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The Conservation on Indonesian Native Chicken Biodiversity Through Primordial Germ Cells Cryopreservation

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ABSTRACT

Primordial germ cells (PGCs) can be cryopreserved and used as a potential tool for the preservation of poultry biodiversity. By transplanting donor PGCs into recipient embryos to produce chimera germline, PGCs can migrate to developing gonads where germ cells are produced, thus enabling reproduction of offspring derived from donor PGCs via mating of chimera germline. Collection of PGCs from the circulation of chickens. Fertile eggs were incubated in a portable incubator for 2.5 days. Purification of circulating PGCs with nycodenz. Then the PGCs were frozen using the 5% DMSO cryoprotectant and to determine the success of cryopreservation, the donor PGCs were transplanted into the recipient embryos. The number of circulating PGCs was 50.58 ± 5.79 cells per embryo, the percentage of PGCs viability after thawing was 80.18 ± 1.25%. From the results of the PGCs transplant by looking at the migration of donor PGCs in recipient embryos with PKH-16 staining, it is clear. In conclusion, PGCs cryopreservation in this study can generally support the survival of good chickens and it is hoped that this PGCs conservation protocol can be used for other native chickens.

Keywords: PGC, cryopreservation, chicken, conservation

INTRODUCTION

Animal germplasm cryopreservation enables the sustainable and economical maintenance of genetic resources for industry and livestock research. In mammals, ex-situ conservation strategies are methodologically possible by integrating reproductive technologies such as semen, egg and embryo cryopreservation, artificial insemination, in-vitro fertilization, somatic nuclear transfer, and embryo transfer. In poultry, semen cryopreservation has not been successful, mainly due to poor fertility rates in frozen or thawed birds, where sperm counts are very low, highly variable, and not sufficiently reliable in commercial production or preservation of genetic resources (Kowalczyk & Lukaszewicz., 2015; Mphaphathi et al., 2016). This is because semen cryobanking is not sufficient as an ex-situ conservation strategy in poultry because genes on the W chromosome and mitochondrial DNA cannot be preserved because males are homogametic (ZZ). Therefore, frozen semen collections can only be used effectively to maintain and increase the genetic diversity for extant chicken breeds (Nandi et al., 2016).

Alternatively, primordial germ cells (PGCs) are the only cells in a developing embryo that have the potential to transmit genetic information to the next generation. Potential PGCs emerge when isolated from donors, manipulated in vitro, and reintroduced into recipient embryos (Szczerba et al., 2016). In the future, PGCs will be a pioneer in the field of stem cell and developmental biology (Anand *et al*., 2018). The development of avian PGCs transplantation technology provides insight into ex-situ conservation as PGCs allow the transfer of all genetic information (Nakamura, 2016).

In chickens, migration of PGCs begins from the center of the blastoderm during early embryo-genesis and into the extra-embryonic tissue (germinal sickle) according to primitive streak formation (Tsunekawa et al., 2000) Then, PGCs enter the dorsal aorta from the germinal crescents and circulate in the bloodstream along the dorsal mesentery and finally collect in the germinal ridges (Nakamura et al., 2013; Kagami, 2016).

This research is the first step to apply PGCs circulation transfer technology in the livestock sector in Indonesia. As the donor animal model, the gaok chicken was used and as the recipient animal model, the White Leghorn (WL) chicken was used. The gaok chicken was used as a donor animal model because the gaok chicken was commonly found in the villages of Gapurana, Poteran, and Palasan, Poteran Island, Sumenep Regency, Madura. The specialty of the gaok chicken is that it has a long voice similar to that of a pelung chicken. Although the population of gaok chickens is not known with certainty, it is estimated that in all districts in Madura there are around 2,000 birds (Sartika & Iskandar, 2007). The spread is also not wide when compared to ordinary free-range chicken. WL chickens are used as recipient models for PGCs-circulation transfer applications because WL chickens are very productive and able to adapt to all environmental conditions.

