In vitro fibroblast cells culture from Pelung chicken embryo and its potential application

E Herawati*, H Wulandari, S Listyawati and N Etikawati

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Jl. Ir. Sutami 36 A, Surakarta, Central Java, Indonesia

Corresponding author: elisahera@staff.uns.ac.id

Abstract. The availability of in vitro cell culture derived from local breed provides an opportunity for tackling problems related to the preservation of its genetic materials and can potentially be applied for downstream in vitro-based studies. Here, we established primary fibroblast cell culture from Pelung chicken, then explored its growth characteristic and potential uses for wound healing assay and cytotoxicity tests of medicinal bioactive compounds. Fibroblast cells were isolated from embryonic skin tissue and maintained in DMEM-FBS media. Wound healing assay was performed by creating a “scratch” in the cell monolayer, followed by capturing periodic images of migrating cells. Cell viability was measured using trypan blue dye exclusion assay in various doses of Centella asiatica L. leaf extract. Cells outgrowth from the skin explant revealed a typical morphology of fibroblast-like cells that reached maximum growth at 7.95x10^4 cells/cm^2 after 5 days. With continuous passage, the population of the cells became more homogeneous and population doubling time increased. In the wound healing assay, cells migrated towards the wound area within 24 hours, suggesting their ability to normally respond to chemical cues. In cytotoxicity test, cells’ viability corresponded in a dose-dependent manner with the amount of C. asiatica extract tested into the culture.

1. Introduction

Fibroblast cell culture technology has become a widely used tool for tackling various biological problems. Chicken fibroblast cell culture has demonstrated successful applications for cytotoxicity screenings of bioactive compounds [1], isolation and identification of avian viruses [2], as well as antibody testing and production of vaccines [3]. Although some earlier studies have reported the establishment of cell culture derived from domestic animals in Indonesia, little was done to use local chicken (i.e., Gallus gallus domestica) as the source of primary cell culture. The availability of fibroblast cell culture from domestic chickens can serve as an alternative way for the preservation of the genetic resources in the long run.

In this study, we isolated fibroblast cells from Indonesian local chicken breed, i.e., Pelung chicken. The isolated cells were characterized for their growth, morphology, cell migration, and cell viability. Pelung chicken was selected as a primary cell culture donor based on its inherent superior characteristics. The chicken is well known as a singing cockerel and resistant to the highly pathogenic H5N1 avian influenza virus (HPAI virus) due to the presence of the antivirus gene Mx + [4,5]. Pelung chicken was often used as superior breeding stock in poultry crossbreeding to increase productivity and improve the genetic quality of local chickens.
The objectives of this research were: (1) to establish and characterize primary fibroblast cell culture from Pelung chicken and (2) to explore its application for downstream in vitro based study, i.e., wound healing assay and cytotoxicity test against bioactive compounds of *Centella asiatica* L. leaf extracts. Wound healing assay serves as a simple method for studying collective cell migration towards chemical stimuli released by the wounded area [6,7]. This method employed cell culture as an in vitro approach to study the wound healing process. The fibroblast cell culture is also versatile for conducting screenings regarding the safety of medicinal compounds such as *C. asiatica*. *C. asiatica* is known as a traditional medicinal herb for its many health benefits. Several studies suggested asiatic acid compounds in *C. asiatica* may help to accelerate the healing process of burn and scratch wounds [8,9].

2. Materials and methods

2.1. Cell isolation and culture

The isolation and culture of chicken embryo primary fibroblast cells were following the previous methods [10]. Chicken embryo age of 8-10 days was rinsed briefly with Phosphate Buffered Saline (PBS). Dermal skin from the dorsal part was excised using pin set and cut into pieces of 1 mm². The explants were placed onto Ø 6 cm culture dish, maintained in DMEM, 10% FBS, amphotericin (25 µg/ml), also 1% penicillin and streptomycin. Cells were grown at 37°C, 5% CO₂, and saturated humidity. The medium was changed every 48 h.

2.2. Immunocytochemistry

Cells were grown on cover glass coated with poly-L-lysine to promote cell attachment. Fixation was done using ice chilled methanol (10 min; −20°C), followed by blocking with 1% bovine serum albumin for 30 min. For cytoskeleton staining, antibody was applied directly onto the cover glass for 1 hour. First and secondary antibodies were mouse anti-α-tubulin (Abcam; 1:1000) and Alexa Fluor-488 anti-mouse, respectively. DAPI was also incorporated in the mixture for visualization of the nucleus. The antibody was rinsed briefly with PBS and cells were mounted. Images were all captured at 60× using Zeiss microscopy.

2.3. Wound healing assay

The wound healing assay was following a protocol described elsewhere [11]. Confluent monolayer cells were scratched using P200 microtips, creating a void area or wound area. Fresh medium was then applied. Cells were further incubated, and the wound area was monitored every 4 hours until nearly recovered. Images were captured at 20×, 40× using Nikon microscopy.

