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Cryopreservation and Biological Characteristics of Bangkok Chicken (Thai Game Fowl) Embryonic Fibroblast Cell Culture

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Abstract

Bangkok chicken, renowned for its exceptional characteristics, presents a valuable option for crossbreeding to enhance the local chicken varieties in Indonesia. Cryopreservation is widely considered a promising approach for protecting the genetic diversity of the Bangkok chicken. This research aims to investigate the biological characteristics of embryonic fibroblast cells from Bangkok chickens, with the goal of preserving the chicken strain's genetic diversity through cryopreservation methods. The fibroblast cells were derived from 8–10-day-old Bangkok chicken embryos and cultivated in DMEM media. Growth curves and Population Doubling Time were observed to assess the rate of fibroblast cell growth. Immunocytochemistry staining was employed to examine the protein expression associated with fibroblast cell adhesion. The results showed that fibroblast cells predominantly exhibited a fusiform morphology rather than an elongated cytoplasmic morphology. The cells proliferated continuously with an average population doubling time of 204 h and the maximum cell count was $7.83\times10^{4}\pm1.7$ cells/ml. The cells exhibited normal detection of protein expression, including integrin, cadherin, actin, and microtubules. It was also noted that after cryopreservation in liquid nitrogen, fibroblast cells took 4-6 days to grow and reach 80% confluency. The data collectively verifies the successful establishment of cell culture and cryopreservation for Bangkok chicken embryonic fibroblast cells.

Introduction

The majority of poultry genetic resources are typically conserved in their natural populations (in situ). However, this approach comes with inherent risks of loss due to factors like genetic issues, breeding cessation, natural disasters, or pathogen outbreaks (Luo *et al*., 2017; Budiharjo *et al*., 2021). There are several methods available in order to protect the genetic variability of living organisms, including in situ conservation within their natural habitat, ex-situ conservation outside their habitat, and preservation through gene banks or cryopreservation (Blackburn, 2018). Cryopreservation is a technique that involves freezing biological materials, such as cells, tissues, or organs, using liquid nitrogen to minimize cellular damage. This method has the potential for long-term germplasm storage spanning several decades (Santiago-Moreno and Blesbois, 2022). One advantage of cryopreservation is its space-efficient nature, as specimens can be stored in tubes. Additionally, it ensures the availability of cells and tissues for future research needs (Jang *et al.*, 2017). Until recently, cryopreservation techniques have been utilized to preserve various components such as embryonic cells, egg cells, ovarian tissue, and testes. This makes germplasm cryopreservation a valuable approach for the poultry industry, aiding in the preservation of existing poultry genetic resources (Rakha *et al.*, 2016; Santiago-Moreno and Blesbois, 2022). However, efforts to conserve domestic genetic resources through cryopreservation in Indonesia remain limited.

This research represents the first endeavor to preserve genetic diversity in vitro, specifically focusing on domestic chicken, Bangkok chicken. The chicken's name refers to its suspected lineage from Thai game fowl (*Gallus gallus domesticus* Linnaeus, 1758), introduced to the Thailand region long ago

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(Winaya *et al*., 2023). In Indonesia, only about 25% of domestic chickens, including Bangkok chicken, have been used for research purposes within the past 20 years (Sulandari *et al*., 2008). Bangkok chicken breed is generally bigger and stockier, often classified as a fighting chicken (Mokodongan *et al*., 2017). Cross-breeding programs employing Bangkok chickens aim to enhance growth (Maliwan *et al*., 2017). Domestic chickens exhibit extensive diversity, making them an excellent foundation for breeding chicken that are well-adapted to various local environmental conditions (Taye *et al*., 2022). While domestic breeds may not be highly productive or well-suited for commercial use, they possess numerous desirable characteristics resulting from their adaptation to local environments. Characterizing the genetic potential of such breeds is essential to understanding their uniqueness and aiding in future poultry breeding selection.

Previous studies have established fibroblast cell cultures from chicken embryos, leading to the development of fibroblast banks (Bai *et al*., 2011; Su *et al*., 2011; Guan *et al*., 2012; Herawati *et al*., 2021). Fibroblast cells are often chosen due to their accessibility for cell culture, wide availability, rapid growth, and regenerative capabilities. These cells are categorized as adherent cell types, meaning they attach to the substrate (Fernandes *et al*., 2016). They play a crucial role in forming the basic components of connective tissue, producing fibers (collagen, reticular, and elastic) and macromolecules (glycosaminoglycans and proteoglycans). Fibroblast cells work in conjunction with specific proteins like integrin and cadherin, which are connected to the cell cytoskeleton, assisting in the attachment and morphing of fibroblast cells (Maitre and Heisenberg, 2013; Manso *et al*., 2010). This study aims to investigate the biological characteristics and cryopreservation techniques for the Bangkok chicken embryonic fibroblast cells.

