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The Establishment of a Fibroblastic Cell Line from Caudal Tissue of *Poecilia reticulata*, Peters 1859

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Authors' contributions

This work was carried out in collaboration between both authors. Author BG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SIÜ managed the analyses of the study and literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Establishment of cell lines from different tissues of different teleost fishes has remarkably increased. Development of fish cell lines has significantly contributed to recent advances in many research areas. *Poecilia reticulata* also known as guppy is a teleost fish which is widely used for ecotoxicological research. In this study to isolate fibroblast cell from caudal tissue of *P. reticulata* and to develop a cell line. For this purpose caudal tissue samples were collected from ten male *P. reticulata* under aseptic conditions. Primary culture was performed with tissue explant technique. Primary cells fed with L15 culture medium supplemented with FBS. To investigate the effects of different incubation temperature on proliferation rate of cells, seven different incubation temperatures (15°C, 20°C, 25°C, 28°C, 30°C, 32°C, 37°C) were assessed. Same experimental design was used for the effects of different FBS concentration (5%, 10%, 15%, and 20%) on proliferation rate of cells. Optimum growth rate was observed at 28°C temperature and 10% FBS concentration. Cells were subcultured succesfuly more than 60 times and this resulted in development of a cell line named as PSF. Cultured cells were also succesfuly cryopreserved. The revival rate of PSF cell line was up to %85-90 and this result could be admitted as a good when compared to other fish cell lines.

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1. INTRODUCTION

In the recent years, establishment of cell lines from different organs or tissues of teleost fishes has remarkably increased. First cell line was reported in 1952 by Wolf and Guimby [1]. Since that time, about 238 cell line have been established from teleost around the World [2]. The development of fish cell culture and cell lines has significantly contributed to recent advances in toxicology [3,4], virology [5] and also cytogenetics [6], biomedical research [7], biotechnology and aquaculture [8], endocrinology [9], fish immunology [10,11], ecotoxicology [12,13,14], disease control [15] and radiation biology [16]. Investigating of environmental pollution and effects of xenobiotics on living organisms, *in vitro* techniques and cell culture methods is widely used for replacement of animals [12,17]. Many cell lines especially isolated from gonads, liver, gills and total embryo tissue of fishes has been used for investigate the effects of various chemicals in cellular level. Fibroblast cells isolated from caudal biopsies of fishes can also be used for that purpose [12].

Poecilia reticulata, also called guppy or milionfish is a world wide distrubuted tropical fish. Because of its smal size, easy acclimation of laboratory conditions, easy maintenance and short live cycle and reproduction, it has become widely used laboratory model for ecotoxicological research [17]. A cell line named as GFT was isolated from fully formed unborn embryos of a female guppy [18], but there is no other cell line has established according to literature [18]. When compared other cultured cells, fibroblast cells can be sampled more easily and provide material for rapid and early genetic screening. Also fibroblast cells yield high quality chromosomes in fish species [19]. Caudal fin is one of the main source of fibroblast tissue and methodologically easy way to isolate fibroblastic cells. The aim of this study was to develop a cell line from caudal fin tissue of guppy and optimize culture conditions.

2. MATERIALS AND METHODS

2.1 Tissue Explant

All experiments have been examined and approved by Animal Experiment Ethics Committee of Ege University. Healthy juveniles (ten male) of *P. reticulata* were obtained local commercial supply and maintained at the Ege University Science Faculty Biology Department Zoology Section, Ecotoxicology laboratory. Fishes were starved for a day and allowed to swim in well-aerated sterile water before the collecting of fin tissue. The specimens were anaesthetized with keeping them on +4°C cold water for 5-7 min. Then surface sterilized by wiped with %70 alcohol.

Primary cell cultures were initiated by collecting caudal fin tissues from the fishes under aseptic conditions. The tissue samples were transferred into phosphate buffered saline supplemented with 10 µg/ml Gentamycin (Gibco). The samples were minced with sterile scissors at room temperature and washed three times with washing buffer.

The minced tissue fragments were seeded into 25 cm^2 tissue culture flasks [TPP, Switzerland] in nearly 1 ml of L15 culture media supplemented with %10 FBS. The explants allowed to attached to the surface of the flask in an incubator at 28°C for 4 h. Care was taken to avoid over drying to the tissue. After observing tissue adherence, the explants were fed with L15 culture media supplemented with %10 FBS.

2.2 Subculture

For proliferation of cells and morphological details, the flasks were observed daily with using inverted microscope (Olympus Tokyo japan). After cells reach %95 confluency they were trypsinized using a trypsin EDTA solution (trypsin 0.25%, EDTA 0.02%) in phosphate buffered saline. The subcultured cells were grown in fresh L15 with %10 FBS.

2.3 Growth Studies

For assessment of growth characteristics of the cell line in L15 media, cells were incubated different incubation temperatures (15°C, 20°C, 25°C, 28°C, 30°C, 32°C, 37°C) for 7 days. On the consecutive days, three flasks from different temperatures at which they were incubated were withdrawn, the cell lines trypsinized and cell counting performed using a hemocytometer. Same procedure were performed for the effects of various concentrations of FBS (5%, 10%, 15%, and 20%) on cell growth at 28°C for 7 days.

