ISSN 2508-755X (Print) / ISSN 2288-0178 (Online)
J. Emb. Trans. (2017) Vol. 32, No. 1, pp. 9~15
https://doi.org/10.12750/JET.2017.32.1.9

Effect of Antioxidant Supplementation in Freezing Extender on Porcine Sperm Viability, Motility and Reactive Oxygen Species

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ABSTRACT

The present study was aimed to determine the effect of green tea extract (GTE) and beta-mercaptoethanol (β-ME) supplementation in boar sperm freezing extender on sperm motility, viability and reactive oxygen species (ROS) level. Experimental groups were allocated into Lactose-egg yolk (LEY) without antioxidant (control), GTE (1,000 mg/L GTE in LEY) and β-ME (50 μM β-ME in LEY). Spermatozoa extended with LEY were cooled to 5°C for 3 h and then kept at 5°C for 30 min following dilution with LEY containing 9% glycerol and 1.5% Equex STM (final sperm concentration: 1 × 10^8/mL). Spermatozoa were loaded into straws and frozen in nitrogen vapor for 20 min. Following thawing at 37°C for 25 sec, sperm viability and ROS level were measured using fluorescent double stain Fertility® and cytometry, respectively. Motility and viability of GTE supplemented-group were higher than those of control and β-ME without significance. ROS level in GTE group showed significantly lower than control (P < 0.05). In conclusion, GTE supplementation in boar sperm freezing extender can reduce ROS generation during freezing.

(Key words: beta-mercaptoethanol, boar spermatozoa, green tea extract, reactive oxygen species)

INTRODUCTION

Recently, oxidative stress has been identified as a significant cause affecting male fertility. Reactive oxygen species (ROS) is the breakdown product of oxygen that causes oxidative stress and can be detrimental to spermatozoa function and survival as well (de Lamirande and Gagnon, 1995).

Sperm cryopreservation is associated with an oxidative stress induced by ROS that exert physical and chemical changes on the sperm membrane (Watson, 1995). Reduction in sperm motility, viability, DNA integrity and fertilizing potential induced by excessive ROS during various procedures in sperm preservation have been reported (de Lamirande and Gagnon, 1992; Hu *et al*., 2008; Waterhouse *et al*., 2010).

Yamaguchi and Funahashi (2012) have demonstrated that supplementation of strong antioxidant in the sperm thawing solution, such as beta-mercaptoethanol (β-ME) would protect against the lipid peroxidation associated with ROS. In light of the above information, we hypothesized that β-ME might be used as an antioxidant in boar sperm freezing. Malo *et al*. (2010; 2011) reported that the effect of natural antioxidant (rosemary) supplementation in freezing extender on boar sperm cryopreservation. Among the various natural antioxidants, green tea (GT: *Camellia sinensis*) contains many types of catechins, which have strong antioxidant activity (Dufresne and Farnworth, 2001). Catechins can inhibit the effect of superoxide, hydrogen peroxide, hydroxyl radicals and nitric oxide produced by various chemicals (Schroeder *et al*., 2003). The (-) epigallocatechin-3-gallate (EGCG) of catechins has been used in *in vitro* fertilization (IVF) media (Kaedei *et al*., 2012). However, there are many components in green tea extract (GTE) such as (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) as well as EGCG. Therefore, we hypothesized that GTE might induce more strong antioxidative effect on boar sperm cryopreservation.

To the best of our knowledge, the present study is the first study to compare the effect of GTE and β-ME on boar sperm freezing. The objective of the present study was to evaluate sperm quality and ROS level following boar sperm cryopreservation using the extender containing GTE or β-ME.

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MATERIALS and METHODS

Chemicals

Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and solutions were prepared with high purity water (6114VF, Sartorius AG, Goettingen, Germany). Equex STM paste was obtained from Nova Chemical Sales Inc. (Scituate, MA, USA).

Preparation of Green Tea Extract

Green tea powder (GTP) was obtained from Bioland (GTP-020968, Seoul, Korea). Green tea extract (GTE) stock was prepared by modifying Yu *et al*. (2002). GTE stock of 100,000 mg/l was prepared by solving GTP in triple distilled water and filtered by 1.2 µm (17593-k, Sartorius, Goettingen, Germany). High performance liquid chromatography analysis of 100,000 mg/l GTE revealed 45.9 mg/l of catechin, 141 mg/l of epicatechin, 297.4 mg/l of gallocatechin, 424.3 mg/l epigallocatechin, 392.7 mg/l of gallocatechin gallate and 392.7 mg/l of epigallocatechin gallate. All were determined by chromatography method (6430 LC/MS/MS, Agilent, Santa Clara, USA) at the Center for University-wide Research Facilities in Chonbuk National University (Park and Yu, 2015; 2016).

