pISSN 1225-4991, eISSN 2288-0178
J. Emb. Trans. (2015) Vol. 30, No. 1, pp. 1~6
http://dx.doi.org/10.12750/JET.2015.30.1.1

Evaluation of Toxicity of Green Tea Extract in Chilled Boar Spermatozoa

Sang-Hyoun Park and Il-Jeoung Yu†

Department of Theriogenology and Reproductive Biotechnology,
College of Veterinary Medicine and Bio-safety Research Institute, Chonbuk National University,
Jeonju 561-756, Republic of Korea

ABSTRACT

The cold shock of spermatozoa is associated with oxidative stress induced by reactive oxygen species. This study was conducted to evaluate the toxicity of natural antioxidant green tea extract (GTE) in lactose-egg yolk (LEY) extender during boar sperm cooling prior to freezing. Spermatozoa were cooled to 5℃ for 3 h in LEY extender containing 0 (control), 1, 10, 100 or 1,000 mg/l of GTE, re-suspended with LEY-glycerol-Equex extender and cooled at 5℃ for 30 min. Sperm progressive motility, viability and phosphatidylserine (PS) translocation were evaluated. PS translocation was assayed by flow cytometry using Annexin V-FITC apoptosis detection kit. The sperm function including progressive motility, viability and PS translocation was not significantly different regardless of GTE concentrations (P>0.05). In conclusion, this study demonstrated non-toxicity of GTE supplement in LEY extender during sperm cooling.

(Key words : boar, spermatozoa, green tea extract, cooling, PS translocation)

INTRODUCTION

Of the sperm from mammalian species, boar spermatozoa are the most susceptible to cold shock damage. This high degree of susceptibility to cold shock is associated with a high ratio of polyunsaturated to saturated fatty acids in the phospholipids and low cholesterol content in the sperm membranes (Darin-Bennett *et al.*, 1976). This peculiar composition causes boar spermatozoa to undergo a more pronounced lipid phase transition, which occurs at temperatures between 15℃ and 5℃ (Drobnis *et al.*, 1993). This temperature range is consistent with the range in which cooling damage is most prominent in boar spermatozoa (Watson, 2000).

In addition, the cold shock of spermatozoa is associated with oxidative stress induced by reactive oxygen species (ROS) generation (Chatterjee and Gagnon, 2001). ROS, which are generated by spermatozoa during cold storage, induce alterations in sperm hyperactivation, capacitation and acrosome reaction (Agarwal *et al.*, 2004). Such stress produces ROS that have been shown to reduce sperm motility (Armstrong *et al.*, 1999) and membrane function (Chatterjee and Gagnon, 2001) and increase nuclear DNA fragmentation (Fraser and Strzezek, 2007; Whitaker and Smith, 2008). Therefore, ROS generated during cooling step might affect function of spermatozoa frozen and thawed.

Recently, there is an increasing interest of natural antioxidants because of the safety and toxicity problems of synthetic antioxidants, butylhydroxyanisole, butylhydroxytoluete that are commonly used in lipid contain in foods (Yanishlieva and Marinova, 1996). Kahl and Kappus (1993) described the positive actions of extracts from herbs. Natural antioxidants are primary plant phenolics which are multifunctional and can act as reducing agents. Natural anti-oxidants such as rosemary (*Rosmarinus officinalis*) and soya have been successfully added into semen freezing extenders in several species, including boar (Malo *et al.*, 2010), canine (González *et al.*, 2010) or ovine (Gil *et al.*, 2010).

Particularly, the main constituents of green tea (GT) flavonoids are catechins. GT also contains gallic acid and other phenolic acid such as chlorogenic acid, caffeic acid and flavonols such as kaempferol, myricetin and quercetin (USDA, 2003). The health benefits of GT are attributed mainly to their antioxidant properties and the ability of its polyphenolic catechins to scavenge ROS.

The aim of this study was to investigate the effect of GTE supplemented into semen extender on boar spermatozoa during cooling. In particular, we focused on examing its toxicity by comparing various concentration of GTE in lactose-egg yolk (LEY) extender. We assessed sperm PS translocation as well as sperm motility and viability.

