ISSN 2508-755X (Print) / ISSN 2288-0178 (Online)
J. Emb. Trans. (2017) Vol. 32, No. 4, pp. 337～342
https://doi.org/10.12750/JET.2017.32.4.337

Effects of Antioxidants Supplement in Porcine Sperm Freezing on *in vitro* Fertilization and the Glutathione and Reactive Oxygen Species Level of Presumptive Zygotes

Sang-Hyoun Park, Yubyoel Jeon and Il-Jeoung Yu†

Department of Theriogenology and Reproductive Biotechnology, College of Veterinary Medicine and Bio-safety Research Institute, Chonbuk National University, Iksan 54596, Republic of Korea

ABSTRACT

The present study was aimed to determine the effects of green tea extract (GTE) and beta-mercaptoethanol (β-ME) supplementation in boar sperm freezing extender on *in vitro* fertilization (IVF) and reactive oxygen species (ROS) and glutathione (GSH) levels of presumptive zygotes (PZs). Experimental groups were allocated into lactose egg yolk (LEY) without antioxidant (control), GTE (1,000 mg/l in LEY) and β-ME (50 μM in LEY). In freezing, 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. The final sperm concentration was 1×10^8/ml. Spermatozoa were loaded into straws and frozen in nitrogen vapor for 20 min. For IVF, oocytes were matured in NCSU-23 medium and co-cultured with spermatozoa following thawing at 37°C for 25 sec. At 12 h following IVF, IVF parameters (sperm penetration and monospermy) were evaluated. In addition, GSH and ROS levels of PZs were determined by Cell Tracker Blue CMF2HC and DCHFDA, respectively. IVF parameters did not show any significant difference among the experimental groups. GSH and ROS levels of PZs were not significantly different between groups. In conclusion, antioxidant supplementation in boar sperm freezing could not influence IVF parameters, ROS and GSH levels of PZs.

(Key Words : Beta-mercaptoethanol, Boar spermatozoa, Green tea extract, Glutathione, Reactive oxygen species)

INTRODUCTION

Oxidative stress has been considered as one of the significant causes to reduce male fertility. Sperm cryopreservation is related to an oxidative stress induced by reactive oxygen species (ROS). ROS generation is harmful to the sperm membrane (Watson, 1995) and reduces the sperm parameters including motility, viability and DNA integrity (de Lamirande and Gagnon, 1992; Hu *et al*., 2008; Waterhouse *et al*., 2010). Moreover, excessive production of ROS is negatively linked to the sperm function and morphology (Agarwal *et al*., 2003; Makker *et al*., 2009; Oborna *et al*., 2010).

Various antioxidants, such as α-tocopherol (Olson and Seidel, 2000; Dalvit *et al*., 2005), L-ascorbic acid (Vitamin C: Tatemoto *et al*., 2004), glutathione (GSH: Luvoni *et al*., 1996) and superoxide dismutase (Tatemoto *et al*., 2004) have been used as ROS scavengers in various species. Yamaguchi *et al*. (2012) reported that beta-mercaptoethanol (β-ME) supplementation in the sperm thawing solution protect sperm against the lipid peroxidation related with ROS. Green tea (GT: *Camellia sinensis*) includes various types of catechins inducing antioxidant activity (Dufresne and Farnworth, 2001). Recently, Park and Yu (2017) have demonstrated that the GT extract supplementation in boar sperm freezing extender significantly reduced sperm ROS level after cryopreservation. In other hand, the sperm cells challenged with high concentrations of ROS have many negative biological effects like blocked sperm-egg fusion (Mammoto *et al*., 1996). However, there is limited information that spermatozoa affected by antioxidants during freezing could affect the oocytes during *in vitro* fertilization. Therefore, we determined whether the spermatozoa exposed in antioxidants during freezing could play a role in *in vitro* fertilization (IVF).

The objectives of the present study were aimed to determine

† Correspondence: Il-Jeoung Yu (ORCID: 0000-0002-5530-5974)
Phone: +82-63-850-0948, Fax: +82-63-850-0910
E-mail: iyu@jbnu.ac.kr

the effects of GTE and β-ME supplementation in boar sperm freezing extender on IVF parameters and ROS and GSH levels of presumptive zygotes (PZs).

