR assays (Table 1). IC50 values calculated by MTT and NR assays for the chlorpyrifos pesticides were 1.66 ± 0.04 and 1.71 ± 0.85 mg/L respectively.

Similarly, the observed MTT and NR IC50 values for malathion were 5.09 ± 0.72 and 5.67 ± 0.61 mg/L respectively (Table.1). IC50 value, which is the concentration of toxicant required to induce cellular cytotoxicity in 50% of the total cell population revealed that chlorpyrifos was more toxic than malathion.

Table 1. *Cytotoxic effects on cell line after 24 h incubation with two pesticides as quantified with the MTT and NR assays.*

S.No	Pesticides	Exposure Period and endpoint	IC50 (mg/L)
1	Chlorpyrifos	24 h MTT assay	1.661 ± 0.04
		24 h Neutral Red uptake assay	1.715 ± 0.85
2	Malathion	24 h MTT assay	5.086 ± 0.72
		24 h Neutral Red uptake assay	5.668 ± 0.61

Discussion

In this study, the PHG cell line was developed by the explant method. The advantages of the explant technique over trypsinization method are adherent capability, requiring minimal amount of tissues for proliferation and also it has no proteolytic activity on cells that damage cell surface proteins. The noticeable part of the explant technique is the ability to maintain the ECM (Extra cellular matrix) in the presence of companion cells to release the essential growth factors and cytokines which are essential for the attachment of the cells . Many continuous cell lines have been developed using explant technique. During the initial stages, the cells radiating from the primary cultures of PHG consisted of a heterogeneous population i.e., epithelial and fibroblast cells. Such heterogeneous population of cells in the initial cultures were reported by number of researchers Heterogenous cell population i.e presence of both the epithelial and fibroblastic cells in the initial cultures later, predominated into fibroblast morphology. This predomination of fibroblastic cells over the epithelial cells also reported by many other researchers

The optimum growth of the cell line PHG in L-15 medium at the temperature of 28ºC with supplementation of 15% FBS identical with other fish cell lines established The relative growth of the cell line increased as the FBS concentration increased from 10% to 20%. However, 15% FBS concentration was used in order, to meet the maintenance cost of the cell line PHG [5].

The authenticity of the cell line ensures that the established cell lines are devoid of cross contamination, accuracy in scientific research and reproducibility. Mitochondrial sequence analysis microsatellite loci profiling and proteomic approaches are the various techniques practised to authenticate cell lines. Among the available techniques, mitochondrial COI subunit-I gene sequence analysis has been used as a gold standard DNA marker for the species identification. In this study, the authentication of the developed cell line was achieved by partial COI subunit-I gene sequences analysis which yielded an amplicon size of 655bp indicating the developed cell line was from the P. hypophthalmus.

Higher transfection efficiency of 27% was achieved in the PCF cell line and the lowest was only 2% recorded in the CSTF cell line indicating the ability and importance of the cell lines in the gene expression studies. This study reports 9% transfection efficiency of the PHG cell line which is in accordance with the result reported earlier. This reveals the importance of the PHG

cell line in assessment of the promoter efficiency or any plasmid construct for the production of recombinant proteins.

The most commonly employed cytotoxic characterization of an established cell lines are MTT and NR. Inhibition of Neutral Red (NR) uptake into cells based on the accumulation of neutral red in lysosomes of viable cells and the tetrazolium salt reduction (MTT) assay based on the mitochondrial metabolic impairment function were found to be most suitable tests, allowing rapid and reliable assessment. Light microscopic inspection in the present study demonstrated a good agreement with the IC50 values obtained with the MTT and NR for the PHG cell line. These observations can thus be used as an adjunct in verifying the results of cytotoxicity assays.

Conclusion

The main aim of the study was to establish and characterize cell line from the sutchi catfish P. hypophthalmus to study the growth of the cell line in response to varying temperature and growth supplements, species authentication of cell line, gene expression studies and also the present study establishes the ability to use the PHG cell line as an in vitro tool for the assessment of the cytotoxicity of two organophosphate pesticides, chlorpyrifos and malathion. In addition to that, testing of other aquatic pollutants would also further increases the efficacy of the PHG cell line as a potential in vitro model for cytotoxicity testing.

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Author Contributions

AS- Benchwork, Manuscript writing

MG- Overall guidance, Designing of experimental setup

NN- Guidance in characterization of cell line

GB- Guidance in transfection

DD- Guidance in cytotoxicity assays

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