Collection of Semen

Eight boars (2 Duroc jersey and 6 Yorkshire) were used for this experiment. The semen collected using gloved-hand technique was filtered through four layers of sterile gauze to remove the gel particle. The sperm-rich fraction of ejaculates with greater than 75% motile and 80% morphologically normal spermatozoa was used in this study. The collected semen was pooled to reduce individual differences. The pooled semen were 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) (Pursel and Johnson, 1975). The diluted spermatozoa were assessed for volume, sperm concentration and percentage of motile spermatozoa. Immediately after evaluation, the diluted spermatozoa were stored at 24℃ for 2 h.

Sperm Freezing-thawing Protocol

The freezing extenders used in the experiments were composed of freezing extender 1 (FE 1) and freezing extender 2 (FE 2). FE 1 was composed of lactose egg yolk (LEY) extender (80 ml of lactose solution, 20 ml of egg yolk and 0.1% antibiotic-antimycotic in 100 ml sterile non-pyrogenic water). FE 2 consisted of LEY extender supplemented with 9% (v : v) glycerol and 1.5% (v : v) Equex STM. Semen was processed according to the freezing procedure (Guthrie and Welch, 2006). Briefly, semen diluted in BTS was centrifuged at 850 × *g* for 15 min at room temperature. Following removing supernatant, sperm pellet was resuspended with FE 1 to a concentration of 1.5×10^8/ml. Sperm suspensions were cooled gradually from 24°C to 5°C for 3 h. The sperm was maintained at 5°C for 30 min after a second dilution step to 1×10^8/ml with FE 2 at 5°C. The cooled sperm was loaded into 0.5 ml straws and sealed.

Ten minutes before liquid nitrogen (LN_2)vapor cooling, a styrofoam box ($29.5 \times 18.7 \times 24$ 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 37°C for 25 sec before use in experiments. The cooling rates were 30.6°C/min from 5°C to -15°C, 88.2°C/min from -15 to -60.0°C and 14.7°C/min from -60°C to -116.0°C (Rahman *et al*., 2016).

Sperm Motility

Semen (10 µl) was placed on a slide and cover-slipped. The percentage of progressive motile sperm was estimated under microscopic examination at 400 × magnification (Yu, 2014). 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 (Rahman *et al*., 2016). The mean of eight successive estimates was recorded as the final motility score.

Sperm Viability

The integrity of the plasma membrane of the sperm was measured (Yu, 2014) using the fluorescent double-stain

Fertilight®(MolecularProbesInc.,Eugene,OR, USA). For each replicate sample, two slides were prepared and appropriately 200 spermatozoa were counted per slide. The number of green or red 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.

Sperm Intracellular ROS Level

2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes Inc.) was used to detect H_2O_2 (Guthrie and Welch, 2006). The working solutions 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 final concentrations of 200 µM. For simultaneous differentiation of living from dead sperm, propidium iodide (PI) (final concentration, 2 µM) was added to H_2DCFDA-treated sperm. Samples stained with H_2DCFDA and PI was incubated at 25°C for 60 min. After incubation, samples were analyzed using flow cytometry. Data were expressed as the percentage of viable sperm with H_2O_2 (high DCF fluorescence). The mean fluorescence (MFI) of DCF was measured to evaluate intracellular mean H_2O_2 per the viable sperm population (Park and Yu, 2016). The samples were analyzed using a FACScalibur flow cytometry (Becton Dickinson, San Jose, CA, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser. A forward- and side-scatter gate was used to separate the sperm cells from debris. All non-sperm events were excluded from further analysis. The MFI, FL1 (DCF) signals were detected through a 530/30 nm band-pass filter. The FL2 (PI) signals were detected through a 585/42 nm band-pass filter. The FACSCalibur is capable of distinguishing 1024 channels and both red and green fluorescence intensities of individual cells. Ten thousand sperm cell events were recorded in the list mode with a flow rate of < 200 events/second. MFI of DCF was analyzed using Cell Quest Pro software (Becton Dickinson, San Jose, CA, USA).

Experimental Design

Effect of GTE and β-ME supplementation in the freezing extender on boar sperm freezing

Experimental groups were allocated into control, GTE and β-ME. GTE stock (100,000 mg/l) or β-ME stock (5 mM) were supplemented into the freezing extender (1 : 100 [v/v]). The final concentration of GTE and β-ME in the freezing extender was 1,000 mg/l and 50 µM, respectively. In an attempt to determine the effect of GTE and β-ME on boar sperm freezing, sperm was frozen in only LEY extender or LEY supplemented with GTE or β-ME. Sperm motility, viability and ROS level were evaluated.

Statistical Analyses

Five replicates were conducted for each experiment. Percentage data were subjected to arcsin transformation before analysis. All data are presented as means ± SE and were analyzed using ANOVA followed by Duncan's multiple range test. Statistical Analysis System ver. 8x software (SAS, Cary, NC, USA) was used. $p < 0.05$ was considered statistically significant.

