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Protective effects of trehalose on frozen-thawed ovarian granulosa cells of cattle

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ABSTRACT

In this study, trehalose was investigated for its cryoprotective effects on ovarian granulosa cells (bGCs) of cattle. Five concentrations of trehalose at 0, 0.2, 0.4, 0.6 and 0.8 mol/L were added to the cryopreservation medium of bGCs, and the effects on the quality of frozen-thawed bGCs were assessed. The results indicate that the use of cryopreservation medium containing 0.2 and 0.4 mol/L of trehalose resulted in a greater rate of bGC viability compared to those of other groups ($P < 0.05$). Culturing with trehalose at 0.2 and 0.4 mol/L increased 17β- estradiol (E_2)and decreased progesterone (P_4)production ($P < 0.05$) in post-thawed bGCs. Compared with the control group, the intracellular Ca^{2+} concentrations of frozen-thawed bGCs were less in all treatment groups ($P < 0.05$), and the least Ca^{2+} concentration was observed in the group containing 0.4 mol/L trehalose. The plasma membrane potentials of frozen-thawed bGCs were greater in the groups with 0.2 and 0.4 mol/L trehalose, and the group treated with 0.4 mol/L trehalose had the greatest membrane potential in comparison to other groups ($P < 0.05$). The relative abundance of the *CYP19* mRNA in frozen-thawed bGCs was greater in the groups containing 0.2, 0.4 and 0.6 mol/L trehalose, and relative abundances of *FSHR* and *BCL2* mRNA were greater in the group of bGCs treated with 0.2 mol/L trehalose ($P < 0.05$). Trehalose treatment at 0.4, 0.6 and 0.8 mol/L had an inhibitory effect on *BAX* gene transcription in frozen-thawed bGCs ($P < 0.05$). In summary, trehalose exhibited a greater cryoprotective effect on bGCs than basic cryopreservation medium.

1. Introduction

In recent years, cryopreservation of tissues has become more important for understanding the communication mechanism in cell-to-cell signaling (Leonel et al., 2018; Devireddy, 2018). As one of the cell types in the ovary, granulosa cells have an important role in follicular growth and atresia through secretions of gonadal steroids, growth factors, and cytokines (Matsuda et al., 2012). Although cryopreservation has been widely used to freeze sperm, embryos and other tissues (Moawad et al., 2017; Fujikawa et al., 2018) in assisted reproduction, only a few studies have addressed the application of this technology to animal ovarian granulosa cells (Choi et al., 2008) due to the cryoinjury on cellular structure and function (Rall and Fahy, 1985). There are reports of successful cryopreservation of granulosa cells in humans (Lee et al., 2018). The cryosurvival rate of granulosa cells, however, is less because of the effects from cryoprotectant agent exposure, cooling rate and stage of development. Furthermore, ovarian granulosa cells are highly

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susceptible to apoptosis during cryopreservation (Bakas and Disalvo, 1991). The inhibition of cryoinjury to granulosa cells is of paramount importance to maintain granulosa cell viability and function after cryopreservation.

Trehalose, a nonreducing disaccharide composed of two glucose molecules, has been used to minimize cryoinjury and improve the quality of post-thawed spermatozoa in rams (Akhtarshenas et al., 2018), boars (Rall and Fahy, 1985), goats (Liu, 2007), bulls (Fujikawa et al., 2018) and rabbits (Zhu et al., 2017). There has also been use of trehalose for stabilization of simple structures in the cells, such as lipids and proteins, as well as more complex biological structures (Vickery et al., 2016). Little, however, is known about the effects of trehalose on frozen-thawed ovarian granulosa cells (bGCs) of cattle, including whether it affects cell viability, the secretion of steroid hormones and plasma membrane potential.

Considering the properties of trehalose, it was hypothesized that the cryopreservation medium containing trehalose would have cryoprotective effects on the survival and function of bGCs. To test this hypothesis, trehalose was added to the cryopreservation medium of granulosa cells at five different concentrations, 0, 0.2, 0.4, 0.6 and 0.8 mol/L, and the characteristics of frozen-thawed bGCs were investigated.

