

EVALUATION OF THE MODIFIED SLOW FREEZING CRYOPRESERVATION PROTOCOL FOR THE BLOOD PRIMORDIAL GERM CELLS (bPGCs) FROM *Gallus gallus domesticus* (Linnaeus, 1758)

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ABSTRACT

Indiscriminate crossbreeding of the Philippine native chicken may pose a biodiversity loss in the future. As such, cryopreservation of blood primordial germ cells (bPGCs) from native chick embryos was targeted as a preventive measure. From 112 eggs, bPGCs were collected and cryopreserved (1 d, 2 d, 7 d, and 14 d) through a modified slow freezing protocol. Afterwards, thawed bPGCs were cultured for a day. Results showed post-thawed bPGCs had a declining viability rate as the period of cryopreservation lengthened ($p < 0.05$). Short-term cell culture of freshly collected and thawed bPGCs showed no significant difference in cell density after 24 h ($p > 0.05$). However, the similar cell densities with their pre-culture values suggest that a long-term cell culture is needed to better support the bPGC recovery and propagation. With this, the modified cryopreservation protocol supplemented with long-term cell culture is recommended to be examined further before it can be used in chicken PGC-based gene banking.

Keywords: chicken, bPGCs, cryopreservation, cell culture

INTRODUCTION

The local animal breeds at risk among farmed and domesticated animals are reported to be at 28.38% globally, with Asia comprising 6.57% of it (FAO, 2024). Among the avian species, chickens (*Gallus gallus domesticus*) are part of the population at risk for biodiversity loss, for which there were already reported 14 extinct breeds globally (Sawicka *et al.*, 2015a). In the Philippines, the native chicken's biodiversity loss is not an immediate concern; however, there is indiscriminate crossbreeding and active upgrading programs for its meat and egg quality that may lead to biodiversity loss (Bondoc & Santiago, 2012). Notably, the chicken inventory in the Philippines for January 2022 to September 2023 showed declining growth rate for layer chickens (4.2% to -1.0%) and broilers (-0.9% to -2.0%) while there is an improving growth rate for native/improved chickens (1.1% to 5%) (PSA, 2024). This suggests that the upgrading programs may be gearing more towards improving egg production and meat quality of native/improved breeds than commercial breeds.

The initiative under United Nations Sustainable Development Goals (SDG) Target 2.5.1b recommends using cryopreservation as a means to conserve animal genetic resources

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for food and agriculture in gene banks (FAO, 2021). For preserving poultry biodiversity, the *ex-situ* method of cryopreserving semen, blastodermal cells, and primordial germ cells (PGCs) was conducted as an economically and sustainable means of maintaining genetic resources (Setioko *et al.* 2007; Sawicka *et al.*, 2015a; Nakamura, 2016). Among these, the PGCs were targeted since they are pluripotent cells with the ability to give rise to the male or female gametes and have migratory capacity. In birds, the PGCs migrate via the vascular network; hence, they can be referred to as blood primordial germ cells or bPGCs (Nakamura *et al.*, 2013).

There are two freezing methods employed in cryopreservation: vitrification and slow freezing. Vitrification is a process of amorphous, non-crystalline solidification of solutions at low temperature by achieving extremely high viscosity ($\sim 10^{13}$ poises) (Pegg, 2007; Elliott *et al.*, 2017). On the contrary, slow freezing involves replacement of intracellular water with cryoprotectants to reduce cellular damage (Jang *et al.*, 2017). It also employs a cooling rate that is typically at $-1^{\circ}\text{C} / \text{min}$ using a freezing machine with a temperature controller (Sawicka *et al.*, 2015a). Slow freezing was the preferred freezing method due to the following advantages: low cryoprotectant toxicity, requires easy manipulation skills, and offers high post-thaw viability of cells preserved (Jang *et al.*, 2017). Since not all laboratories have a freezing machine, a liquid nitrogen tank and a modified slow freezing protocol were used as a substitute for it. In this regard, this study was designed to aid in the genetic conservation of the Philippine native chicken through a modified slow freezing of bPGCs.