† Correspondence : iyu@jbnu.ac.kr

MATERIALS AND METHODS

1. 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). Green tea powder (GTP) was obtained from Bioland (GTP-020968, Seoul, Korea). GTE was prepared by modifying Yu *et al.* (2002). GT solution with different concentration (0, 1, 10, 100 and 1,000 mg/L) were prepared by solving GTP in 3rd distilled water and were filtered by 1.2 μm (Sartorius, Goettingen, Germany). GTE was analyzed by spectrophotometer (UV-1601, Shimadzu Co., Kyoto, Japan) for its flavonoid concentration at Research Center for industrial Development of Biofood materials in Chonbuk National University. Flavonoid concentrations of GTE are shown in Table 1.

2. Collection of Semen

Semen was collected from six fertile boars (1 ejaculate/boar) by the gloved-hand technique and filtered through four layers of sterile gauze to remove the gel particle and then transferred into a 15 mL tube. The semen samples of the sperm-rich fraction 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 sperm was assessed for volume, sperm concentration and percentage of motile spermatozoa. The sperm-rich fraction of ejaculates with greater than a minimum of 75% motile and of 80% morphologically normal spermatozoa was used in this study. Immediately after evaluation, the diluted sperm-rich fractions were stored to 24℃ for 2 h.

Table 1. Flavonoid concentration in GTE

GTE (mg/l)	Total flavonoid (mg/100g)
1	1.11
10	17.74
100	171.87
1,000	1,712.67

GTE: Green tea extract

3. Sperm Cooling

The extenders used in experiments were composed of extender 1 and extender 2. Extender 1 was composed of LEY extender (80 mL of lactose solution, 20 mL of egg yolk and 0.1% antibiotic-antimycotic in 100 mL sterile non-pyrogenic water) supplemented with different concentration of GTE (0 [control], 1, 10, 100 and 1,000 mg/L). Extender 2 consisted of LEY extender supplemented with 9% (v:v) glycerol and 1.5% (v:v) Equex STM.

Semen was processed according to the cooling procedure (Guthrie and Welch, 2006). Briefly, semen diluted in BTS was centrifuged at 850 × g for 15 min at room temperature. The supernatant was then removed and the semen pellet was re-suspended with extender 1 to a concentration of 1.5×10^8/mL. Sperm suspensions were cooled gradually from 24℃ to 5℃ for 3 h. At this temperature, a second dilution step to 1×10^8/mL was performed with extender 2 and the sperm were maintained at 5℃ for 30 min.

4. 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.

5. Sperm Viability

The integrity of the plasma membrane of the sperm was measured (Yu, 2014) using the fluorescent double stain Fertilight® (Molecular Probes Inc., 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 membrane-intact spermatozoa (green fluorescence in sperm head) was calculated.

6. Phosphatidylserine (PS) Translocation

An Annexin V-FITC apoptosis detection kit I (BD Pharmingen, San Diego, CA, USA) was used according to the manufacturer's instructions. The sperm suspension was centrifuged

at 300 × g for 5 min and the supernatant was removed. The sperm pellet was resuspended in 1× Annexin V binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl and 2.5 mM $CaCl_2$) at room temperature to a concentration of 1 × 10^6 sperm/mL. Aliquots (100 μL of 1 × 10^5 cells) of the sperm suspension were transfer red to 5 mL culture tubes. Annexin V-FITC (5 μL) and 5 μL of propidium iodide (PI) or nothing was added to the samples. The tubes were gently mixed and incubated at room temperature for 15 min in the dark. After incubation, an additional 1× binding buffer (400 μL) was added to each tube.

All fluorescence signals of labeled spermatozoa were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser. FL1 (Annexin V) signals were detected through a 530/30 nm band-pass filter and 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 and analyzed using Cell Quest Pro software. The sperm population was gated on the basis of the log forward (FSC) and side-scatter (SSC) properties of the sperm.

