Primordial Germ Cells Quality of Chicken Embryo from Post Cryopreservation Blood Samples

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Abstract

The optimal cellular reservoir for the conservation and propagation of endangered poultry is acknowledged to be primordial germ cells (PGCs). This study aimed to evaluate and compare the efficacy of cryopreserving PGCs derived from purified sources and blood samples. The experimental design encompassed two treatment groups, each iterated six replication. Blood samples that had been filled with 500 µL of phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) underwent centrifugation at 1200 rpm for six minutes. The supernatant was discarded and the pellet was mixed with 500 µL 10% FBS-PBS and was then subdivided for cryopreservation and PGCs purification. Cryopreservation included the controlled freezing of blood and purified PGCs samples in Nalgene cryogenic vials with the incorporation of banker cells. Following thawing, statistical analyses revealed no significant variance in the mean PGCs count between purified PGCs and blood samples ($p > 0.05$). The percentages of PGCs mortality and the recovery rate demonstrated statistical significance ($p < 0.05$). Further investigations indicated that cryopreserving blood samples significantly increased the recovery rate of PGCs while decreasing post-thaw mortality. Morphology of PGCs from blood cryopreservation showed similar morphology to PGCs in general. The potential application of cell-based cryo-banking will contribute to the preservation of poultry genetic diversity for sustainability and adaptation to future poultry demands.

Keywords: blood, cryopreservation, endangered poultry, primordial germ cells, purified

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INTRODUCTION

Primordial germ cells (PGCs) stand as the exclusive carriers of genetic information across generations. These cells can be extracted from individual embryos and cultured in vitro using specialized media. PGCs exhibit significant potential in modeling embryonic development, supporting animal health, conducting gene editing, and preserving genetic material in avian species. Their adaptability to year-round cultivation makes them suitable for effective utilization and optimization in diverse conditions (Chaipipat *et al*., 2022). In chickens, PGCs are conveniently isolated from embryos at stages 14–17 (Kostaman *et al*., 2013). Circulating PGCs or cPGC offers advantages over other cell types (such as gonadal tissue or gPGC) due to their isolation method, which can be performed while maintaining or rescuing the donor animal. However, cryopreservation is essential for preserving other genetic materials.

Cryopreservation involves the preservation of cells and tissues at low temperatures. In avian species, the significance of storing PGCs in gene banks has grown, as it now represents the most viable approach to safeguarding genetic material present in the female W chromosome and mitochondrial DNA. Unlike mammals, where males typically have homogametic ZZ chromosomes, avian females possess heterogamous ZW chromosome pairs, enabling

the conservation of the entire chicken genome (Hamai *et al*., 2023).

Cryopreservation of PGCs involves the use of either slow freezing or vitrification techniques (Lazar *et al*., 2021; Nakajima *et al*., 2022). Slow freezing of PGCs typically employs serum-containing media supplemented with dimethyl sulfoxide (DMSO) and ethylene glycol. Alternatively, commercially prepared media are now available and have demonstrated successful outcomes. Although there is limited study data on PGCs cryopreservation using vitrification, this method is generally considered more effective than slow freezing, particularly for preserving oocytes, stem cells, and embryos, ensuring improved cell viability and stability (Tonus *et al*., 2017).

The cryopreservation of PGCs represents a crucial aspect of cryo-banking, offering the potential to preserve essential characteristics for meeting present and future demands in chicken production. The success of PGCs cryo-banking initiatives is contingent upon factors such as post-thaw recovery, viability, and quality of chicken PGCs (Nakamura, 2017). Consequently, PGCs cryopreservation emerges as a viable strategy to facilitate the generation of both male and female PGCs lines, contributing to the restoration of diverse chicken breeds (Mathan *et al*., 2023).