Materials and Methods

Isolation and Culture of Bangkok Chicken Embryonic Fibroblast Cells Chicken embryos aged 8–10 days were carefully extracted from the egg and rinsed with warm Phospate Buffered Saline (PBS) and treated according to ethical guidelines of Faculty of Medicine, Sebelas Maret University. The embryo's head and visceral organs were removed. The skin from the dorsal part of the embryo was then thinly sliced into pieces measuring 1-2 mm^2. These small pieces of skin tissue were hereinafter referred to as explants. The explants were cultured on culture dish containing Dulbecco's Modified Eagle Medium (DMEM) (Gibco, New York, U.S) with 10% Fetal Bovine Serum (FBS) (Gibco, New York, U.S), supplemented with 100 U/mL penicillin-streptomycin (Thermofisher Scientific, MA, U.S), and 2.5 µg/mL amphotericin B (Gibco, New York, U.S). The culture dishes were incubated at 37°C with 5% CO_2. Media were replaced every other day (Herawati *et al*., 2021).

Cell Viability

Fibroblast cells were harvested with 0.25% trypsin-EDTA (Sigma-Aldrich, Missouri, U.S), and then 1 mL of PBS was added and homogenized with a pipette. A cell suspension of 10 µL was mixed with trypan blue solution in a 1:1 ratio, and then transferred to a counting chamber. Live cells and dead cells were distinguished based on the intensity of the blue color, where living cells appear clear as they do not absorb the dye, while dead cells appear dark blue due to loss of cell membrane integrity and absorption of the dye (Freshney, 2011; Master and Stacey, 2007). Subsequently, the percentage of living cells and dead cells, total cells/mL, and cell viability were calculated using a counting chamber. The cell count was calculated daily using the formula below and then plotted on a growth curve.

Eq. 1. Total cells/ml = (Total cells counted×Dilution factor×10,000 cells/ml)/ Number of squares counted

Meanwhile, population doubling time (PDT) was calculated using this equation:

Eq. 2. (t2 - t1)/3, 32×(log n2 - log n1) where t is time and n number of cells.

Immunocytochemistry

Cells were grown on a cover glass coated with poly-L-lysine to aid cells attachment. Immunocytochemical stages were categorized into two: fixation and staining. Fixation was done with 4% formalin for the rhodamine phalloidin antibody, while for other antibodies, methanol fixative was used. Before the staining stage, blocking was performed to minimize the reaction of unspecified antibodies using 1% Bovine Serum Albumin (BSA) (Sigma-Aldrich, Missouri, U.S) for 30 minutes at room temperature. Each antibody was diluted 500 times from stock using 1% BSA. Cover glass overgrown with cells incubated with a primary antibody solution for 1 hour at room temperature.

Primary antibodies used include acetylated tubulin anti-mouse (Sigma-Aldrich, Missouri, U.S) for α-tubulin detection, rhodamine phalloidin (Thermofisher Scientific, MA, U.S) for actin detection, K-cadherin anti-rabbit (Abcam, Cambridge, UK) for cadherin detection, and anti-mouse integrin (Merck, Massachusetts, U.S) for integrin detection. After that, the coverglass was rinsed with PBS three times. The process was followed by staining with Alexa fluor fluorescent-conjugated secondary antibodies from the respective species of the primary antibodies (Thermofisher Scientific, MA, U.S). The secondary antibody stock was diluted 500 times with 1% BSA and then incubated with cells for 1 hour at room temperature. Nuclei were counterstained with DAPI (Thermofisher Scientific, MA, U.S). The last step was coating the cells with Dako fluorescence mounting

medium. This mounting medium not only serves to protect the specimen physically but also contains a special material that protects the fluorescent color from fading easily.

Staining with Giemsa

Fibroblast cells were fixed with formalin, rinsed with PBS, then incubated with Giemsa solution for 2 min at 25ºC. Fibroblast cells were rinsed briefly with aquades and then dried overnight.

Cryopreservation

Fibroblasts cells in the logarithmic growth phase were harvested, rinsed with PBS, and then centrifuged for 5 minutes at 5000 ×g. The pellets were resuspended with Bambanker (Nippon Genetics, Tokyo, Japan) cell freezing medium (cryoprotectant) and transferred into sterile cryovials. Cell concentration at the time of storage was $5–10\times10^6$ cells/mL. The process of cryopreserving fibroblast cells involved sequential stages, starting with overnight cooling at a temperature of -70 °C and subsequent storage in liquid nitrogen (Mehrabani *et al*., 2016).

In preparation for use, frozen cells were taken out of liquid nitrogen and quickly immersed in a water bath at 37 °C for less than one minute to allow the cell suspensions to thaw promptly. Afterward, the cryoprotectant medium was replaced with fresh growth media, rendering the cells ready for planting (Mahesh *et al*., 2012).

