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Original article

EVALUATION OF NON-PENETRATING SUGAR CRYOPROTECTANTS AND ANTIOXIDANTS DURING CHICKEN BLASTODERMAL CELL CRYOPRESERVATION

M. SHANMUGAM & N. ANAND LAXMI

ICAR – Directorate of Poultry Research, Rajendranagar, Hyderabad, India

Summary

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Chicken egg blastodermal cell (BC) cryopreservation is an effective strategy for conservation of complete genome of a breed. The present study evaluated non-penetrating sugars sucrose, trehalose and raffinose, and antioxidants tempol, betaine and ascorbic acid at different concentrations during Kadaknath chicken BC cryopreservation. BCs isolated from fresh Kadaknath eggs were cryopreserved in DMEM containing 10% dimethylsulfoxide (DMSO) and 10% FBS. In independent trials non-penetrating sugars sucrose (0, 0.1, 0.2 M), trehalose (0, 0.1, 0.2, 0.4 M) and raffinose (0, 0.1, 0.2 M), and tempol (0, 1, 5 mM), betaine (0, 0.1, 0.2, 0.4 M), ascorbic acid (0, 25, 50, 100 µM) were added to the cryopreservation mixture and evaluated. The medium containing BCs was filled in 0.25 mL plastic straws and exposed to liquid nitrogen vapours for 30 min. The straws were then plunged into liquid nitrogen and stored at least for fifteen days before thawing at 20 °C for 20 seconds and evaluation. The post-thaw percentage of live cells was assessed using 0.4% trypan blue staining. Both evaluated concentrations of sucrose significantly ($P<0.05$) increased the post-thaw live cell percentage. Betaine at 0.2 M concentration increased whereas ascorbic acid at 100 µM concentration decreased the post-thaw live cells. Trehalose, raffinose and tempol did not affect the post thaw BC live cells percentage. In conclusion, the results demonstrate that addition of sucrose and betaine during blastodermal cell cryopreservation improves the post thaw live cells.

Key words: antioxidant, blastodermal cell, chicken, cryopreservation, cryoprotectant, sucrose

INTRODUCTION

Fresh laid chicken egg contains stage X embryo with approximately 40,000–80,000 blastodermal cells (Eyal-Giladi & Kochav, 1976) that can be easily harvested and cryopreserved. The cryopreserved blastoderms can be transferred to recipient eggs for production of chimeric chicken. The damaging effects of cryopreservation are overcome with use of cryoprotectants. Based on the cell membrane permeability, the cryoprotectants are majorly classified as penetrating or non-penetrating. Dimethylsulfoxide is a widely used cell wall penetrating cryoprotectant. Chicken BCs were cryopreserved using 10% DMSO during slow freezing or vitrification and post-thaw the dead cells increased to approximately 79% from 5% in fresh samples (Svoradová *et al.*, 2018). The cryoprotectant DMSO was also used at higher concentration of 20% (Kino *et al.*, 1997). However, depending on concentration and cell exposure duration DMSO may be toxic based on the type of cryopreserved cells. To overcome this problem, DMSO is used at lower concentration and combined with other cryoprotectants. Sugars are non-toxic to cells and act as non-penetrating cryoprotectants. The disaccharide trehalose has been used to cryopreserve human pluripotent stem cells (Ntai *et al.,* 2018). Highest survival of zebrafish blastomeres after cryopreservation using 0.1 M sucrose and DMSO has been reported (Lin *et al.,* 2009). A multicomponent cryoprotectant which had sucrose and glycerol in addition to other cryoprotectants showed better post-thaw viable chicken BCs (Sawicka *et al.*, 2015).

During the process of cryopreservation of gonadal tissues and gametes the mitochondrial function is affected resulting in increase in intracellular oxidative stress that may affect the functional competency of the cells (Gualtieri *et al.,* 2021). To overcome the damaging effects of the reactive oxygen species, antioxidant compounds are included in the cryopreservation medium. N-Acetyl-L-Cysteine and catalase have been reported to increase blastodermal cell post thaw viability (Sun *et al.,* 2018).