Therefore, this study aimed to establish a protocol for the collection of PGCs derived from gaok chicken embryos and to cryopreserve PGCs to be used for the production of germline chimeras in the future.

MATERIALS AND METHODS

Preparation of Gaok and White Leghorn Chickens Eggs

A total of 200 fertile eggs of gaok chickens as donors and 100 fertile eggs of WL chickens as recipients, eggs were placed in a portable incubator (Biotype P-008B; Showa Furanki, Saitama, Japan) at 37.7^{o}C and 70% humidity and rotated 45^{o} every 15 minutes. Gaok and WL chicken eggs were incubated until they reached embryonic age at the stage of 14-15 or 2.5-3 days (Hamburger & Hamilton, 1951).

Isolation and Purification of Circulating PGCs

Gaok chicken eggs that have reached the embryonic development stage 14-15, the eggshells are broken and the contents of the eggs are transferred to a petri dish (90 x 15 mm, LBS60001PT, BIOLAB). Blood was collected from the dorsal aorta using a micropipette (inner diameter = 30 mm) under a microscope (Olympus SZ30, Japan) (figure 1). The blood collection was placed in a 1.5 ml eppendorf tube containing 1.000 µl of PBS solution (-) in 10% fetal bovine serum (FBS, 26140 Gibco). PGCs-circulation purification procedure used gradient centrifugation method using nycodenz (Prod. No. 1002424, Axis-Shield Poc AS) (Zhao & Kuwana, 2003).

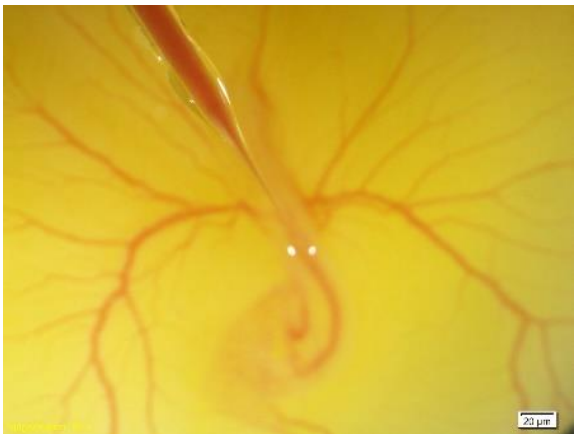

Figure 1. Embryonic Blood Sampling in the Dorsal Aorta

Freezing of Circulating PGCs

The circulating PGCs were purified and suitable for freezing (round, not deformed, the same size, symmetrical and transparent), then diluted with the cryoprotectant DMSO 5%. PGC-circulation was put into a 0.25 ml straw, which contained 100 cells. The freezing process uses a freezing machine (FHK Fujihara brand: ET-1, Japan). The frozen circulation of PGCs to be transferred is first thawed according to the procedure (Setioko et al., 2007). After thawing, the frozen circulating PGCs were evaluated to determine the live and dead circulating PGCs, then the live circulating PGCs were transferred to the recipient embryo.

Circulating PGCs Transfer

Recipient embryos were transferred with circulating frozen PGCs donors (≤ 100 cells). The criteria for the recipient embryo to be used are the embryo must be normal, the blood vessels are clearly visible, the yolk is large, and not broken. The stages of transferring frozen circulating PGCs donors to recipient embryos are carried out by in the recipient egg (WL), a small hole is made in the blunt eggshell, with the aim that the air in the air chamber is released so that it will be easier to manipulate the recipient embryo. Then the egg is

turned over so that the blunt part of the shell is on the bottom and the pointed part of the shell is on the top. The pointed part of the shell is then made a window or hole (diameter 1.5 to 2 cm) until the embryo is visible in order to facilitate manipulation of the embryo because the pointed part of the eggshell is where the embryo falls. After the recipient embryo was visible, using a micropipette (30 m) under a microscope (Olympus SZ30, Japan) all of the recipient embryo's blood was taken from the dorsal aorta. Then through the same spot, the circulating PGCs donor (which has been prepared) is transferred to the recipient embryo. Next, the window of the recipient's egg is closed with cling film, recipient embryo eggs were incubated at 37.7^{o}C with 60% humidity and rotated 45^{o} every 15 minutes using a portable incubator (P-008B Biotype; Showa Furanki, Saitama, Japan) for 21 days.