2. Materials and methods

2.1. Ethics statement

The present study was approved by the ethics committee of Northwest A & F University, Shaanxi, China.

2.2. Chemicals and reagents

Unless stated differently, all chemicals and reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trehalose was purchased from Shanghai Solarbio Chemical Co. Ltd. (Shanghai, China) and stored at 4 ℃.

2.3. Cell culture

Ovaries were obtained from adult Qinchuan cattle, irrespective of estrous cycle stage, at a local slaughterhouse and transported to the laboratory at 30 °C in 0.9% NaCl containing penicillin (100 IU/mL), streptomycin (100 μg/mL) and fungizone (1 μg/mL) (Jiang et al., 2013). Granulosa cells were harvested from follicles 2 to 6 mm in diameter, and the cell suspension was filtered through a 150 mesh (106 μm) steel sieve (Sigma-Aldrich, China). Cell viability was assessed using the Trypan blue dye exclusion procedure.

The BGCs were cultured in serum-free conditions that maintain estradiol secretion and responsiveness to FSH was determined using the procedures previously described by Jiang et al. (2013) with a few modifications. Briefly, cells were cultured in 24-well tissue culture plates (Corning Inc., China) at a density of 10^6 viable cells in 1 mL of DMEM/F12 containing sodium bicarbonate (10 mM), sodium selenite (4 ng/mL), bovine serum albumin (BSA) (0.1%, W/V, Sigma-Aldrich), penicillin (100 U/mL), streptomycin (100 μg/mL), transferrin (2.5 μg/mL), nonessential amino acid mix (1.1 mmol/L), bovine insulin (10 ng/mL), androstenedione (10^{-7} M) and bovine FSH (10 ng/mL, BIONICHE INC. Ontario, Canda). Cultures were maintained at 37 °C in 5% CO_2 and 95% air for 2 days. Treatments were then applied on day 2.

2.4. Treatment of bGCs

To maintain the viability of granulosa cells during freezing and thawing, DMEM/F12 containing 5% FBS, 5% DMSO and 5% ethylene glycol (EG) was prepared as the basic cryopreservation medium. Trehalose was added to the basic medium to make the cryopreservation medium with final concentrations of 0, 0.2, 0.4, 0.6 or 0.8 mol/L.

On day 2, 95% of the granulosa cell culture medium was replaced with the cryopreservation medium containing one of five concentrations of trehalose. After 30 min, granulosa cells were collected by gentle aspiration with a pipette. The granulosa cell mixture was centrifuged at 800 x *g* for 5 min to remove the medium. The granulosa cells were re-suspended in the same volume of cryopreservation medium and were centrifuged at the same speed and duration as previously described in this manuscript. The final sediment was diluted with 500 μL of cryopreservation medium and was immediately transferred to the liquid nitrogen tank.

2.5. Assessment of cell viability

The MTT assay kit was used to assess the effect of trehalose on the viability of cryopreserved granulosa cells according to the manufacturer's guidelines. Briefly, the samples were removed from the liquid nitrogen tank and thawed in a water bath at 37 °C for 5 min. After thawing, the cells were washed twice using cell culture medium at 37 °C and transferred to 96-well tissue culture plates with a density of 10^4 cells per well at 37 °C and 5% CO_2 for a 4 h incubation. The MTT solution (20 μL; 5 mg/mL) was added to each well and mixed gently. The cells with MTT solution were then incubated for 4 h at 37 °C and 5% CO_2, after which 160 μL of DMSO was added to each well to dissolve the formazan crystals by collection into and aspiration from a pipette several times. A microplate reader (BioTek, USA) was used to measure the absorbance of samples at 450 nm compared to 620 nm, and the standard curve of absorbance related to viable cell number was prepared by culturing and measuring granulosa cells at different plating densities in quintuplicate (from 1×10^3 to 5×10^5 per 200 μL) for 4 h. The number of viable cells/well was estimated from the resulting linear regression equation.