The Indian native chicken breed Kadaknath is a dual-purpose bird characterised by dark melanin pigmentation in all parts of the body. This breed demonstrates climatic adaptability and can survive well in adverse climatic conditions of its breeding tract (Haunshi *et al.,* 2011). In the field the purity of the breed is threatened by genetic dilution and erosion because of propagation of high yielding varieties having higher productivity for production under backyard poultry production system. Therefore, efforts are made to conserve Kadaknath breed under *ex situ* conditions. Kadaknath semen has been cryopreserved with improved post-thaw fertility (Balusa *et al.,* 2022). However, BC cryopreservation provides a way for conservation without losing the W chromosome. In addition to conservation preserved BCs can further be used for other gene manipulation activities such as using CRISPR/cas techniques for production of designer chicks.

The present study was conducted to elucidate the effect of the inclusion of non-penetrating sugar cryoprotectants (sucrose, trehalose and raffinose) and antioxidants (tempol, betaine and ascorbic acid) during BC cryopreservation on post thaw livability.

MATERIALS AND METHODS

The study was carried out using Kadaknath chicken eggs. The experimental protocol was approved by the Institutional Animal Ethics Committee.

Blastodermal cell isolation

Freshly laid Kadaknath chicken eggs were used for isolation of stage X BCs as described by Kino *et al.* (1997) with few modifications. The eggs were broken open and contents placed on a sterile glass Petri dish. The yolk with germinal disc was transferred to another glass Petri dish. Filter paper rings were placed over the germinal disc, cut out and removed by cutting perivitelline membrane around the filter paper. The adhering yolk was removed by washing repeatedly with PBS (Calcium Magnesium Free). The germinal disc was punched through the hole using a sterile Pasteur pipette and collected in PBS (CMF). The tube containing the germinal disc in PBS (CMF) was centrifuged at 1000×g for 3 min. The supernatant was discarded and the pellet was resuspended in 0.5 mL of PBS (CMF) supplemented with 0.25% (w/v) trypsin and 0.04% (w/v) EDTA. The tube was incubated at 37 °C for 15 min. After incubation the tube was centrifuged at 1000×g for 3 min, the supernatant removed and resuspended in DMEM medium. The tube content was centrifuged once again and finally suspended in a known volume of DMEM and used for further experiments.

Treatments

The BCs were cryopreserved in the presence of non-penetrating sugars sucrose (0, 0.1, 0.2 M), trehalose (0, 0.1, 0.2, 0.4 M), raffinose (0, 0.1, 0.2 M), and antioxidants 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol) (0, 1, 5 mM), betaine (0, 0.1, 0.2, 0.4 M), ascorbic acid (0, 25, 50, 100 µM) in independent experiments. The non-penetrating sugars and antioxidants were added to the cell mixture just before freezing, filled in straws and proceeded for cryopreservation. The BC collection and cryopreservation procedure for each compound was repeated six times for collection of data. During each repetition of the trial 8-10 blastodiscs were pooled and processed.

Freezing and thawing

The BCs in DMEM were frozen using 10% DMSO and 10% FBS along with the test compounds in 0.25 mL plastic straws. The straws were exposed to liquid nitrogen vapours for 30 min by placing it 4 cm above liquid nitrogen on a floating raft and then plunged and stored in it till further thawing and evaluation. The straws were stored for a minimum of fifteen days before evaluation. The straws were thawed in cold water 20 ^{o}C for 20 seconds.

Viability assessment

The percent of viable BCs was calculated after staining with 0.4% trypan blue and examining under a microscope. The viability of BCs was assessed before (fresh cells) and after cryopreservation treatments. A minimum of 200 cells were counted classifying as live or dead for each sample.

Statistical analyses

The data were analysed by one-way ANOVA using SAS 9.2 software and $P<0.05$ was considered significant. Percent value data were arcsine transformed and analysed. Tukey's HSD *post-hoc* test was used for pairwise comparisons between group means in each experiment.