Flow cytometric analysis was conducted within 1 h. The different labeling patterns in the Annexin V (AN)/PI analysis were classified as follows: viable (AN−/PI−); viable but phosphatidyl serine (PS) translocated (AN+/PI−); nonviable and PS translocated (AN+/PI+); and nonviable and late necrotic sperm (AN−/PI+). We defined the ratio between AN+/PI− sperm and total living (PI−) sperm as the PS translocation index.

7. 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 by Duncan's multiple range test using Statistical Analysis System ver. 8× software (SAS, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

RESULTS

1. Effect of GTE in LEY Extender on Viability and Progressive Motility of Boar Spermatozoa during Cooling

Sperm parameters according to GTE concentrations following cooling are shown in Table 2. The viability and progressive motility after cooling were not significantly different among groups.

2. Effect of GTE in LEY Extender on the PS Translocation of Boar Spermatozoa during Cooling

There were no statistically significant difference in the number of non-PS translocated live sperm and dead sperm among groups (Table 3, Fig. 1).

DISCUSSION

To the best of our knowledge, the present study is the first to examine the toxicity of GTE in boar sperm cooling before applying GTE to freezing extender. We assessed sperm motility, viability and PS translocation following cooling of boar

Table 2. Effect of GTE in LEY extender on viability and progressive motility of boar spermatozoa during cooling

GTE (mg/l)	Viability (%)	Motility (%)
C	75.9 ± 5.4	25.8 ± 3.0
1	72.0 ± 6.0	27.2 ± 3.6
10	75.6 ± 4.5	34.4 ± 2.9
100	77.3 ± 5.8	33.3 ± 1.6
1,000	79.8 ± 3.7	37.7 ± 1.4

GTE: Green tea extract, C: no GTE.
Values are expressed as means ± SE.
Values are not significantly different (P>0.05).

Table 3. Effect of GTE in LEY extender on the PS translocation of boar spermatozoa during cooling

GTE (mg/l)	Apoptotic index
C	17.5 ± 6.6
1	18.1 ± 5.9
10	17.9 ± 6.2
100	15.9 ± 5.8
1,000	15.6 ± 7.2

GTE: Green tea extract, C: no GTE.
Values are expressed as means ± SE.
Values are not significantly different (P>0.05).

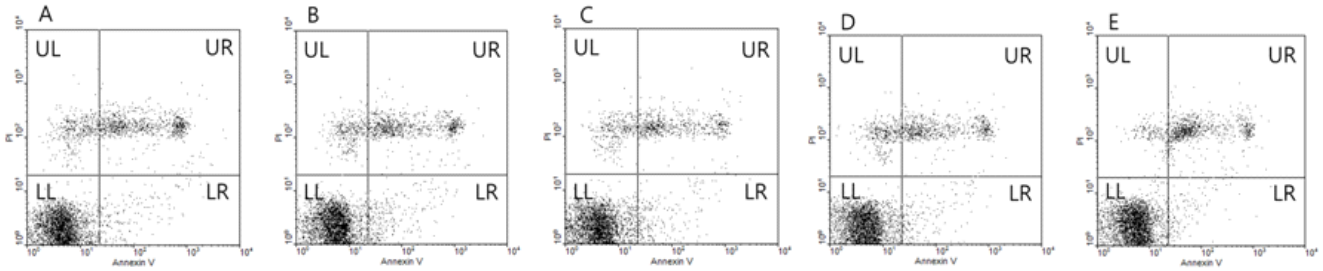

Fig. 1. Phosphatidylserine (PS) translocation of boar spermatozoa during cooling. Flow cytometry analysis of a sperm population sample. (A) no treatment group (control), (B) 1 mg/l group, (C) 10 mg/l group, (D) 100 mg/l group, (E) 1,000 mg/l group. LL: viable spermatozoa with no signs of PS translocation. LR: viable spermatozoa showing PS translocation. UL: dead spermatozoa with no signs of PS translocation. UR: dead spermatozoa showing PS translocation. Values are not significantly different (P>0.05).

spermatozoa in LEY extender supplemented with different concentrations of GTE.