Staining and Examination of Donor PGCs Transferred to Recipient Embryos

To determine the presence of donor PGCs in recipient embryos, the donor cells were visualized by staining using the PKH-26 Red Fluorescent Cell Linker Mini Kit (Sigma-Aldrich Inc., St. Louis, MO). PKH-26 is a cell marker that colors cell membranes so that these cells will glow red when observed under a red filter fluorescent microscope. The PGCs staining with the following stages first step the collected PGCs cell suspension was stored in a 1.5 ml eppendorf tube, then centrifuged at 1,070 rpm for 5 minutes, supernatant discarded (reserve 30 µl with pellet). Wash the pellets by adding 1.000 µl PBS (-) and centrifugation at 1.070 rpm for 5 minutes (first washing) then remove the supernatant (reserve about 30 µl with the pellet), then add 1 ml of diluent C (control diluent/stock of PKH-26) (referred to as solution a).Prepare a new 15 ml tube, add 1 ml of diluent C and 2.4 µl of PKH 26 dye (referred to as solution b), add solution a (diluent C + PGCs) into solution b (diluent C + PKH 26) and incubate at 37°C for 5 minutes in an incubator. Centrifugation at a speed of 1.070 rpm for 5 minutes. Wash the pellets by adding 1.000 µl PBS (-) and centrifugation at 1.070 rpm for 5 minutes (second washing), the labeled primordial germ cells are injected into the recipient embryo. After the 6th day of incubation, the gonads were taken from the recipient embryo, and the injected PGCs were seen to detect PGCs migration using a fluorescence microscope.

Determination of the Number of Circulating PGCs

The number of circulating PGCs gaok chickens per embryo was calculated using the formula from (Zhao and Kuwana, 2003) as follows: the number of PGCs isolated was divided by the number of embryos used.

Viability PGCs-circulation

The frozen PGCs that had been evaluated were then assessed for viability using trypan blue staining (Freshney, 2005), that is, ± 20 µl of PGCs cells were mixed with 10 µl of 0.4% trypan blue (Sigma-Aldrich Corporation, St. Louis, MO, USA) and then stirred until homogenized and allowed to stand for 2 minutes at room temperature. Then, the viability was measured under a microscope (Olympus CKX41, Japan). Life PGCs is PGCs which does not absorb blue stains and the dead are PGCs that absorb blue stains. The calculation is as follows: the number of live PGCs minus dead PGCs divided by live PGCs multiplied by 100%.

RESULTS AND DISCUSSIONS

PGCs-circulation Isolation and Purification

In this study, embryos at developmental stage 15 according to (Hamburger & Hamilton. 1951) were used for circulating PGCs collection. This is in line with the report of Tajima et al. (1999) where the number of circulating PGCs was found to be maximum at stages 14-15 because at stage 15 the circulating PGCs were very actively migrating and it was possible that all PGCs were already in the embryo's circulatory system, and after that, the number of circulating PGCs would decrease. The decrease in the number of circulating PGCs after stage 15 may mean that some PGCs have left the circulatory system because the presence of PGCs in the blood circulation is only for a short time and after that, they form colonies in the germinal ridge. Similar results were reported by Li et al. (2001) on quail, Zhao et al. (2003) on Rhode Island Red chickens, Kuwana et al. (2006) on WL and Kureko Dori chickens, Nakamichi et al. (2006) on Barred Plymouth Rock chickens, Atsumi et al. (2008) on WL chickens, and Qian et al. (2010) on Silky chickens, namely that as the age of the embryo increases, the number of circulating PGCs decreases or decreases.