RESULTS

The average live BCs before carrying out cryopreservation experiment during different trials was 95.3%. Sucrose at both the concentrations evaluated significantly ($P<0.05$) increased post-thaw live cells (Fig. 1). Sucrose at 0.2M increased the post-thaw live cells by 63% in comparison to DMSO alone. The other non-penetrating sugars – trehalose and raffinose – had no effect on the post-thaw live cells (Fig. 2 and 3). Similarly, the antioxidant tempol had no influence on the post-thaw live cells (Fig. 4). Betaine at 0.2 M concentration significantly ($P<0.05$) increased post-thaw live cells (Fig. 5), with no signi-

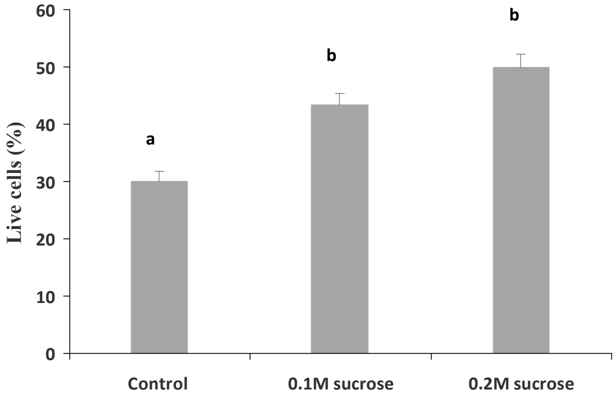

Fig. 1. Effect of different concentrations of sucrose on post-thaw live chicken blastodermal cells. Data are mean ± SE; bars with different letters differ significantly (P<0.05).

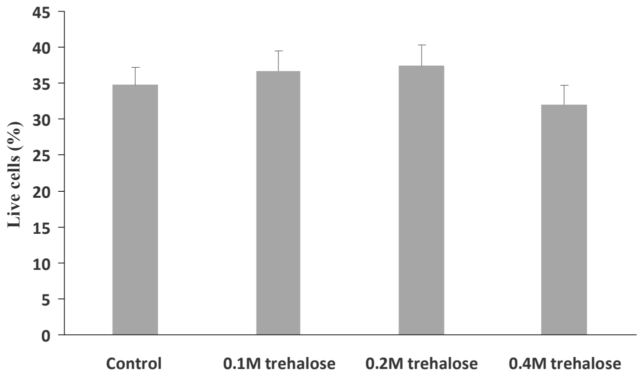

Fig. 2. Effect of different concentrations of trehalose on post-thaw live chicken blastodermal cells. Data are mean±SE.

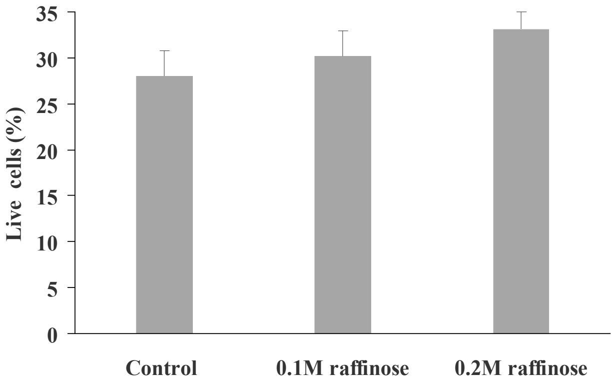

Fig. 3. Effect of different concentrations of raffinose on post-thaw live chicken blastodermal cells. Data are mean±SE.

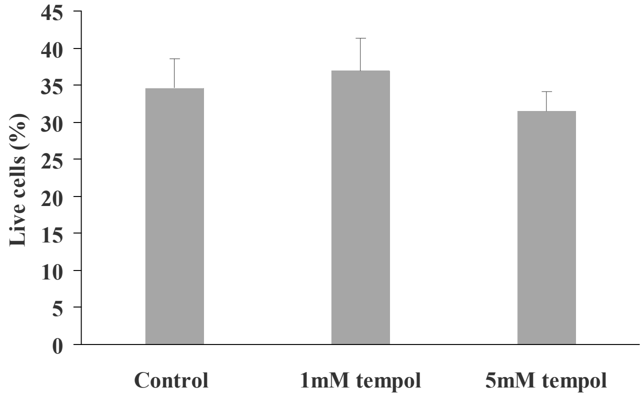

Fig. 4. Effect of different concentrations of tempol on post-thaw live chicken blastodermal cells. Data are mean ± SE.