2.4. Treatment with extract of *Centella asiatica* leaves

*Centella asiatica* leaves were oven-dried, finely powdered, then macerated with 70% ethanol for 24 hours with occasional stirring. The mixture was then filtered using Whatman filter paper and the supernatant was evaporated using a rotary evaporator at 40°C to obtain extract/paste. The extract was diluted in aqua dest at a two-fold concentration to obtain final concentrations of 250, 500, 1000, 2000, 4000 µg/ml. Fibroblast cells were grown on 24-well microplate until 80-90% confluent. Each dose of extracts was applied in triplicates. After 24 h of incubation, cell viability was measured using the trypan blue dye exclusion assay. Cell viability was counted using 0.4% trypan blue solution in a haemocytometer.

3. Results and discussion

Cells outgrowth from the explant adhered firmly to the dish surface by 24 h after isolation. The typical fibroblast-like cells exhibited a spindle-shape structure that reached 60-70% confluent at 72 h and covered all surfaces in 96 h. As the cell density increased, the cell morphology appeared more aligned and parallel to each other (Figure 1). The cells used for further analyses were from passage 4.
The cell growth was examined to check whether the culture conditions support healthy cell proliferation and development. The exponential phase lasts for 2 days (Figure 2), with an average maximum cell number reached $7.95 \times 10^4$ cells/cm$^2$ ($79.5 \times 10^4$ cells/ml). The number of fibroblast cells of Pelung chicken in this study is higher compared to the ones found from other chickens [12,13]. A possible explanation is due to the differences in the species (strain) and the media used. The routine culture showed no contamination of mycoplasma (Figure 2B-C), meanwhile, bacterial and fungi growth were prevented using antibiotic and antifungal.

The population doubling time (PDT) was 184 hours (Figure 2A), which is quite slow. Possibly, the cell population is not yet fully homogeneous in the first sub-culture, thus causing prolonged PDT. Primary cell culture generally has slower growth characteristics and a limited cell cycle compared with cell lines with more homogenous cell populations. A previous study mentioned that PDT became shorter when fibroblast cells were sub-cultured several times. The first sub-culture showed quite similar PDT (i.e., 4.2 days) to this study. Meanwhile, the ninth sub-culture PDT was obtained for 1.9 days [14].

Figure 1. Cells outgrowth from skin explant of Pelung chicken embryo. Asterisk marks the explant.

Fibroblast cells have an important role in the wound healing phase [15]. The potential of Pelung chicken fibroblast cells to be used as a wound-healing model in this study was based on the contractile and migratory nature of cells. Figures 3 and 4A show the width of the wound area of 390,143 µm which begins to narrow up to 245,087 µm after 4 hours. Four hours later (8 h after the scratch), the width of the wound area was reduced by 68% to 122,608 µm. The process of closing the wound area was almost complete at 24 hour, although some spaces between cells remained open (Figure 3). This proved that the Pelung embryo fibroblast cell culture has the migration ability to cover the wound (scratched area) within 24 hours.

Figure 2. The growth of Pelung chicken fibroblast cells. (A) Growth curve and Population Doubling Time, $n=3$. (B, C) Morphology of fibroblast cells. Cytoskeleton was stained with $\alpha$-tubulin (B). DAPI staining revealed a clear cytoplasmic area confirming no mycoplasma contamination (C).

The migration of fibroblast cells to the wound area marks the starting point of the proliferation phase of the wound healing. In general, cells need a minimum of 8-18 hours to migrate and cover the injured area [11]. After 24 hours of wound formation, fibroblasts will proliferate and produce extracellular matrix to cover and repair injured tissue. Collective cell migration is crucial in the
process of wound healing. Fibroblasts will synthesize new extracellular matrices and regenerate damaged cells; however, fibroblasts must first migrate to the injured area to carry out this role. The ability of cells to migrate is regulated continuously by the dynamic actin reshuffle activity. At first, cells will form a membrane bulge at the front of the cell called lamellipodia, which is the filament produced by actin. Lamellipodia will form an attachment to the extracellular matrix so that the cell body can move forward [16].

In vitro cell viability and cytotoxicity assay has been widely used for testing cytotoxicity of chemicals and screening drugs. In this study, the cytotoxicity of C. asiatica extract was tested on the culture of Pelung chicken fibroblast cells. Fibroblast cells were treated with the C. asiatica extract for 24 hours at various doses (250-4000 µg / ml). The results showed that the cell viability decreased in a dose-dependent manner, where the increase in the C. asiatica dose resulted in lower cell viability. A dose of 250 µg/ml C. asiatica caused a 16% decrease in cell viability compared to control, and cells treated with 1000 µg/ml of C. asiatica showed a 51% cell viability decrease (Figure 4)

Figure 3. Wound healing assay of Pelung chicken fibroblast cells. Arrows show the observed wound area after different periods (h) since the scratch was done.

