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by Gratiana E. Wijayanti

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The Primary culture of caudal fin, gill lamella, hepatopancreas and spleen of *Osteochilus vittatus*

Gratiana E. Wijayanti and Atang

Laboratory of Animal Structure and Development, Biology Faculty, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia

Corresponding author 1.bugrat@gmail.com

Abstract. The cell lines play an important role in the development of basic research and applied research. Most of the available cell lines derived from mammalian tissue and only 5% of cell lines derived from fish tissue. This experimental study was conducted to evaluate the potency of caudal fin, gill lamella, hepatopancreas and spleen explant of the *Osteochilus vittatus* as resources for cell line development. The explants were culture in DMEM/-Ham's 12 with 15mM HEPES supplemented with 5% L-glutamine, 5% penicillin-streptomycin, and 10% Fetal Bovine Serum for 9 days at 25°C. The results showed that the caudal fin, hepatopancreas, spleen and gill filament cell outgrowth formed confluent monolayer. In addition to monolayer cells, floating life cells were found in the culture media of spleen ($1.74x10^6$ cells) and gill lamella ($1.54x10^5$cells). The time needed to reach confluent was significantly different among explant type ($p<0.05$) with the fastest growth was observed in spleen explant culture. These results indicated that the four tested explants have good potency as resources for cell line development in *Osteochilus vittatus*.

1. Introduction

Cell lines are cells had been cultured in many passages while maintaining their stable characters. Animal Cell line plays an important role in the development of basic research as well as applied research. In the basic research, the cell lines are used in evaluating gene expression, metabolic pathway, cell to cell interaction, cell to extra cellular matrix for adhesion or cell migration[1,2]. In applied research, the cell lines are used to isolate and propagate virus [3], virus production, drug development [3], and tissue engineering [4].

Cell lines serve as alternative to overcome the complexity of animal model physiology also overcome the limitation in providing enough number of animal model for experimental research. This has triggered many researches to develop various cell lines. By 2010, the American Type Culture Collection (ATCC) recorded there were 3400 cell lines available, yet only 43 cell lines developed from aquatic animals and only 17 of them were used for research[5]. The development of fish-origin cell line was initiated by Wolf and Quimby [6] using the gonad of *Salmo gairdneri*. Other tissues had also been cultured such as skin, gill lamella [7], cardia, liver, kidney, spleen, swim bladder and brain [5]. Considering the plasticity of fish, more and more cell lines are needed.

One of the proposed fish model is the bony lip barb (*Osteochilus vittatus*). This species had been studied as animal model in our laboratory since 1995. Preliminary researches showed that cells originated from dissociated hepatopancreas tissue cultured in DMEM supplemented with either autologous or heterologous serum could be maintained up to three passages[8,9].

This study was conducted to evaluate the potency of other hard-lipped barb tissues as other resources to develop cell line. The first step in achieving this goal is conducting primary culture. In this study, the primary culture was conducted using fragment of caudal fin, gill lamella, hepatopancreas, and spleen.

2. Materials and Method

2.1. Experimental design

A complete randomized design was applied in this study. The types of explants serve as independent variable while cell confluence and cell viability serve as dependent variables; 6 explants of each type were provided as replicates.

2.2. Explant Preparation

The explants were obtained from adult female bony lip barb weight of 100g and 20 cm length. The fish were purchased from the local farmer. Upon arrival the fish were kept in aquaria with clean water then were disinfected by 70% ethanol swab. The fish were immobilized in cold water then were decapitated according to routine laboratory protocol. The caudal fin, gill lamella, hepatopancreas, and spleen were aseptically excised then were placed on a sterile petri dish containing a handling medium consisted of DMEM/-Ham's 12 with 15mM HEPES (Capricorn Scientific) and 5% penicillin-streptomycin (Sigma, #P4333). The fragments were washed three times then were cut into 3mm using sterile scalpel blade for explants culture.

2.3. Explant Culture

The explants were cultured in Corning 35mm petri dish containing 1ml of DMEM/Ham's 12 with 15mM HEPES supplemented with 5% L-glutamine (Sigma, #G7513), 5% penicillin-streptomycin, and 10% Fetal Bovine Serum (Sigma, #F0804). On the second day, when the explants have attached to the growth area, another 1ml culture media was added to each petri dish. The explants were culture at 25ºC with saturated humidity for 9 days. The media were refreshed every 3 days, the replaced media were collected into sterile 15ml conical tubes. The explants were evaluated daily under a light microscope for the presence of outgrowth, the confluency and cell morphology.

The area of cells outgrowth were measured using ocular graticule. The confluency was determined using the following formula:

$$Confluency = \frac{\text{area covered by cells outgrowth}}{\text{growth area}} x\ 100\%$$

Due to the presence of refractive clump from the spleen and gill culture, the media containing the floating clump cells were collected for morphological examination and calculation of cell viability and cell density. The media was put in a 15ml conical tube then was centrifuged at 300 g force. Upon discarding the media, the cell pellet was resuspended in D-PBS.