Gaok chicken PGCs were collected and isolated from the embryonic circulation (figure 2A) and purified by the gradient centrifugation method using nycodenz (figure 2B). In whole blood samples collected from embryos, PGCs could be

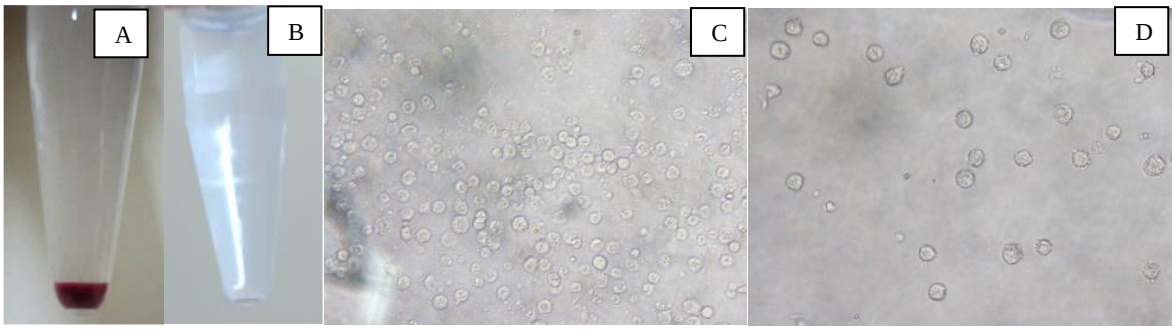

Figure 2. PGCs collection and purification. Blood sample before purification (A), blood sample after purification (B). A small amount of PGCs was observed in blood samples collected from gaok chicken embryos (C). After purification by gradient centrifugation method using nycodenz, most of the blood cells were removed resulting in a significant increase in PGCs concentration (D).

Distinguished from blood cells with different morphology under an inverted phase-contrast microscope (figure 2C). PGCs are spherical and larger in size with a large core reflecting the oblique illumination observed under an inverted phase-contrast microscope. The PGCs population in whole blood samples was found to be relatively small. After purification with a nycodenz density gradient, large numbers of blood cells were removed giving a much higher proportion of PGCs (figure 2D) for further use in cryopreservation.

In this study, using the nycodenz method will reduce the number of red blood cells, so that circulating PGCs can be isolated easily. The advantage of isolating circulating PGCs using nycodenz is that a collection of circulating PGCs with high purity (up to 90%) will be obtained, simple, and more efficient than previously reported methods (Zhao and Kuwana, 2003). This is because nycodenz is a non-ionic chemical, has a molecular weight of 821 and a density of 21 g/ml in powder form. Other advantages of nycodenz are low toxicity, high water solubility, and more stability when autoclaved. In addition, nycodenz has a low viscosity and can be used to separate blood cells (Boyum & Scand, 1976; Rickwood et al., 1982). The number of PGCs purified from whole blood samples of each embryo was at 50.58 ± 5.79 cells, slightly lower than that of PGCs isolated from commercial broiler embryos. Rhode Island Red chicken embryos at stage 15, the number of circulating PGCs varied between 67-73 cells per embryo (Nakamura et al., 2011). In addition, in general, blood volume and blood cell count increased linearly with embryo development (Zhao et al., 2003).

Circulating PGCs Freezing

The quality of PGCs to be used in the freezing process was evaluated based on morphological characteristics. Evaluation of the morphological characteristics of fresh PGCs that deserve to be frozen was assessed based on round shape, no defects, the same size and symmetrical and transparent, while those that were not eligible were not symmetrical and not the same size (figure 3).