Microscopy

We employed several microscopes for image capture, including a fluorescence microscope (Olympus BX51) equipped with a DP12 digital camera (Olympus), a light microscope from Nikon, and an inverted microscope (Nikon Eclipse Ti). The image processing for stained samples using immunocytochemical techniques (Herawati *et al*., 2021) was carried out using MetaMorph 7.8 software from Molecular Devices.

Data Analysis

The cell count data was graphed on a semi-logarithmic curve (Microsoft Excel) to visualize the exponential growth phase of fibroblast cells. This growth curve was then used to calculate the growth rate and PDT. Additionally, the cell morphology and immunostaining were analyzed descriptively.

Results

Isolation of Bangkok Chicken Embryonic Fibroblast Cells

To the best of our knowledge, this study is the first to report the isolation of fibroblast cells from Bangkok chicken embryos. Despite their widespread use as a foundation for crossbreeding to produce superior offspring, the potential for preserving genetic diversity in the form of a fibroblast bank, as well as the characterization of these cells, has not been extensively explored.

The explant technique was chosen because it is easier and carries no risk of "over digestion," which may occur with enzymatic cell dissociation methods. Additionally, the explant technique allows for the transfer of cells from in vivo conditions to in vitro conditions together with tissue fragments, enabling cells to maintain stable surroundings and become more tolerant to stress during the isolation process.

Twenty-four hours after the explants were seeded, fibroblast-like cells started to emerge and attached to the substrate outside their original tissue (Figure 1A). The cell's number continued to increase and reached its peak on the fourth day. At this stage, the cell confluence reached 80%, and the cells were ready for passaging. The cells' ability to multiply and grow daily indicated that the in vitro conditions used in this culture were favorable for preserving fibroblast cells outside their tissue of origin. Further characterization of the cell population involved analyzing aspects such as morphology, viability, growth curve, and the expression of proteins related to morphogenesis.

Morphology

The cells that have been passaged exhibited consistent morphological characteristics resembling fibroblast cells, but they also showed heterogeneity. These cells appeared flat-elongated (spindle-shaped), bipolar, or multipolar, and strongly adhered to the culture dish surface. The elongated and bipolar (fusiform) cell shapes dominated the multipolar cells with branching cytoplasmic extensions (Figures 1B-C).

The fibroblast cell population obtained from the primary cell culture displayed different morphologies from one another, depending on the donor species and donor age used (Repiská *et al*., 2010).

Viability and Growth Curve

Fibroblast cell growth was monitored to investigate their proliferation and differentiation. Based on daily observations over a period of 7 days, a sigmoid growth curve was obtained, indicating distinct phases of the cells: the lag phase, exponential phase, and stationary phase (Figure 2). The average cell viability was 96.55±10.32%. Day 0 to day 1 represent the lag phase, or cell adaptation phase. During this phase, the cells adjust to their surrounding environment. Day 2 to 5 is the exponential phase, characterized by rapid and constant cell division following a logarithmic curve. In the exponential phase, the growth rate is greatly influenced by environmental conditions such as nutrient content, temperature, and humidity. Cells require more energy during this phase compared to others. Moreover, the exponential phase is sensitive

to changes in the environment. The end of the exponential phase is marked by a decline in the growth rate of the cell population. On day 6 to day 7, the stationary phase occurred, and the cell population began to decrease. During this phase, cells continued to divide even though nutrients were depleted. The exponential phase of the Bangkok fibroblast cells lasted for 2 days, and during this time, the average cell count reached its peak at $7.83\times10^4\pm1.7$ cells/ml (Figure 2).