Currently, obtaining PGCs from avian embryos is conveniently achieved through the blood collected between 48–65 hours or at stages 14–17. PGCs present in embryonic blood have been a focal point for isolation studies, given their accessibility, reduced presence of contaminating cell types (in comparison to gonadal tissue), and the understanding that PGCs are actively migratory. This method is swift and yields a substantial number of viable PGCs (Kostaman *et al*., 2013; Wade *et al*., 2014; Ecker *et al*., 2023). However, challenges arise when dealing with large blood samples that require immediate processing, and even if stored at 4ºC, they are generally suitable for short-term use (≤ 24 hours). In this study, fresh blood samples were directly cryopreserved due to the inherent issue of fresh whole blood being prone to damage and challenging to use, especially when analyzing a large batch of samples simultaneously to minimize variability (e.g., different operators during PGCs purification). The study aimed to compare the quality of PGCs obtained after cryopreservation from purified PGCs samples and blood, with the expectation that cryopreserving blood samples would maintain PGCs quality.

MATERIALS AND METHODS

Ethical Approval

This study did not involve animals directly and stored biological materials so it did not require research ethics clearance according to the Research Ethics Committee of the National Research and Innovation Agency.

Study Period and Location

This study took place at the Genomic Laboratory of Cibinong Science Center of the National Research and Innovation Agency (BRIN), Cibinong, Indonesia. This study was carried out in June–July 2023.

Blood Collection and PGCs Purification

Embryo blood collection utilized the fertile eggs of White Leghorn chickens. The eggs underwent incubation at 37.5°C, with a rotation of 65° every 30 minutes facilitated by a portable incubator (P-008B Biotype; Showa Furanki, Saitama, Japan). Following 54 hours of incubation (corresponding to stage 14 as per Hamburger and Hamilton, 1951), the egg shells were carefully cracked, and the contents were transferred to a plastic petri dish.

Embryonic blood retrieval was conducted from the dorsal aorta using a micropipette, as illustrated in Figure 1 under microscopic observation. The obtained blood was carefully transferred into a 1.5 mL Eppendorf tube pre-filled with 500 µL of phosphate-buffered saline solution without Ca^{2+} and Mg^{2+} (PBS). After the blood collection, centrifugation at 1200 rpm for 6 minutes took place, followed by the removal of the supernatant. The resulting pellet was then combined with 500 µL of 10% FBS-PBS and divided into four portions. Two of these portions

were allocated to Nalgene cryogenic vials, where 1000 cell bankers were introduced, initiating an immediate freezing process. Concurrently, the remaining two portions were placed in separate 1.5 mL Eppendorf tubes, proceeding to the primordial germ cell (PGC) purification process through gradient centrifugation using Nycodenz, with slight adjustments as per the methodology proposed by Kostaman *et al*. (2013). Upon quantifying the PGCs, they were mixed with 100 µL of cell banker and deposited into a Nalgene cryogenic vial for subsequent cryopreservation.

Freezing and Thawing PGCs

The cryotubes, housing PGCs, were positioned within a NALGENE Cryo 1ºC Freezing Container (Catalog No. 5100-0001). This container was filled with 250 mL of isopropyl alcohol (Sigma Aldrich) to ensure a controlled cooling rate of -1°C per min. The NALGENE Cryo apparatus was then stored at -80°C and allowed to remain overnight. Following this period, the cryotube was extracted from the NALGENE Cryo and carefully placed into an aluminum cryo-cane. Subsequently, the cryo-cane, along with the cryotube, was immersed in liquid nitrogen at -196°C.

Following one week, the cryotube containing purified PGCs and blood was retrieved from liquid nitrogen and subjected to immediate thawing by immersion in a 37ºC water bath until partial ice melting ensued. Subsequently, the blood samples underwent centrifugation at 1200 rpm for 5 minutes, with the resultant supernatant discarded, and the pellet washed with 500 µL PBS at 1200 rpm for 6 minutes. The washed pellets were then subjected to a purification process involving gradient centrifugation utilizing Nycodenz, incorporating slight modifications from the protocol outlined by Kostaman *et al*. (2013). Concurrently, the PGCs (post-purification) underwent a wash step to eliminate cryoprotectants. This entailed mixing 1000 µL of 10% FBS in PBS and subsequent centrifugation at 1200 rpm for 5 minutes. The supernatant was then discarded, and approximately 20 µL of the cell suspension was combined with 100 µL of 10% FBS in PBS, placed in a petri dish, and utilized for quantifying the number of PGCs after thawing under microscopic observation.