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).

2. Preparation of antioxidants

Green tea powder (GTP) was obtained from Bioland (GTP-020968, Seoul, Korea). GTE and the β-ME stock were prepared by modifying the stock prepared by Park and Yu (2017). The stock solution of GTE (100,000 mg/l) was prepared by solving GTP in triple distilled water and filtered by 1.2 μm (17593-k, Sartorius, Goettingen, Germany). GTE stock (100,000 mg/l) or β-ME stock (5 mM) was supplemented into the freezing extender (1 : 100 [v/v]). The final concentration of GTE and β-ME in the freezing extender were 1,000 mg/l and 50 μM, respectively.

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 (Park and Yu, 2013).

3. 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 were 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.

4. 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 x *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 spermatozoa were maintained at 5°C for additional 30 min after a second dilution step to 1×10^8/ml with FE 2 at 5°C. The cooled spermatozoa were loaded into 0.5 ml straws and sealed.

Ten minutes before liquid nitrogen (LN_2) vapor freezing, 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 to 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. 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.

Straws from each group were thawed by immersion in a circulating water bath at 37°C for 25 sec before use in experiments.

5. Oocyte collection and *In vitro* maturation (IVM)

Porcine ovaries were collected from a local abattoir and transported to the laboratory at 34 to 36℃ 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 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℃ 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 x g for 30 min and filtration through a 1.2 µm syringe filter, aliquoted and stored at - 80℃ until use.

6. *In vitro* fertilization (IVF) and *In vitro* culture (IVC)

Percoll solutions and gradients were prepared as follows. A two-layer discontinuous gradient was formed by layering 1 ml of a 45% Percoll solution on top of 1 ml of a 90% Percoll solution in a 15 ml conical tube. Aliquants of extended semen (3 ml) were layered onto Percoll gradient and centrifuged for 20 min at 850 × g at room temperature. The pellet recovered after aspiration of the supernatant 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. After the supernatant was discarded, motile spermatozoa were collected. The sperm concentration (spermatozoa/ml) was diluted to 10 × 10^5 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).

For IVF, the COCs after IVM were denuded by gently pipetting with 0.1% hyaluronidase. Denuded oocytes were washed three times with mTBM and transferred to a 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℃ in a humidified atmosphere of 5% CO_2 in air. 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℃ in a humidified atmosphere of 5% CO_2 in the air.

7. Assessment of sperm penetration and presence of two pronuclei (PN)

Twelve hours after insemination, the percentages of spermatozoa penetrating oocytes and monospermy were assessed as previously described (Gil et al., 2007). 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] and 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 (percentage of the number of penetrated oocytes/total inseminated) and monospermy (percentage of the number of monospermic oocytes/total penetrated).

8. Measurement of intracellular ROS and 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 a blue fluorescence. At 12 h after insemination, PZs were incubated for 30 min in TL-HEPES-PVA supplemented with 10 µM Cell Tracker. PZs were washed in TL-HEPES-PVA and placed on a glass slide with a 10 µl drop of TL-HEPES-PVA.

The levels of ROS in the PZs were measured in green fluorescence by dichlorodihydrofluorescein diacetate (DCHFDA). After IVC, PZs were transferred into IVC medium containing 10 µM DCHFDA. After 30 min of culture, PZs were washed in DPBS-PVA. The PZs were then placed on a glass slide with a 10 µl drop of DPBS-PVA.

The fluorescent emissions from the PZs were recorded as TIFF files using a cooled CCD camera attached to a fluorescence microscope (Axio-observer A1, Charl Zeiss, Goettingen, Germany) with filters (excitation: 450-490 nm, emission: 515-565 nm for ROS, UV filter: 370 nm for GSH). The recorded fluorescent images were analyzed using ImageJ software 1.33u (National Institutes of Health, Bethesda, MD, USA) by the intensity of fluorescence in each PZ picture.