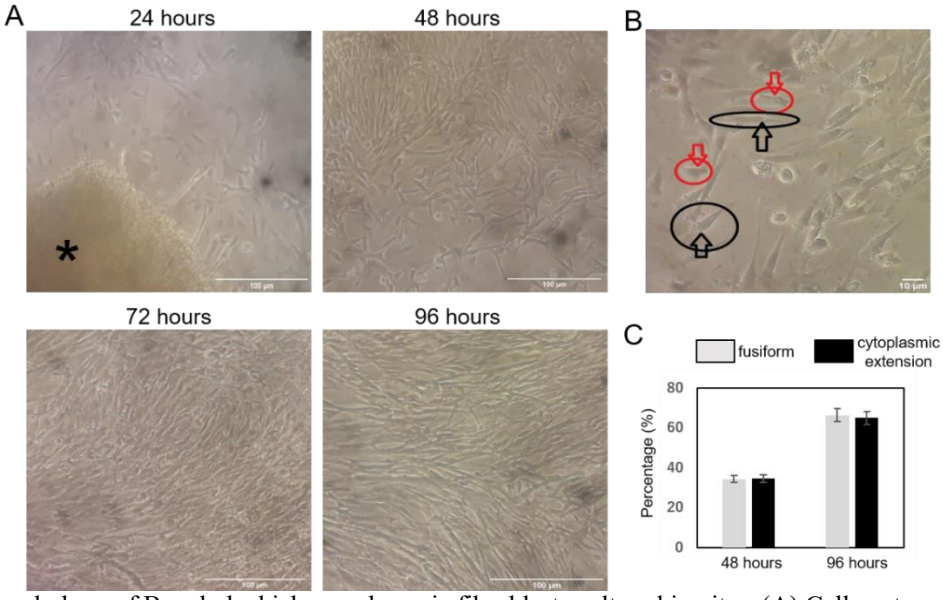

Figure 1. Morphology of Bangkok chicken embryonic fibroblasts cultured in vitro. (A) Cells outgrowth from the explants (asterisk) (B) Fibroblast cells after passage exhibited fusiform cells (black arrows), and multipolar cells with cytoplasmic extension (red arrows). The percentages of the two cell types are shown in C. There were no significant differences (p = 0.959); n=5. Scale bars 100 μm.

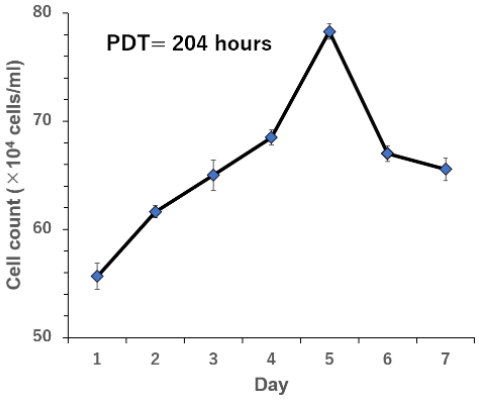

Figure 2. Growth and population doubling time of Bangkok chicken embryonic fibroblast cells.

Population Doubling Time (PDT) is the time required for cells to double their population within a certain period. PDT is calculated during the exponential phase (Boucher, 2015). It serves to monitor cell growth, cell count, the dilution factor required for sub-culturing (Freshney, 2011), and cell characteristics under controlled culture conditions.

The population size of Bangkok chicken embryonic fibroblast cells doubles within 8 days (Figure 2). During the initial passage, it is probable that the cell population is not entirely uniform, resulting in an extended Population Doubling Time (PDT). Primary cell cultures typically display slower growth attributes and restricted cell cycles when compared to cell lines with homogeneous cell populations. Previous research reports indicate that with each passage, the time required for cells to divide (PDT) decreases. The doubling time of fibroblast primary cells isolated from chicken embryo ranged from 25 to 150 h (Pasitka *et al*., 2022).

Subcelullar expression of proteins associated with cell morphogenesis

Fibroblasts continuously synthesize extracellular matrix to maintain tissue integrity. Their capacity as the largest source of extracellular matrix production allows fibroblasts to live in constant interaction with the matrix they produce. In this study, we observed the expression of integrin, a transmembrane protein that acts as a mediator for cell adhesion to the extracellular matrix. The subcellular localization of this integrin protein appears evenly distributed on the membrane of Bangkok chicken fibroblast cells (Figure 3A).

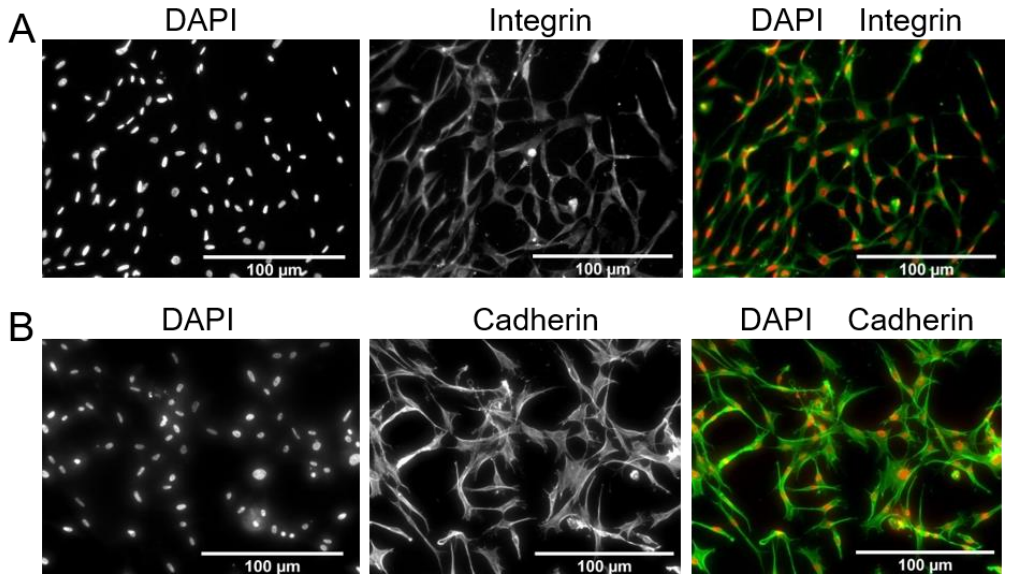

Figure 3. Expression of integrin (A) and cadherin (B) proteins in the Bangkok chicken embryonic fibroblast cells.

