# EFFECT OF SUPPLEMENTATION OF CRYOPROTECTANT SOLUTION WITH HYDROXYPROPYL CELLULOSE FOR VITRIFICATION OF BOVINE OOCYTES

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## Abstract

**BACKGROUND:** Successful cryopreservation of bovine oocytes is very important for research and commercial applications. However, the survival and development rate of vitrified-thawed (VT) oocytes are lower than those of non-vitrified-thawed (non-VT) oocytes. **OBJECTIVE:** To investigate the effect of adding hydroxypropyl cellulose (HPC) to the vitrification solution for bovine oocytes. **MATERIALS AND METHODS:** For vitrification, bovine metaphase II oocytes were pretreated with a solution containing 10% ethylene glycol supplemented with 0, 10, 50, or 100 µg/mL HPC for 5 min, exposed to a solution containing 30% ethylene glycol supplemented with 0, 10, 50, or 100 µg/mL HPC for 30 s, and then directly plunged into liquid nitrogen. **RESULTS:** The survival rate of oocytes was significantly higher in the 50 HPC group than in the 0, 10, and 100 HPC groups. The reactive oxygen species level was lower in the non-VT and 50 HPC groups than in the other groups. The mRNA levels of proapoptotic genes (Bax) were lower in the non-VT, 0, and 50 HPC groups than in the other groups. The mRNA levels of antiapoptotic genes (BCl2) were higher in the non-VT than in the other groups. The development rates of embryos (day 8) obtained via parthenogenetic activation (PA) were determined in the non-VT, 0 HPC, and 50 HPC groups. The cleavage rate was significantly higher in the non-VT group. **CONCLUSION:** Supplementation of vitrification solution with HPC improves the survival of VT bovine oocytes and the development capacity of embryos derived from these oocytes via PA.
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**Keywords:** bovine oocytes; cryoprotectant; hydroxypropyl cellulose; solution vitrification.

## INTRODUCTION

Cryopreservation ensures a consistent and steady supply of oocytes. Oocyte cryopreservation is useful not only for long-term storage of female genetic materials, but also for reproduction of endangered species and livestock with high economic value. It is particularly useful for the production of embryos via in vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT). Oocyte quality greatly affects embryo development in vitro.

Oocyte vitrification has been successfully used to cryopreserve oocytes of humans and many other species. Various technologies have been described for vitrification of bovine (1), pig (2), mouse (3), and human (4) oocytes. However, oocytes are inevitably damaged during vitrification.

Previous studies about the effect of cryopreservation on in vitro matured oocytes

reported that vitrified-thawed (VT) oocytes have a normal spindle and karyotype, but exhibit cortical granule exocytosis, swelling of smooth endoplasmic reticulum vesicles, and mitochondrial damage (5, 6). In general, oocytes are more susceptible to cooling damage than embryos because the integrity of metaphase spindle microtubules is perturbed during cooling and high concentrations of cryoprotective agents damage oocytes (7). In addition, the mechanism underlying vitrification of oocytes is complex and likely involves disruption of cellular structures and premature release of cortical granules, leading to zona hardening and impairment of normal fertilization, and changes in oocyte gene expression and apoptosis (8). Oocyte damage upon freezing negatively affects development of embryos generated via IVF and SCNT. Therefore, this study attempted to minimize oocyte damage during vitrification by supplementing vitrification solution with the synthetic polymer hydroxypropyl cellulose (HPC) and investigated the effects on development of embryos generated via parthenogenetic activation (PA).

HPC is a polysaccharide with a variable length that can form a viscous gel at low temperatures because it has very similar physical properties as albumin-based formulations with certain molecular weights (9). It is listed in a pharmacopeia and can be safely used because it is a general food additive and drug excipient. Recent studies reported that HPC increases the viscosity of vitrification solution and reduces the amount of time that an embryo spends attached to the Cryotop surface during thawing, indicating that it can improve vitrification by reducing the risk of cryogenic injury and increasing the survival rate (10). HPC has been added to vitrification solution for freezing of human oocytes (11) and embryos (10). This study investigated the effect of supplementation of vitrification solution with HPC on vitrification of bovine oocytes and development of embryos derived from these oocytes via PA.

