The primary culture and subculture of kidney-derived cells of the bony lip barb fish (*Osteochilus vittatus*) using different concentrations of serum

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Abstract. Cell and tissue culture have advanced and developed in a short time yet continuous improvement needs to be conducted for better results. This research was conducted to obtain suitable culture conditions of kidney-derived cell culture from bony lip barb kidney (*Osteochilus vittatus*). The development of primary cell culture from the kidney fragments was performed in a cell culture medium composed of DMEM supplemented with various concentrations (0%, 5%, 10%, and 15%) of Fetal Bovine Serum, Penicillin-Streptomycin, and 2Mm L-Glutamine, and incubated at 29°C with 0.8% CO_2. The results showed that the primary cell culture achieved confluence on day four and subsequently subculture. The addition of 10% serum increased cell density and cell survival. The cell morphological evaluation using a phase-contrast microscope showed two dominant cell types for both primary and subcultures, i.e. erythrocytes and small-rounded cells. The highest cell yield was obtained with the addition of 10% serum concentration with Population Doubling Time (PDT) and Population Doubling Level (PDL) for 69 h 23 min and 0.47 times, respectively. Statistical analysis indicated that serum addition significantly increased cell density of the primary culture ($p<0.05$). A better proportion of serum supplementation to culturing success is essential to establish an auspicious cell line.

Keywords: cell culture, kidney, Osteochillus vittatus, serum concentration, subculture

1. Introduction

Fish-derived cell has become an emerging model in studying various aspects of science. Information and scientific data obtained from the cell culture system can be used to get insight into various biological process such as reproduction, growth, disease, [1, 2] and provide tool in many research fields including immunology, biotechnology, ecotoxicology [3]. In addition to its wide range of usage, cell culture getting more preferences because cells can be genetically manipulated, quantified, and characterized using molecular techniques and can be cryopreserved and revived for future applications [4].

The type of cell culture used in research could be a primary culture or established culture. The primary cultures are biologically and physiologically closer to the host, therefore they are appropriate for in vivo model [5] and potentially used as substitute or replacement for the living fish. Established cells have advantage in term of generation and maintenance. Now days there are a number of fish-derived cell have been developed and commercially available. There are 13 fish cell line derived from muscle tissue of 11 species of teleost [6], and at least 30 fish cell lines listed in American Type Culture Collection (ATCC), and European Collection of Authenticated Cell Cultures (ECACC) [3]. Amongst those fish cell lines, there is a kidney derived cell line i.e. ASK (CRL-2747) which is derived from Atlantic salmon (*Salmon salar*).

The primary cultures of the fish kidney have been studied in several species including the spotted scat

(*Scatophagus argus*) [7]. The spotted scat kidney cells (SK cells), were cultured in L-15 media supplemented with 10% FBS and were maintained at 28C. Kidney-derived cells of freshwater grass carp (*Ctenopharyngodon idella*) were cultured in TC199 medium supplemented with 10% FBS [8]. Our preliminary study showed that the primary culture of *Osteochilus vittatus* kidney have been successfully cultured in DMEM with 5% L-glutamine (Sigma, #G7513), 5% penicillin-streptomycin, and 10% heterologous and autologous sera [9]. In the culture medium, FBS is known as the best supplements to be used, as it is mass produced and contains many known and unknown growth factors [10]. FBS also promotes cell growth and growth factors, such as EGF and HGF, which are necessary for cell growth [11]. The hepatocytes of Klunzinger's mullet (*Liza klunzingeri*) were cultured in FBS free medium did not adhere to culture plate, which indicate that FBS also important to promote cell adhesion and proliferation [12]. However, a very high concentration of FBS may hinder cell growth [13].
Several studies suggested that the proportion of serum in the culture media could affect cell growth and survival. This study was conducted to determine the optimum serum concentration to support kidney-derive cell culture in *Octeochilus vittatus*.

