Effectiveness of DMSO Concentration on Recovery Rate and Viability of Primordial Germ Cell of Gaok Chicken

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ABSTRAK

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Perkembangan teknologi terbaru untuk memproduksi *germline chimera* dengan transfer *primordial germ cell* (PGC) ke dalam embrio penerima telah memungkinkan konservasi dan pengambilan kembali sumber daya genetik ayam dalam bentuk yang lengkap. Penelitian dilakukan untuk mendapatkan persentase krioprotektan dimethyl sulfoxide (DMSO) yang paling efektif terhadap *recovery rate* dan viabilitas PGC ayam Gaok sesudah pembekuan yang nantinya layak untuk ditransferkan. Dalam penelitian ini, telur fertil ayam Gaok diinkubasi selama sekitar 2,5 - 3 hari untuk mendapatkan embrio pada tahap 14 - 16. Pengambilan darah embrio dilakukan melalui aorta dorsalis dengan menggunakan mikropipet di bawah mikroskop. Prosedur isolasi PGC ayam Gaok dengan gradien sentrifugasi menggunakan nycodenz. Krioprotektan yang tersedia secara komersial digunakan untuk pembekuan PGC. PGC ayam Gaok hasil isolasi dan yang layak dibekukan diencerkan dengan krioprotektan yang mengandung 2,5; 5; dan 10 % DMSO dalam *fetal bovine serum* (FBS). Nilai *recovery rate* dari perlakuan DMSO 2,5; 5; dan 10 % berturut-turut adalah 36,4; 48,2 dan 48 %. Viabilitas PGC setelah pembekuan secara signifikan lebih tinggi untuk DMSO 5 % dibandingkan dengan perlakuan DMSO 2,5 % (P<0,05), akan tetapi tidak berbeda dengan perlakuan DMSO 10 %. Dari hasil penelitian dapat disimpulkan bahwa konsentrasi DMSO sebanyak 5 % pada pembekuan PGC ayam Gaok merupakan tingkat konsentrasi efektif.

Kata Kunci: Krioprotektan, DMSO, PGC, Ayam Gaok

ABSTRACT

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Recent technological developments to produce germ line chimeras with primordial germ cell (PGC) transfer into the recipient embryo provide an opportunity to conserve and retrieval of chicken genetic resources in complete form. The study was conducted to obtain the most effective DMSO percentage to recovery rate and viability of Gaok chicken PGC after freezing which will later be feasible to be transferred. In this study, the eggs of Gaok chicken were incubated for about 2.5 - 3 days to obtain embryos at stages 14 - 16. Blood retrieval was done through the dorsal aorta using micropipettes under microscope. The procedure of PGC isolation of Gaok chicken with centrifugation gradient was using nycodenz as a substance. Commercially available cryoprotectants (dimethyl sulfoxide = DMSO) were used for PGC freezing. Isolated and frozen PGCs of Gaok chicken were diluted with cryoprotectants containing 2.5; 5 and 10% DMSO in *fetal bovine serum* (FBS). The recovery rate of 2.5; 5 and 10% DMSO concentration were 36.4; 48.2 and 48 % respectively. The viability of PGC after freezing was significantly higher for 5% DMSO compared with 2.5% DMSO (P<0.05), but not different from 10% DMSO. It can be concluded that the concentration DMSO of 5 % was effectve contration in freezing Gaok chicken PGC.

Key Words: Cryopreservation, DMSO, PGCs, Gaok Chicken

INTRODUCTION

It is reported by the International Union for Conservation of Nature (IUCN 2015) that more than 13% of bird species were endangered. Recent adopted strategies to conserve the bird biodiversity both *in situ* and *ex situ* were not effective (Sawicka et al. 2015). Therefore, the development of bird biodiversity conservation was indispensable.

In chicken, freezing sperm was one of the methods commonly used on genetic diversity (Blesbois et al. 2007; Santiago-Moreno et al. 2011), but might not guarantee the reconstruction of poultry species (Sawicka et al. 2015). Furthermore, freezing embryo and oocyte might not be performed due to its big size and high lipid content. As an alternative, primordial germ cell (PGC) freezing had been developed to preserve female and male animal germplasm (Liu et al.