To determine the appropriate concentration of HPC, oocytes were exposed to 0, 10, 50, and 100 µg/mL HPC (called the 0, 10, 50, and 100 HPC groups, respectively). We investigated the survival rate, reactive oxygen species (ROS) level, and mRNA expression (Bax, Bcl2, Hsp70, and Dnmt3a) of non-VT oocytes and oocytes in the 0, 10, 50, and 100 HPC groups. Next, we parthenogenetically activated oocytes and determined the development rate at day 2 and 8, total cell number per blastocyst, and number of apoptotic cells. We compared the mRNA expression levels of developmental potential-related genes (Glut-5, Interferon-tau, Caspase-3, Hsp70, HSF, Bax, and Dnmt3a) between blastocysts produced from non-VT oocytes and those produced from oocytes in the 0 and 50 HPC groups.

We previously successfully vitrified bovine oocytes using the minimum volume cooling (MVC) method (12) and confirmed that embryos derived from these VT oocytes can develop to full-term following SCNT (13). The objective of this study was to modify this method using HPC as a supplement to improve vitrification.

## MATERIALS AND METHODS

Unless stated otherwise, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA)

### *Oocyte preparation and in vitro maturation (IVM)*

Bovine ovaries were transported from a slaughterhouse to the laboratory in saline buffer (9 g/L NaCl and 0.1 g/L penicillin–streptomycin). Cumulus-oocyte complexes (COCs) were aspirated from visible follicles measuring 2–6 mm using an 18-gauge needle and washed with HEPES-buffered Tyrode's medium (TL-HEPES). They were cultured in an incubator in IVM medium, which comprised TCM-199 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco), 0.2 mM sodium pyruvate, 1 µg/mL follicle-stimulating hormone (Folltropin™; Bioniche Animal Health, Belleville, ON, Canada), 1 µg/mL estradiol-17β, and 25 µg/mL gentamycin sulfate. Sets of 10 COCs were matured in 50 µL IVM medium under mineral oil at 38.8°C in a humidified atmosphere of 5% $CO_2$ in air for 20–22 h.

### *Vitrification and thawing*

The basic medium used for pretreatment, vitrification, and dilution was Dulbecco's phosphate-buffered saline (D-PBS, Gibco) containing 10% FBS. The pretreatment solution contained 10% ethylene glycol (EG10). The vitrification solution contained 30% ethylene glycol and 0.5 M sucrose (EG30). For serial dilution after thawing, D-PBS containing 1.0,

0.5, 0.25, or 0.125 M sucrose and 10% FBS was used. Oocytes were freeze-thawed according to the previously reported MVC method (12). After incubation for 20–22 h in IVM medium, cumulus cells were partially removed from metaphase II (MII) oocytes by treatment with 0.1% hyaluronidase and mechanical pipetting. Oocytes were washed with TL-HEPES and incubated in a droplet of previously cultured IVM medium for 1 h to recover. Freezing procedures were performed at room temperature. MII oocytes were washed three times in TL-HEPES and then equilibrated in D-PBS for 5 min. For vitrification, oocytes were pretreated with EG10 containing 0, 10, 50, or 100 µg/mL HPC for 5 min, exposed to EG30 containing 0, 10, 50, or 100 µg/mL HPC for 30 s, and then individually loaded onto the inner wall of a modified French ministraw (total length, 2.5–3.0 cm) that had been coated with a minimum volume of vitrification solution. The straw was plunged directly into liquid nitrogen, and 4–5 straws were placed into a pre-chilled cryovial, which was stored in a freezing cane and placed in a liquid nitrogen tank. For thawing, cryopreservation solution was removed via a five-step procedure using thawing solutions warmed to 37°C. Straws stored in liquid nitrogen were moved rapidly to D-PBS containing 1.0 M sucrose. Thereafter, oocytes were sequentially transferred to D-PBS containing 0.5, 0.25, and 0.125 M sucrose, and then into D-PBS lacking sucrose. They were incubated in each solution for 1 min. Finally, VT oocytes were cultured in TCM-199 containing 10% FBS for 1 h.

***PA and in vitro culture***

Non-VT and VT oocytes were activated with 10 µM calcium ionophore in Charles Rosenkrans 1 medium with amino acids (CR1aa) containing 3 mg/mL bovine serum albumin (BSA) for 5 min, immediately placed in 2 mM 6-dimethylaminopurine, and incubated under mineral oil at 38.8°C in a humidified atmosphere of 5% $CO_2$ in air for 3 h. After activation, reconstructed embryos were cultured in CR1aa medium supplemented with 0.03% BSA (fatty acid-free) for 4 d. On day 4, they were switched to CR1aa medium containing 10% FBS and incubated for a further 4 d in an incubator containing 5% $CO_2$ at 38.8°C. Embryo development was examined in the non-VT, 0 HPC, and 50 HPC groups.