2.6. Steroid production

After thawing, bGCs were cultured in 96-well plates (1×10^5 viable cells/200 µL of media/well) in cell culture medium containing 10 ng/mL of FSH for 2 days at 37 °C and 5% CO_2. The cell culture medium was subsequently collected, frozen and stored at −20 °C until progesterone (P_4) and 17β-estradiol (E_2) concentrations were determined using ELISA kits (DIAsource, Louvain-la-Neuve, Belgium) in accordance with the manufacturer's guidelines. The inter- and intra-assay CVs averaged 15% and 10.5%, respectively. The data represent three independent cultures with each treatment conducted in triplicate.

2.7. Determination of intracellular Ca^{2+} concentration

After thawing, the cells were washed twice using cell culture medium at 37 °C and were diluted to a density of 4×10^5 cells/mL. Fura3-AM, a fluorescent Ca^{2+} indicator, was used to monitor the concentration of intracellular Ca^{2+} (Bakas and Disalvo, 1991). The Ca^{2+}-free Krebs solution (140 mM NaCl, 3 mM KCl, 1 mM $MaCl_2$, 11 mM D-glucose, 0.5 mM EGTA, 10 mM HEPES, pH 7.4) was used to suspend the Fura3-AM-loaded cells. The intracellular Ca^{2+} changes were measured with the ratio of fluorescence intensities excited at 488 nm and 530 nm using flow cytometry (CyFlow Cube, PARTEC, Germany).

2.8. Measurement of plasma membrane potential

After thawing the bGCs, the plasma membrane potential was measured by a fluorescence spectrofluorometer (CyFlow Cube, PARTEC, Germany) using the procedures described by Magalhães et al. (2017). An anionic slow-response membrane potential fluorescent reporter, DiBAC4(3), was used for this experiment. The cells were washed twice with 20 mL of assay buffer (120 mM NaCl, 2 mM KCl, 5 mM D-glucose, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, pH 7.4) containing 5 µM DiBAC4(3), and then incubated with 20 mL of assay buffer for 30 min at 25 °C. The incubation medium was replaced with 18 mL of assay buffer containing 5 µM DiBAC4(3), and the whole-plate fluorescence was assessed for 15 min (basal fluorescence). Fluorescence was assayed in a spectrofluorometer (CyFlow Cube, PARTEC, Germany) with the reaction mixture in 96-well plates at 37 °C. The excitation wavelength was 488 nm, and the emission wavelength was 530 nm. The emitted radiation was quantified by determining the AUC (area under the curve) of the fluorescence intensity variation from the control.

2.9. RNA extraction and real-time PCR

Total RNA was extracted from frozen-thawed bGCs using Trizol reagent using the manufacturer's intructions. The concentration of RNA was quantified on a NanoDrop 2000 (Thermo, USA) at 260-nm absorbance. Reverse transcription PCR was performed on 1 µg of DNase-treated total RNA in the presence of 1 mmol/L oligo-(dT) primer, 4 U Omniscript RTase, 0.25 mmol/L dNTP mix and 19.33 U RNase inhibitor in a volume of 20 µL at 37 °C for 1 h. The reaction was terminated by incubation at 93 °C for 5 min.

Relative abundances of CYP19, FSHR, BAX and Bcl-2 mRNA were determined using the 7500 Real-Time PCR system (Applied Biosystems, USA) with Power SYBR Green I PCR Master Mix. The bovine-specific primers for target genes are listed in Table 1. The quantitative real-time PCR parameters were as follows: 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C. Histone H2AFZ was used as a housekeeping gene, and samples were assessed in duplicate. Values for relative abundance of mRNA were calculated for all samples.

2.10. Statistical analysis

All experiments were performed in three replicates (control and experimental groups). All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, USA). Experimental data are presented as the means ± SEM, and the significant differences between treatments and interactions were calculated with multifactorial one-way ANOVA using statistical software. When there were significant differences, means were compared by the *F*-test. Differences with *P* values of less than 0.05 were considered statistically significant. The median fluorescence intensity variation was analyzed by FlowJo V10 (Leonard Herzenberg, USA) using the manufacturer's instructions.