2010; Silversides et al. 2013). PGC had been reported as a valuable starting material for cell-based genetic engineering, germplasm expansion and genetic preservation (Tonus et al. 2017). Furthermore, with the PGC cryopreservation as the genetic material seems effective for the conservation strategy.

Chicken PGC collected from embryo blood and gonad might be maintained in the form of frozen PGC in the liquid nitrogen using a medium containing dimethyl sulfoxide (DMSO) (Setioko et al. 2007; Nakamura et al. 2011) without changing its biological characteristics (Nandi et al. 2016; Tonus et al. 2016). Notman et al. (2006) reported that the DMSO that was used as cryoprotectant on phospholipid membranes caused cell membrane become more floppy and it was able to facilitate membrane fusion process. The cell became better in preparation of recieving the effect of stress caused by cryopreservation, thus reducing the molecule transport obstacles and helps the formation of a pore.

Freezing PGC of commercial chicken in a medium consisting of 10% serum and 5-10% DMSO had been reported to have various recovery rates (RR) and viabilities of PGC. Research of Moore et al. (2006) resulted in the viability of White Leghorn (WL) chicken PGC with DMSO addition by 10%, was 76.5%. Setioko et al. (2007) reported that percentage of RR and viability with DMSO addition by 10% on WL chicken PGC each was 49.9% and 83.5%, respectively. While, Nakamura et al. (2011) reported that addition of the same concentration of DMSO on Barred Plymouth Rock (BPR) showed recovery rate and viability by 54.3% and 86.8%, respectively. Kohara et al. (2008) reported the vitrification and slow freezing methods of RR value by 36.8 and 56.7 % respectively.

This study was expected to obtain optimal concentration of DMSO for RR and viability of Gaok chicken PGC after freezing and further feasible to be transferred.

MATERIALS AND METHODS

Isolation and collection of the primordial germ cell (PGC) on 14-16th level

As much as 60-70 fertile eggs of Gaok chicken were used in this study. Those eggs were incubated under 37.8°C with 60-65% of humidity using portable incubator (P-008B Biotype; Showa Furanki, Saitama, Japan). After reaching 14-16 stadium of 50-59 hours of incubation, the egg shell was cracked and the embryos were transferred into a petri dish (90 x 15 mm, LBS60001PT, BIOLAB).

Embryo blood collection through aorta dorsalis used micropipette under a microscope (Olympus SZ30, Japan). The collected blood was placed into eppendrof 1.5 ml tube which was filled by 1000 µl phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS-) in 10% fetal bovine serum (FBS, 26140 Gibco). Isolation procedure of Gaok chicken PGC with centrifugation gradient used nycodenz (Prod. No. 1002424, Axis-Shield Pos AS) (Kostaman 2014).

Selection of the primordial germ cell (PGC)

Evaluation of amount and quality of morphology of the PGC resulted from PGC purification process obtained from Gaok chicken embryo blood, was performed under microscope (Olympus CKX41, Japan). The PGC morphology is divided into two categories: feasible to be frozen and not feasible to be frozen. The PGC criteria that are feasible to be frozen were round; non-defective; equally large; symmetrical and transparent, while for the not feasible one were not symmetrical and not in the same size (Kostaman 2013) (Figure 1).

Stages of PGC freezing were (1) entering straw into methanol pool of embryo freezing tools under 25 ºC – (-7)°C with temperature decrease speed of 1 ºC per minute (for 32 minutes), (2) maintaining the temperature of -7°C for 10 minutes. Seeding was conducted in 2-3 minutes after reaching -7°C of temperature. It was performed to initiate the forming of ice crystal by pressing the of a dipped nipple into liquid nitrogen to the end of the straw to freeze the whole straw content, (3) re-entering straw into freezing and cooling tools until reaching -30°C of temperature with temperature decrease speed by 0.5°C per minute (for 46 minutes), and (4) entering and keeping the -30°C straw into liquid nitrogen with -196°C of temperature (Kostaman et al. 2011).