RESULTS and DISCUSSION

The effect of GTE and β-ME supplementation in freezing extender of boar sperm on progressive sperm motility is presented in Fig. 1. The percentage of sperm motility in GTE group was higher than the control and β-ME groups without significance. Sperm viability between the groups is shown in Fig. 2. There was similar pattern between the results of viability and motility. On the other hand, intracellular hydrogen peroxide in sperm cryopreserved with the extender containing GTE showed significantly lower level ($p < 0.05$) than control (Fig. 3, Fig. 4). However, there were no significant differences between β-ME and GTE groups or β-ME and control. Boar spermatozoa among mammalian animal's sperm are highly sensitive to the freeze-thaw process, which causes considerable cell damage. Bailey *et al*. (2000) reported that cryoinjury may be induced by ROS generated during the process of freezing. Antioxidants in freezing extender are usually used to reduce this damage (Breininger *et al*., 2005; Hu *et al*., 2008). In this study, we estimated the antioxidative effects of GTE and β-ME in boar sperm cryopreservation. GTE supplementation in freezing extender showed significantly ($p < 0.05$) lower ROS level than the control group. Dufresne and Farnworth (2001) reported that GT as a natural antioxidant has strong antioxidant capability by inhibiting the activity of superoxide, hydrogen peroxide and

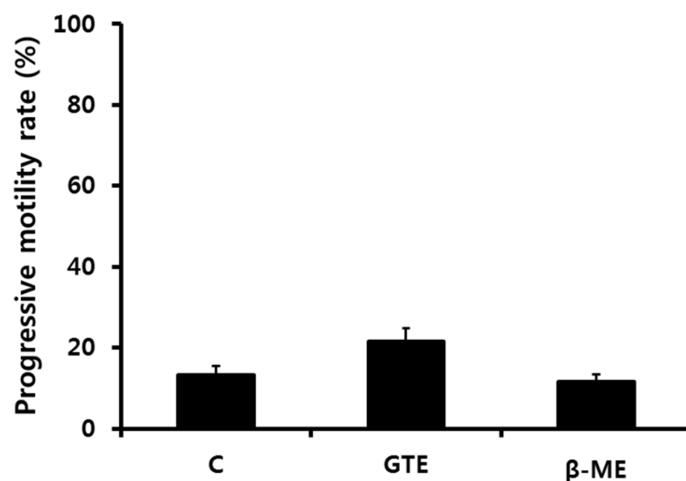

Fig. 1. Effect of antioxidant supplementation on progressive motility during boar sperm freezing. C: control, GTE: green tea extract, β-ME: beta-mercaptoethanol. The data represent means ± SE.

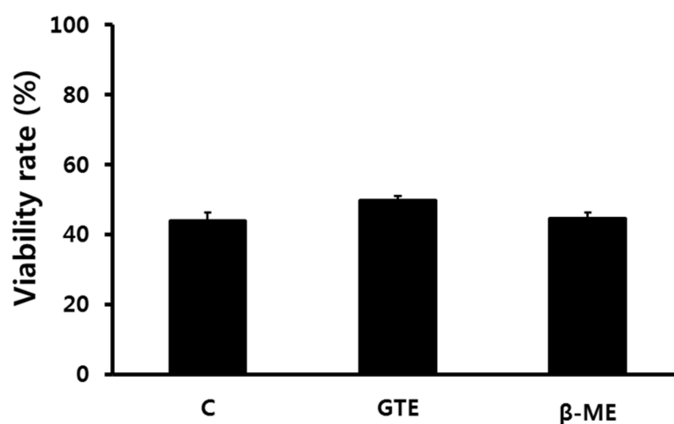

Fig. 2. Effect of antioxidant supplementation on viability during boar sperm freezing. C: control, GTE: green tea extract, β-ME: beta-mercaptoethanol. The data represent means ± SE.

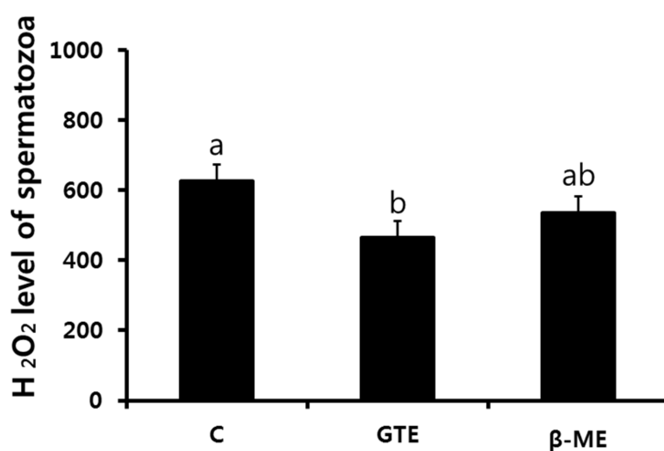

Fig. 3. Effect of antioxidant supplementation on the hydrogen peroxide (H_2O_2) level during boar sperm freezing. C: control, GTE: green tea extract, β-ME: beta-mercaptoethanol. The data represent means ± SE. Values with different superscript are significantly different ($p < 0.05$).