Table 1
Bovine specific primer sequences for real-time PCR.

Genes	Forward primer	Reverse primer	Reference
H_2A	5'-GAGGAGCTGAACAAGCTGTTG-3'	5'-TTGTGGTGGCTCTCAGTCTTC-3'	Jiang et al. (2011)
CYP19	5'-GTGTCCGAAGTTGTGCCTATT-3'	5'-GGAACCTGCAGTGGGAAATGA-3'	Gasperin et al. (2015)
FSHR	5'-AGCCCCTTGTCACAACTCTATGTC -3'	5'- GTTCCTCACCGTGAGGTAGATGT -3'	Gasperin et al. (2015)
BAX	5'-AACATGGAGCTGCAGAGGAT-3'	5'-CAGTTGAAGTTGCCGTCAGA-3'	Jiang et al. (2011)
BCL2	5'-ATGACTTCTCTCGGCGCTAC-3'	5'-CTGAAGAGCTCCTCCACCAC-3'	Jiang et al. (2011)

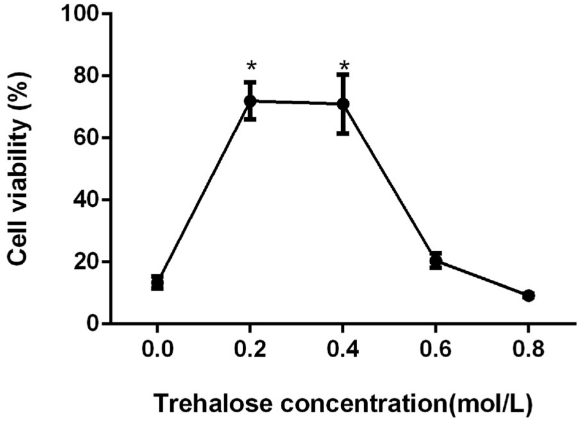

Fig. 1. Percentage of viable cells in frozen-thawed bGCs; Viability of bGCs was assessed using the MTT kit after cells were freeze-thawed and incubated at 37 °C and 5% CO_2 for 4 h; *indicates a difference ($P < 0.05$).

3. Results

3.1. Effects of trehalose on the viability of frozen-thawed bGCs

The effects of trehalose in the cryopreservation medium on the viability of frozen-thawed bGCs were evaluated, and the results are depicted in Fig. 1. The use of cryopreservation medium containing 0.2 and 0.4 mol/L trehalose resulted in a greater percentage of viable cells in comparison to the other treatments and control group ($P < 0.05$). There, however, was no difference in the percentage of viable cells between the groups treated with 0.6 and 0.8 mol/L trehalose.

3.2. Effects of trehalose on steroid production in frozen-thawed bGCs

The frozen-thawed bGCs were incubated for 2 days at 37 °C and 5% CO_2, and the culture medium was collected to quantify steroid production. In the presence of FSH, treatments with trehalose at concentrations of 0.2 and 0.4 mol/L resulted in greater E_2 production ($P < 0.05$) in frozen-thawed bGCs compared to the other treatment groups and the control group, whereas treatment with 0.6 and 0.8 mol/L trehalose had no effect on E_2 production ($P > 0.05$; Table 2). Treatment with 0.2 and 0.4 mol/L trehalose resulted in lesser P_4 production ($P < 0.05$) in frozen-thawed bGCs, and cells treated with 0.6 and 0.8 mol/L trehalose had a similar P_4 production compared to that of the control group ($P > 0.05$; Table 2).