***Measurement of intracellular ROS***

Intracellular ROS were measured in oocytes by the 2,7-dichlorofluorescein assay as described previously (13). Briefly, oocytes were incubated with 100 µM 2,7-dichlorodihydrofluorescein diacetate (DCHFDA; Molecular Probes, Eugene, OR, USA) for 20 min at 38.8°C, washed three times in TL-HEPES to remove excess dye, and immediately analyzed by epifluorescence microscopy (Olympus, Tokyo, Japan) using excitation and emission wavelengths of 450–490 and 515–565 nm, respectively. Grayscale images were acquired using a digital camera (Nikon, Tokyo, Japan) attached to the microscope, and mean grayscale values were measured using ImageJ (NIH, Bethesda, MD, USA). Background fluorescence values were subtracted from the final values prior to statistical analysis. Experiments were repeated three times, with 10–20 oocytes per experiment.

***TUNEL assay***

The numbers of apoptotic cells in blastocysts produced by PA on day 8 were determined with an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Briefly, blastocysts were fixed in phosphate-buffered saline containing 3.7% paraformaldehyde for 1 h, permeabilized in 0.3% Triton X-100 for 1 h, and incubated with fluorescein-conjugated deoxyuridine triphosphates and terminal deoxynucleotidyl transferase in the dark for 1 h. This process was followed by incubation in 50 µg/mL RNase A for 1 h at 37°C. Nuclei were simultaneously counterstained with 40 µg/mL propidium iodide. Stained blastocysts were loaded onto glass slides and observed using a fluorescence microscope equipped with a UV filter. Red, green, and yellow (merged) staining

**Table 1.** Survival of VT bovine MII oocytes (n=5).

| Treatment group * | No. (%) of oocytes | |
|---|---|---|
| | Thawed | Survived |
| 0 HPC | 183 | 138 (75.4) |
| 10 HPC | 204 | 164 (80.4) |
| 50 HPC | 202 | 170 (84.2) [a] |
| 100 HPC | 204 | 154 (75.5) |

* 0 HPC, VT MII oocytes treated with 0 µg/mL HPC; 10 HPC, VT MII oocytes treated with 10 µg/mL HPC; 50 HPC, VT MII oocytes treated with 50 µg/mL HPC; 100 HPC, VT MII oocytes treated with 100 µg/mL HPC.

[a] $p < 0.05$.

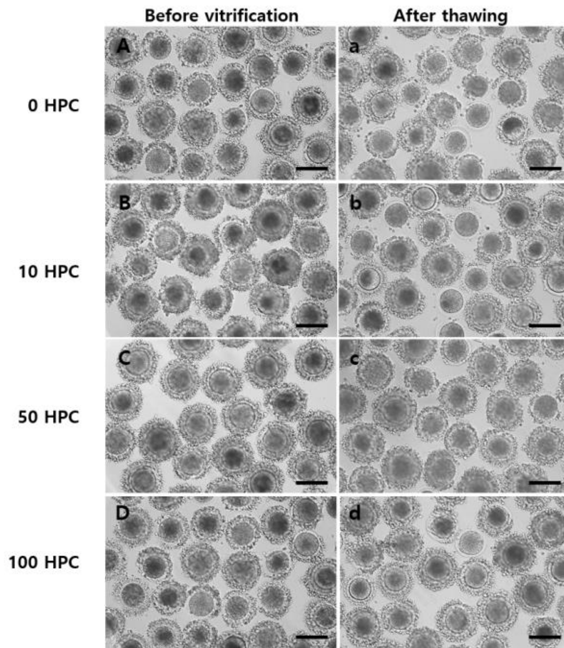


**Figure 1.** Morphologies of oocytes in the 0 (A and a), 10 (B and b), 50 (C and c), and 100 (D and d) HPC groups. A–D: before vitrification, a–d: after thawing. Bar, 100× in A–D and a–d.

indicated chromatin, fragmented DNA, and fragmented DNA of an apoptotic blastomere, respectively. The apoptotic index was determined as the percentage of yellow blastomeres among the total number of red blastomeres.