9. 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

IVF parameters including sperm penetration and monospermy did not show any significant difference among the groups (Table 1).

The intracellular GSH and ROS levels of PZs are shown in Table 2. Likewise, as the results of IVF parameters, GSH and ROS levels of PZs following IVF with boar spermatozoa cryopreserved with GTE or β-ME or without GTE and β-ME had no significant difference among the groups. However, GTE group showed slightly lower ROS level.

The increased ROS level causes poor motility, viability, morphology and acrosome integrity of frozen-thawed spermatozoa (Chatterjee and Gagnon, 2001). Bailey *et al*. (2000) reported that cryoinjury may be induced by ROS activity generated

Table 1. Effects of antioxidants supplementation in sperm freezing extender on fertility parameters

Extender	No. of presumptive zygote	Penetration (%)	Monospermy (%)
Control	98	39.7±5.9	71.7±5.3
GTE	90	41.1±7.1	72.9±7.3
β-ME	92	41.3±6.7	73.6±4.9

GTE: green tea extract, β-ME: beta-mercaptoethanol
The data represent mean ± SE.

Table 2. Effects of antioxidants supplementation in sperm freezing on GSH and ROS levels in presumptive zygotes

Groups	GSH		ROS	
	No. of zygote	Levels	No. of zygote	Levels
Control	53	55.8 ± 7.8	52	62.7±14.8
GTE	46	48.6 ± 6.6	48	45.8±10.1
β-ME	70	52.8 ± 6.9	68	55.2±12.1

GTE: green tea extract, β-ME: beta-mercaptoethanol, ROS: reactive oxidative species
The data represent mean ± SE.

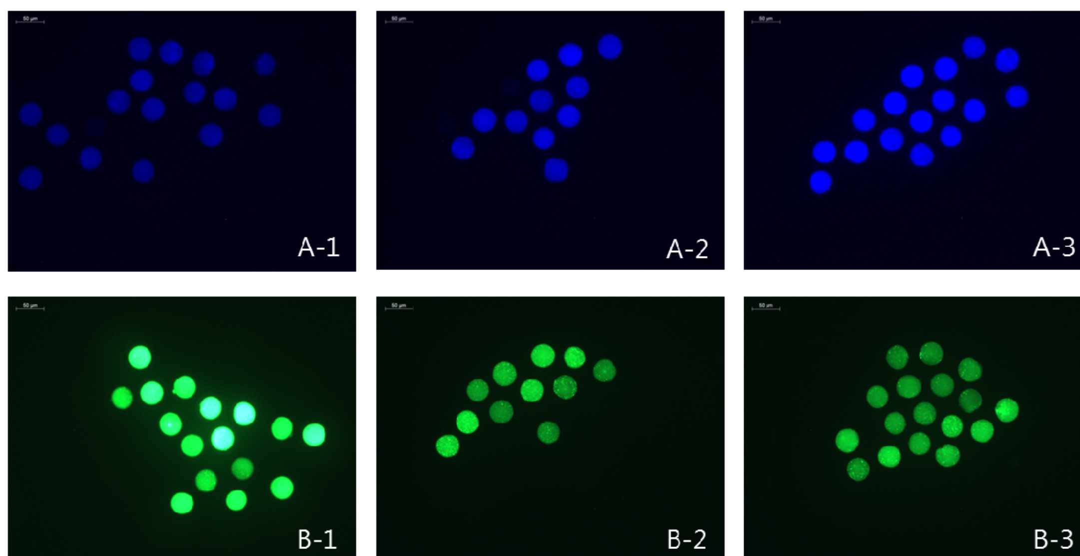

Fig. 1. Representative images of the intracellular glutathione (A: GSH) and reactive oxygen species (B: ROS) levels of porcine presumptive zygotes by staining with Cell Tracker Blue CMF_2HC and DCHFDA, respectively. A-1 and B-1: control, A-2 and B-2: GTE, A-3 and B-3: β-ME.