The cells viability and cell density was determined manually using Neaubauer hemocytometer, 50μl of trypan blue was added to 50 μl cell suspension then was mixed by pipetting. The cell suspension was loaded to the counting chamber. The number of living cells and dead cells on 5 center counting chambers were counted. The cell density and cell viability were determined using the following formula.

$$Cell\ density = \text{Average number of cells from 5 square } x \text{ dilution } x\ 10.000 \text{ cells/ml}$$

$$Cell\ viability = \frac{\text{number of living cells}}{\text{number of all counted cells}} x\ 100\%$$

2.3. Data Analysis

The cell confluence and cell viability data were examined for normality using Kolmogorov-Smirnov and homogeneity using Levene's test then were analyzed using one way Anova.

3. **Results**

Evaluation at 24 hours of culture showed that all of the explants have attached to the growth surface area, no sign of outgrowth was observed from caudal fin, gill lamella nor hepatopancreas explants but there was a significant amount of outgrowth from the spleen explants (Figure 1).

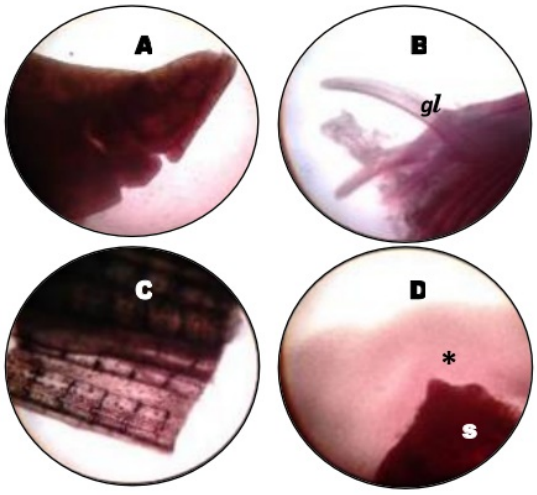

Figure 1. Photomicrograph of explants morphology cultured in DMEM/-Ham's 12 with 15mM HEPES supplemented with 5% L-glutamine, 5% penicillin-streptomycin, and 10% Fetal Bovine Serum for 24 hours. A. hepatopancreas, B. gill lamella, C. caudal fin, D. spleen; *gl*. gill lamella. s. explant. Magnification 40x

3.1. The caudal fin explants.

At 24 hours of culture the caudal fin explants showed a thin layer of white tissue sprouted from certain edge of the cut fragment. This tissue was resembled the wound epidermis in *in vivo* fin regeneration. At day 3, the white tissue has extended outward and at day 5 the cells outgrowth were spread over the growth area. The cells were relatively homogenous in shape. At day 9 the cells out growth have confluent. The cells showed several morphological features and size. Some cells have spherical shape with three size categories large, medium and small. The medium size spherical cells have obvious nucleus. Some cells were ovoid with prominent ovoid nucleus, their size were comparable to the medium size spherical cells. Others cells showed fibroblast-like morphology. Some cells of the proximal outgrowth were pigmented in yellowish brown and some black (Figure 2.A).

3.2. Hepatopancreas explants

At 24 hours of culture the explants have attached to the growth area. At day 3, some clear cells sprout out from the surface of the explants and cells outgrowth were identified surrounding the explants. At day 5, the cells outgrowth expanded on the growth area. At day 9, the cell outgrowth showed epithelial-like characterized by polygonal shape and fibroblast-like feature characterized by their bipolar shape (Figure 2.B).

3.3. Spleen explants

At 24 hours of culture, the spleen explants have attached to the growth area and the cells outgrowth with homogenous shape was very obvious. At day 3 of culture the cell outgrowth expanded and at day 5 the cells have confluent. The outgrowth cells of the spleen have spherical and oval shape. The oval cells has distinct oval nucleus similar to the fish erythrocyte. In addition to the adherent cells, there were some floating cells appear in clumps (Figure 2.C). At day 9, the number of floating cells was 1.74×10^6 with cell viability of 90.3%.

3.4. Gill explants

At 24 hours of culture the gill explants have attached to the growth area. The branchial vein and artery were visible. At day 5, some cells formed clump and floated in the medium. The floating cells

were refractive indicating the cells were alive. At day 9, the amount of the floating cells was $1.54x10^5$ with cell viability of 90,8%. The adherent cells showed several morphological features, some cells have epithelial-like feature, some have spherical shape and the other cells showed fibroblast-like feature (Figure 2.D).