According to Malo *et al.* (2011), sperm motility following freezing and thawing increased as concentration of rosemary in LEY extender increased. However, our study demonstrated that GTE concentration in LEY extender was not related to sperm motility during cooling. Contradictory reports exists regarding the effect of antioxidant supplementation on sperm motility in both liquid and freezing-thawed semen from various species, including human (Askari *et al.*, 1994; Maxwell and Stojanov, 1996; Aurich *et al.*, 1997; Donnelly *et al.*, 1999), the effect being dependent of the antioxidant dose.

It is nowadays generally accepted that cooling induces the formation of ROS, detrimental for subsequent sperm performance (Watson, 2000). The process of peroxidation induces structural alterations, a fast and irreversible loss of motility, a deep change in metabolism and an increase in the rate of release of intra-cellular components (Jones and Mann, 1977). In this study, GTE in LEY extender did not affect motility of spermatozoa during cooling. In other words, GTE with various concentrations was not toxic to spermatozoa cooled before freezing. Moreover, the result of PS translocation, earliest indicator of cell apoptosis demonstrated that GTE was not toxic to spermatozoa. The sperm plasma membrane is one of the key structures affected by cooling, with evaluation of sperm membranes thus being of utmost importance when evaluating freezing protocols. The numerous functions of the membrane are related to the cell metabolism for maintaining sperm motility, capacitation, acrosome reaction and interactions between the spermatozoa and the epithelium of the female genital tract and sperm-egg interactions (Rodríguez-Martínez, 2003).

Boar sperm membrane is particularly sensible to oxidative stress (Cerolini *et al.*, 2000). Sperm spontaneously produce a variety of ROS, including the superoxide anion, hydrogen peroxide and nitric oxide (Aitken *et al.*, 2010). Produced in small amounts, ROS are important in the regulation of normal cellular functions. In sperm cells, they also play a key role in essential events such as sperm capacitation, acrosome reaction, hyperactivation and oocyte interaction (Agarwal and Allamaneni, 2004). However, when ROS production exceeds the sperm's limited antioxidant defenses, a state of oxidative stress is induced, characterized by peroxidative damage to the sperm plasma membrane, sperm chromatin and DNA strand breakage in the sperm nucleus. Such oxidative stress not only disrupts the fertilizing potential of human sperm, but also the ability of these cells to create a normal healthy embryo (Aitken *et al.*, 2010). H_2O_2 is considered the most toxic species (Griveau*et al.*, 1995; Aitken *et al.*, 2010) because of its ability to cross membranes freely and inhibit enzyme activities and cellular functions, thus decreasing the antioxidant defenses of the sperm (Griveau *et al.*, 1995). Likewise, H_2O_2 is known as the major ROS responsible for oxidative damage in boar spermatozoa (Guthrie and Welch, 2006).

Unfortunately, this did not attempt to determine the effect of GTE in LEY extender on ROS including H_2O_2 generated during cooling. So, further study needed to examine the direct effect of GTE on ROS. In addition, the effect of GTE supplementation during cooling on freezing of boar spermatozoa should be investigated.

In conclusion, non-toxicity of GTE in LEY extender during boar sperm cooling was demonstrated by assessing sperm function according to GTE concentrations in LEY extender.

REFERENCES

Agarwal A and Allamaneni SS. 2004. Role of free radicals in female reproductive diseases and assisted reproduction. Reprod. Biomed. Online 9:338-347.

Agarwal A, Nallella KP, Allamaneni SS and Said TM. 2004. Role of antioxidants in treatment of male infertility: An overview of the literature. Reprod. Biomed. Online 8:616-627.

Aitken RJ, De Iuliis GN, Finnie ZM, Hedges A and McLachlan RI. 2010. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: Development of diagnostic criteria. Hum. Reprod. 25: 2415-2426.

Armstrong JS, Rajasekaran M, Chamulitrat W, Gatti P, Hellstrom WJ and Sikka SC. 1999. Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. Free Radic Biol. Med. 26: 869-880.

Askari HA, Check JH, Peymer N and Bollendorf A. 1994. Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. Arch. Androl. 33:11-15.

Aurich JE, Schönherr U, Hoppe H and Aurich C. 1997. Effects of antioxidants on motility and membrane integrity of chilled-stored stallion semen. Theriogenology 48:185-192.