Thawing process of the frozen primordial germ cell (PGC)

Thawing process of the frozen PGC was performed by carrying out the straws from the liquid nitrogen container and then placed in the 39°C water (Setioko et al. 2007). As soon as the cryoprotectant media in the straw melted (± for 20 seconds), circulated-PGC was removed from the straw and then put into eppendorf tube and added by 1000 µl PBS-FBS 10% then was centrifuged under 1200 rpm for 7 minutes.

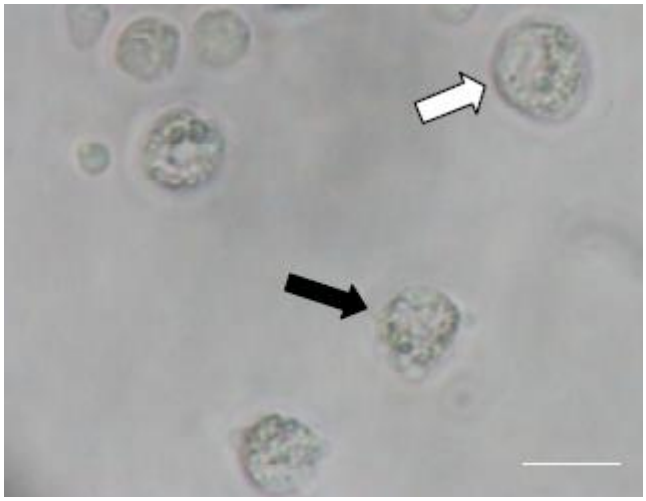

Figure 1. Morphology characteristic of Gaok chicken PGC. The feasible PGC to be frozen (showed by white arrow). Frozen PGC (showed by black arrow). Bar=40 µm. **Source:** Kostaman (2013).

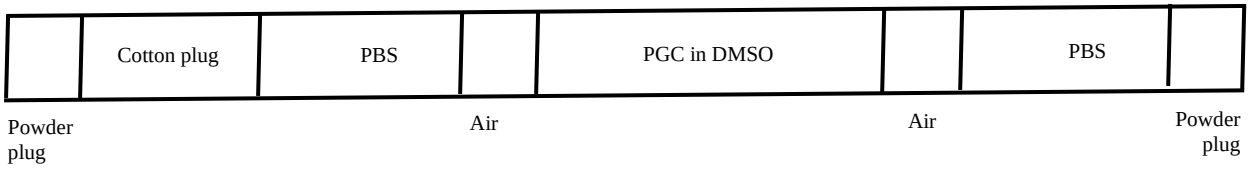

Figure 2. PGC in the mini straw

Viability of the primordial germ cell (PGC)

Frozen PGC that had been evaluated was then assessed for its viability using trypan blue staining (Freshney 2005): ± 20 µl cell PGC was mixed with 10 µl trypan blue 0.4% (Sigma-Aldrich Corporation, St. Louis, MO, USA) then stirred until homogeneous and aged for 2 minutes at room temperature. Then, its viability was measured under a microscope (Olympus CKX41, Japan). Life PGC was the PGC that did not absorb the blue stain and the dead one was the PGC that absorb the blue stain.

Variables observed

Variables observed were:

1. *Recovery rate* (RR) of circulated-PGC after thawing was the number of PGC that could be re-activated after being frozen divided by the number of PGC before being frozen multiplied by 100%.
2. PGC viability after thawing was the number of life PGC reduced by dead PGC divided by life PGC multiplied by 100%.

Analysis

Data were analyzed using complete randomized design (CRD) with concentration of DMSO as treatment and repeated five times. Data were also analyzed using ANOVA and continued by Duncan test when there was a difference between treatments based on Steel & Torrie (1995).