2. Materials and Methods

2.1. Experimental Design
This study was conducted according to a completely randomized design. The independent variable was serum concentration consisted of 5%, 10%, and 15%. 9 replicates were provided for each treatment. The dependent variables were cell density and cell viability.

2.2. Ethics statement
The present study had been approved by the Medical Research Ethics Committee of the Faculty of Medicine, Jenderal Soedirman University under the registration number of 021/KEPK/PE/XII/2021. All the experiments were carried out according to the "Declaration of Helsinki" (2008).

2.3. Media preparation
Three different media compositions were used in this study consisted of handling medium, washing medium and culture medium. The handling medium composed of DMEM with 5% antibiotic, washing medium composed of DMEM with 5% antibiotic and 10% FBS, and the cultured culture medium composed of DMEM Low Glucose (1 g/L) supplemented with serum (5-15%), antibiotic (5%), and L-glutamine (5%).

2.4. 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 corpus kidney was aseptically excised then were placed on a sterile petri dish containing a handling medium consisted of DMEM Low Glucose (1 g/L) (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 [14].

2.5. Primary cell culture
The explants were cultured in Corning 35mm petri dish containing 1mL supplemented with 5% L-glutamine (Sigma, #G7513), 5% penicillin-streptomycin, and 5%, 10%, or 15% 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 [14]. The explants were cultured at 29ºC with saturated humidity and 0.8% CO_2 until the cell outgrowth has reached 80% confluence.
Upon reaching confluence, the medium was removed from the culture dish and put into sterile microcentrifuge tube. The remnant of the fragment was removed then the cell outgrowth forming monolayer was washed in D-PBS (Phosphate-buffered saline). The cells were detached from the substrate by adding 0.25% Trypsin EDTA. Cell detachment was monitor under a light microscope.

Once the cells have detached, the cells were transferred into sterile microcentrifuge. The tubes were centrifuged at 300g force for 5 minutes. The supernatant was removed, the cell pellet from the medium was resuspended in 1mL culture medium, while the cell pellet from monolayer was washed three times using medium containing 10% of FBS. The cell yield was examined in a hemocytometer to calculate cell density and cell viability.
The cells viability and cells density was determined manually using Neaubauer hemocytometer, 20µl of trypan blue was added to 20 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 centers counting chambers were counted. The cell density and cell viability were determined using the the formula presented in point 2.8.

2.6. Sub-Culture

The cells obtained from the primary culture were subsequently sub-cultured in 24-well plates containing culture medium as used in the primary culture. The cells obtained from the medium were cultured separately from those monolayer-origin. The cells were plated at initial density of 10^6 cells/mL. The cells were cultured for 3 days at 29ºC with 0.8% CO_2 and saturated humidity.
The culture condition was monitored daily by observing the temperature, chamber humidity, CO_2 concentration, and color of the media. The microscopic evaluation was conducted to evaluate the presence of cell outgrowth, cells attachment, confluency, and the presence of contamination. The culture showing any sign of contamination were discharged after decontamination.

2.7. Culture evaluation

The cell morphology evaluated using a phase-contrast microscope with unopened culture dishes. The observation was conducted with 100x and 400x magnification. Some microphotographs were taken for documentation.

2.8. Data analysis

The cell density and cell viability were determined using the following equation:

$$\text{Density} = \frac{X \times 2.5 \times 10^5 \times \text{df}}{\text{mL}}$$

$$\text{Viability} = \frac{\Sigma \text{ living cells}}{\Sigma \text{ all cells}} \times 100\%$$

The data in form of cell density and cell viability were subjected to normality test using Kolmogorov-Smirnov and homogeneity test using Levene's test, followed by one-way ANOVA with p-value < 0.05.