***Real-time RT-PCR***

Real-time RT-PCR was performed as described previously (13). The primers are described listed in Table 3. A Dynabeads mRNA Direct Kit (Invitrogen, Carlsbad, CA, USA) was

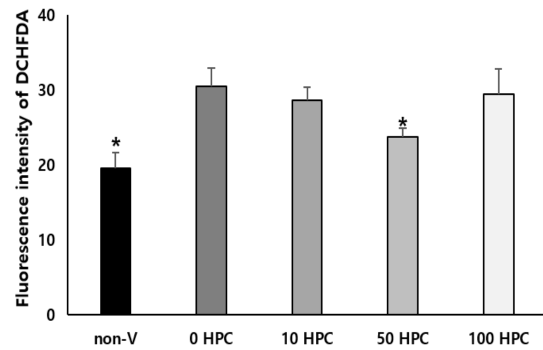


**Figure 2.** Levels of ROS (DCHFDA intensity) in the 0, 10, 50, and 100 HPC groups were compared with that in the non-VT group.

used to prepare mRNA from 20 oocytes and 20 blastocysts per group. cDNA was synthesized from 2 μg mRNA per sample using an oligo (dT)20 primer and SuperScript III reverse transcriptase (Invitrogen). Real-time RT-PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Warrington, UK) with a final reaction volume of 20 μL containing SYBR Green PCR Master Mix (Applied Biosystems). The PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 50–60°C. Samples were then cooled to 12°C. Relative gene expression was analyzed by the 2-ΔΔCt method (14). Expression levels were normalized against the mRNA level of β-actin. The experiment was independently repeated 4–5 times.

**Table 2.** Effect of HPC supplementation during freezing on the in vitro developmental potential of VT bovine oocytes following PA (n=5).

| Treatment group * | No. (%) of oocytes | | | | | Total no. of cells per blastocyst | No. (%) of apoptotic cells per blastocyst |
|---|---|---|---|---|---|---|---|
| | Thawed | Survived | PA | Cleaved (Day 2) | Blastocysts (Day 8) | | |
| Non-VT | - | - | 147 | 139 (94.6) | 31 (21.1) | 120.2±6.4 | 2.0 (1.7) |
| 0 HPC | 242 | 179 (74.0) | 170 | 127 (74.7) [a] | 12 (7.1) [a] | 82.7±2.5 [a] | 4.0 (4.8) |
| 50 HPC | 201 | 161 (80.1) | 161 | 121 (75.2) [a] | 21 (13.0) | 100.2±10.0 | 4.0 (4.0) |

* Non-VT, non-vitrified MII oocytes; 0 HPC, VT MII oocytes treated with 0 μg/mL HPC; 50 HPC, VT MII oocytes treated with 50 μg/mL HPC.

[a] $p < 0.05$.

***Statistical analysis***

The general linear model procedure embedded in the Statistical Analysis System (SAS User's Guide, 1985, Statistical Analysis System Inc., Cary, NC, USA) was used to analyze all data. Significant differences were determined by Tukey's multiple range test. The paired Student's t-test was used to compare relative gene expression. P values < 0.05 were considered significant.

## RESULTS

### *Comparison of the survival rate and ROS level of oocytes between the various groups*

Table 1 shows the survival rates of VT bovine MII oocytes treated with vitrification solution containing various concentrations of HPC. The survival level was 75.4%, 80.4%, 84.2%, and 75.5% in the 0, 10, 50, and 100 HPC groups, respectively, and was significantly higher in the 50 HPC group than in the other groups. The morphologies of oocytes in the 0, 10, 50, and 100 HPC groups are shown in Figure 1. There was no marked morphological difference between the groups under a microscope. The level of ROS was compared between the non-VT and VT groups (Fig. 2A). The ROS level in the 50 HPC group was significantly lower than that in the 0, 10, and 100 HPC groups, and was similar to that in the non-VT group (Fig. 2B).

### *Comparison of mRNA expression levels of oocytes between the various groups*

mRNA expression levels of genes related to apoptosis (Bax), antiapoptotic (Bcl2), demethylation (Dnmt3a), and stress (Hsp70) were compared between VT and non-VT oocytes. The mRNA expression level of Bax was significantly higher in the 0 and 100 HPC groups than in the non-VT, 10 HPC, and 50 HPC groups. The mRNA expression level of Bcl2 was significantly lower in the 0 HPC group than in the non-VT and 10, 50, and 100 HPC groups. The mRNA expression level of Hsp70 was significantly lower in the 50 HPC group than in the non-VT and 0, 10, and 100 HPC groups. However, the mRNA expression level of Dnmt3a did not differ between the groups.