RESULTS AND DISCUSSION

The primordial germ cell (PGC) of Gaok chicken after

The quality of purified PGC that would be used in the freezing process was evacuated based on morphological characteristics. The evaluation of the morphological characteristics of feasible fresh PGC to be frozen was assessed according to round shape, no defect, in the same size and symmetrical and transparent, while the not feasible one was not symmetrical and not in the same size (Figure 1).

The number of PGC collected was 2,125 cells, where 1,513 cells (71.2%) were feasible to be frozen and the 612 cells (28.8%) were not feasible to be frozen. The feasible to be frozen PGC were divided into

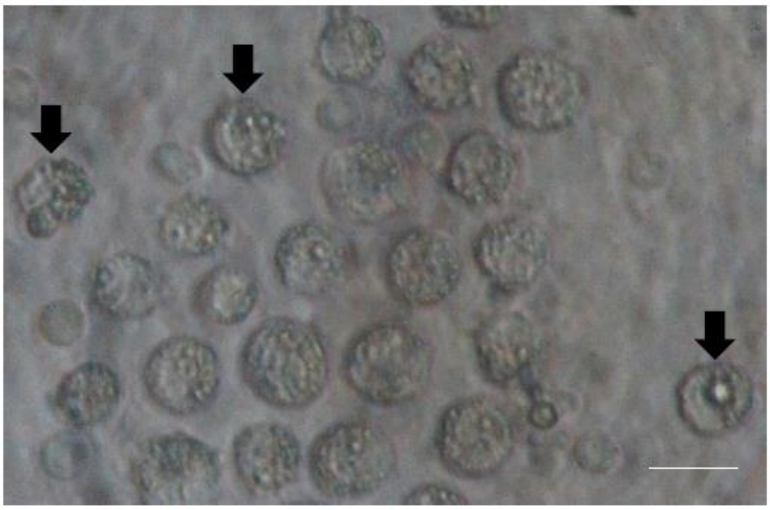

Figure 3. Morphological characteristics of Gaok chicken PGC after thawing. Dead PGCs are shown by the black arrow. Bar=40 µm. Source: Private documentation.

three treatments, where each treatment was repeated five times. PGC packaging used mini straws and was filled with 100 cells.

The success of a method in cryopreservation process might be determined through microscopic evaluation of PGC morphology. The PGC after freezing had almost similar morphology with the one before freezing. The cells were still intact, in a large round shape and the edge looks like a bright ring beneath the cell membrane. Meanwhile, the dead PGCs were dark with very little cytoplasmic mass (Figure 3). It is believed due to several changes and damages of membrane and organelles of PGC cytoplasm. Mohr & Trounson (1981) reported that structural changes following cryopreservation process were nucleus damage and plasma membrane.

Kohara et al. (2008) reported that the morphology of WL chicken did not show a difference between the PGC before and after being frozen. Life PGC looked like fresh PGC and cannot be distinguished from unfrozen PGC. The cells were still intact and had similar size and shape with the fresh PGC. In the other word, the morphology of frozen PGC was normal.

The comparison of concentration of DMSO on recovery rate (RR) of Gaok chicken primordial germ cell (PGC) after freezing

According to the result of morphological selection, the feasible to be frozen PGC then was observed its influence to concentration of DMSO resulted in difference effect on the RR percentage. Hafez (2000) reported that RR was observed to evaluate the effect of cryoprotectant on cell viability after cryopreservation process.

Average RR percentage of PGC after freezing in this study was relatively low. However, RR percentage with DMSO treatment by 5% showed a better role by the RR value of 48.2% (241 cells), which was higher compared to 10% and 2.5% DMSO concentration each by 48% (240 cells) and 36.4% (182 cells), respectively (Figure 4).

Statistical analysis result showed that DMSO concentration by 5 and 10% was not significantly different ($P>0.05$), while the 2.5% DMSO concentration was significantly lower ($P<0.05$). It was allegedly that the addition of 2.5% DMSO could not protect PGC during washing and centrifugation process leading to PGC damage. Damage cells during washing and centrifugation process would affect the RR. This condition was allegedly due to some changes and damages of membrane and PGC cytoplasm organelles. Damage level of PGC was showed by the damage of membrane, marked by intracellular ice crystals formation in the fast cooling process, as the osmotic effect and the attack mechanism of extracellular ice crystals during the slow cooling process. The formation of ice crystals during cryopreservation process lead to electrolyte buildup in the cell causing mechanical cell damage, in which the accumulated electrolyte would damage the cell wall, so that permeability of plasma membrane at the thawing process would change and the cell would die (Watson 2000).