Despite its advantages, the cryopreservation of the bPGCs may have negative consequences. Not all of the post-thawed PGCs remain viable or healthy, with some becoming necrotic and apoptotic due to the ice nucleation and dehydration caused by cryopreservation (Sawicka *et al.*, 2015b). Hence, it is important to determine the viability of the thawed PGCs. Currently, there are no reports on the viability of post-thawed bPGCs from the Philippine native chickens. Considering the research gaps, this study determined the efficiency of the modified slow freezing cryopreservation protocol for the bPGCs from native chicken through assessment of the viability rates and cell density of thawed and cultured bPGCs.

MATERIALS AND METHODS

The procedures conducted in this study were approved by the Institute of Animal Care and Use Committee (IACUC) of the University of the Philippines Los Baños (UPLB) under the protocol number CAFS-2018-006. The study was conducted from January 2020 to June 2022. A total of 112 unincubated eggs from mixed-breed Philippine native chickens were collected from the University Animal Farm, UPLB. The eggs were incubated at 37°C for 50 - 56 h at the Animal Physiology Laboratory, Villegas Hall, UPLB. The chick embryos were subsequently staged according to parameters by Hamburger and Hamilton (1951), also known as HH staging.

The collection protocols for bPGCs of Chojnacka-Puchta *et al.* (2015) and Sawicka *et al.* (2015a) were followed with modifications. Instead of a glass micropipette, blood was collected from the dorsal aorta of embryos at the HH 14 -16 stages (50 - 56 h of incubation) using a 34G needle (JBP® Nanoneedle Premier Super Ultra-Thin Walled, FEEL TECH Co., Ltd.® Busan, Korea) in 1 cc syringe [1 cc tuberculin syringe, Terumo® (Philippines) Corporation; Laguna, Philippines]. The HH 14-16 stages were selected as they covered

the migration to settlement of the bPGCs from the area pellucida to the embryo proper, with the genital ridge as the endpoint. The visualization of the embryos was done under a microscope (DM1000 LED Ergonomic system microscope, Leica® Microsystems (SEA) Pte Ltd., Singapore) with a camera (C-WF2 WIFI camera, OPTIKA® Srl, Bergamo, Italy). The collected blood from the chick embryos was pooled (5 embryos per 1.5 ml microtube) to obtain ample amounts to examine.

Erythrocyte lysis was accomplished by treating the pooled embryonic blood with 1.5 ml lysing buffer (Gibco™ ammonium chloride-potassium (ACK) lysing buffer, Thermo Fisher Scientific, Inc., Massachusetts, USA). Afterwards, the samples were incubated (10 min, room temperature) with mild shaking. The suspension from the lysis contained the bPGCs; thus, the samples were centrifuged (3000 rpm, 12 min, room temperature) to isolate the bPGCs. The supernatant was discarded, and the white pellets containing the bPGCs were suspended in Dulbecco's modified Eagle medium (DMEM) and 10% dimethyl sulfoxide (DMSO) in fetal bovine serum (FBS) inside the 1.8 ml cryogenic vials. In this study, DMEM served as the freezing and culture media that provided glutamine and glucose to the bPGCs compared to OptiMEM in other studies (Chojnacka-Puchta *et al.*, 2015; Sawicka *et al.*, 2015a; Sawicka *et al.*, 2015b). The cryoprotectant used was 10% DMSO based on its favorable cryoprotection of blastodermal cells from the work of Sawicka *et al.* (2015a). Aside from some changes in the pre-cryopreservation, there were also steps altered in the slow freezing due to the lack of a freezing machine with a temperature controller. The modified steps were as follows: 1) The bPGC suspension in the cryogenic vials was cooled inside a refrigerator (4°C, 2 h); 2) Afterwards, the vials were exposed to liquid nitrogen vapor (10 cm elevation, 15 min); and 3) The vials were submerged into liquid nitrogen (-196°C) for cryopreservation under different durations: 1 d (Sawicka *et al.*, 2015a), 2 d, 7 d (Tonus *et al.*, 2017), and 14 d (Setioko *et al.*, 2007).