3. Result and Discussion

3.1. Serum Concentration

The results of primary cell culture showed that the cell density was ranged from 3.13 x 10^6/mL to 7.46 x 10^6/mL. The cell viability was ranged from 72.5%±11.72% to 83.55%±9.82%. Meanwhile, the cell density of sub culture was ranged from 1.56 x 10^6/mL to 3.11 x 10^6/mL. The cell viability of sub-culture was ranged from 66%±7.99% to 79.87%±7.51%. The cells were mainly harvested from the medium.
Statistical analysis showed that there was no significant different on cell density ($p>0.05$) nor cell viability among culture supplemented with 5%, 10%, or 15% serum ($p>0.05$). This result suggested that the addition of normal bovine serum up to 15% did not improve cell growth and cell survival of the hard-lipped barb kidney derived cells sub-culture.
Based on the cell yield, the addition of fetal bovine serum concentrations of 5% and 15% did not significantly affect cell growth and development, but with 10% serum concentration, there was 1% cell growth of subculture (Table 1). These results suggested that 10% serum concentration was quite suitable for kidney-derived cell culture.

Table 1. Cell Yield of the *O. vittatus* Kidney-Derived Cell Cultured in DMEM Supplemented with

Different Serum Concentrations

Serum Concentration	Cell Yield (%)	PDT	PDL
0%	0.57±0.14	-	-
5%	0.91±0.08	-	-
10%	1.01±0.57	69 h 23 min	0.47 times
15%	0.88±0.27	-	-

Other researcher recommended that the minimum serum concentration to culture fish kidney was 10% [15]. Serum concentration over 10% in form of FBS was also recommended for primary cultures, as well as for the initial passages, whereas a concentration of 5% is enough in later stages [16]. The initial cell viability also plays an important role for all forms of cell culture [17]. It was recommended that to grow and maintain subculture it needs at least 90% viability with 5 x 10^5 to 2 × 10^6 cells/mL cell density [18]. The effect of serum supplementation in culture media is varied according to the taxa of the cell sources and cell type. Research using mammalian cell culture showed that addition of 5% to 15% serum is sufficient to support cell growth, provide buffering system and protection of cultured cells [13]. The suitable serum concentration to support cell growth, proliferation and function of Mouse L929 fibroblast was 20%-60%. Addition of serum at concentration of less than 20% or more than 80% decrease cell proliferation [19]. The presence of 5% fetal bovine serum optimally promotes the formation of tight junction in immortal cell line representing organ model for gut (Caco-2), liver (HepG2), and kidney (HK-2) as indicated by biomarker of albumin, kidney injury molecule-1, and alkaline phosphatase [20]

Even though the addition of serum concentrations were accordance with the references, the result obtained from kidney-derived cell culture was not as expected. This can be caused by the type of fish kidney cells which is hematopoietic.

3.2. Cell Morphology

Cell morphology of the primary culture was presented in Figure 1. Three types of cells were identified from the primary culture. The most dominant cells have an ovoid shape with a prominent nucleus resembling the fish red blood cells. In the second place was cells with a rounded shape, small in size, and translucent. Other cell type has rounded shape but are larger in size.

Cell morphology of the sub-cultured presented in Figure 2. showed different types of cells, including ovoid bipolar cell with an ovoid nucleus, non-bipolar ovoid cell with an ovoid nucleus, medium size rounded cell with a rounded nucleus, small size rounded cells with unclear nucleus, and large rounded cell with several nuclear lobes (Figure 2.).

Evaluation based on the cell behavior showed two different types, adherent and non-adherent cells. The adherent cells attached to the substrate and the non-adherent cells were floating on the medium. It is highly recommended to characterize both cell types which grow in both monolayer and suspension culture. It is also possible that the addition of FBS affect the morphology and attachment of cells. The morphological of the goldfish fin cells exposed with FBS were typically bipolar and fibroblastic, and sharply outlined. However, the morphological result of the goldfish fin cells exposed with carp serum were epithelial-like and less clearly outlined, extending dendritically [21]. The result of cultured hepatocytes of Klunzinger's mullet (*L. klunzingeri*) also showed that the addition of 20% FBS lead to complete cell attachment to culture plate in the form of fibroblastic-like cells [12].