Conversely, cadherin is involved in facilitating the attachment of fibroblast cells to one another. Cadherin exhibits uniform expression on the cell membrane and displays robust green fluorescence signals at the tips of cytoplasmic extensions (Figure 3B). This aligns with its role as a facilitator of adherens junction structures, which promote stable interactions between cells. Adherens junctions contribute to cell shape and tension while also acting as a site for intercellular signaling. Direct physical contact between fibroblast cells is crucial during growth in in vitro culture. These connections enable cells to communicate and respond to changes in the microenvironment, ensuring their survival.

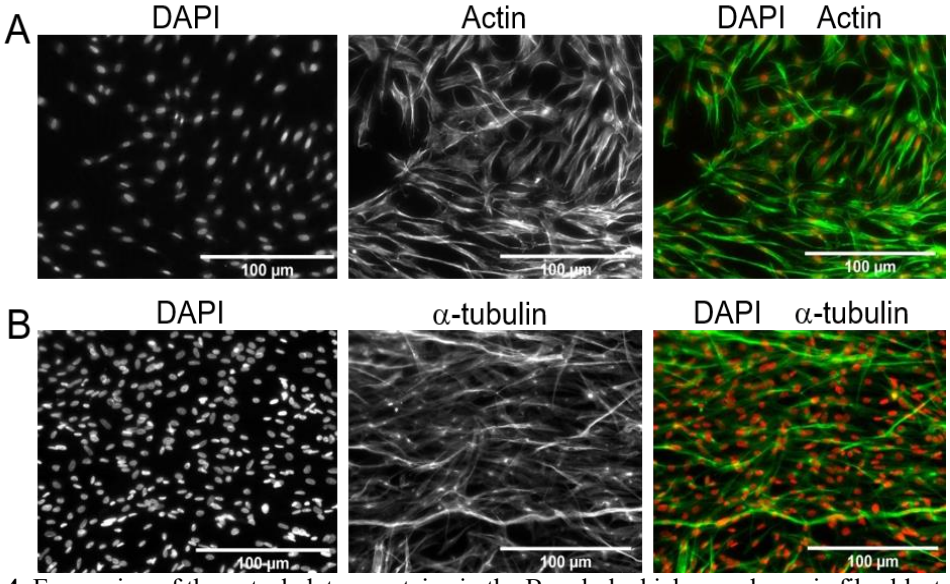

Figure 4. Expression of the cytoskeleton proteins in the Bangkok chicken embryonic fibroblast cells. (A) Actin stained with phalloidin. (B) Microtubules stained with α-tubulin.

At the tissue level, integrin and cadherin coordinate with the cytoskeleton (actin and microtubules) to regulate several fundamental biological processes such as cell proliferation, tissue morphogenesis patterns, and collective cell migration. Immunocytochemistry staining results show that all cells express actin and microtubules, both of which appear as fine cytoplasmic fibers (Figure 4). The Bangkok chicken fibroblast cells normally express integrin, cadherin, actin, and microtubules (Figure 3-4). Aberrations in the expression of these proteins can result in uncontrolled cell proliferation and cancer (Ramage, 2012; Bianconi *et a*l., 2016).

Cryopreservation

Fibroblast cells that have been routinely cultured can be stored for a long time using cryopreservation techniques. The principle of cryopreservation involves using an extremely low temperature (-196°C) in liquid N_2 to maintain the biological material's structure intact. With proper cryopreservation techniques, fibroblast cells that have been frozen can be "revived" without significant changes in their phenotype.

Prior to cryopreservation, measures were taken to ensure that the Bangkok chicken fibroblast cells were free from mycoplasma contamination. The "revived" cells, after being stored in liquid nitrogen, took a minimum of 2 days to display normal growth activity and morphology, as evidenced by cytoplasmic extensions. On the 3rd day following cell seeding, the confluency reached approximately 30%, and by the 6th day, it reached around 80% (Figure 5). This indicates that the cells were harvested in a healthy state and that extreme temperature conditions do not adversely affect cell metabolism upon reactivation.