2.4 Cryopreservation

To observe the ability of cells to survive in liquid nitrogen and stability of them were assessed in freezing medium using the method that described by Freshney [20]. Logarithmically growing cells were harvested and re-suspended at $3x10^6$ to $4x10^6$ cells ml^{-1} densities. The cell suspensions were carefully mixed with equal volume of recovery medium (Invitrogen) which is suitable complete cryopreservation medium for freezing cells (Invitrogen) at $1x10^6$ cells mL^{-1} according to manufacturer instructions. The recovery medium could increase cell viabilities average of 25% in cryopreservation both adherent and suspension cell lines and it is an optimized fully supplemented formulation which avoids the messy mixing of DMSO. Cell suspensions were aliquoted (1ml) and distributed into 1,5 sterile cryovials (Nunc) held at 4°C for 2 h, -20°C for 2 h and -70°C overnight then transferred into liquid nitrogen (-196°C). The frozen cells were thawed after 6 months of post storage at 37°C water bath. Freezing medium was removed by centrifugation and cells were suspended in L15 with 10% FBS. Cell viability measured by trypan blue staining and number of cells was counted using hemocytometer. Cells were seeded into 25 cm^2 tissue culture flasks for further subculturing.

3. RESULTS AND DISCUSSION

The cell line named as PSF from caudal fin tissue of *P. reticulata* was established by explant technique. The explant technique has many advantages such as speed, easy maintenance of cellular interactions and no enzymatic damage of cell surface proteins over the trypsinization method [21].

For morphological and other examinations, cells were observed by inverted microscope every day during subsequent passages (Fig. 1). Culture medium was changed every 3 day. Initially, fibroblastic and epithelial cells were found at the beginning of the culture, but after 4th passage, fibroblastic cells dominated over the epithelial cells (Fig. 1). PSF cells were predominantly observed as fibroblastic cells at 10^{th} passages (Fig. 1). During early passages, the heterogeneity nature of cells during early passages was also reported by other researchers [22,23,24]. The predomination of fibroblastic cells over epithelial cells in cell cultures from fin tissue of fishes also has been reported before [25,26,27]. A fibroblast like cell line [LJH-2] was developed from *Lateolabrax japonicus* [28]. In contrast cells isolated from heart tissue of sea perch, have been reported to be epithelial morphology with no change during subsequent culture [29]. Cells started to spread after 4-5 days of explant preparation from fins. Confluent monolayer cells were observed 10-12 days of culture (Fig. 1) Cells were subcultured in L15 with %10 FBS at 5-7 day of interval and split at ratio of 1:2. Cells were subcultured more than 60 times regularly and this resulted in development of a cell line named as PSF.

The effects of different FBS concentration on proliferation rate have been examined and shown in Fig. 2. For this aim, four different (5%, 10%, 15%, 20%) FBS concentration have been examined. Relatively, growth rate of cells increased as the FBS portion increased from %5 to %20 at 28°C incubation temperature. However, growth rate was observed as minimum at %5 concentration of FBS and maximum at %10 and 15 FBS. Comparatively fish cell lines have been exhibited good growth at %10 FBS but maximum growth at %15 and %20 FBS concentrations [30,31,28].

For observation of different growth patterns of PSF cell line at different incubation temperatures between 15°C and 37°C was examined and shown in Fig. 3. The minimum growth rate was observed at 15°C incubation temperature. The monolayer detachment was observed 37°C incubated cells. However optimum culture temperature was found to be 28°C. This temperature also reported for other fish cell lines in the literature before [22,32,33,34,35,36,37,24].

As described before, ideal growth temperature for cultured fish cells is a few degrees above environmental living temperature of fish. [38], because of selection or adaptation of cells to growth of higher than normal temperature [39]. For tong et al. [40], temperatures between 35-37°C has been found lethal fort o many fish cells. As seen in the literature, fish cells could be growth at over wide temperature range. This could be an advantage for isolating both warm water and coldwater fish viruses [41].

The cells that grow in medium containing %20 FBS has showed higher proliferation rate than cells in medium containing %5-15 FBS (Fig. 2). The growth rate of the cells increased paralel with FBS concentration from %5 to %20. Nevertheless %10-15 concentration of FBS also

provided good growth. So it would be an advantage to use %10 FBS to maintain the cell line because of low cost. In the literature, relatively good growth of the fish cell observed at %10 FBS but maximum growth also observet at the concentration of %15 to %20 [30,27,28].