PGCs Assessment

To enumerate viable and non-viable PGCs, the PGCs were placed within Eppendorf tubes containing 15 µL of 10% FBS-PBS and gently mixed. Subsequently, 5 µL of 0.05% trypan blue was introduced into the tube and slowly mixed using a pipette. The resulting solution was then loaded onto a hemocytometer and subjected to microscopic examination. The viability percentage was computed by dividing the number of PGCs post-thawing by the number of PGCs pre-thawing, multiplied by one hundred percent. The percentage of non-viable PGCs was determined by dividing the number of non-viable PGCs by the sum of viable PGCs and non-viable PGCs, multiplied by one hundred percent. Concurrently, the recovery rate percentage, denoted as the post-thawing recovery rate, was calculated by dividing the number of viable PGCs by the sum of viable PGCs and non-viable PGCs, multiplied by one hundred percent.

Statistical Analysis

The average count of post-thawed PGCs, the percentage of viability and mortality of PGCs, and the recovery rate were analyzed. The analysis was conducted using the T-test with the assistance of the SPSS version 25 software.

RESULTS AND DISCUSSION

This study pioneered the examination of cryopreserved blood samples to assess the quality of chicken PGCs. Chicken PGCs hold substantial promise as a model for various vertebrate studies. Research indicates that cPGC in green-footed partridges reach their zenith at stage 14 (Szczerba *et al*., 2021). Therefore, in this investigation, blood samples from chicken embryos were obtained at stage 14.

In this investigation, both purified PGCs and blood samples were effectively isolated and cryopreserved, suggesting the potential of PGCs for preserving chicken germplasm. Based on the average PGCs numbers post-thawing for both

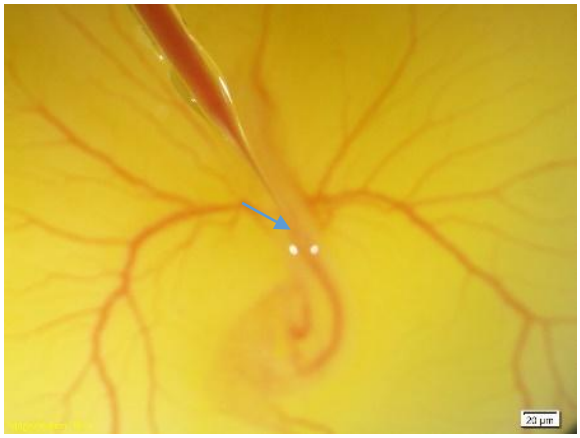

Figure 1. Collecting blood samples from chicken embryos through the dorsal aorta.