### *Effect of HPC treatment during freezing of oocytes on development of embryos obtained via PA*

The developmental potentials of embryos derived from non-VT and VT oocytes following PA were compared. The percentage of thawed oocytes that survived was higher in the 50 HPC group (80.1%) than in the 0 HPC group (74.0%) (Table 2). At day 2 post-activation, the percentage of embryos that underwent cleavage was significantly higher in the non-VT group

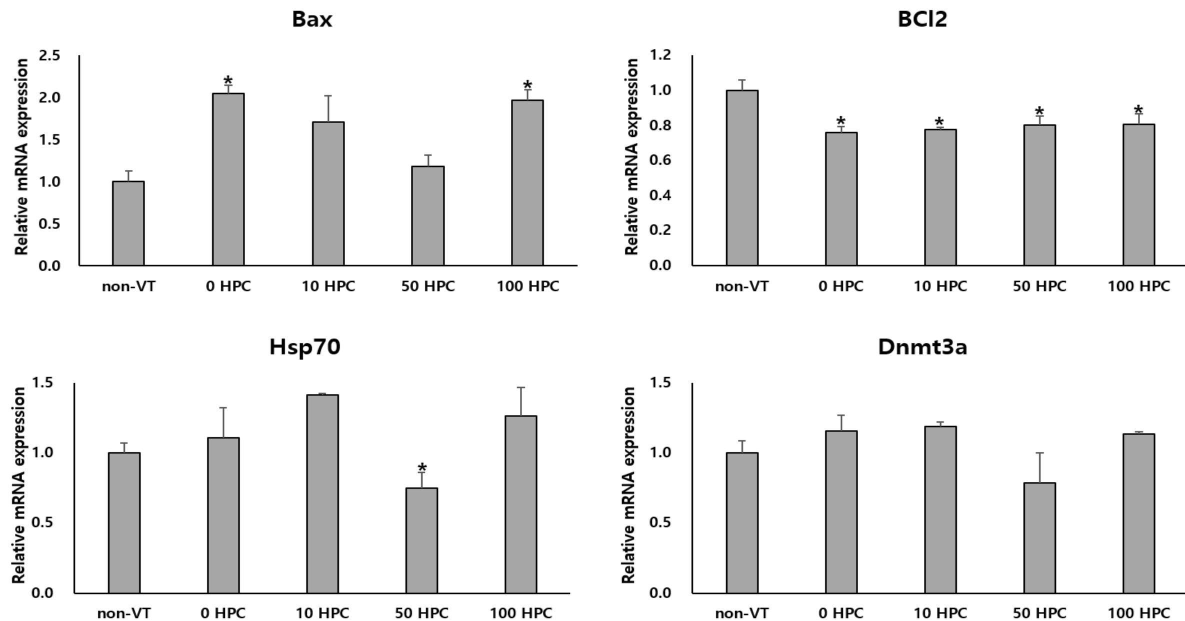


**Figure 3.** Relative mRNA expression levels of genes related to apoptosis (*Bax* and *Bcl2*), demethylation (*Dnmt3a*), and stress (*Hsp70*) in non-VT and VT oocytes. * p < 0.05 compared with the non-VT group.

**Table 3.** Sequences of primers used for real-time RT-PCR.

| Gene | Primer sequence | Annealing temperature (℃) | Product size (bp) |
|---|---|---|---|
| *Bax* | 5'-GCTCTGAGCAGATCAAG-3'<br>5'-AGCCGCTCTCGAAGGAAGTC-3' | 56 | 201 |
| *Bcl2* | 5'-TTGGAGAGCCAGTGAACAGT-3'<br>5'-TGCTGATAACTGTCTGCGCT-3' | 54 | 203 |
| *HSF1* | 5'-GAGCGAGGACATAAAGATTC-3'<br>5'-GAGATGAGGAACTGGATGAG-3' | 54 | 207 |
| *Glut-5* | 5'-TTGGAGAGCCAGTGAACAGT-3'<br>5'-TGCTGATAACTGTCTGCGCT-3' | 60 | 292 |
| *Interferon-tau* | 5'-ATGGCCTTCGTGCTCTCTCT-3'<br>5'-AGGTCCTCCAGCTGCTGTTG-3' | 55 | 356 |
| *Caspase-3* | 5'-CGATCTGGTACAGACGTG-3'<br>5'-GCCATGTCATCCTCA-3' | 50 | 359 |
| *Hsp70* | 5'-GACAAGTGCCAGGAGGTGATTT-3'<br>5'-CAGTCTGCTGATGATGGGGTTA-3' | 51 | 117 |
| *Dnmt3a* | 5'-TGATCTCTCCATCGTCAACCCT-3'<br>5'-GAAGAAGGGGCGGTCATCTC-3' | 54 | 221 |
| *β-actin* | 5'-GTCATCACCATCGGCAATGA-3'<br>5'-GGATGTCGACGTCACACTTC-3' | 56 | 111 |