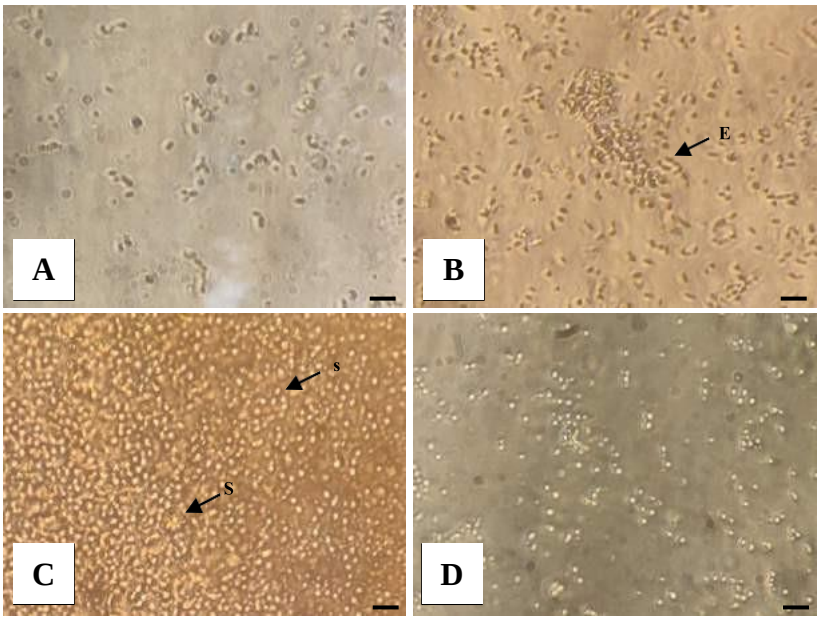

Figure 1. Microscopic Observation of *O. vittatus* Kidney-Derived Primary Culture with 0%, 5%, 10%, and 15% Serum Concentration on Day 4 using Phase-Contrast Microscope with 100x magnification.
Details: (A) 0% serum concentration; (B) 5% serum concentration; (C) 10% serum concentration; (D) 15% serum concentration. (E, fish red blood cells; s, cells with rounded shape, small in size, and translucent; S, rounded shape but larger in size), barr represent 50µm.