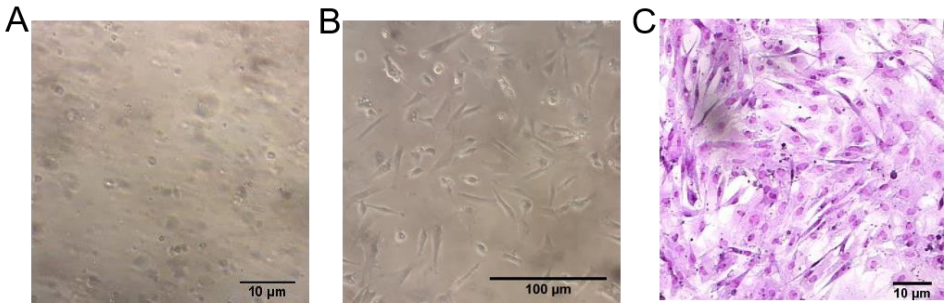

Figure 5. Bangkok chicken embryonic fibroblast cell growth post-cryopreservation. Morphological changes of the fibroblast cells on day 0 (A) and day 3 (B) after seeding. Monolayer condition of fibroblast cells 6 days after seeding (B). Cell confluence reaches 80%. Cells stained with Giemsa.

Discussion

There are currently 13.3% of avian species worldwide that are classified as critically endangered, endangered, or vulnerable (IUCN Red List version 2020). Additionally, there are 3,689 avian breeds recorded globally, with 2,222 being indigenous breeds, and approximately 28% of these breeds are facing endangerment, vulnerability, or have already become extinct. Concerning domesticated animals, there are around 8,800 recorded breeds of 40 species, and 7% of these breeds are already extinct, with 24% at risk of extinction (GenRes Bridge, 2021).

Examining this data highlights a notable threat to both wild birds and native poultry varieties. As a result, it becomes imperative to conserve the genetic resources of local chicken breeds, like the Bangkok chicken. This not only ensures the preservation of poultry biodiversity but also secures valuable materials that could be utilized in upcoming crossbreeding strategies.

Efforts to isolate fibroblast cell lines from various domestic chicken breeds have been primarily focused on characterizing these cells. This characterization process involves understanding their growth requirements, specific culture conditions, as well as investigating any known genetic or phenotypic features, as documented in several studies (Na *et al*., 2010; Guan *et al*., 2010; Bai *et al*., 2011; Herawati *et al*., 2021). While fibroblast typically exhibit consistent morphological characteristics, variations in growth rates have been observed, such as the case of Bangkok chicken embryonic fibroblast, which appears to have a delayed population doubling time (PDT) when compared to other breeds. This delay may be attributed to heterogeneity within the cell population, particularly in passages 2-3. Moreover, to ensure the authenticity of fibroblast cells' origin and prevent potential cross-contamination, it is imperative to employ a combination of isoenzyme polymorphisms and karyotyping, particularly in the context of Bangkok chicken fibroblast cell culture in the future. Overall, the success rate of the isolation methods employed in these studies has effectively demonstrated their feasibility and efficiency, further underscoring the importance of thorough characterization and quality control in the maintenance of fibroblast cell lines from diverse chicken breeds.

Fibroblast cells exhibit morphological plasticity which reflects collagen matrix synthesis activity. Active fibroblasts possess a high amount of rough endoplasmic reticulum and collagen secretion granules

located close to the Golgi apparatus (Eyden, 2004). In mature skin tissue, where collagen production decreases, fibroblasts transform into fibrocytes, and their nucleus size reduces. To differentiate between fibrocytes and fibroblasts, apart from observing ultrastructure under an electron microscope as mentioned above, a combination of type I pro-collagen and leukocyte-specific protein (LSP-1) markers can also be used (Bucala, 2007).

Fibroblast cells cultivated in the same medium may exhibit varying morphological plasticity, but they can still be induced to undergo character changes. During the wound-healing process, fibroblast cells alter the expression of actin genes and adopt some contractile properties similar to smooth muscle cells (they differentiate into myofibroblasts). These cells collectively contribute to the wound-healing process by reducing the size of the injury area and secreting extracellular matrix proteins (Tracy *et al*., 2016).

In the study of Bai *et al*. (2011), fibroblast cells from embryonic Jingning chickens were stored by cryopreservation techniques for a long storage time, while re-growing takes 2 days to reach ~ 80–90% confluence. Cells show a typical fusiform morphology. Cell viability before storage was 96.23 ± 0.58%, and after storage it was 90.51 ± 0.74%. Research by Li *et al*. (2009) also reported that fibroblast cells from silkie Bantam chicken embryos had cell viability before storage of 96% and cell viability after storage of 90.5%.

The success of cryopreservation is greatly influenced by cryoprotectants. Commercial cryoprotectants were employed in this study. These substances prevent freezing, which could otherwise harm organelles and lead to cell death. Additionally, cryoprotectants preserve the integrity of the cell membrane during the dehydration process. The effective preservation of cell membranes by cryoprotectants indicates a favorable interaction between these substances and cell membranes. Cryoprotectants consist of dimethylsulfoxide (DMSO), which prevents cell death during freezing, and glycerol, which has a strong water-binding capacity that allows it to replace some of the water released during the process (Liu and Li, 2020).

