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Effects of Green Tea (*Camellia sinensis*) Extract Supplementation at Different Dilution Steps on Boar Sperm Cryopreservation and *in vitro* Fertilization

Sang-Hyoun Park, Yubyeol Jeon* and Il-Jeoung Yu*[1]**

**Department of Theriogenology and Reproductive Biotechnology, College of Veterinary Medicine and Bio-safety Research Institute, Chonbuk National University, Iksan 54596, Republic of Korea*
***Animal Biotechnology Division, National Institute of Animal Science, Rural Development Administration, Jeollabuk-do 55365, Republic of Korea*

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Abstract : We evaluated the effects of green tea extract (GTE) supplementation at different dilution steps on boar sperm freezing and *in vitro* fertilization. Sperm intracellular hydrogen peroxide (H_2O_2), motility, viability, acrosome integrity and morphology were determined. In addition, sperm IVF parameters (penetration and monospermy) and glutathione (GSH) levels of presumptive zygotes (PZs) were evaluated. Semen was diluted in lactose egg yolk (LEY) and cooled at 5°C for 3 h (first dilution step) and then diluted in LEY with 9% glycerol and maintained at 5°C for 30 min (second dilution step). Four experimental groups were compared: first and second dilution steps without GTE (control), first dilution step with GTE (Step 1), second dilution step with GTE (Step 2) and first and second dilution step with GTE (Step 1+2). The spermatozoa were frozen in nitrogen vapor. Higher sperm motility, viability and acrosome integrity after thawing were observed in Step 1, Step 2 and Step 1+2 groups compared with the control ($P < 0.05$). Lower H_2O_2 level was observed in Step 1+2 compared with control and Step 1 ($P < 0.05$). For IVF, matured oocytes were co-cultured with spermatozoa frozen according to the experimental groups. GSH levels of PZs were significantly higher in Step 2 and Step 1+2 than in control and Step 1 ($P < 0.05$) without a significant difference in IVF parameters. In conclusion, supplementation with GTE in both first and second dilution steps during the freezing process resulted in better boar sperm cryopreservation and might be beneficial for further embryo development.

***Key words* :** Dilution step, Freezing, Green tea extract, Glutathione, Reactive oxygen species.

Introduction

Freezing of semen causes physical and chemical stresses to spermatozoa (20). These stresses hasten production of reactive oxygen species (ROS). The ROS reduce the sperm motility (6) and change the sperm morphology (7) and membrane function (13). Spermatozoa consist of a small volume of cytoplasm and are primarily dependent on the antioxidant resource of seminal plasma. Removal of seminal plasma by centrifugation during cryopreservation process increases the exposure of spermatozoa to oxidative stress (2). In addition, researchers suggest that the antioxidant activity of the spermatozoa themselves may be decreased by cryopreservation (9,22). In particular, boar sperm has a higher content of unsaturated fatty acids in phospholipids in their plasma membrane (10,12) and is therefore easily damaged by excessive ROS during cryopreservation.

The steps in cooling, freezing and thawing may damage the plasma membrane and cellular structure of spermatozoa (25,39,41). During freezing process, spermatozoa have to undergo three different and successive steps of temperature changes and the addition of cryoprotectant (CPA). The steps include ① a preliminary cooling step to 5°C; ② addition of CPA and equilibration at 5°C and ③ freezing to −196°C. The major phase change occurs in the vicinity of 15°C to 5°C, which might be the prime temperature range for temperature-dependent injury (18,42). Increased ROS production by sperm during cooling to 4°C was reported (40). For this reason, the antioxidants have been added during the first dilution step in the freezing process (26,27). In addition, the cryoinjury may be triggered by the ROS that is produced during the freezing process (8). Furthermore, adding glycerol in the second freezing step of boar spermatozoa increased the osmolality of the extender (16) and consequently enhanced ROS production (30).