3.3. Effects of trehalose on intracellular Ca^{2+} in frozen-thawed bGCs

Flow cytometry was used to monitor the variation of intracellular Ca^{2+} concentration (Fig. 2), and the values were represented by median fluorescence intensity (MFI), which was calculated using FlowJo (V10). Compared with the control group, the MFI values were less in all trehalose treatment groups ($P < 0.05$), and the cells treated with the cryopreservation medium containing 0.4 mol/L trehalose had the smallest MFI value compared to the control and other treatment groups ($P < 0.05$) (Table 3). There were no differences for MFI values among the groups treated with 0.2, 0.6 and 0.8 mol/L trehalose in the cryopreservation medium (Table 3).

3.4. Effects of trehalose on the membrane potential of frozen-thawed bGCs

In the present study, DiBAC4(3), an anionic slow-response membrane potential fluorescent reporter, was used to quantify the plasma membrane potential of frozen-thawed bGCs (Fig. 3), and the values are represented as MFI (Table 4). The MFI values of frozen-thawed bGCs were greater in cryopreservation medium when there was treatment with 0.2 and 0.4 mol/L trehalose ($P < 0.05$) in comparison to other treatment groups and the control group. The greatest MFI value occurred when cells were treated with 0.4 mol/L trehalose. There were no differences observed for the MFI values in the cryopreservation medium containing 0.6 or

Table 2
Production of E_2 and P_4 in frozen-thawed bGCs.

Items	Control	Trehalose (mol/L)			
		0.2	0.4	0.6	0.8
E_2 (pg/mL)	7.25 ± 0.85^{c}	39.08 ± 6.22^{b}	56.33 ± 4.87^{a}	14.66 ± 3.51^{c}	10.43 ± 3.67^{c}
P_4 (ng/mL)	83.26 ± 8.41^{c}	68.18 ± 5.56^{b}	52.35 ± 8.17^{a}	72.71 ± 4.36^{bc}	75.28 ± 5.25^{bc}

Values within a row without a common superscript (a–c) indicate differences ($P < 0.05$).

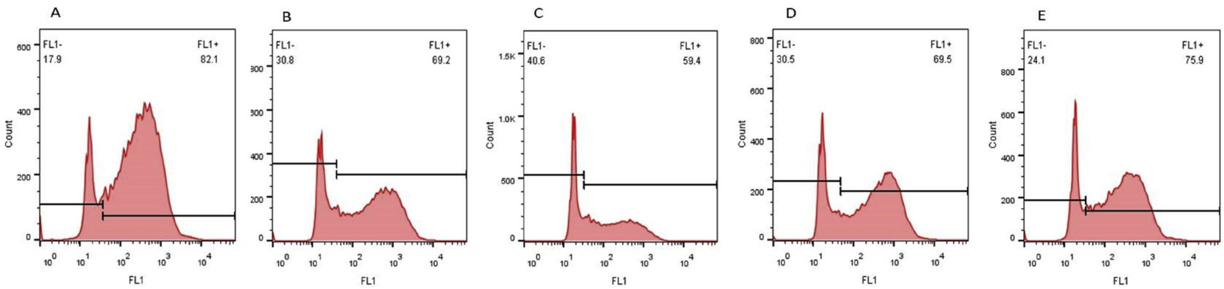

Fig. 2. Median fluorescence intensity of intracellular Ca^{2+} in frozen-thawed bGCs. The red area in the diagram represents the value of MFI measured by a spectrofluorometer. A–E indicate the groups of trehalose concentrations ranging from the control on the left to 0.8 mol/L on the right (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3
Effects of trehalose on intracellular Ca^{2+} concentration in frozen-thawed bGCs.

Items	Control	Trehalose (mol/L)			
		0.2	0.4	0.6	0.8
MFI	28.60 ± 0.49^{c}	16.60 ± 0.26^{b}	13.30 ± 0.18^{a}	18.10 ± 0.96^{b}	19.70 ± 0.52^{b}

Values within a row without a common superscript (a–c) indicate differences ($P < 0.05$).