Frozen bPGCs were thawed by transfer from -196°C to 4°C (Sawicka *et al.*, 2015b). The frozen bPGCs were left to thaw for 30 min inside the refrigerator (4°C). Thawed bPGCs were washed with phosphate-buffered saline, PBS (without Ca²⁺ and Mg²⁺), then centrifuged (3000 rpm, 6 min). The supernatant was discarded, and the cell suspension was used in the viability assessment (Setioko *et al.*, 2007). About 10 µl of cell suspension was mixed with 10 µl of 0.4% Trypan blue stain. The number of cells stained and unstained by Trypan was noted for the determination of viability. A hemocytometer was used in the counting of bPGCs. Viability rate was computed using the formula below:

$$Viability\ rate(\%) = \left(\frac{\text{number of bPGCs unstained by Trypan}}{\text{number of bPGCs stained} + \text{bPGCs unstained by Trypan}} \right) \times 100\%$$

The protocol for cell culture was adapted from Chojnacka-Puchta *et al.* (2015). Freshly collected and cryopreserved bPGCs (thawed after 24 h) were cultured to check the bPGCs' mitotic capacity. The bPGCs were added with DMEM supplemented with neomycin and fetal bovine serum (FBS). The cells were grown for 24 h at 37°C and 5% CO₂ inside the incubator. Cell density was determined for both freshly collected and cryopreserved bPGCs before and after the cell culture. About 10 µl of the cell suspension was examined, and the average number of bPGCs counted for the four large squares of the Neubauer hemocytometer was multiplied by 10,000 to get the final cell count.

A one-way analysis of variance was conducted on the viability rates and cell densities

obtained from freshly collected, post-thawed, and cultured post-thawed bPGC specimens. Post-hoc analyses were done using Tukey's honest significant difference test to determine specific bPGC groups that caused the effect on viability and cell density. A paired t-test was done to assess the cell densities obtained from before and after culture samples of freshly collected bPGCs. Levels of significance were set at $p < 0.01$ and $p < 0.05$. All statistical analyses were performed using the online statistical tool, VassarStats.

RESULTS AND DISCUSSION

HH staging

Before the blood collection, HH staging of the unincubated eggs was done to ensure that unfertilized and non-targeted HH stages were not included in the samples for the PGC isolation. Only 52% (58/112 eggs) yielded the targeted stages of HH 14 – 16 among the 112 unincubated eggs that were staged. The results suggest that not all unincubated eggs develop according to their age of incubation nor there is assurance that they form an embryo at all, i.e., 29% (33/112 eggs) were in the blastodisc/blastoderm stage and 8% (9/112 eggs) were younger or older than HH 14-16 in the 50-56 h marks. This may be attributed to how the chick embryo development is affected by the egg storage and age of the hen as pre-incubation factors, along with temperature, turning conditions, and gaseous environment as incubation factors. Changes in these factors may influence the embryo's metabolic and growth hormone profile (Decuypere & Bruggeman, 2007). In this study, pre-incubation factors may have more weight in the observed stages than the incubator factors. The gaseous environment was not controlled in the setup, but the temperature, turning, and humidity were properly monitored. Furthermore, several hens served as sources of eggs, and their different age, body conditions, and genetic variations might have affected the quality of eggs used, i.e., varied embryonic development in eggs from young versus old hens (Fasenko *et al.*, 1992).

Isolation and purification of the blood primordial germ cell (bPGC)

According to studies, there are initial differences in the number of PGC population in chick embryos, with numbers ranging from 50 - 400 for HH 4 - HH 26 embryos (Fujimoto *et al.* 1976; Bernardo *et al.*, 2012). The differences are linked to genetic differences among hens. Any effect on the variation of initial PGC counts was limited by the pooling of bPGC donors during the blood collection stage (5 donors/tube), which serves as a standardization step in leveling the initial PGC counts per sample. In this study, there was no difference in the viability rates of freshly collected PGCs ($p = 0.087035$), although Tube 2's bPGC viability rate was notably higher than those in other tubes (Figure 1a). Low viability rate (39%) for freshly collected bPGCs was observed despite the considerations in careful purification of PGCs before cryopreservation. This indicated that there was possible damage incurred through centrifugation and exposure to reagents during PGC processing, unlike the absence of damage in bPGCs collected via Percoll gradient centrifugation in the study of Chojnacka-Puchta *et al.* (2015).