For inhibition of ROS generation, the use of herbal antioxidants has attracted attention from several researchers who have reported the useful effect of antioxidant treatment on the oxidative stress in mammalian spermatozoa (27,28). In recent times, natural antioxidants are of great interest on account of the safety and toxicity problems of synthetic antioxidants (5). The green tea extract (GTE) is primarily beneficial for health due to its antioxidant properties and the ROS scavenging ability of its polyphenolic catechins (45). As a natural antioxidant, green tea has been used in cancer chemoprevention and cosmetics (48,49). Therefore, we focused on investigation of the antioxidative effect of GTE as a supplement in the freezing extender in the first (24°C to 5°C for 3

[1]Corresponding author.
E-mail : iyu@jbnu.ac.kr

h), the second (5ºC for 30 min) and both the first and second dilution steps, respectively, during boar sperm freezing. To ascertain the antioxidative effect of GTE, we assessed intracellular hydrogen peroxide (H_2O_2) level as well as sperm motility, viability, morphology and acrosome integrity. In addition, we examined other aspects of sperm function such as sperm-oocyte fusion and glutathione (GSH) levels of presumptive zygotes (PZs).

Materials and Methods

Chemicals

All the chemicals were bought from Sigma-Aldrich (St. Louis, MO, USA) except stated otherwise. High-purity water (6114VF, Sartorius AG, Goettingen, Germany) was used to prepare solutions.

Preparation of green tea (*Camellia sinensis*) extract

Green tea powder (GTP) was obtained from Bioland (GTP-020968, Seoul, Korea). Green tea extract (GTE) stock was prepared according to the methods described by Park and Yu (31). GTE stock of 100,000 mg/L was prepared by dissolving GTP in triple distilled water (DW) and filtration through 1.2 µm filter (17593-k, Sartorius, Goettingen, Germany). The working solution of GTE (1,000 mg/L) was prepared by adding DW to stock solution (v:v).

High-performance liquid chromatography analysis of 100,000 mg/L GTE revealed 45.9 mg/L catechin, 141 mg/L epicatechin, 297.4 mg/L gallocatechin, 424.3 mg/L epigallocatechin, 392.7 mg/L gallocatechin gallate and 392.7 mg/L epigallocatechin gallate. Chromatography (6430 LC/MS/MS, Agilent, City, USA) was performed at the Center for University-wide Research facilities in Chonbuk National University, Republic of Korea (31,33).

Collection of semen

Five boars (two Duroc jersey and three Yorkshire) were used for this experiment. Semen was collected using the gloved-hand technique and filtered through four layers of sterile gauze to remove gel particles. The sperm samples had to fulfill minimal quality criteria before use: sperm rich fraction volume ≥ 75 mL, concentration ≥ 200 × 10^6 sperm/mL, motility ≥ 70% and viability ≥ 85%. The collected semen was pooled to reduce the effect of individual differences. The pooled semen was extended (1:1 [v:v]) in Beltsville thawing solution (BTS; 37 mg/mL glucose, 1.25 mg/mL EDTA, 6 mg/mL sodium citrate, 1.25 mg/mL sodium bicarbonate, 0.75 mg/mL potassium chloride, 0.6 mg/mL penicillin and 1 mg/mL streptomycin) (36). The diluted spermatozoa were stored at 24ºC for 2 h (31,33).

Sperm freezing-thawing protocol

The freezing extenders used in these experiments were composed of freezing extender 1 (FE 1) and freezing extender 2 (FE 2). FE 1 was made of lactose egg yolk (LEY) extender (80 mL lactose solution, 20 mL egg yolk and 0.1% antibiotic-antimycotic in 100 mL sterile non-pyrogenic water). FE 2 composed of LEY extender supplemented with 9% (v:v) glycerol and 1.5% (v:v) Equex STM (Nova Chemical Sales Inc., Scituate, MA, USA). Semen was processed according to the freezing procedure (4,24). Briefly, the semen diluted in BTS was centrifuged at 850 × *g* for 15 min at room temperature. The supernatant was then removed and the sperm pellet was suspended with FE 1 to a concentration of 1.5 × 10^8/mL. Sperm suspensions were cooled gradually from 24ºC to 5ºC for 3 h. At 5ºC, a second dilution step to 1 × 10^8/mL was performed with FE 2 and the spermatozoa were maintained at 5ºC for 30 min (31). The cooled sperm was loaded into 0.5 mL straws and sealed. At ten minutes before liquid nitrogen (LN_2) vapor cooling, a styrofoam box (29.5 × 18.7 × 24 cm^3) was filled with LN_2 to a depth of 5 cm and a rack with two bars was set on 7 cm from the surface of the LN_2. The straws were then aligned horizontally for 20 min on the rack in the LN_2 vapor and then plunged into LN_2 for storage. Straws from each group were thawed by immersion in a circulating water bath at 37ºC for 25 sec before use in experiments. The cooling rates were 30.6ºC/min from 5ºC to −15ºC, 88.2ºC/min from −15 to −60.0ºC and 14.7ºC/min from −60ºC to −116.0ºC (37).