(94.6%) than in the 0 (74.7%) and 50 (75.2%) HPC groups, but did not significantly differ between the 0 and 50 HPC groups. At day 8 post-activation, the percentage of embryos that had reached the blastocyst stage was higher in the non-VT group (21.1%) than in the 0 (7.1%) and 50 (13.0%) HPC groups, and was significantly higher in the 50 HPC group than in the 0 HPC group. The mean total cell number per blastocyst was significantly lower in the 0 HPC group (82.7±2.5) than in the non-VT (120.2±6.4) and 50 HPC (100.2±10.0) groups. The apoptotic index of blastocysts was determined by fluorescence microscopy and the TUNEL assay. The apoptotic index was lower in the non-VT group (1.7%) than in the 0 (4.8%) and 50 (4.0%) HPC groups, and did not significantly differ between the 0 and 50 HPC groups (Table 2). The morphologies of embryos at day 2 and blastocysts at day 8 in the non-VT, 0 HPC, and 50 HPC groups are shown in Figure 4.

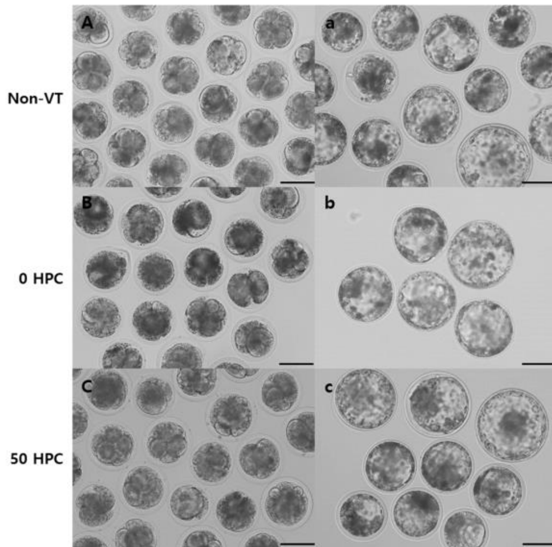


**Figure 4.** Morphologies of embryos obtained via PA in the non-VT, 0 HPC, and 50 HPC groups. Images of cleaved embryos at day 2 (A, non-VT; B, 0 HPC; and C, 50 HPC) and blastocysts at day 8 (a, non-VT; b, 0 HPC; and c, 50 HPC). Bar, 100 μm.