Conclusion

The establishment of cell culture and cryopreservation for Bangkok chicken embryonic fibroblast cells was accomplished. These cells exhibited robust proliferation and exhibited the usual expression of proteins linked to adhesion and cellular morphogenesis. Cryopreservation of Bangkok chicken fibroblast cells resulted in good cell viability, as they were able to survive, expand, and achieve 80% confluence upon regrowth. By preserving this valuable genetic resource at the cellular level, the cells can serve as a valuable biological material for research and biotechnology, as well as conservation efforts.

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References

in Surakarta area, Central Java. IOP Conference Series: Earth and Environmental Science, 902: 012035. DOI:10.1088/1755-1315/902/1/012035

Eyden B. 2004. Fibroblast phenotype plasticity: relevance for understanding heterogeneity in “fibroblastic” tumors. Ultrastructural Pathology, 28: 307-319. DOI: 10.1080/019131290882204

Fernandes IR, Russo FB, Pignatari GC, Evangelinellis MM, Tavolari S, Muotri AR & Beltrão-Braga PC. 2016. Fibroblast sources: where can we get them?. Cytotechnology, 68(2): 223–228. DOI: 10.1007/s10616-014-9771-7

GenRes Bridge. 2021. Animal genetic resources strategy for Europe. https://www.animalgeneticresources.net/wp-content/uploads/2021/11/AnGR_strategy_FinalDraft2111.pdf. Accessed in October, 2022.

Freshney RI. 2011. Culture of animal cells : A manual of basic technique and specialized applications 6th Ed. John Wiley & Sons, Inc. New Jersey. 732 Pages.

Guan W, He X, Li L, Liang H, Zhao Q, Pu Y, & Ma YH. 2010. Establishment and biological characterization of fibroblast cell line from the

Bai C, Wang D, Li C, Jin D, Li C, Guan W & Ma Y. 2011. Establishment and biological characteristics of a Jingning chicken embryonic fibroblast bank. European Journal of Histochemistry, 55(1):19-23. DOI: 10.4081/ejh.2011.e4

Bianconi D, Unseld M & Prager GW. 2016. Integrins in the spotlight of cancer. International Journal of Molecular Sciences, 17 (2037): 1-27. DOI: 10.3390/ijms17122037

Blackburn HD. 2018. Biobanking genetic material for agricultural animal species. Annual Review of Animal Biosciences, 6: 69-82. DOI: 10.1146/annurev-animal-030117-014603

Boucher L. 2005. What is doubling time and how is it calculated?. populationeducation.org/what-doubling-time-and-how-it-calculated/ Accessed on May 4. 2023.

Bucala R. 2007. Fibrocytes: new insights into tissue repair and systemic fibroses. 1st Ed. World Scientific Publishing Company. Singapore. 268 Pages.

Budiharjo A, Prabawati AWF & Herawati E. 2021. Seroprevalence of avian influenza virus subtype H5N1 in chickens of the two main poultry market

2284. DOI: 10.1038/nprot.2007.319

Mehrabani D, Tajedini M, Tamadon A, Dianatpour M, Parvin F, Zare S & Rahmanifar F. 2016. Establishment, characterization and cryopreservation of fars native goat fetal fibroblast cell lines. Asian Pacific Journal of Reproduction, 5(3): 247-251. DOI: 10.1016/j.apjr.2016.04.013

Mokodongan AR, Nangoy F, Leke JR & Poli Z. 2017. Penampilan pertumbuhan ayam Bangkok starter yang diberi pakan dengan level protein berbeda. Jurnal Zootek, 37(2): 426-435. [INDONESIAN]. DOI: 10.35792/zot.37.2.2017.16268

Na RS, Bai CY, Jin DP, Su XH, Feng, BG, Guan, WJ & Ma YH. 2010. Establishment and biological characteristics of Qingyuan partridge chicken fibroblast line. Poultry Science, 89(6), 1207–1216. DOI: 10.3382/ps.2009-00558

Pasitka L, Cohen M, Ehrlich A, Gildor B, Reuveni E, Ayyash M, Wissotsky G, Herscovici A, Kaminker R, Niv A, Bitcover R, Dadia O, Rudik A, Voloschin A, Shimoni M, Cinnamon Y & Nahmias Y. 2022. Spontaneous immortalization of chicken fibroblasts generates stable, high-yield cell lines for serum-free production of cultured meat. Nature Food, 4: 35–50. DOI: 10.1038/s43016-022-00658-w

Rakha BA, Ansari, MS, Akhter S, Hussain I & Blesbois E. 2016. Cryopreservation of Indian red jungle fowl (Gallus gallus murghi) semen. Animal Reproduction Science, 174: 45-55. DOI: 10.1016/j.cryobiol.2017.07.006

Ramage L. 2012. Integrins and extracellular matrix in mechanotransduction. Cell Health and Cytoskeleton, 4: 1-9. DOI: 10.1101/cshperspect.a005066

Repiská V, Varga I, Lehocký I, Böhmer D, Blaško M, Polák Š, Adamkov M & Danišoviče Ľ. 2010. Biological and morphological characterization of human neonatal fibroblast cell culture B-HNF-1. Biologia, 65(5): 919-924. DOI: 10.2478/s11756-010-0095-6

Santiago-Moreno J & Blesbois E. 2022. Animal board invited review: germplasm technologies for use with poultry. Animal, 16(3): 100475. DOI: 10.1016/j.animal.2022.100475

Su X, Jin D, Li C, Yue H, Pu Y, Ma Y & Guan W. 2011. Establishment of big bone chicken fibroblast cell bank and study of its biological characteristics. Belgian Journal of Zoology, 141 (1): 14-23.