Sperm motility

Semen (10 µL) was placed on a slide and cover-slipped. The percentage of progressive motile spermatozoa was estimated under microscopic examination at 400 × magnification (46). The mean of six successive estimations was recorded as the final motility score. Sample identity was hidden from the operator and the samples were randomly numbered to avoid operator bias in subjective evaluation. Samples in each group were assessed in duplicate (37). The mean of eight successive estimates was recorded as the final motility score.

Sperm viability

The integrity of sperm plasma membrane was measured (46) using the fluorescent double-stain [SYBR-14/propidium iodide (PI)] Fertilight® (Molecular Probes Inc., Eugene, OR, USA). Sperm samples were incubated at room temperature for 5 min with SYBR-14 at a final concentration of 100 nM, and then with PI at a final concentration of 10 µM for 5 min at the same temperature. For each replicate sample, two slides were prepared and appropriately 200 spermatozoa were counted per slide. The number of green (SYBR-14) or red (PI) fluorescent spermatozoa was counted under a fluorescence microscope (Axio, Carl Zeiss, Goettingen, Germany) fitted with a 488 nm excitation filter and the percentage of spermatozoa with an intact membrane (green fluorescence in sperm head) was calculated (31,32).

Sperm morphology

The morphology of spermatozoa was evaluated by the Diff-Quik kit (International Reagents Corp., Kobe, Japan). In Diff-Quik staining, a drop on a glass slide was drawn out as for a blood smear and allowed to air dry. The slide was dipped 10 times in each of the first (fixative) and second (anionic/acidic dye) solutions and final (cationic dye) solution. The slide was rinsed with wiping of the back of the slide and allowed to dry. At least 200 spermatozoa were evaluated by light microscopy (DM2500, Leica, Wetzlar, Ger-

many) at 1,000 × magnification.

Spermatozoa were classified into one of the following categories: normal morphology and abnormal morphology including tail defects (folded and coiled) and head defects (giant, double, narrow) on the evaluation guidelines of Gadea *et al* (21). The percentage of spermatozoa with normal morphology was calculated.

Sperm acrosome integrity

Acrosome integrity was determined using the method described by Yu and Leibo (46). Spermatozoa were stained with *Pisum sativum agglutinin* (PSA) conjugated to fluorescein isothiocyanate (FITC) [50 μL of FITC-PSA (100 μg/mL; Sigma, L-0770)] for 15 min then stained sample were washed with DW for 15 min. For each replicate sample, two slides were examined under a fluorescence microscope (Axio, Carl Zeiss) and approximately 200 spermatozoa were counted per slide. The percentage of spermatozoa with an intact acrosome (green fluorescence on sperm anterior acrosomal region) was calculated.

Sperm intracellular hydrogen peroxide (H_2O_2) level

2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA (DCF); Molecular Probes Inc.) was used to detect H_2O_2 (24). The working solution of 20 mM H_2DCFDA was prepared in dimethyl sulfoxide. Aliquots of 500 μL of semen sample (1×10^6 sperm/mL) were mixed with H_2DCFDA to a final concentration of 200 μM. For simultaneous differentiation of living from dead spermatozoa, PI (final concentration, 2 μM) was added to H_2DCFDA-treated spermatozoa. Samples stained with H_2DCFDA and PI were incubated at 25°C for 60 min and analyzed using flow cytometry (FACScalibur, Becton Dickinson, San Jose, USA). Data were expressed as the percentage of viable spermatozoa with H_2O_2 (high DCF fluorescence). The mean fluorescence intensity (MFI) of DCF was measured to evaluate intracellular mean H_2O_2 per the viable sperm population (33).