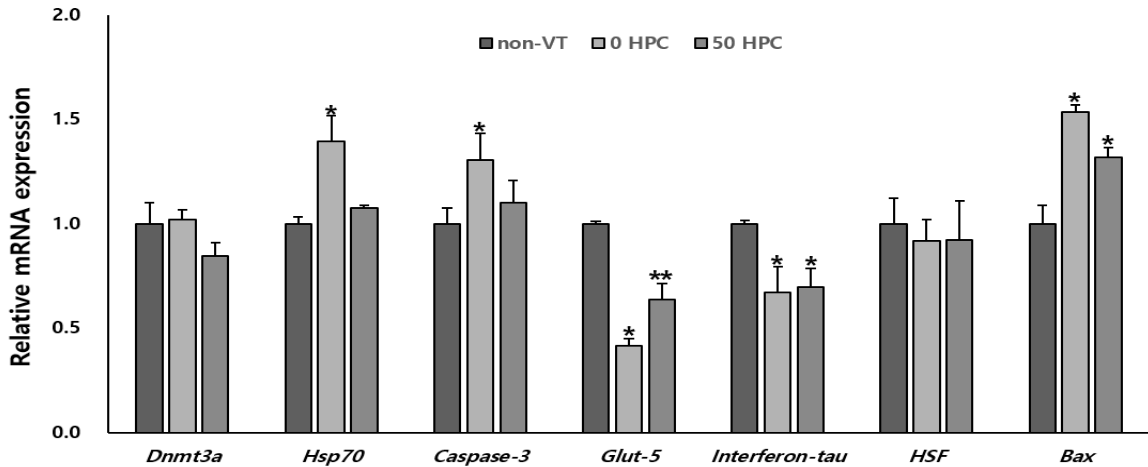


**Figure 5.** mRNA expression levels of developmental potential-related genes (*Dnmt3a*, *Hsp70*, *Caspase-3*, *Glut-5*, *Interferon-tau*, *HSF*, and *Bax*) in blastocysts produced via PA in the non-VT, 0 HPC, and 50 HPC groups. *β-actin* was used as an internal standard. * $p < 0.05$ compared with the non-VT group.

***Comparison of the mRNA expression levels of developmental potential-related genes in blastocysts between the groups***

The mRNA levels of developmental potential-related genes (Dnmt3a, Hsp70, Caspase-3, Glut-5, Interferon-tau, HSF, and Bax) in in vitro produced 8-day-old blastocysts generated via PA were compared between the groups. The mRNA levels of Dnmt3a (methylation-related) and HSF (stress-related) did not differ between the non-VT, 0 HPC, and 50 HPC groups. The mRNA levels of the proapoptotic genes Bax and Caspase-3 were significantly higher in the 0 HPC group than in the non-VT and 50 HPC groups. The mRNA levels of Glut-5 (metabolism-related) and Interferon-tau (implantation-related) were significantly lower in the 0 and 50 HPC groups than in the non-VT group, but the mRNA level of Glut-5 was higher in the 50 HPC group than in the 0 HPC group. The mRNA level of Hsp70 (stress-related) was significantly lower in the non-VT and 50 HPC groups than in the 0 HPC group (Fig. 5).

## DISCUSSION

Cryopreservation can provide a stable source of oocytes for production of embryos via IVF and SCNT. IVF is a basic technology used for animal production, while SCNT is a useful technology for preservation of endangered species and production of transgenic animals. In addition, cryopreservation of human oocytes has advantages over cryopreservation of human embryos. Specifically, it preserves the fertility of women who are at risk of becoming infertile due to diseases, facilitates donation of oocytes, and allows women to choose when they have children. Furthermore, freezing of oocytes eliminates the legal and ethical concerns associated with freezing of embryos. During cryogenic retention, cells are exposed to numerous mechanical, thermal, and chemical stresses (15), which can lead to cell dysfunction and cell death. In general, oocytes are more sensitive to damage caused by exposure to low temperatures than embryos (16). Oocytes exhibit low permeability to water and cryopreservation solution due to their large size and are therefore very sensitive to low temperature-mediated preservation (17). To solve these problems, vitrification is a simple, fast, cost-effective, and reliable method, and many vitrification methods have been reported (18, 19). We previously studied the suitability of the MVC method for vitrification (12) and developed an efficient vitrification method for production of SCNT embryos (13). Based on these studies, we sought to identify a supplement that can be added to vitrification solution, and improve the survival rate of bovine oocytes and the development ability of embryos derived from these oocytes. HPC increases the viscosity of vitrification solutions and reduces the risk of cryodamage by improving the efficiency of solidification during the vitrification process (20). We added HPC to

vitrification solution and investigated the survival rate of VT bovine oocytes and embryo development after PA.