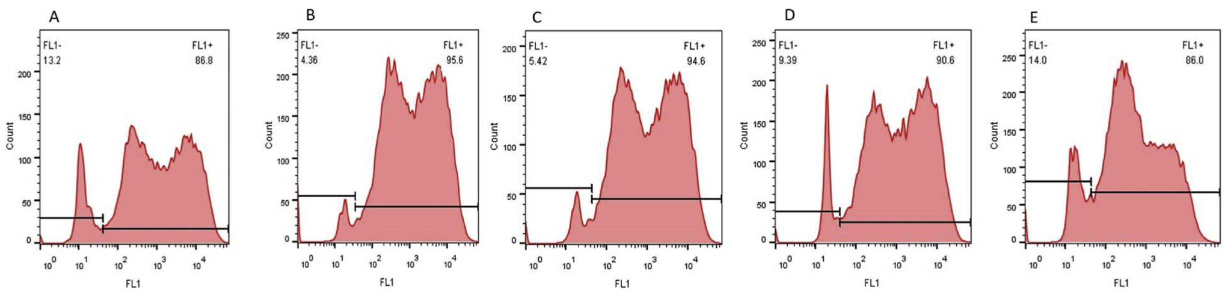

Fig. 3. Median fluorescence intensity of plasma membrane potential in frozen-thawed bGCs; Red area in the diagram represents the value of MFI measured by a spectrofluorometer; A–E indicate the groups with trehalose concentrations ranging from the control on the left to 0.8 mol/L on the right (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 4
Effects of trehalose on plasma membrane potential in frozen-thawed bGCs.

Items	Control	Trehalose (mol/L)			
		0.2	0.4	0.6	0.8
MFI	76.60 ± 5.00^{c}	103.12 ± 10.03^{b}	124.00 ± 4.72^{a}	80.90 ± 8.65^{c}	64.70 ± 4.26^{c}

Values within a row without a common superscript (a–c) indicate differences ($P < 0.05$).

0.8 mol/L trehalose compared with the control group ($P > 0.05$; Table 4).

3.5. Effects of trehalose on –relative abundance of mRNA in frozen-thawed bGCs

The effects of trehalose on relative abundance of mRNA in frozen-thawed bGCs were measured. Compared with the control group, *CYP19* mRNA relative abundance was greater in groups treated with 0.2, 0.4, and 0.6 mol/L trehalose ($P < 0.05$), but there was no effect when there was treatment with 0.8 mol/L trehalose ($P > 0.05$; Fig. 4). With trehalose treatment, there was a greater relative abundance of *FSHR* and *BCL2* mRNA ($P < 0.05$), with maximal effects when there was treatment with 0.2 mol/L trehalose (Fig. 4). Trehalose treatment at 0.4, 0.6 and 0.8 mol/L, however, resulted in a lesser relative abundance of *BAX* mRNA in frozen-thawed bGCs compared to the control and group treated with 0.2 mol/L trehalose ($P < 0.05$) (Fig. 4).

4. Discussion

Cryopreservation of ovarian tissue has many advantages for the preservation of the female germline in animals as it has an