Oocyte collection and *in vitro* maturation (IVM)

Oocyte collection and IVM were processed according to methods described by Park and Yu (32). Porcine ovaries were collected from a local abattoir and transported to the laboratory at 34-36°C in 0.9% saline supplemented with 100 IU/mL penicillin G and 100 μg/mL streptomycin. Cumulus-oocyte complexes (COCs) were aspirated through an 18-gauge needle. Oocytes with compact cumulus mass and a dark, homogenous cytoplasm were washed three times in Tyrode's lactate-HEPES-polyvinyl alcohol (TL-HEPES-PVA: 114 mM NaCl, 3.2 mM KCl, 0.4 mM $Na_2H_2PO_4$, 2 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 5 mM $NaHCO_3$, 20 mM HEPES, 16.6 mM sodium lactate [60% syrup], 0.5% PVA, 10 IU/mL penicillin and 10 μg/mL streptomycin). COCs were cultured in North Carolina State University (NCSU)-23 medium supplemented with 10% porcine follicular fluid (PFF), 0.6 mM cysteine, 10 ng/mL epidermal growth factor, 10 IU/mL pregnant mare serum gonadotropin and 10 IU/mL human chorionic gonadotropin for 22 h and then for another 22 h in maturation medium without hormones at 39°C in a humidified atmosphere of 5% CO_2 in air. PFF was collected from ovarian follicles 3-6 mm in diameter by centrifugation at 1,600 × g for 30 min and filtration through a 1.2 μm syringe filter, aliquoted and stored at −80°C until use (32).

In vitro fertilization (IVF) and *in vitro* culture (IVC)

Percoll solutions and gradients were prepared as described previously (47). A two-layer discontinuous gradient was formed by layering 1 mL of 45% Percoll solution on top of 1 mL of 90% Percoll solution in a 15 mL conical tube. Aliquots of frozen-thawed spermatozoa (3 mL) were layered onto Percoll gradient and centrifuged for 20 min at 850 × g at room temperature. After aspiration of the supernatant, the recovered pellet was washed twice by centrifugation at 350 × g for 3 min with 5 mL D-PBS supplemented with 0.1% BSA, 10 IU/mL penicillin and 10 μg/mL streptomycin. The supernatant was discarded and motile spermatozoa were collected and diluted to a concentration of 10×10^5 spermatozoa/mL with Tris-buffered medium (mTBM: 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, 11 mM D-glucose, 5 mM sodium pyruvate, 2 mM caffeine and 0.2% BSA). After IVM, cumulus cells were removed as described (23). Denuded oocytes were washed three times with mTBM and transferred to an mTBM insemination drop (45 μL). A 5 μL volume of spermatozoa was added to each insemination drop to give a final concentration of 1×10^5 spermatozoa/mL. Oocytes and spermatozoa were co-cultured for 6 h at 39°C in a humidified atmosphere of 5% CO_2 in the air (32).

Following IVF, presumptive zygotes (PZs) were washed three times, transferred to IVC medium (D-glucose free NCSU-23 supplemented with 0.17 mM sodium pyruvate, 2.73 mM sodium lactate and 0.4% BSA) and incubated for 12 h at 39°C in a humidified atmosphere of 5% CO_2 in the air (32).

Assessment of sperm penetration and monospermy

Twelve hours after insemination, the percentages of spermatozoa penetrating oocytes and monospermy were assessed as previously described (23). Briefly, the presumptive zygotes were mounted on slides, fixed for 7 days with acetic acid in ethanol (1:3, v:v) at room temperature, stained with aceto-orcein [1% (w:v) orcein in 45% (v:v) acetic acid], destained with glycerol:acetic acid:DW (1:1:3, v:v) and examined under a light microscope at 400 × magnification. The fertilization parameters evaluated were: penetration (the percentage of penetrated oocytes/total oocytes inseminated with sperm) and monospermy (the percentage of oocytes with two pronuclei or with one pronucleus and one decondensed sperm head/penetrated oocytes).

Measurement of intracellular glutathione (GSH) levels of PZs

Cell Tracker Blue CMF2HC (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin, Invitrogen) was used to detect GSH levels in PZs as blue fluorescence. After IVC, PZs were incubated for 30 min in TL-HEPES-PVA supplemented with 10 μM Cell Tracker. The PZs were then placed on a glass slide with a 10 μL drop of DPBS-PVA and observed under a fluorescence microscope (Axio, Charl Zeiss) with a UV filter (370 nm). The fluorescence density was measured and the

intensity of fluorescence in each PZ image was analyzed using Image J software 1.33u (National Institutes of Health, Bethesda, MD, USA) (32).

Experimental design

Four experimental groups were defined according to the dilution step supplemented with GTE during boar sperm freezing as follows: first and second dilution steps without GTE (control), first dilution step with GTE (Step 1), second dilution step with GTE (Step 2) and both first and second dilution steps with GTE (Step 1+2). Spermatozoa were frozen according to the freezing protocol described above.