during the process of freezing. Therefore, most researchers have studied the effect of antioxidants during the freezing process for reduction of ROS, (Yamaguchi and Funahashi, 2012). In other word, Gadea *et al*. (1998) and Sellés *et al*. (2003) reported that IVF is the most precise tool for evaluating both the refrigerated and frozen-thawed boar semen. Spermatozoa exposed with higher ROS levels could affect on sperm-egg fusion (Mammoto *et al*., 1996). The reduced ROS level by GTE in frozen-thawed boar spermatozoa was demonstrated (Park and Yu, 2017). Though, the reduced ROS level of frozen-thawed spermatozoa did not make any significant change in IVF parameters of presumptive zygote. Therefore, in the present study, we suggest that the sperm penetration following IVF were not significantly affected by reduced sperm ROS regardless of antioxidant. The effect of antioxidant supplementation during freezing might have limited influence on sperm function in IVF process (giving to porcine oocytes). Similarly, Gadea *et al*. (2004) reported that supplementation of GSH in sperm freezing extender had no effect on sperm fertilizing ability.

GSH level is an important indicator of embryo development (Abeydeera *et al*.,1998; de Matos and Furnus, 2000). GSH supplementation in oocyte maturation medium showed a positive effect on male pronuclear formation during fertilization (Yoshida, 1993; Funahashi *et al*., 1994). Recent studies also reported that treatment of oocytes with antioxidants during IVM increased embryonic development by reducing ROS and increasing GSH synthesis (Maya-Soriano *et al*., 2013). Therefore, we inferred that the use of antioxidant in oocyte maturation can induce strong antioxidative effect in the embryo compared to antioxidant used in sperm freezing. In other words, the greater amount of cytoplasm in oocyte than that of sperm head might be more affected by antioxidant. Therefore, use of an antioxidant in sperm freezing extender might not affect embryo development compared to the treatment of antioxidant in oocyte maturation.

In contrast, addition of GSH to the freezing extender would result in a high rate of ROS-induced GSH oxidation during thawing (Gadea et al 2004). Therefore, we will evaluate the effect of addition of GTE and β-ME in post-thawed spermatozoa to know the latent antioxidant effect on IVF parameters and ROS and GSH level of presumptive zygote.

On the other hand, we should not overlook the generation of ROS during IVF. Recently the addition of antioxidants to IVF medium exclusively increased mouse blastocyst cell numbers (Truong and Gardner, 2017). The ROS of PZs might be generated from the IVF environment. Therefore, ROS level reduced in only sperm freezing could not influence the ROS and GSH levels of PZs.

It is concluded that there is no positive effect of supplementation of GTE and β-ME in freezing extender on IVF parameters and ROS and GSH levels of PZs.

REFERENCES

Abeydeera LR, Wang WH, Cantley TC, Prather RS and Day BN. 1998. Presence of beta-mercaptoethanol can increase the glutathione content of pig oocytes matured *in vitro* and the rate of blastocyst development after *in vitro* fertilization. Theriogenology 50: 747-756.

Agarwal A, Saleh RA and Bedaiwy MA. 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil. Steril. 79: 829-843.

Bailey JL, Bilodeau JF and Cormier N. 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J. Androl. 21: 1-7.

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

Dalvit G, Llanes SP, Descalzo A, Insani M, Beconi M and Cetica P. 2005. Effect of alpha-tocopherol and ascorbic acid on bovine oocyte in vitro maturation. Reprod. Domest. Anim. 40: 93-97.

Dufresne CJ and Farnworth ER. 2001. A review of latest research findings on the health promotion properties of tea. J. Nutr. Biochem. 12: 404-421.

Funahashi H, Cantley TC, Stumpf TT, Terlouw SL and Day BN. 1994: Use of low-salt culture medium for in vitro maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced male pronuclear formation after in vitro fertilization. Biol. Reprod. 51: 633-639.

Gil MA, Almiñana C, Cuello C, Parrilla I, Roca J, Vazquez JM and Martinez EA. 2007. Brief coincubation of gametes in porcine in vitro fertilization: role of sperm: oocyte ratio and post-coincubation medium. Theriogenology 67: 620-626.

Gadea J, Matás C and Lucas X. 1998: Prediction of porcine

semen fertility by homologous in vitro penetration (hIVP) assay. Anim. Reprod. Sci. 54: 95-108.