Meanwhile, PGC treated by 5 and 10% of DMSO on the freezing medium was able to protect Gaok chicken PGC from any adverse effects. It could protect PGC by preventing ice crystals formation during the

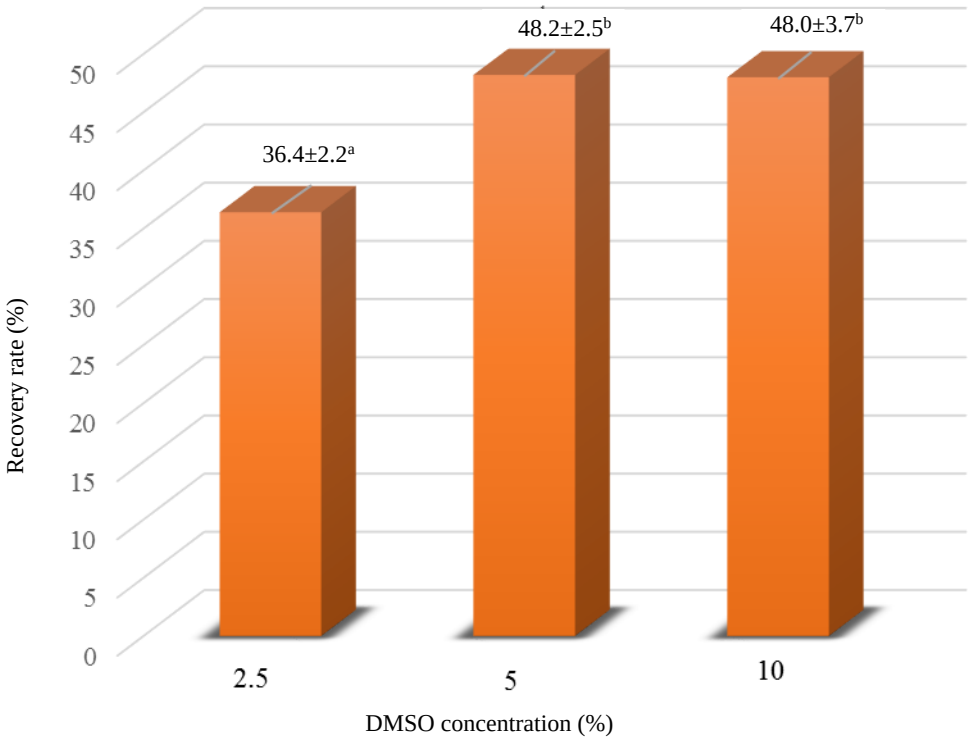

Figure 4. Recovery rate (RR) of circulated-PGC of Gaok chicken after freezing treated with DMSO.

freezing process due to excessive dehydration of cells and stabilizing cell plasma membrane that may prevent physical and functional damages during the freezing process and modify ice crystal structure.

It might prevent damage to PGC cytoplasm organelles. DMSO's cryoprotectant work process was by replacing the water in the cell so that dangerous ice crystals were not be formed (Valerdi et al. 2009). DMSO was able to penetrate the cell before the liquid cell was fully compacted during the freezing process, in which the electrolyte concentration inside and outside the cell was reduced. Meanwhile, the humidity inside the cell wold not be too much to penetrate outside and the dehydration could be avoided. In another word, DMSO served to protect the cell from damage lead by high concentration due to freezing process (Yan et al. 2016).

Average RR percentage of PGC after thawing in this study was lower than the PGC of WL (49.9%) and BPR (54.3%) chickens reported by Setioko et al. (2007) and Nakamura et al. (2011). The PGC in this study had allegedly experienced a lysis during the freezing process.