Sperm motility, viability, morphology, acrosome integrity and ROS level were evaluated. In addition, IVF parameters and GSH level of PZs were assessed.

Statistical analyses

Five replicates were conducted this experiment. Percentage data were subjected to arcsine transformation before analysis. All data are presented as mean ± SE and were analyzed using One-way ANOVA followed by Duncan's multiple range test. Statistical Analysis System ver. 8x software (SAS, Cary, NC, USA) was used and $P < 0.05$ was considered statistically significant. Correlation between motility and ROS level was investigated using Pearson's correlation test using SPSS for Windows version 22, statistical software.

Results

Sperm parameters

The effects of GTE supplementation of boar sperm freezing extender on sperm parameters according to dilution step in freezing process are presented in Table 1. Higher sperm motility was observed in Step 1, Step 2 and Step 1+2 groups compared with the control group ($P < 0.05$). The percentage of sperm viability in Step 2 and Step 1+2 groups was higher than that in the control group ($P < 0.05$). However, no significant differences in viability were found between the control and Step 1 groups. GTE supplementation did not affect sperm morphology in any group. Sperm acrosome integrity of Step 1, Step 2 and Step 1+2 groups was higher than that of the control group ($P < 0.05$).

Sperm intracellular H_2O_2 level

Lower H_2O_2 levels in viable sperm were observed in the Step 1+2 group compared with the control and Step 1 groups ($P < 0.05$; Table 2; Fig 1). However, no significant differences in H_2O_2 levels were found between the control and Step 1 groups.

Table 2. Effect of GTE supplementation on hydrogen peroxide (H_2O_2) level according to dilution steps in boar sperm freezing

Group	hydrogen peroxide (H_2O_2) level
C	52.1 ± 6.0^{a}
Step 1	51.1 ± 8.3^{a}
Step 2	40.9 ± 1.5^{ab}
Step 1+2	30.7 ± 3.6^{b}

GTE: 1,000 mg/L green tea extract (final concentration), C: control, first and second dilution step without GTE, Step 1: only first dilution step with GTE, Step 2: only second dilution step with GTE, Step 1+2: first and second dilution step with GTE.
Values with different superscript are significantly different ($P < 0.05$).
The data represent means ± SE.

Fertility parameters and GSH level of PZs following IVF

Fertility parameters did not show any difference in sperm penetration and monospermy rate according to dilution step of GTE supplementation (Table 3). The GSH levels of Step 2 and Step 1+2 groups were significantly higher than those of control and Step 1 groups ($P < 0.05$; Fig 2).

Discussion

Generally, boar spermatozoa undergo two different dilution steps for cryopreservation. In the first dilution step, dilution extender without CPA is used to cool the sperm from 17 to a 5ºC (cooling step) whereas in the second dilution step, the extender including CPA is used to equilibrate spermatozoa at 5ºC. Temperature changes during freezing process produce a rapid phase transition and hence there is a radical increase in ROS. The increased ROS level causes poor motility, viability, morphology and acrosome integrity of frozen-thawed spermatozoa (13,41). For reduction of ROS, most researchers have studied the effect of antioxidants during the first dilution step of the freezing process (43,44). However, Bailey *et al* (8) reported that cryoinjury might be induced by ROS activity generated during all freezing processing steps. Therefore, the present study evaluated the antioxidative effect of GTE added during either one or both of two dilution steps. This is the first report of antioxidative and other related effects of GTE

Table 1. Effect of GTE supplementation on sperm parameters according to dilution steps in boar sperm freezing

Group	Motility	Viability	% of sperm with normal morphology	Acrosome integrity
C	26.2 ± 1.6^{b}	42.2 ± 1.1^{b}	98.1 ± 0.4	81.1 ± 4.8^{b}
Step 1	40.6 ± 3.8^{a}	45.8 ± 1.6^{ab}	98.1 ± 0.4	94.0 ± 0.9^{a}
Step 2	38.7 ± 0.7^{a}	47.6 ± 2.0^{a}	97.7 ± 0.4	93.5 ± 1.4^{a}
Step 1+2	38.7 ± 1.6^{a}	47.8 ± 1.7^{a}	98.1 ± 0.4	93.5 ± 1.3^{a}

GTE: 1,000 mg/L green tea extract (final concentration), C: control, first and second dilution step without GTE, Step 1: only first dilution step with GTE, Step 2: only second dilution step with GTE, Step 1+2: first and second dilution step with GTE.
Values with different superscript are significantly different ($P < 0.05$).
The data represent means ± SE.