The results obtained in this study were similar to the other C. asiatica cytotoxicity test using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) reduction assay on three kidney cells (i.e., African green monkey, baby hamster, and rabbit). C. asiatica extract in methanol solvent showed non-toxic concentration (LC50) within the range of 100-250 µg/ml, while extract with distilled water showed LC50 above 200 µg/ml [17]. This indicates that Pelung fibroblast cells as primary cell culture showed similar sensitivity and response with cell lines, at least when compared to the three cell types mentioned above.

Figure 4. (A) Width of wound area of Pelung chicken fibroblast cell culture at several time intervals. (B) Viability of Pelung chicken fibroblast cells against various doses of Centella asiatica ethanol extract.

Pelung chicken fibroblast cells were isolated directly from the skin tissue; thus, their physiological state mimics the original tissue in vivo. The result of this study showed that Pelung chicken fibroblast cells has a potential use as an in vitro testing device for studies on skin damage due to cosmetic or drug applications. A previous study by [18] has explained the molecular mechanism of C. asiatica
bioactive compounds on stimulating wound healing in human fibroblast cells. Triterpenoids in *C. asiatica* can modulate the gene expression involved in the wound healing process, i.e., genes that induce angiogenesis, extracellular matrix remodelling, and genes for growth factors [18]. It also contains asiaticoside from the saponin group that can stimulate cell proliferation and collagen synthesis, two very important processes in the skin and tissue regeneration [19].

Cytotoxicity testing of a bioactive compound against cancer cells has been widely carried out, however testing the same compound for normal cells is equally crucial. Cytotoxicity testing of medicinal herbs such as *C. asiatica* using normal cell culture will be useful to determine the safety level of the preparation of herbal medicines consumed by the people.

**Acknowledgments**

The authors thanked Ika Listnayati and Laela Naisaroh for providing the plant extract. This work was supported by PNBP Research Grant of Universitas Sebelas Maret.

**References**

[1] Kakad S B and Dhembare A J 2014 *Arch. Appl. Sci. Res.* **6** 139–42

[2] Liu C, Jiang L, Liu L, Sun L, Zhao W, Chen Y, Qi T, Han Z, Shao Y, Liu S and Ma D 2018 *Front. Microbiol.* **9** 751

[3] Freire M S, Mann G F, Marchevsky R S, Yamamura A M Y, Almeida L F C, Jabor A V, Malachias J M N, Coutinho E S F and Galler R 2005 *Vaccine* **23** 2501–12

[4] Sartika T, Sulandari S and Zein M S A 2011 *BMC Proc.* **5** S37

[5] Sulandari S and Zein M S A 2009 *J. Vet.* **10** 50–6

[6] Johnston S T, Ross J V., Binder B J, Sean McElwain D L, Haridas P and Simpson M J 2016 *J. Theor. Biol.* **400** 19–31

[7] Shabbir A, Cox A, Rodríguez-Menocal L, Salgado M and Badiavas E Van 2015 *Stem Cells Dev.* **24** 1635–47

[8] Somboonwong J, Kankaisre M, Tantisira B and Tantisira M H 2012 *BMC Complement. Altern. Med.* **12** 103

[9] Azis H A, Taher M, Ahmed A S, Sulaiman W M A W, Susanti D, Chowdhury S R and Zakaria Z A 2017 *South African J. Bot.* **108** 163–74

[10] Mehrabani D, Booyash N, Aqababa H, Tamadon A, Zare S and Dianatpour M 2016 *Ital. J. Zool.* **83** 306–11

[11] Liang C-C, Park A Y and Guan J-L 2007 *Nat. Protoc.* **2** 329–33

[12] Wu H, Guan W, Li H and Ma Y 2008 *Cell Biol. Int.* **32** 1478–85

[13] Bai C, Wang D, Li C, Jin D, Li C, Guan W and Ma Y 2011 *Eur. J. Histochem.* **55** e4

[14] Harlystiarini 2010 Kultur In Vitro Sel-sel Fibroblas Fetus Tikus (Bogor: Fakultas Kedokteran Hewan IPB)

[15] Kanazawa S, Fujiwara T, Matsuzaki S, Shingaki K, Taniguchi M, Miyata S, Tohyama M, Sakai Y, Yano K, Hosokawa K and Kubo T 2010 *PLoS One* **5** 22–28

[16] Adıloğlu S, Fajardo S, García-Galvan, R. F, Barranco V, Galvan J C and Batlle S F 2016 *Intech* **13**

[17] Hanisa H, Mohdazmi M L, Suhaila M and Hakim M N 2014 *Int. J. Pharm. Pharm. Sci.*

[18] Coldren C D, Hashim P, Ali J M, Oh S K, Sinskey A J and Rha C K 2003 *Planta Med.* **69** 725–32

[19] Yuliati L, Mardliyati E, Bramono K and Freisleben H J 2015 *Universa Med.* **34** 96