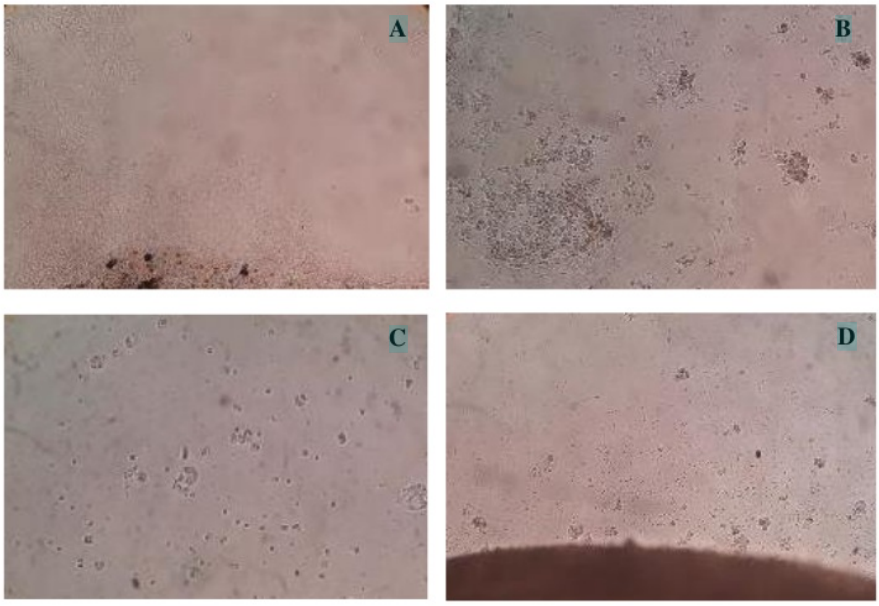

Figure 2. Photomicrograph showing morphology of cells derived from A. caudal fin, B. hepatopancreas, C. spleen, and D. gill lamella explants cultured in DMEM/-Ham's 12 with 15mM HEPES supplemented with 5% L-glutamine, 5% penicillin-streptomycin, and 10% Fetal Bovine Serum for 9 days. Magnification 400 x

Caudal fin cells culture had been conducted in *Danio rerio* [10] and *Cyprinus carpio* [11]. The common carp fin explant cultured at 28ºC in FBS for 8 hours followed by L-15 medium supplemented with 20%FBS and 10ng/ml bFGF showed fibroblast-like and epithelial-like cell type in the initial growth then gradually changed into epithelial-like cell type. A monolayer was form 3 weeks after seeding the explant[11]. In the current study several type of cells were observed, the differences type of cells might be due to the medium used and the supplement added to the media which support the survival and growth of different type of cells. The times needed to achieve confluent were not comparable between the two species since the culture condition and the size of growth area were different.

Preliminary culture of bony lip hepatopancreas cell had been initiated using heterogenous cell suspension from dissociated hepatopancreas [8,9]. In these cultures, various cell types were observed even after several passages [8,9]. Therefore in this study hepatopancreas explants were cultured since several study showed that explant culture produce more homogenous cells [12]. In the current study, the cells outgrowth form a monolayer mostly consisted of epithelial-like and fibroblast-like cells. This result suggests that explant culture is a privilege in obtaining more homogenous cell type.

During spleen culture, some of the outgrowth cells form clamp composed of spherical cell and sometimes float in the media and some cells were attached to the growth surface area. The spleen contains two groups of cells, the hematopoietic and non-hematopoietic tissue. It is possible that the floating cells were the originated from the hematopoietic region of the spleen while the adherent cells

were originated from the non-hematopoietic region of the spleen as evidence in the rainbow trout [13].

Gill primary culture had been conducted in Atlantic salmon in which the gill explants were culture in $L15^{3+}$ medium at 15°C. Under such condition, the cells outgrowth with varying morphology formed a confluent monolayer after two weeks of culture [14]. In Atlantic salmon gill primary culture no floating cells was reported. In the current study, some floating cells were observed in gill explants culture. Initially these cells were thought as dead cells, however due to their refractive appearance these cells were subjected for cell viability test using trypan blue. The results confirmed that those cells were alive with cell viability of 90%. The cells were spherical in shape with diameter of 12-15μm. These cells have not been characterized. The gill lamella is covered by epithelial cells [15] which commonly form a monolayer. Studies across fish species including common carp (*Cyprinus carpio*), a closed related species to the bony lip barb used in the current study, showed the presence of gill-associated lymphoid tissue (GIALT), this structure contains a large number of lymphocytes cell [15]. It is interesting to evaluate if the floating cells seen in the gill lamella culture are lymphocytes.

The presence of cells outgrowth and achievement of cells confluence from all of the cultured explants suggesting that the culture condition and the media used in this experiment is suitable to support the survival of the explants and to stimulate cells proliferation. The time needed to achieved confluent monolayer was significantly different among explants ($p<0.05$) with spleen explant is the fastest to achieve confluent. Based on these results, it could be concluded that the caudal fin, gill lamella, hepatopancreas and spleen explant culture of the *Osteochilus vittatus* are potential resources for cell line development. The follow up of this finding is currently underway by passaging the harvest cells from the primary culture.

Acknowledgement

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