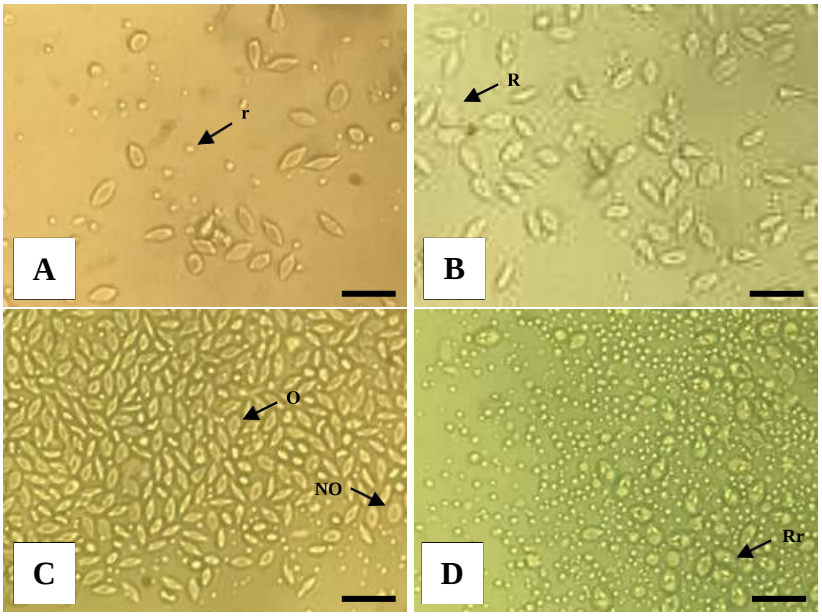

Figure 2. Microscopic Observation of O. vittatus Kidney-Derived Subculture with 0%, 5%, 10%, and 15% Serum Concentration on Day 3 using Phase-Contrast Microscope with 400x magnification.
Details: (A) 0% serum concentration; (B) 5% serum concentration; (C) 10% serum concentration; (D) 15% serum concentration. (O, ovoid bipolar cell with ovoid nucleus; NO, non-bipolar ovoid cell with ovoid nucleus; R, medium size rounded cell with rounded nucleus; r, small size rounded cells with unclear nucleus; Rr, large rounded cell with several nuclear lobes), barr represent 30µm.

Fish do not have bone marrow; hematopoiesis occurs primarily in the kidney and spleen [22]. Hematopoietic tissue is found in the anterior kidney, and because of its structural resemblance to bone marrow from higher vertebrates, this tissue is referred to be the teleost analogue of bone marrow [23]. Hematopoietic stem cells (HSC) are considered to initiate the process of hematopoiesis, which is the process by which blood cells are produced. HSCs develop through several differentiation intermediates and give rise to all blood lineages [24].

The cell populations of the anterior kidney are divided into the myeloid lineage and the lymphoid lineage. Megakaryocytes and erythrocytes (MegE), granulocytes, monocytes, macrophages, and dendritic cells (DCs) are the main components of the myeloid lineage, while B cells, T cells, and non-specific cytotoxic cells compensate the lymphoid lineage (NCCs). These cell types can be classified into subsets with various functions based on the distinct expressed cytokines and transcription factors (TFs) [25]. The anterior kidney contains mostly proliferating B cell precursors and plasma cells, whereas the posterior kidney contains abundant B cells, some of which are activated, and in addition plasma blasts [23]. It is possible due to various types of cell population; the cell types of bony-lip barb kidney cell-derived culture were also morphologically varied.

The most abundant cell type from our research was the erythrocytes-like cells. Fish erythrocytes are ellipsoidal and contain a nucleus. Their size varies considerably long diameter of 8.8–17.1 μm and a short diameter of 6.9–12.9 μm. Erythrocyte count in fish strongly depends on environmental conditions (mainly temperature and dissolved oxygen level). Ambient temperature is the most crucial factor affecting metabolic rate in poikilotherm animals, and it causes seasonal changes in fish red blood cell parameters: higher RBC is observed at higher temperatures [26].

3.3. Population Doubling Time and Population Doubling Level

Based on data depicted in Table 1, the cell harvested from the culture containing 10% serum concentration shows the highest cell yield with 1% cell growth, therefore it is possible to calculate the PDT and PDL. The value of the PDL was 0.47 times with PDT for 69 h 23 min. PDT and PDL are important to accurately calculate the cellular age to avoid miscalculation during cell culture.

Fewer cells that were adhered to the substrate and a large number of floating cells suggested that the primary and subculture cells of the kidney were unable to grow. Adhesion factor, type of medium, trypsinization time period, and contamination are common factors influencing the cell growth. Since cultured cells live in the media, the composition of the media has an impact on how well cells grow. An appropriate composition and medium type for a certain cell is typically needed for culture media [27]. Previous study in hard-lipped barb, however, showed that living some cells from gill outgrowth detached from the monolayer. These cells behave as lymphoid-like cells and therefore did not form a monolayer [14]. Further study is necessary to obtain a suitable condition for hard-lipped bard kidney derived cell as such cells is important for various studies.

4. Conclusion

Based on all of the result and discussion, the serum concentration capable of supporting bony lip barb kidney as a source cell line is 10% and the obtained population doubling time of the dominant cell derived from bony lip barb kidney were 0.47 times with PDT for 69 h 23 min. The dominant morphology of cells derived from bony lip barb kidney are erythrocytes and small-rounded cells.

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