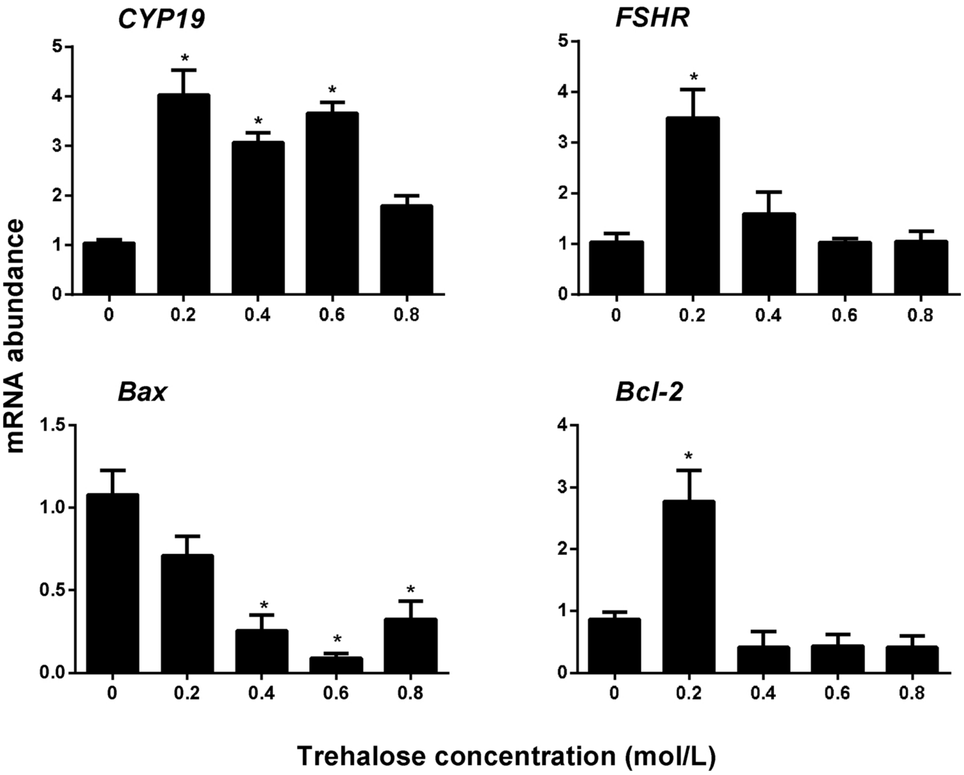

Fig. 4. Relative abundance of *CYP19*, *FSHR*, *BAX* and *BCL2* mRNA in frozen-thawed bGCs; *indicates a difference ($P < 0.05$).

important role in supporting oocyte maturation in in vitro conditions, which are similar to those occurring in vivo (Perego et al., 2017). Nevertheless, it is still a challenge with the susceptibility of granulosa cells to apoptosis during cryopreservation (Bakas and Disalvo, 1991). The cryoprotectants and protocols should be optimized (Horvath et al., 2005), and there will be biological variability among species (Wildt and Wemmer, 1999). The present study is the first to investigate whether the addition of trehalose to the cryopreservation medium protects bGCs from cryoinjury. In the present study, use of cryopreservation medium that had trehalose added resulted in an improvement in the values for frozen-thawed bGC variables. The addition of 0.2 and 0.4 mol/L trehalose to the basic cryopreservation medium resulted in a greater percentage of viable cells and decrease in the plasma membrane potential and intracellular Ca^{2+} concentration. Trehalose also had a protective role in steroid production and gene expression related to the function and survival of bGCs in the present study. The results have confirmed our hypothesis that use of the cryopreservation medium containing trehalose provides for greater cryoprotective effects of bGCs.

Viability is one of the most important bGC variables during the process of freezing and thawing, and it is reduced by any damage to the cells. There have been several reports that trehalose (100 mM/L) has cryoprotective effects on sperm (Vickery et al., 2016; Zhu et al., 2017). In the present study, the cryopreservation medium with trehalose had greater cryoprotective effects on the viability of bGCs. The percentages of viable cells of post-thawed bGCs were greater with 0.2 and 0.4 mol/L trehalose supplementation ofin the cryopreservation medium than supplementations in the other treatment groups or the control group. Results of the present study are the first to suggest that the addition of trehalose to cryopreservation medium has a protective action on the viability of bGCs.