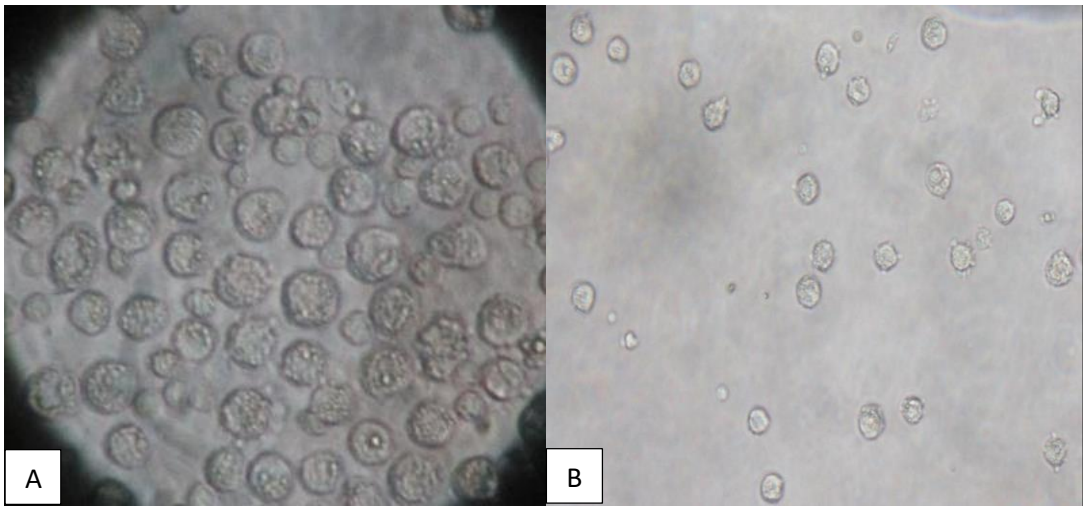

Figure 2. (A) Morphological quality of fresh PGCs and (B) after cryopreservation of PGCs.

Table 1. Primordial germ cells quality of White Leghorn chicken embryos from purified (P) and non-purified (NP) samples

Sample	Number of PGCs before thawing	Number of PGCs after thawing	Viability (%)	Mortality (%)	Recovery rate (%)
P 1	371	193	52.02	3.85	96.15
P 2	267	120	44.94	12.64	87.36
P 3	315	204	64.76	14.12	85.88
P 4	406	253	62.32	10.65	89.35
P 5	551	284	51.54	5.56	94.44
P 6	449	259	57.68	5.95	94.05
Mean ± SD*	393.17 ± 100.71	218.83^{a} ± 59.47	55.54 ± 7.43	8.79^{a} ± 4.23	91.21^{a} ± 4.23
NP 1	-	227	-	5.45	94.55
NP 2	-	178	-	4.85	95.15
NP 3	-	266	-	1.53	98.47
NP 4	-	347	-	5.68	94.32
NP 5	-	430	-	6.76	93.24
NP 6	-	294	-	1.87	98.13
Mean ± SD*	-	290.33^{a} ± 89.39	-	4.36^{b} ± 2.15	95.64^{b} ± 2.15

*SD= standard deviation, different superscripts in the same column indicate significant ($p < 0.05$).

sample types indicated no significant difference ($p > 0.05$). However, there were notable distinctions in the percentage of PGCs deaths and recovery rates between the two sample types ($p < 0.05$) (Table 1). These findings demonstrate that cryopreservation of blood samples yields PGCs quality that is nearly equivalent to, or even superior to, purified PGCs. Notably, for White Leghorn chickens, blood samples showed fewer dead PGCs compared to purified PGCs. Similar outcomes were observed by Langenskiold *et al*. (2018), where frozen blood samples produced results comparable to unprocessed ones. The kind and concentration of cryoprotectants were among the factors influencing cryopreservation success (Fernández-Santos MR *et al*., 2006).

There are two categories of cryoprotectants: permeable and non-permeable. Permeable cryoprotectants are tiny, non-ionic compounds that can flow through cell membranes and into cells. As a result, permeable cryoprotectants can replace intracellular fluids and inhibit the production of ice crystals when freezing. In contrast, non-penetrating cryoprotectants do not penetrate cell membranes. Non-permeable cryoprotectants can prevent extracellular ice crystal formation through direct water contact and intracellular dehydration (Hamai *et al*., 2023). Furthermore, the concentration of cryoprotectants can impact the quality of frozen cells. Osei-Bempong *et al*. (2018) found that raising the concentration of cryoprotectant above 5% degraded cell quality.