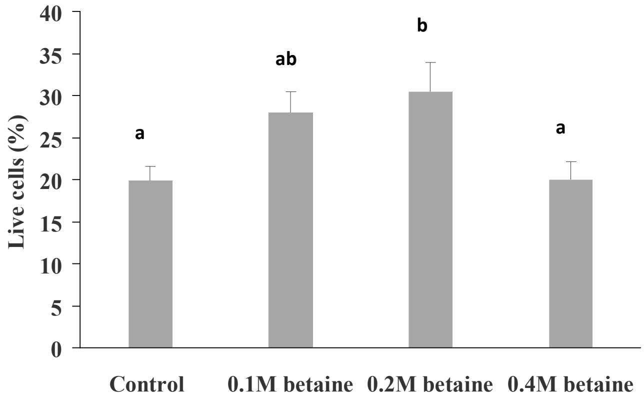

Fig. 5. Effect of different concentrations of betaine on post-thaw live chicken blastodermal cells. Data are mean ± SE; bars with different letters differ significantly (P<0.05).

ficant effect in the other evaluated concentrations. The increase in live cells by 0.2M betaine was by 53% in relation to the control group with only DMSO. Ascorbic acid at the lower concentrations did not affect the post-thaw live cells, however, the highest concentration of 100 µM decreased significantly (P<0.05) the percentage of live cells (Fig. 6) – by 29.25% from the control value.

DISCUSSION

Chicken BCs have been cryopreserved using DMSO at different concentrations (Kino *et al.,* 1997; Sawicka *et al.,* 2015; Sun *et al.,* 2018). There are meagre reports on the effect of non-penetrating cryoprotectants and other additives during chicken BCs cryopreservation. Increasing the post-thaw viable cells will be of use in

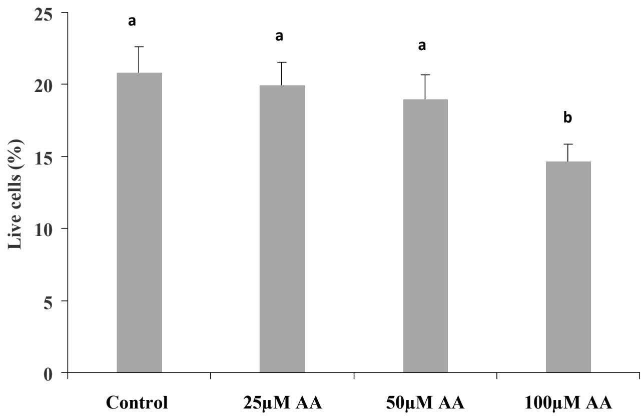

Fig. 6. Effect of different concentrations of ascorbic acid (AA) on post-thaw live chicken blastodermal cells. Data are mean ± SE; bars with different letters differ significantly (P<0.05).

production of chimeras after injection of these cells into recipient eggs.

Sucrose increased the post-thaw live BCs at both levels evaluated in the present study. This is similar to the results obtained in cryopreserving zebrafish blastomeres where 0.1M sucrose in combination with DMSO produced highest post-thaw survival of blastomeres (Lin *et al.,* 2009). However, a higher concentration of 0.2M sucrose decreased the survival of zebrafish blastomeres. Sucrose has been shown to reduce the area of salmon blastomeres after cryopreservation and authors have suggested that this change might be due to dehydration and toxicity to the cells (Kusuda *et al.,* 2002). Sucrose affects the cell lipid membrane properties thereby enhancing the diffusion of cryoprotectant into the multipotent stromal cells and efficiency of cryopreservation (Arutyunyan *et al.,* 2021). Addition of sucrose and trehalose to cryopreserve adherent human tumour cell aided in reduction of DMSO and improved the functional capacity of cells (Myagmarjav & Liu, 2022). Trehalose in combination with other cryoprotectants helped maintain the functional properties and higher post-thaw viability of human pluripotent stem cells (Ntai *et al.,* 2018) indicating its usefulness during cryopreservation. However, other reports did not find a positive effect of trehalose during cell cryopreservation. During zebrafish blastomere cryopreservation, trehalose had no positive effect, and at a higher concentration (0.2M) decreased cell survival (Lin *et al.,* 2009). In the present study, trehalose had no effect on the post-thaw viability of BCs. Raffinose has been used for cryopreservation of ram semen (Bucak *et al.,* 2013), chicken semen (Balusa *et al.,* 2022) and mouse oocytes (Eroglu, 2010) with improved post thaw results. In contrast, the results of the present study indicated no effect of raffinose on the post-thaw viability of the BCs. No effects observed with the use of trehalose and raffinose might be due to inappropriate concentration for the cell type used in the present study.