The freshly collected and isolated post-thawed bPGCs followed the description for the morphology of chicken bPGCs (Figure 1b) observed in related studies. The bPGCs appear to be spherical or oval (average diameter = 14µm), with a large nucleus (average diameter = 8µm), with a fragmented nucleolus and a rough cellular surface (Fujimoto *et al.*, 1976; Yu *et al.*, 2019). The rough surface may be attributed to various vacuoles in PGCs that contain

glycogen granules (Sawicka *et al.*, 2015a). Similarly, no morphological differences were observed between freshly collected and post-thawed PGCs from White Leghorn chickens (Kohara *et al.*, 2008) and Gaok chickens (Kostaman *et al.*, 2011).

Freezing and thawing of bPGCs

There was a significant decline in the viability rates of thawed bPGCs ($p=0.012168$) as the duration of cryopreservation became longer (Figure 1c). There was a lower viability rate in the bPGCs thawed after 1 d, 2d, and 7d [18-22.4% versus 70-90% reported by Sawicka *et al.* (2015a) and Tonus *et al.* (2017)] and after 14 d [10.8% in comparison to

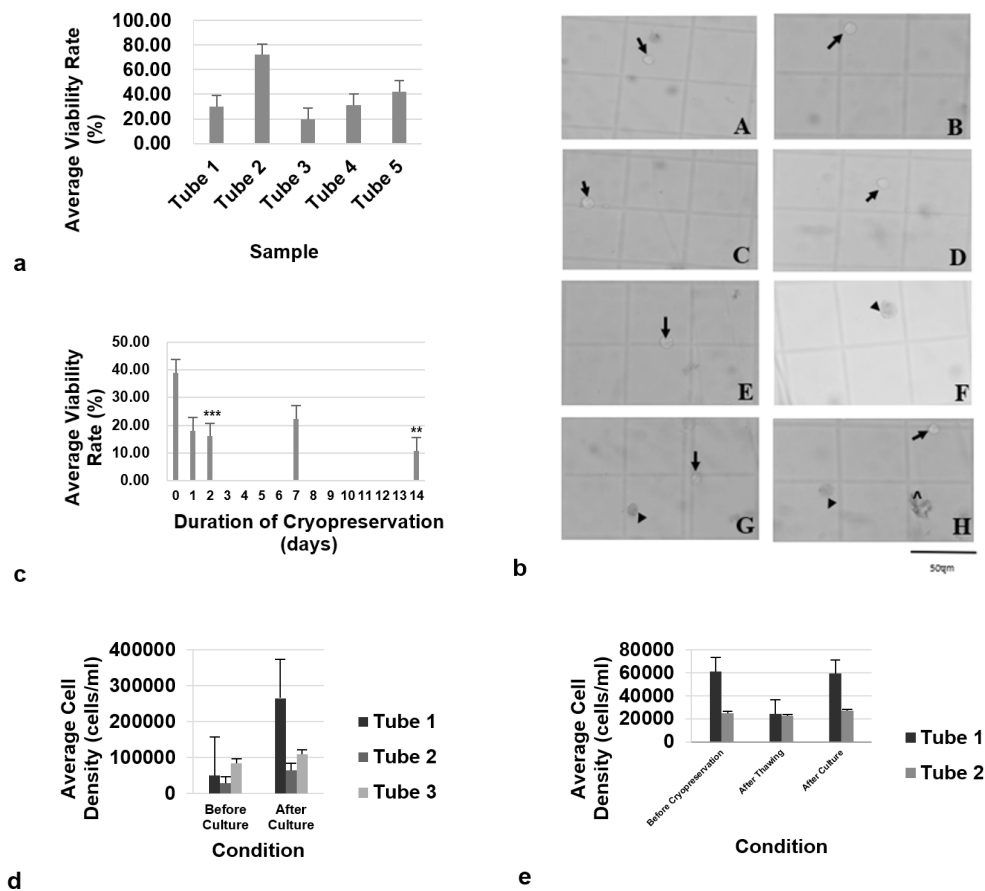


Figure 1. Viability rates (%), cell densities (cells/ml) and morphological observations of PGCs: **a**) % viability of freshly collected chicken bPGCs (5 blood donors/tube); **b**) appearance of freshly collected (A-D) and post-thawed (E- H) bPGCs after trypan blue staining (400x); **c**) % viability of thawed chicken bPGCs (from Tubes 1-5 in a) under different duration of cryopreservation: 1 d, 2 d, 7 d, 14 d; **d**) cell density of freshly collected chicken PGCs before and after cell culture (5 blood donors/tube) [37°C, 5% CO₂, 24 h incubation] and **e**) cell density of cryopreserved chicken PGCs before cryopreservation, after thawing and after cell culture (5 blood donors/tube)[37°C, 5% CO₂, 24 h incubation]. Vertical bars are standard errors. Legend: arrowhead - dead, arrow- alive, circumflex – debris; ** - $p<0.01$ and *** - $p<0.05$ with Day 0: viability rate before cryopreservation.