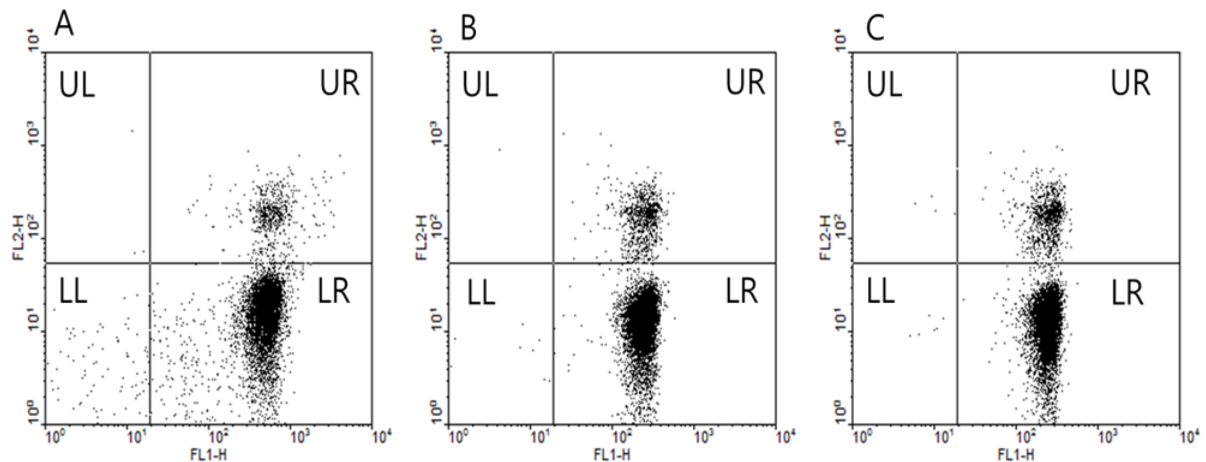

Fig. 4. Flow cytometric analysis of sperm labeled with DCFDA/PI. A: control, B: GTE C: β-ME. The upper panel shows dot plot cytograms to measure the sperm population upon the level of intracellular H2O2 following cryopreservation. The LL quadrant represents viable sperm with a low intracellular H_2O_2 level, while the LR shows viable sperm with a high intracellular H_2O_2 level. The UL quadrant shows dead sperm with a low intracellular H_2O_2 level. The UR shows dead sperm with a high intracellular H_2O_2 level.

hydroxyl radicals (Schroeder *et al*., 2003). Ponglowhapan *et al*. (2004) reported that polyphenols of GT bind to the components of the sperm membrane and subsequently prevent the membrane lipid peroxidation induced by ROS. Moreover, the polyphenols inhibit egg yolk oxidation in a semen extender and preserve semen quality (Hyon, 2004). Wittayarat *et al*. (2012) reported that polyphenols of GTE are more potent antioxidants than Vitamin C, Vitamin E and rosemary extract. More detailed research for the mechanisms of ROS reduction in future study could provide more evidence about the action of GTE as an antioxidant during boar sperm cryopreservation.

On the other hand, β-ME acts as an antioxidant by increasing intracellular enzymatic synthesis of glutathione (GSH) in many species cell (Ishii *et al*., 1981; Ohmori and Yamamoto, 1983; de Matos *et al*., 1996). This GSH synthesis is highly dependent on the availability of cysteine (Rathbun and Murray, 1991). To accomplish intracellular enzymatic synthesis of GSH, there is the suitable environment. That is, enzyme has its own optimal temperature for its proper function. β-ME was commonly used in media for oocyte maturation and embryo development at the temperature between 37.5°C and 39°C depends on species (Abeydeera *et al*., 1998; Funahashi,2005). In the present study, we added β-ME in freezing medium in which exposed at lower temperature from 4°C to -196°C. Therefore, temperature might not be appropriate for GSH synthesis. Gadea *et al*. (2004) reported that cryopreservation process reduces the GSH content in sperm. Moreover, the thawing process increases the oxidation of GSH into oxidized GSH (Chatterjee and Gagnon, 2001). In considering the temperature sensitivity of such antioxidant, its function might not be acting at lower temperature during freezing.

It is concluded that GTE supplementation in freezing extender can reduce ROS generation during boar sperm freezing. Therefore we suggest GTE might be used as an antioxidant in porcine sperm freezing.

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Received March 02 2017, Revised March 03, 2017, Accepted March 15, 2017