Rosato & Iaffaldano (2013) explained that cryoprotectant that penetrated to the cell membrane was needed to improve fluidity of membrane and partially dehydrated cells. DMSO would provide effective protection to the PGC when its concentration in the freezing medium was optimum. Not optimum DMSO concentration in the freezing medium leads to quality decease of the PGC. Administration of 2.5% DMSO into freezing medium in this study resulted in the lowest RR percentage after thawing. Therefore, the 2.5% DMSO treatment had not been able to provide optimal protection for PGC freezing of Indonesian native chickens.

Cell freezing optimization to maximize RR value after thawing depended on the formation of intracellular ice crystals and cryogenic damage due to the high concentration of solute when the intracellular water freezed. This could be achieved using hydrophilic cryoprotectant to absorb the water and fast thawing to minimize ice crystal forming (Freshney 2005).

As a comparison, Gautam (2008) reported that by using the slow freezing method, the addition of DMSO cryoprotectant on buffalo oocyte, the damage of RR was less than the addition of ethylene glycol (EG) or propylene glycol. Meanwhile, for the zebrafish embryo stadium, DMSO toxicity was not as much as glycerol, EG, methanol and N,N-dimethylacetamide (Lahnsteiner 2008).

The comparison of concentration of DMSO on viability of Gaok chicken primordial germ cell (PGC) after freezing

In this study, the highest percentage level of PGC viability after thawing was on 5% DMSO by 79.2 % (191 cells) followed by 10% DMSO concentration by 79 % (190 cells) and 2.5% DMSO concentration by 75.7% (138 cells) (Figure 5). This study result was in accordance with the study conducted by Moore et al. (2006) which showed that gonadal germ cell (GGC) of WL chicken frozen by DMSO cryoprotectant addition and ethylene glycol (EG) was less than 5% and resulted in low viability percentage.

Statistical analysis result showed that the viability level of 5% DMSO was not different significantly ($P>0.05$) from the 10% DMSO concentration. Those two concentrations were significantly different with the 2.5% DMSO, which described optimum cryoprotectant concentration in protecting PGC viability on the cryopreservation process. Use of the proper cryoprotectant concentration was a necessity in cryopreservation to avoid cell damage. Therefore, the addition of 5 and 10% DMSO on the freezing medium was able to protect PGC from cold stress during the freezing process. Its protection effect was by managing the balance of intracellular and extracellular electrolyte, so that biochemical process occurring on the PGC persisted and reduced the excessive dead of PGC. The existence of DMSO on the freezing medium was expected could increase electrolyte concentration to avoid adverse damages.

During the freezing process, PGC experienced changes in osmotic pressure due to the cryoprotectant addition or temperature change. A drastic change of temperature occured during circulated-PGC from room temperature to storage temperature (-196°C) or when the thawing process at 39°C lead to damage of PGC plasma membrane. The damage to plasma membrane lead to loss of necessary enzymes in the metabolic process so there was no energy generated which resulted in low viability (Rizal et al. 2003). The whole change of osmotic pressure or temperature would directly influence the quality of PGC. The difference of temperature and osmolarity between freezing medium and PGC caused a huge different in the water volume in the cell that lead to stress mechanism in the cell (Watson 2000).

Pegg (2002) reported that osmotic stress during thawing was caused by the effect of excessive cryoprotectant, causing the cell to swell and busted. It was allegedly due to the extortionate osmotic pressure of the medium liquid, so the water in the cell would break out and cause dehydration. (Best 1990). Furthermore, Best (1990) reported that cell water consisted of bulk water that filled about 90% of the cell and bound water which only filled 10% of the cell. Bulk water is the water that can be frozen and will come out due to osmotic pressure change. Whereas, bound water is water molecule which 20-100 times more viscous than bulk water. Its hydrogen bond is very tightly bound to hydrophilic surface and macromolecule. During the freezing process, the exterior cell will freeze first and will draw the water into the cell out.