The number of PGCs from the purified samples closely resembled that reported in previous studies on green-footed partridges at stage 14 (Szczerba *et al*., 2021). The PGCs count from blood samples exhibited higher values compared to purified PGCs samples (Table 1). This highlights the advantages of blood samples in preserving cell condition during cryopreservation. This observation is supported by Tonus *et al*. (2016), who noted a decrease in the viability of purified chicken PGCs one to two days post-thawing, potentially attributed to apoptosis. PGCs apoptosis can primarily occur due to three processes: the absence of specific growth factors, loss of contact with somatic cells or particular extracellular matrix (ECM) molecules (anoikis), and DNA damage (De Felici and Klinger, 2015).

The mean percentage of PGCs deaths from both samples remains relatively low, leading to a notably high recovery rate percentage (Table 1). This indicates the effectiveness of the cryoprotectant utilized in safeguarding cells during the freezing process, as cells frozen at temperatures below 0°C without cryoprotectant assistance typically incur lethal damage. This underscores the importance of cryoprotectants in mitigating structural alterations caused by unprotected freezing (Whaley *et al*., 2021). The cooling and thawing of unprotected cells represent processes incompatible with cellular viability. To mitigate this effect, two protective measures are imperative: (1) the application of cryoprotectants and (2) the adoption of appropriate cooling and thawing rates (Whaley *et al*., 2021).

In this study, a cryoprotectant utilizing a cell banker based on dimethyl sulfoxide (DMSO) was employed. DMSO typically added to cell media at a concentration of 2 M, serves to enhance cell membrane permeability, facilitating the freer movement of water across the membrane (Ock and Rho, 2011). Moreover, DMSO functions to shield cells from damage caused by high concentrations during the freezing process (Yan *et al*., 2016). The research findings indicate that all parameters attest to the continued quality of PGCs even after undergoing freezing and subsequent thawing. Essentially, the cell banker, acting as a cryoprotectant, effectively permeates cells before the complete solidification of cell fluid during freezing, thereby reducing electrolyte concentrations inside and outside the cell. Consequently, excessive moisture within the cells is prevented from escaping, thus averting dehydration (Whaley *et al*., 2021). Consequently, DMSO has demonstrated its efficacy as a fundamental component for optimal cryoprotectant utilization in chicken PGCs preservation (Setioko *et al*. 2007).

In the study, a controlled cooling rate was employed, set at -1°C per minute. Typically, a cooling rate of approximately -1°C per min

proves effective for various cells and organisms, barring very large cells, as the cooling process becomes more critical with increasing cell size (Whaley *et al*., 2021). Following successful cryopreservation, the intention is to store the cells for future use or testing, with the hope of subsequent thawing.

After undergoing freezing and subsequent thawing, PGCs originating from blood exhibit a morphology that closely resembles typical PGCs. These cells maintain their integrity and retain a rounded shape (Figure 2), consistent with findings reported by various researchers investigating the freezing and thawing of chicken PGCs (Yön and Akbulut, 2015; Kostaman *et al*., 2017; Tagami *et al*., 2017). This indicates that freezing PGCs derived from whole blood does not induce cellular damage, and PGCs morphology remains intact. The research results highlight a notably low average percentage of dead PGCs in comparison to purified PGCs.

The findings of this study have provided valuable insights into various crucial aspects concerning blood cryopreservation. This study has illustrated the feasibility of identifying, isolating, cryopreserving, and subsequently recovering PGCs with excellent viability from chicken genetic material.

CONCLUSION

Blood samples exhibit the capability to uphold the quality of chicken PGCs on par with that of purified PGCs after cryopreservation. Subsequent investigations have shown that cryopreservation of blood samples enhances the recovery rate percentage of PGCs and diminishes PGCs mortality following thawing. The blood samples developed in this study present a streamlined protocol for the prolonged cryopreservation of chicken PGCs. The implementation of cell-based cryobanking applications will play a crucial role in conserving the genetic diversity of chickens, ensuring sustainability, and facilitating adaptation to future poultry demands.

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AUTHORS' CONTRIBUTIONS

NA and TK: Conceptualization, methodology, investigation, and writing the manuscript. DAK, S, ZM, and EK: investigation and writing the manuscript. YAT and MG: formal analysis and statistical analysis. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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