83.5% by Setioko *et al.* (2007)]. Post-hoc analysis showed significant differences between the viability rates of bPGCs under 0 and 2 d at $p < 0.05$ and 0 and 14 d of cryopreservation at $p < 0.01$. Despite following the working cryopreservation and thawing protocols in other chicken breeds, a low average viability rate was still observed, from the ideal 80-100% post-thaw viability. This may be related to genetic differences among native chickens, resulting in varied responses and sensitivities to cryoprotectant, freezing, and thawing temperatures. Overall, the results suggest that the current freezing protocol needs to be further optimized to increase the viability rates of thawed PGCs. Furthermore, it is still recommended to conduct a longer duration of cryopreservation as there might be advantages to it, *i.e.*, 85.7% viability rate in bPGCs thawed after one month (Nakamura *et al.*, 2011).

Cell culture of bPGCs

Because of cryoinjury, death, and low numbers upon collection, it is imperative to ensure that PGCs can be propagated before and after cryopreservation. The proliferation of bPGCs was examined in this study, with results showing that average cell density had no significant change from before and after cell culture of freshly collected bPGCs ($p = 0.272393$; Figure 1d) and among before cryopreservation, post-thaw, and cultured post-thawed bPGCs ($p = 0.408163$; Figure 1e). It is notable, however, that the cultured bPGCs from freshly collected and post-thawed samples had similar cell densities to their pre-culture values.

The declining trend observed in the viability rate of cryopreserved PGCs is to be expected and can be reversed via a subsequent medium - to long-term cell culture using proper media. Interestingly, Tonus *et al.* (2017) noted a continued increase in both cell density and viability rate of thawed PGCs as the culture was extended from 1 to 7 d, with an average doubling time for control PGCs of 2.05 d and slow-frozen PGCs of 2.25 d. This is something to look further into, since in Tube 1 in Figure 1e, the cultured post-thawed bPGCs (59,375 cells/ml) had doubled their cell density compared to thawed bPGCs (24,375 cells/ml). Moreover, the *in vitro* self-renewal of avian PGCs in media with only growth factors and no feeder cells is well-documented. For example, freshly collected bPGC cultures from a chick embryo donor resulted in abundant cell densities after long cell culture durations with growth factors, *i.e.*, > 50,000 cells after 3 weeks (Whyte *et al.*, 2015) and >100,000 cells after 4 weeks (Woodcock *et al.*, 2019).

CONCLUSION

The HH staging results suggest that pre-incubation factors have more weight than incubation factors in terms of influencing chick embryo development. Initial pooling of blood is also a must to ensure that there is a standardization of the number of PGCs to be examined for viability tests and cell culture densities. Post-thawed bPGCs from the modified slow freezing protocol also do not lose their characteristic appearance when compared to freshly collected bPGCs, but their viability rate decreases as the duration of cryopreservation lengthens, with 2 d ($p < 0.05$) and 14 d ($p < 0.01$) post-thaw bPGCs showing significant reductions from bPGCs at 0 d. Short-term culture of freshly collected and thawed bPGCs yielded low viability rates of recovered bPGCs; however, overall cell densities showed that cell culture may be done before cryopreservation to increase the bPGC population, and thawed bPGCs can also be initiated to recover and self-renew after freeze-thaw. Longer

durations of cryopreservation and of cell culture, as well as selection of optimal media and growth factors, need to be further examined in order to maximize the efficiency of the presented modified slow freezing protocol. The results of the study support that the modified slow freezing of bPGCs of the native chicken eggs has the potential to be an affordable way to do PGC-based gene banking and therefore, can become a tool to push forward the conservation efforts for the prevention of biodiversity loss for Philippine poultry, particularly the Philippine native chicken.

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