Cerolini S, Maldjian A, Surai P and Noble R. 2000. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Anim. Reprod. Sci. 58: 99-111.

Chatterjee S and Gagnon C. 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing and thawing. Mol. Reprod. Dev. 59:451-458.

Darin-Bennett A, Poulos A and White IG. 1976. The fatty acid composition of the major phosphoglycerides of ram and human spermatozoa. Andrologia 8:37-45.

Donnelly ET, McClure N and Lewis SE. 1999. The effect of ascorbate and alpha-tocopherol supplementation *in vitro* on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. Mutagenesis 14:505-512.

Drobnis EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet JW and Crowe JH. 1993. Cold shock damage is due to lipid phase transitions in cell membranes: A demonstration using sperm as a model. J. Exp. Zool. 265:432-437.

Fraser L and Strzezek J. 2007. Is there a relationship between the chromatin status and DNA fragmentation of boar spermatozoa following freezing-thawing? Theriogenology 68:248-257.

Gil L, Mascaró F, Mur P, Gale I, Silva A, González N, Malo C and Cano R. 2010. Freezing ram semen: The effect of combination of soya and rosemary essences as a freezing extender on post-thaw sperm motility. Reprod. Dom. Anim. 45:91.

González N, Gil L, Martinez F, Malo C, Cano R, Mur P and Espinosa E. 2010. Effect of natural antioxidant rosemary in canine soya freezing extender. Reprod. Dom. Anim. 45:88.

Griveau JF, Dumont E, Renard P, Callegari JP and Le Lannou D. 1995. Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. J. Reprod. Fertil. 103:17-26.

Guthrie HD and Welch GR. 2006. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. J. Anim. Sci. 84:2089-2100.

Jones R and Mann T. 1977. Damage to ram spermatozoa by peroxidation of endogenous phospholipids. J. Reprod. Fertil. 50:261-268.

Kahl R and Kappus H. 1993. Toxicology of the synthetic antioxidants BHA and BHT in comparison with the natural antioxidant vitamin E. Z. Lebensm. Unters. Forsch. 196:329-338.

Malo C, Gil L, Cano R, Martínez F and Galé I. 2011. Antioxidant effect of rosemary (*Rosmarinus officinalis*) on boar epididymal spermatozoa during cryopreservation. Theriogenology 75:1735-1741.

Malo C, Gil L, Gonzalez N, Martinez F, Cano R, de Blas I and Espinosa E. 2010. Anti-oxidant supplementation improves boar sperm characteristics and fertility after cryopreservation: Comparison between cysteine and rosemary (*Rosmarinus officinalis*). Cryobiology 61:142-147.

Maxwell WM and Stojanov T. 1996. Liquid storage of ram semen in the absence or presence of some antioxidants. Reprod. Fertil. Dev. 8:1013-1020.

Pursel VG and Johnson LA. 1975. Freezing of boar spermatozoa: Fertilizing capacity with concentrated semen and a new thawing procedure. J. Anim. Sci. 40:99-102.

Rodríguez-Martínez H. 2003. Laboratory semen assessment and prediction of fertility: Still utopia? Reprod. Dom. Anim. 38:312-318.

USDA. 2003. USDA Database for the flavonoid contents of selected foods. US Department of Agriculture, Beltsville.

Watson PF. 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60-61:481-492.

Whitaker M and Smith J. 2008. Introduction. Calcium signals and developmental patterning. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 363:1307-1310.

Yanishlieva NV and Marinova EM. 1996. Antioxidative effectiveness of some natural antioxidants in sunflower oil. Z. Lebensm. Unters. Forsch. 203:220-223.

Yu IJ. 2014. Canine sperm cryopreservation using glucose in glycerol-free Tris. Cryo. Letters. 35:101-107.

Yu YG, Shin MH and Choi JW. 2002. The anti-oxidative stress effect of antioxidants in the cell using DCFH-DA. J. Soc. Cos. Sci. Ko. 28:42-57.

Received January 26, 2015, Revised February 28, 2015, Accepted March 9, 2015