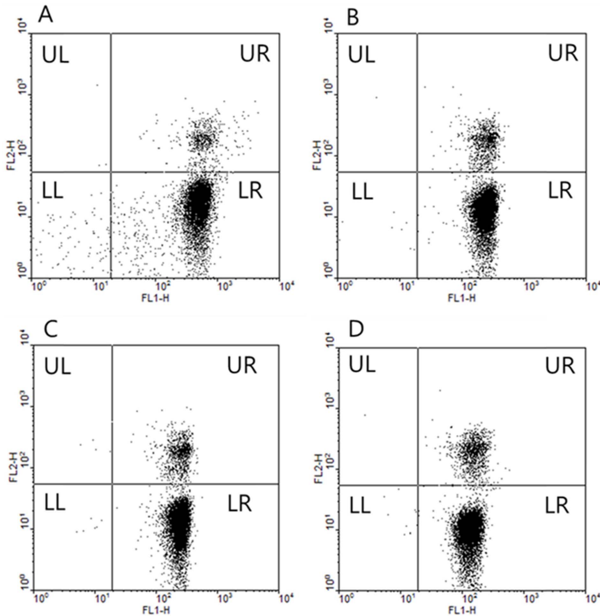

Fig 1. Flow cytometric analysis of sperm labeled with DCFDA/PI. GTE: 1,000 mg/L green tea extract (final concentration), A: control, first and second dilution step without GTE, B: only first dilution step with GTE (Step 1), C: only second dilution step with GTE (Step 2), D: first and second dilution step with GTE (Step 1+2). The upper panel shows dot plot cytograms to measure the sperm population upon the level of intracellular H_2O_2 during cryopreservation. The LL quadrant represents viable sperm with a low intracellular H_2O_2 level, while the LR shows viable sperm with a high intracellular H_2O_2 level. The UL quadrant shows dead sperm with a low intracelluar H_2O_2 level. The UR shows dead sperm with a high intracellular H_2O_2 level.

on boar sperm freezing and subsequently IVF parameters.

The change in ROS due to cryopreservation may be related to an imbalance between ROS production and detoxification by sperm antioxidants. Moreover, the CPA used during the second dilution step might aggravate the induction of harmful factors like ROS (30,35). In this study, the Step 1+2 group showed significantly ($P < 0.05$) lower H_2O_2 levels than the control and Step 1 groups, whereas there was no significant difference between control, Step 1 and Step 2 groups. We infer that addition of GTE in the first dilution step might initiate a potential ROS reducing activity; however, GTE supplementation to the first dilution (Step 1) alone was not sufficient because the ROS reducing activity might be inhibited by the glycerol added in the second dilution step. Additional GTE supplementation in the second dilution step might activate and maintain ROS reducing activity. Moreover, the addition of GTE in the second dilution step might suppress the oxidative stress generated by the glycerol that induces osmotic stress and is toxic to spermatozoa. Thus, boar sperm extended with GTE in both steps 1 and 2 may have sufficient antioxidative capacity to reduce ROS during freezing. In other words, the addition of GTE in both the first and second dilution steps might have a synergistic effect on ROS reducing activity during freezing of boar spermatozoa.

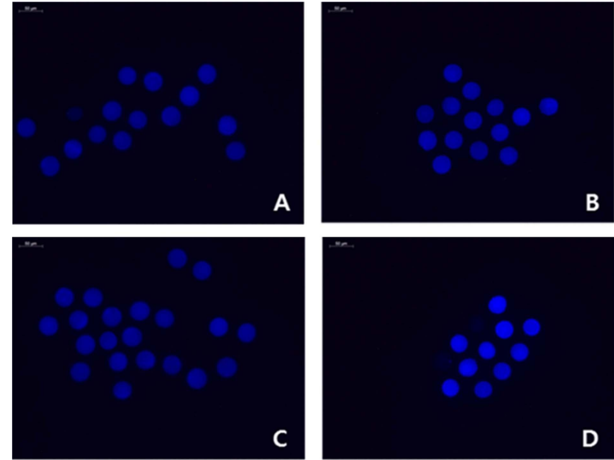

Fig 2. Representative images of intracellular glutathione (GSH) of porcine presumptive zygotes by staining with Cell Tracker Blue CMF2HC.
A: control, first and second dilution step without GTE, B: only first dilution step with GTE (Step 1), C: only second dilution step with GTE (Step 2), D: first and second dilution step with GTE (Step 1+2).