We supplemented vitrification solution with 0, 10, 50, and 100 µg/mL HPC in an attempt to increase the survival rate of bovine oocytes. The survival rate of VT oocytes was significantly higher than that in the 0, 10 and 100 HPC groups. Oxidative stress is mediated by ROS, which are byproducts of normal mitochondrial metabolism and key signaling molecules in various physiological and pathological processes (21). An increase in the level of ROS can reduce the intracellular adenosine triphosphate concentration and the glutathione/glutathione disulfide ratio, and concomitantly increase the cytosolic concentration of calcium ions, which can damage oocytes. We expected freezing to affect the ROS level in oocytes. The level of ROS in the 50 HPC group was lower than that in the 0, 10, and 100 HPC groups, and was similar to that in the non-VT group. This result suggests that oocytes in the various groups were exposed to different levels of oxidative stress, and that among VT oocytes, those in the 50 HPC group were exposed to the lowest level of oxidative stress. To investigate damage of VT oocytes and the effect of HPC, the mRNA expression levels of genes related to apoptosis (Bax and Bcl2), stress (Hsp70), and methylation (Dnmt3a) were determined. Bax regulates mitochondrial membrane permeability and induces apoptosis by disrupting the mitochondrial membrane (22), while Bcl2 is an anti-apoptotic protein that promotes survival (23). Expression of Bax is increased (24) and expression of Bcl2 is decreased (25) after vitrification. Alterations in expression of apoptosis-related genes in oocytes upon vitrification may interfere with embryo development because the majority of apparently normal oocytes fail to develop during the first few days of culture after vitrification and undergo degeneration (26). In this study, mRNA expression of Bax did not significantly differ between the non-VT, 10 HPC, and 50 HPC groups, but was higher in the 0 and 100 HPC groups than in the non-VT group. mRNA expression of Bcl2 did not significantly differ between the non-VT and HPC-treated groups, but was significantly lower in the 0 HPC group. These results suggest that HPC prevents apoptosis. mRNA expression of the stress-related gene Hsp70 was significantly lower in the 50 HPC group than in the non-VT group. We expected Hsp70 expression to increase upon vitrification due to oocyte damage, and further research is required to elucidate the mechanism by which HPC reduces Hsp70 expression. Oocyte vitrification is accompanied by alterations in DNA methylation in bovine oocytes and embryos, which may contribute to the impaired embryo development and reduced embryo quality observed after vitrification (27, 28). A previous study reported that Dnmt3a expression is significantly reduced in vitrified oocytes (29). However, we found that Dnmt3a expression did not significantly differ between non-VT and VT oocytes.

To investigate the effect of HPC on embryo development, oocytes in the non-VT, 0 HPC, and 50 HPC groups underwent PA and were cultured for 8 days. The cleavage rate at day 2, blastocyst formation rate at day 8, and total cell number per blastocyst significantly differed between the non-VT and VT groups. The cleavage and blastocyst formation rates were significantly higher in the non-VT group. The cleavage rate did not significantly differ between the 0 and 50 HPC groups, but the blastocyst formation rate was higher in the 50 HPC group (13.0%) than in the 0 HPC group (7.1%). The cleavage rate of reconstructed embryos generated using VT oocytes was reported to be ~50–70% (30, 31), while the blastocyst formation rate was reported to be ~4–8% (32, 33, 34). In addition, these studies reported that varying percentages (7–13%) of reconstructed embryos obtained via PA developed to the blastocyst stage. The TUNEL assay found no significant difference in apoptosis between the 0 and 50 HPC groups, but proapoptotic gene expression levels were significantly higher in the 0 HPC group than in the non-VT and 50 HPC groups. VT oocytes are repeatedly exposed to heat shock stress. Heat shock induces apoptosis in preimplantation embryos in a developmentally regulated manner (35). mRNA expression of Hsp70 expression was significantly lower in the non-VT and 50 HPC groups than in the 0 HPC group. This is similar to the differences in mRNA expression of the proapoptotic genes Caspase-3 and Bax. These results indicate that supplementation of vitrification solution of oocytes with HPC reduces stress in embryos derived from these oocytes via PA. To investigate the developmental potential of embryos obtained via PA, the expression level of various factors was determined. The mRNA expression level of Glut-5 (metabolism-related) was significantly

lower in the 0 and 50 HPC groups than in the non-VT group, but was higher in the 50 HPC group than in the 0 HPC group. mRNA expression of Dnmt3a did not significantly differ between non-VT and VT oocytes. These results indicate that oocyte damage caused by vitrification perturbs embryo development. Similar results have been previously reported (13). However, this study did not significantly differ between the 0 and 50 HPC groups. This indicates that HPC does not markedly affect DNA methylation, but further research is needed. Supplements must be identified that reduce oocyte damage during vitrification and thawing, and thereby improve embryo development in vitro. We propose that HPC is one such supplement.

In conclusion, this study demonstrates that supplementation of vitrification solution with HPC improves the survival rate of VT oocytes, decreases ROS production, reduces cell death, and improves development of embryos obtained via PA. Our results indicates that HPC is suitable for use as a vitrification solution supplement.

**Acknowledgements:** This work was supported by the Agriculture, Food and Rural Affairs Convergence Technologies Program for Educating Creative Global Leaders (#715003-07), Ministry of Agriculture, Food and Rural Affairs.

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