Tempol, a nitroxide compound has superoxide dismutase enzyme mimetic activity, reduces oxidative damage and preserves mitochondrial function. This has been used during semen cryopreservation in chicken (Najafi *et al.,* 2022), alpaca (Santiani *et al.,* 2013) and humans (Azadi *et al.,* 2017) with improved post thaw semen parameters. In contrast to the reports of its use during semen cryopreservation, the addition of tempol had no effect on the post-thaw livability of BCs in the present study. There are no reports on use of tempol during cryopreservation of somatic cells for comparing the results of the present study. Lower concentrations of the antioxidant enzyme catalase increased the chicken blastodermal cell viability after cryopreservation (Sun *et al.,* 2018). Thus, tempol at lower concentrations than those used in the present study may be evaluated for any beneficial effects.

Betaine, a zwitterionic molecule is a nontoxic and efficient cryoprotectant that enters the cells rapidly during the freezing process and provides protection against the cryoinjury (Yang *et al.,* 2016). Addition of betaine during semen cryopreservation improved the post thaw motility and viability in turkey (Blanco *et al.,* 2011) or there was no effect (Lindeberg *et al.,* 1999). In horses, the effect of betaine depended on the concentration – no effect was observed at lower concentrations and inhibitory effect on sperm motility at higher concentration (Trimeche *et al.,* 1999). In chicken, betaine had an inhibitory effect on post-thaw semen parameters and fertility (Balusa *et al.,* 2022). Thus, most studies indicate a nil or adverse effect when betaine is included in the cryomixture. In contrast, studies using betaine as cryoprotectant for preserving other types of cells indicated beneficial effects. Betaine in combination with other compounds has been shown to be useful for cryopreserving red blood cells (Sui *et al.,* 2019). Betaine in combination with electroporation maintained the normal viability and functions of mesenchymal stem cells after thawing and also reduced the level of reactive oxygen species (Gao *et al.,* 2021). In the present study betaine at 0.2M concentration increased the post-thaw viability of BCs. Betaine has osmotic regulation capacity and can help in preventing cell injury due to ice crystals. The action of betaine may be different in sperm and other cells and the propable reason for the better post thaw effect in stem cells or BCs.

Ascorbic acid, a water-soluble vitamin, has free radical scavenging activity and stimulates the biosynthesis and activation of antioxidant enzymes (Gęgotek & Skrzydlewska, 2022). Murine spermatogonial stem cells cryopreserved in the presence of ascorbic acid had lower proliferation rate and mitochondrial activity (Ha *et al.,* 2016). Similar to this result, the cell viability in the present study was reduced at the highest ascorbic acid concentration (100 µM). It was observed that umbilical cord blood cells cryopreserved in the presence of ascorbic acid alone did not improve post-thaw colony forming units or other parameters (Motta *et al.,* 2010). However, ascorbic acid in combination with disaccharides (trehalose and sucrose) could improve the post-thaw parameters of the umbilical cord blood cells. In another study utilising trehalose and vitamin C during freezing ovine spermatogonial stem cells, the post-thaw quality and viability of cells were improved when both the compounds were in combination rather than alone (Asadpour *et al.,* 2022). Therefore, ascorbic acid should be evaluated in future experiments along with disaccharides or other sugar cryoprotectants during BCs cryopreservation to evaluate potential beneficial effects.

In conclusion, the addition of sucrose or betaine during chicken BCs cryopreservation improves the post-thaw cell viability. The combination of non-penetrating cryoprotectants and antioxidants should be evaluated along with penetrating cryoprotectants during BC cryopreservation.

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Correspondence:

M. Shanmugam
ICAR – Directorate of Poultry Research,
Rajendranagar, 500030 Hyderabad, India
e-mail: shanmugam.murugesan@icar.org.in