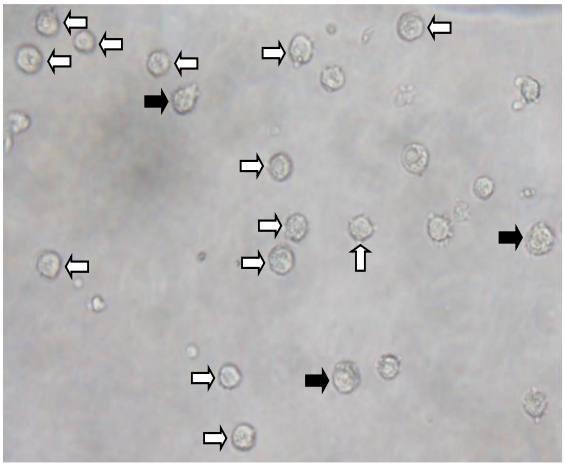

Figure 3. Morphological characteristics of the gaok chicken PGCs. PGCs eligible for freezing (indicated by white arrows). PGCs that do not deserve to be frozen (indicated by black arrows). Bars = 40 m.

Success in the cryopreservation of PGCs can be determined by evaluating the morphology of PGCs microscopically. PGCs after freezing have a morphology that is almost similar to before freezing. The cells are intact, large round shape, and the edges look like a bright ring under the cell membrane. Meanwhile, the dead PGCs were dark in color with very little cytoplasmic mass (Figure 3). Reinforced by Kohara *et al*., (2008) reported that the morphology of WL chickens did not show any difference between PGCs before and after freezing. Live PGCs looked like fresh PGCs and were indistinguishable from unfrozen PGCs. The cells

were intact and similar in size and shape to fresh PGCs. In other words, the morphology of frozen PGCs is normal.

The average percentage of PGCs viability after thawing was 80.18 ± 1.25%. The results of this study are in accordance with research conducted by More et al. (2006) which showed that gonad germ cells (GGC) of WL chickens frozen with the addition of the cryoprotectant DMSO and ethylene glycol (EG) of less than 5% resulted in a low percentage of viability.

The use of the right concentration of cryoprotectants is necessary for cryopreservation to avoid cell damage. Therefore, the addition of 5% DMSO to the freezing medium was able to protect PGCs from cold stress during the freezing process. The protective effect is to regulate the balance of intracellular and extracellular electrolytes so that the biochemical processes that occur in PGCs continue and reduce excess death of PGCs. The presence of DMSO in the freezing medium is expected to increase the electrolyte concentration to avoid harmful damage. In general, the results of this study are lower than those reported by Setioko et al. (2007) who obtained a percentage of 83.5%, respect, and Nakamura et al. (2011) amounted to 86.8%. This is strongly suspected because of the different methods used in each study. The method used in this study is slow freezing with setting the freezing speed.

Apart from different freezing methods, another factor that affects the percentage of viability is the packaging material used for PGCs storage. In this study, PGCs were packaged in mini straws, whereas in previous studies they were packaged in cryovials. The type of packaging will affect the decrease in temperature and the rate of cell re-melting (Mohamad et al., 2005). Straw packaging is more practical to provide clearer sample identification (Benesova and Trefil, 2016). PGC of zebrafish packaged in straw showed the viability of 70%, better than PGC packaged in microcapsules which showed the viability of 20% (Riesco *et al.*, 2012).

PGCs Transfer and Detection of Donor PGCs Colonization in Recipient Embryos

Confirmation of donor PGCs colonization in recipient gonads aged 7 days after transplantation was carried out because the gonads of 7-day-old WL chickens had developed and if the gonads were taken, the gonads were clearly visible and easy to retrieve. Seven days is thought to be the right time to determine whether donor cells can develop or not in the gonads of the recipient chicken (figure 4).

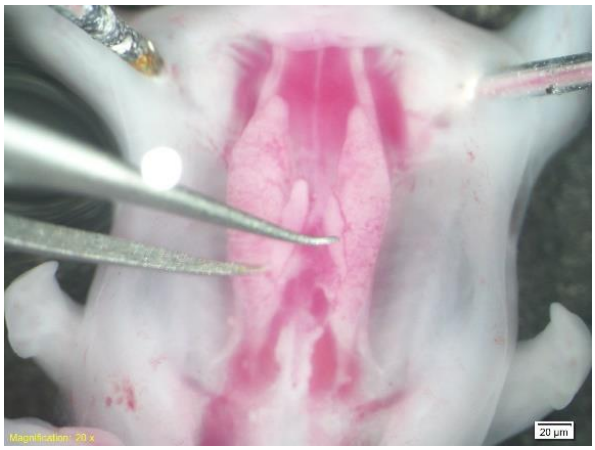

Figure 4. Recipient chicken gonads aged 7 days. The gonads attach to the mesonephros indicated by tweezers