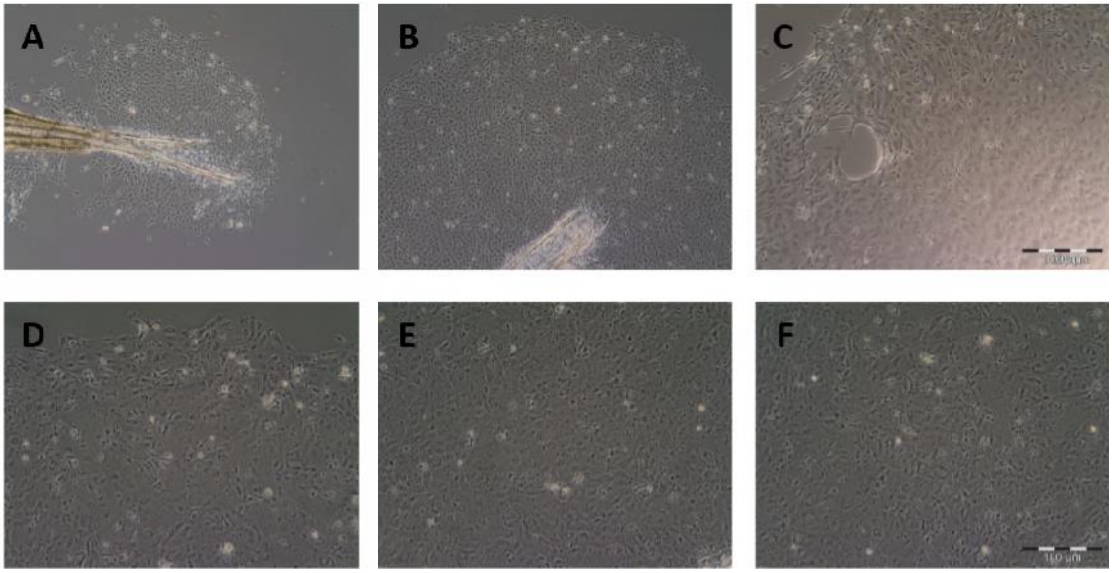

Fig. 1. Microscopic observation of cells, A: 3th day of culture (spreading cells from fin tissue), B: 5th day of culture (proliferating and increasing of cells), C: 10 day of culture (Confluency of cells), D: 4th passage of cells (dominating of fibroblast cells), E: 10th passage of cells (fibroblast cells are become stabilized), F: 60th day of culture (dominated and stabilized fibroblast cells)

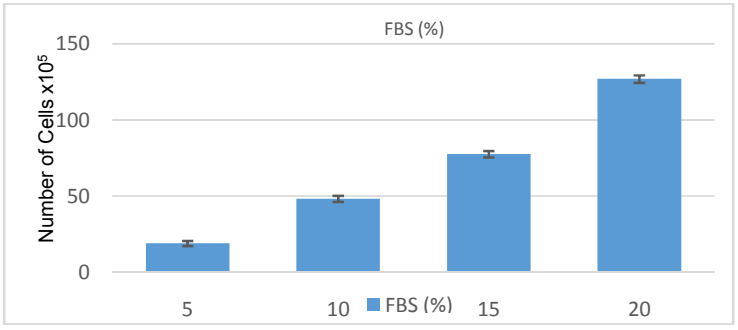

Fig. 2. Proliferation of cells on different FBS concentration

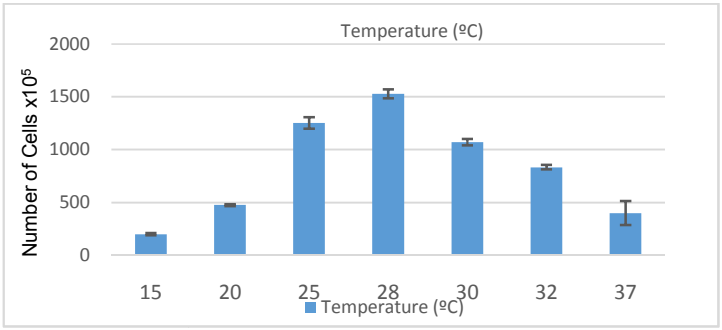

Fig. 3. Proliferation of cells on different incubation temperatures

Protection of cell lines from genetic change and avoid aging and transformation, cryopreservation is needed in long term storage [20]. The PSF cell line freezed in liquid nitrogen and storage during 6 months. When cells were thawed, they showed %85-90 viability and continued to grow and became confluency within 7 days. The revival rate of the PSF cell line was up to %85-90. This result could be admited as a good when compared to that of other fish cell lines reported by Parameswaran et al. [42] who could revive SISE cell succesfully after cryopreservation with the survival rate of %70-80. The GFM and GSFB cell lines showed %93 and %92 survival rate after cryopreservation according to Roughee et al. [43]. The cell line named as MFF-1 from mandarin fish, *Siniperca chuatsi* revived %80-90 after cryopreservation [44]. Cells were cryopreserved and succesfully revived with the survival rates of %75, %70 and %72 for RHi RF and RSB cell lines [2]. Swaminthan et al. [33] succesfully cryopreserved and revived with the survival rate of %70. Cell line named as PSF succesfully cryopreserved and revived with the survival rate of %75 by Lakra and Goswami [31].

4. CONCLUSION

In conclusion, the present study results in the development of cell line PSF from the caudal fin of *P. reticulata*. The development of cell line PSF from *P. reticulata* has not been reported before. Therefore, development of a cell line form tissues of guppy (*P. reticulata*) could open new horizons of *in vitro* research in fish genetics, conservation and fish biotechnology. Also it could further be used for developing cellular models for toxicological, ecotoxicological and genotoxicological studies to replace whole animals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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