Sulandari S, Zein MSA & Sartika T. 2008. Molecular characterization of Indonesian indigenous chickens based on mitochondrial DNA displacement (D)-loop sequences. Hayati Journal of Biosciences, 15(4): 145-154. DOI: 10.4308/hjb.15.4.145

Taye S, Goshu G & Abegaz S. 2022. Effect of crossbreeding on growth performance of improved Horro Crosses with Koekoek and Kuroiler chicken breeds. Poultry Science Journal, 10(1): 35-44. Langshan chicken. Cell Proliferation, 43(2), 157–163. DOI: 10.1111/j.1365-2184.2010.00666.x

Guan W, Wang D, Bai C, Zhang M, Li C & Ma Y. 2012. Biological characteristic of an embrionic fibroblast line from Xiaoshan chicken for genetic conservation. Journal of Cell and Animal Biology, 6(4): 46-53. DOI: 10.5897/JCAB11.089

Herawati E, Wulandari H, Listyawati S & Etikawati N. 2021. In vitro fibroblast cells culture from Pelung chicken embryo and its potential application. IOP Conference Series: Earth and Environmental Science, 637(1): 012007. DOI: 10.1088/1755-1315/637/1/012007

IUCN. 2020. The IUCN red list of threatened species. Accessed on December 2. 2020.

Jang TH, Park SC, Yang JH, Kim JY, Seok JH, Park US, Choi CW, Lee SR & Han J. 2017. Cryopreservation and its clinical applications. Integrative Medicine Research, 6: 12-18. DOI: 10.1016/j.imr.2016.12.001

Li IF, Guan WJ, Li H, Bai XJ & Ma YH. 2009. Establishment and characterization of the fibroblast line from Silkie Bantam. Asian Australian Journal Animal Science, 22(4):492-499. DOI: 10.5713/ajas.2009.80480

Liu S & Li F. 2020. Cryopreservation of single-sperm: where are we today?. Reproductive Biology and Endocrinology, 18(1): 41. DOI: 10.1186/s12958-020-00607-x

Luo S, Xie Z, Xie Z, Xie L, Huang L, Huang J, Deng X, Seng T, Wang S, Zang Y & Liu J. 2017. Surveillance of live poultry markets for low pathogenic avian influenza viruses in Guangxi Province, Southern China, from 2012–2015. Scientific Reports, 7: 17577. DOI: 10.1038/s41598-017-17740-0

Mahesh YU, Rao BS, Katari VC, Komjeti S, Christo D, Lakshmikantan U, Pawar RM & Shivaji S. 2012. Cell cycle synchronization of bison (*Bos Gaurus*) fibroblasts derived from ear piece collected post-mortem. Reproduction in Domestic Animals, 47: 799-805. DOI: 10.1111/j.1439-0531.2011.01970.x

Maitre JL & Heisenberg CP. 2013. Three functions of cadherins in cell adhesion. Current Biology, 23 (14): 626-633. DOI: 10.1016/j.cub.2013.06.019

Maliwan P, Khempaka S & Molee W. 2017. Evaluation of various feeding programmes on growth performance, carcass and meat qualities of Thai indigenous crossbred chickens. South African Journal of Animal Science, 47(1): 16-25. DOI: 10.4314/sajas.v47i1.4

Manso AM, Kang SM & Ross RS. 2010. Integrins, focal adhesion, and cardiac fibroblasts. Journal of Investigative Medicine, 57 (8): 856-860. DOI: 10.231/JIM.0b013e3181c5e61f

Master JR & Stacey GN. 2007. Changing medium and passaging cell lines. Nature Protocols, 2(9): 2276-

& Jaganathan R. 2023. Morphometric diversity and genetic relationship of "Bangkok" chicken (Thai Game Fowl) in East Java, Indonesia. Jordan Journal of Biological Sciences, 16 (2): 189 -197. DOI: 10.54319/jjbs/160203

DOI: 10.22069/psj.2022.19503.1729

Tracy LE, Minasian RA & Caterson EJ. 2016. Extracellular matrix and dermal fibroblast function in the healing wound. Advance Wound Care, 5(3): 119-136. DOI: 10.1089/wound.2014.0561

Winaya A, Fahmiady DI, Suyatno S, Malik AM