The process of colonization of donor cells into the recipient gonad begins with the migration of donor cells to the gonad tissue (genital ridge) of the recipient chicken. According to Yoshizaki *et al.*, (2010) the PGCs migration process begins with the secretion of chemokine stromal-derived factor-1 (SDF-1) by somatic cells of the recipient gonad, the PGCs (donor cell) then expresses the CXC-chemokine receptor 4 (CXCR-4) receptor and migrate to future gonads using pseudopodia. When it reaches the area of the future gonads, the PGCs undergo colonization with the recipient gonad. Based on the detection of recipient gonadal cells by labeling and examination under a fluorescent microscope, it can be said that colonization was successful by injecting 2.5-3 days old recipients (figure 5). This success is presumably because the recipient's immune system is not fully developed so that the recipient can still accept donor cells from outside that are inserted into the recipient's blood vessels. In addition, this study used PGCs of the same age as the donor and recipient embryos. It is suspected that the age of the recipient has an important influence in providing a microenviron-ment capable of directing the migration of donor cells to the genital ridge so that donor cells can colonize.

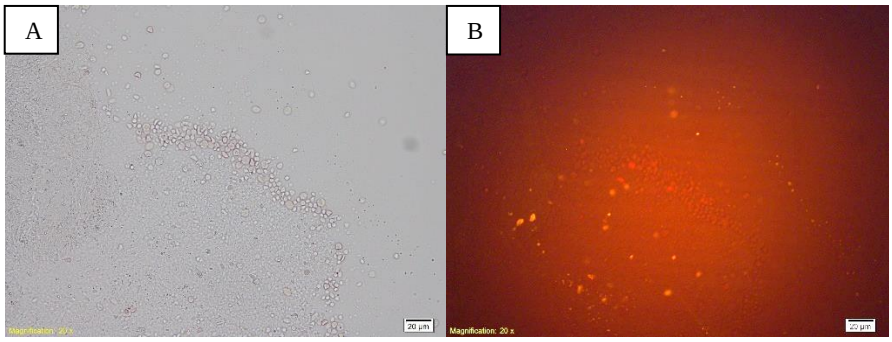

Figure 5. Detection of donor embryo migration in recipient embryos. Embryo migration is seen under an inverted microscope (A) and embryo migration is viewed under a fluorescent microscope

The results for the formation of germline chimera are still relatively low, this may be influenced by the number of circulating PGCs donors transferred to recipient embryos, only ≤ 100 cells. While the research results of Kuwanai *et al.,* (2006); Nakamura *et al.,* (2009) transferred fresh donor PGCs to recipient embryos of 100 to 232 cells; while for frozen PGCs donors, there are 100 to 500 cells (Kino *et al.,* 1997; Kim *et al*., 2010). According to Kim *et al.,* (2010) the number of PGCs donors transferred to recipient embryos is one of the critical factors to increase the success of germline chimera formation. Apart from the number of PGCs donors, a factor that also greatly influences the success of the formation of germline chimera is the high consistency of skills (Nakamura *et al.,* 2009). Technical ability in the micro-injection method itself has an important role in the successful entry of donor cells into the recipient's blood channel. In addition, technical mastery of injection can also indirectly affect the survival rate of the recipient at the time the injection is carried out. According to Athar *et al.,* (2008), using the microinjection technique on embryos, it is possible to have injection techniques that damage certain tissues, causing the eggs to not hatch after injection.

CONCLUSION

PGCs cryopreservation used in research, in general, can support the survival of gaok chickens which can be used in other free-range chicken conservation protocols with PGCs.

CONFLICT OF INTEREST

The authors whose names are listed have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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