Generally, results of this study both the RR percentage and the viability of PGC were lower than study reported by Setioko et al. (2007) who obtained RR percentage and viability by 49.9% and 83.5%, respe and Nakamura et al. (2011) by 54.3% and 86.8%, respectively. It is strongly allegedly due to the different method used in each study. The method used in this study was slow freezing with freezing speed setting.

Total time needed to freeze the PGC was 1 hour and 28 minutes to reach -30°C, before entered into liquid nitrogen (-196°C). Meanwhile, the freezing method used by Setioko et al. (2007) and Nakamura et al. (2011) was freezing PGC for 24 hours to reach -80°C, to enter into liquid nitrogen (-196°C). Slow freezing with freezing speed setting had an advantage of control at every step and limiting the effect of cryoprotectant toxicity using low concentration (Santos et al. 2006).

Apart from the different methods of freezing, the other factor affecting RR percentage and viability was packaging material used for PGC storage. In this study, the PGC was packed in the mini straw, while the previous study was packed in cryovial. Mohamad et al (2005) reported that the type packaging would influence temperature decrease and re-liquefaction rate of the cell. Straw packaging was more practical to provide clearer sample identification (Benesova & Trefil 2016). Kostaman et al. (2011) reported that PGC freezing packed with straw 0.5 ml resulted in the highest recovery rate and viability of 44.9 and 7.4%, respectively. Then Riesco et al. (2012) reported that zebrafish PGC packed in straw showed viability by 70%, better than PGC packed in microcapsule that showed viability by 20%.

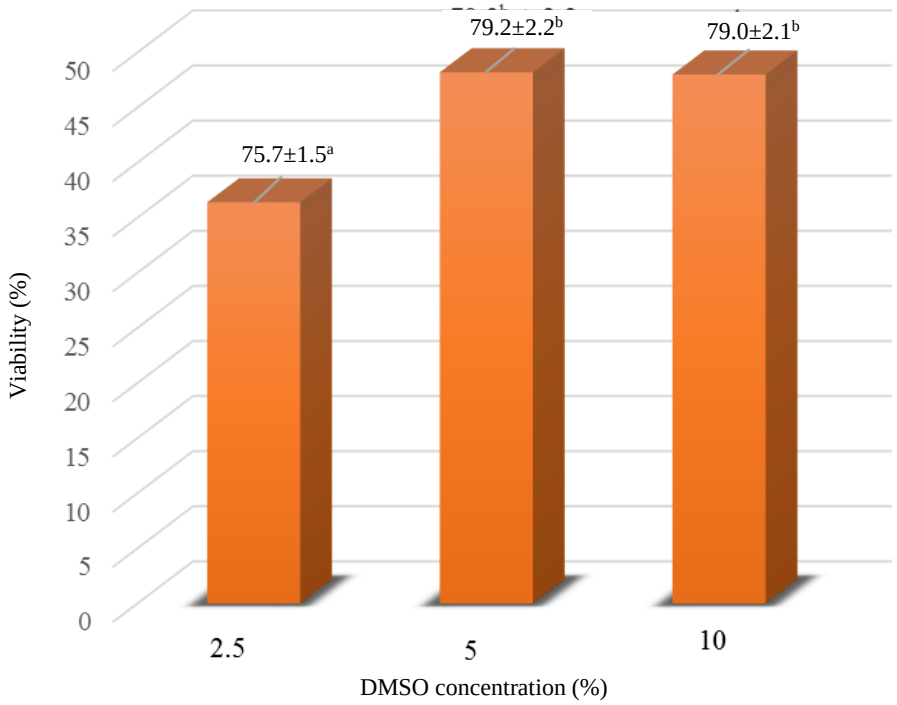

Figure 5. Viability of Gaok chicken PGC after freezing, treated with DMSO concentration.

CONCLUSION

It can be concluded that DMSO concentration by 5 or 10% on Gaok chicken PGC showed better role in protecting PGC quality (recovery rate and viability) during the freezing process compared to the 2.5% DMSO. The morphology characteristics of Gaok chicken after the freezing and thawing were similar with the fresh PGC.

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