Sperm motility in Step 1, Step 2 and Step 1+2 groups supplemented with GTE was significantly higher than that of the control. The reduction of sperm motility due to cryopreservation may be because of oxidative damage from undue or ridiculous formation of ROS (34,38). However, we could not find a statistically significant correlation between motility and

Table 3. Effects of GTE supplementation on fertility parameters and GSH levels of presumptive zygotes according to dilution steps in boar sperm freezing

Group	No. presumptive zygotes	Penetration (%)	Monospermy (%)	GSH levels (N)
C	128	39.8 ± 7.5	74.5 ± 10.3	71.1 ± 2.9^b (31)
Step 1	125	40.8 ± 5.9	68.6 ± 12.1	70.6 ± 2.5^b (29)
Step 2	130	40.7 ± 7.1	75.4 ± 9.3	81.7 ± 4.9^a (28)
Step 1+2	124	42.7 ± 6.3	73.5 ± 8.9	88.9 ± 2.8^a (29)

GTE: 1,000 mg/L green tea extract (final concentration), GSH: glutathione, N: No. presumptive zygotes assayed. C: control, first and second dilution step without GTE, Step 1: only first dilution step with GTE, Step 2: only second dilution step with GTE, Step 1+2: first and second dilution step with GTE.
Values with different superscript are significantly different ($P < 0.05$).
The data represent means ± SE.

sperm ROS level (r = −0.396). Armstrong *et al* (6) reported that ROS reduces sperm motility as a result of decreased ATP production. In the present study, ATP production in Step 1, Step 2 and Step 1+2 groups might not be reduced because GTE has potential ROS reducing action compared to control as discussed above. The addition of GTE improved the sperm quality parameters including motility, viability and acrosome integrity. Chen *et al* (14) reported that catechins present in GTE significantly conserved cell viability and enhanced mitochondrial membrane potential of cells. GTE contains several types of phenolic components including catechins that might have a positive effect on sperm parameters.

The sperm cells challenged with high concentrations of ROS have many negative biological effects like blocked sperm-egg fusion (29), decreased sperm movement (6) and sperm DNA damage (3). The post fertilization embryonic development is related to sperm functionality (11). Therefore, we assumed that ROS production in the frozen-thawed spermatozoa would have a negative effect on IVF. We, therefore, expected that the use of GTE in both steps of the freezing process would improve the sperm IVF parameters. However, our results demonstrated that there were no significant differences between the experimental groups in terms of sperm penetration and monospermy rates. Similar results have been reported by other researchers using antioxidants such as N-acetyl-L-cysteine and GSH. These antioxidants did not improve the IVF parameters (42) and fertilizing ability in sperm penetration and did not affect polyspermy (22). Nevertheless, we observed that the addition of GTE resulted in higher ($P < 0.05$) GSH levels of PZs in Step 2 and Step 1+2 groups than the control and Step 1 group. Dilution and freezing of sperm with GTE in Step 2 or Step 1+2 prior to IVF might have a latent effect on GSH level in the PZs following IVF. In other words, sperm with lower H_2O_2 might result in higher GSH in PZs. GSH is a tripeptide that is ubiquitously distributed in living cells and plays an important role in the intracellular defense mechanism against the oxidative stress and maintains redox homeostasis. The oxidative stress and imbalanced redox are hazardous for the cellular processes, including apoptosis (15). GSH level is an important indicator of embryo development (1,17,19). Our results demonstrated that addition of GTE to the sperm freezing extender at Step 2 or Steps 1 and 2 enhanced the GSH levels of PZs, thus porcine oocytes might be affected by the intracellular ROS of the frozen boar sperm used for IVF. To our knowledge, this is the first evaluation of GSH level in PZs with respect to the antioxidative activity after boar sperm cryopreservation using GTE.

Conclusion

In conclusion, the present results suggest that the supplementation with GTE in dilution step 1 from 17-5°C as well as dilution step 2 at 5°C during boar sperm cryopreservation produced better sperm quality with significantly lower ROS levels and might also be beneficial for development of the embryo following IVF on the basis of increased GSH levels in PZs.

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