The use of trehalose should facilitate maintenance of the functional integrity of bGCs after freezing and thawing to ensure that these cells can be used for further study. Because E_2 production represents one of the main functions of granulosa cells (McNatty et al., 1975), the production of E_2 and P_4 by frozen-thawed bGCs was investigated in the present study to assess the cryoprotective potential of trehalose on bGC functions. In the present study, treatment with 0.2 and 0.4 mol/L trehalose led to a marked increas in E_2 production and a lesser P_4 production by frozen-thawed bGCs, whereas treatment with 0.6 and 0.8 mol/L trehalose had no significant effects on steroid production compared to the control group. The trehalose-induced variations in E_2 and P_4 production may possibly be due to the positive effects of trehalose on bGC viability. These variations, however, may also be due to the stimulatory effect on protein kinase A (PKA)-cAMP and the activation of the extracellular signal-regulated kinase (ERK; Zeng et al., 2005), a member of the mitogen-activated protein kinase (MAPK) group (Su et al., 2008), which appears to be induced by low temperatures (Iwata et al., 2005). We hypothesize that trehalose enters the cells and increases aromatase activity, which is responsible for estrogen synthesis (McNatty et al., 1975), because trehalose is present on both sides of the cell membrane when used as a cryoprotectant (Motta et al., 2014). Further research will be required, however, to understand how trehalose affects the E_2 and P_4 production in frozen-thawed bGCs (Tirelli et al., 2005; Perego et al., 2017).

The mitochondria and endoplasmic reticulum are intracellular Ca^{2+} storage sites, and alterations in these organelles are involved in cell senescence and apoptosis (Mcconkey and Orrenius, 2010). Once cellular Ca^{2+} channels open, intracellular Ca^{2+} can be released through the plasma membrane leading to relatively greater concentrations of cellular Ca^{2+} which can result in cell death (James et al., 1988). In the present study, the use of the cryopreservation medium with trehalose resulted in lesser intracellular Ca^{2+} concentration in bGCs in all treatment groups compared with the control group. Further research should address whether trehalose activates the release of intracellular Ca^{2+}.

All plasma membranes have a transmembrane potential difference generated by electrochemical ion gradients across the bilipid bilayer (Coster, 1999). The plasma membrane potentials, which are related to the fluidity of lipid and proteins of the cell membrane, are known to influence the conformation and function of some membrane proteins (Magalhães et al., 2017). Furthermore, plasma membrane potential appears to be useful in predicting sperm fertilization capacity in humans (Li et al., 2016). In the present study, use of trehalose at 0.2 and 0.4 mol/L had protective effects on the plasma membrane potential of bGCs compared to the other groups. This may also suggest that trehalose stabilizes the plasma membrane during freezing by forming hydrogen bonds with the polar head groups of phospholipids, in addition to functioning as an excellent inducer of the glass state in cells (Mori et al., 2010).

In ovarian granulosa cells, FSHR and CYP19 are important factors that ensure the integrity of granulosa cells and the secretion of estrogen (McNatty et al., 1975). Based on the results of the present study, the activation of *CYP19* and *FSHR* gene expression in frozen-thawed bGCs by trehalose is likely the cause for increased E_2 production. In similar studies, treatment with FSH induced cell proliferation (Spicer et al., 2006) and increase in relative abundance of *CYP19A1* and *FSHR* mRNA (Albonico et al., 2017) in cultured bGCs, but the effects of trehalose on gene transcription in bGCs have been minimally studied. The BCL-2 family is associated with the activity of mitochondrial and intracellular apoptosis pathways and includes apoptosis-inhibiting (BCL-2) and apoptosis-promoting (BAX) members (Teijido and Dejean, 2010). In the present study, results indicate that trehalose has positive effects on the expression of the *CYP19* and *FSHR* genes and suppresses expression of the *BAX* gene in contrast to the increased expression of th e*BCL2* gene in frozen-thawed bGCs. Nevertheless, further research is necessary to explain the potential mechanism of trehalose effects on gene transcription related to the survival and/or apoptosis of frozen-thawed bGCs.

In conclusion, cryopreservation medium supplemented with trehalose had protective effects on frozen-thawed bGCs. The addition of trehalose in the cryopreservation medium of bGCs may be recommended to facilitate the improvement of bGC preservation for cattle reproduction. Although the results of the present study provide useful information for future studies, the molecular mechanisms of the protective effects of trehalose on bGCs need to be more clearly understood. Further research is required to obtain more information on frozen-thawed bGCs and to find more effective preservation methods for animal cells.

5. Conclusion

Trehalose had a greater cryoprotective effect on bGCs than basic cryopreservation medium.

Conflict of interest

None of the authors have any conflicts of interest.

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