Gadea J, Sellés E, Marco MA, Coy P, Matás C, Romar R and Ruiz S. 2004: Decrease in glutathione content in boar sperm after cryopreservation: Effect of the addition of reduced glutathione to the freezing and thawing extenders. Theriogenology 62: 690-701.

Hu JH, Li QW, Jiang ZL and Li WY. 2008. Effects of different extenders on DNA integrity of boar spermatozoa following freezing-thawing. Cryobiology 57: 257-262.

de Lamirande E and Gagnon C. 1992. Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. J. Androl. 13: 368-378.

Luvoni GC, Keskintepe L and Brackett BG. 1996. Improvement in bovine embryo production *in vitro* by glutathione-containing culture media. Mol. Reprod. Dev. 43: 437-443.

Makker K, Agarwal A and Sharma R. 2009. Oxidative stress & male infertility. Indian. J. Med. Res. 129: 357-367.

Mammoto A, Masumoto N, Tahara M, Ikebuchi Y, Ohmichi M, Tasaka K and Miyake A. 1996. Reactive oxygen species block sperm-egg fusion via oxidation of sperm sulfhydryl proteins in mice. Biol. Reprod. 55: 1063-1068.

de Matos DG and Furnus CC. 2000. The importance of having high glutathione (GSH) level after bovine *in vitro* maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. Theriogenology 53: 761-771.

Maya-Soriano MJ, Taberner E and López-Béjar M. 2013. Retinol improves in vitro oocyte nuclear maturation under heat stress in heifers. Zygote 21: 377-384.

Oborna I, Wojewodka G, De Sanctis JB, Fingerova H, Svobodova M, Brezinova J, Hajduch M, Novotny J, Radova L and Radzioch D. 2010. Increased lipid peroxidation and abnormal fatty acid profiles in seminal and blood plasma of normozoospermic males from infertile couples. Hum. Reprod. 25: 308-316.

Olson SE and Seidel GE Jr. 2000. Culture of in vitro-produced bovine embryos with vitamin E improves development in vitro and after transfer to recipients. Biol. Reprod. 62: 248-252.

Park SH and Yu IJ. 2015. Evaluation of Toxicity of Green Tea Extract in Chilled Boar Spermatozoa. J. Emb. Trans. 30: 1-6.

Park SH and Yu IJ. 2017. Effect of antioxidant supplementation in freezing extender on porcine sperm viability, motility and reactive oxygen species. J. Emb. Trans. 32: 9-15.

Sellés E, Gadea J, Romar R, Matás C and Ruiz S. 2003. Analysis of in vitro fertilizing capacity to evaluate the freezing procedures of boar semen and to predict the subsequent fertility. Reprod. Domest. Anim. 38: 66-72.

Tatemoto H, Muto N, Sunagawa I, Shinjo A and Nakada T. 2004. Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid. Biol. Reprod. 71: 1150-1157.

Truong T and Gardner DK. 2017. Antioxidants improve IVF outcome and subsequent embryo development in the mouse. Hum. Reprod. Nov. 10:1-10 doi: 10.1093/humrep/ex330. Equb ahead of print

Watson PF. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their ost-thawing function. Reprod. Fertil. Dev. 7: 871-891.

Waterhouse KE, Gjeldnes A, Tverdal A, De Angelis PM, Farstad W, Håård M & Kommisrud, E. 2010. Alterations of sperm DNA integrity during cryopreservation procedure and in vitro incubation of bull semen. Anim. Reprod. Sci. 117: 34-42.

Yamaguchi S and Funahashi H. 2012. Effect of the addition of beta-mercaptoethanol to a thawing solution supplemented with caffeine on the function of frozen-thawed boar sperm and on the fertility of sows after artificial insemination. Theriogenology 77: 926-932.

Yoshida M, Ishigaki K, Nagai T, Chikyu M and Pursel VG. 1993. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol. Reprod. 49: 89-94.

Received November 29 2017, Revised December 15, 2017, Accepted December 26, 2017