Anim. Reprod., v.5, n.3/4, p.127-131, Jul./Dec. 2008

Effect of exogenous glutathione supplementation on motility, viability, and DNA integrity of frozen-thawed boar semen

B.D. Whitaker[1,3], B. Carle[2], T. Mukai[2], A. Simpson[2], L. Vu[2], J.W. Knight[1]

[1]Department of Animal and Poultry Sciences, Virginia Tech, Blacksburg, VA, 24061, USA.
[2]Governor's School for Agriculture, Virginia Tech, Blacksburg, VA, 24061, USA.

Abstract

This study evaluated the effects of supplementing 5.0 mM glutathione (GSH) to the media during semen thawing and culture on forward progressive spermatozoa motility, viability, and DNA fragmentation. Pellets of frozen boar semen were thawed using a PBS wash procedure at 39°C. Spermatozoa were analyzed for forward progressive motility, viability, and DNA fragmentation by the Comet assay at 0.5 h and 6.0 h post-thawing. Supplementation of 5.0 mM GSH had no significant effect on forward progressive motility, viability, or DNA fragmentation at 0.5 h post-thawing compared to the control. At 6.0 h after thawing there was no significant difference in viability between the control and the 5.0 mM GSH supplemented group. Forward progressive spermatozoa motility was significantly less ($P < 0.05$) at 6.0 h after thawing in the 5.0 mM GSH supplemented group compared to the control. DNA fragmentation was significantly higher ($P < 0.05$) at 6 h after thawing in the 5.0 mM GSH supplemented group compared to the control. These results indicate that supplementing 5.0 mM of GSH to the thawing and culture media significantly decreases ($P < 0.05$) spermatozoa forward progressive motility and significantly increases ($P < 0.05$) DNA fragmentation.

Keywords: Comet assay, glutathione, pig, sperm, viability.

Introduction

Swine are valuable to science because they are a suitable model for human anatomy and physiology research. Swine are routinely utilized in medical trials, organ transplant procedures, and skin grafting to burnt victims. Researchers have also produced transgenic pigs that produce several specific human therapeutic proteins (Abeydeera, 2002). Research using swine as the experimental model often utilizes *in vitro* techniques to limit the experimental variability observed *in vivo*. *In vitro* fertilization (IVF) uses spermatozoa from boars that are considered optimal for research and medical applications by freezing and thawing semen from a single ejaculate, thus minimizing variability among trials and animals (Abeydeera, 2002).

However, there is a decrease in fertilization success using frozen-thawed semen in addition to a reduction in viable IVF-derived embryos. These shortcomings have been associated with oxidative stress during the freezing and thawing of semen. Oxidative stress produces large amounts of reactive oxygen species (ROS) that effect spermatozoa motility (Armstrong *et al*., 1999) and nuclear DNA (Fraser and Strzezek, 2005). Reactive oxygen species pull electrons from the spermatozoon and damage the membrane through lipid peroxidation. The negative effects of ROS on spermatozoa have been observed in boar, rhesus monkey, and humans where cryopreservation has led to DNA fragmentation, chromosome fragmentation, and aneuploidy (Donnelly *et al*., 1999; Li *et al*., 2007).

The antioxidant glutathione (GSH) facilitates decondensation of the sperm pronucleus during fertilization. Decondensation occurs when the chromatin becomes uncoiled due to histone activation inside the penetrated oocyte. It is thought that GSH facilitates the breakdown of protamines, which keep DNA condensed by changing the redox environment of the oocyte (Funahashi and Sano, 2005). This change in environment breaks the disulfide bonds that maintain the structure of the protamines. Glutathione supplementation has elicited beneficial effects in many facets of *in vitro* production: increasing the IVF success rate (Abeydeera, 2002; Jiang *et al*., 2007), acting as a cryoprotectant during semen freezing (Munsi *et al*., 2007), and assisting in antioxidant defense mechanisms during semen thawing (Gadea *et al*., 2005).

Research has shown that supplementing GSH in the thawing medium increases IVF success (Gadea *et al*., 2005) and semen thawing causes DNA fragmentation (Fraser and Strzezek, 2007b). Research has not studied these results together; therefore, the objective of this study was to determine the effects of glutathione supplementation during the thawing of frozen boar semen on motility, viability and DNA fragmentation prior to IVF.

Materials and Methods

Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The thawing medium was Dulbecco's phosphate

[3]Corresponding author: bwhitaker@ferrum.edu
Phone: +1(540)365-4363
Received: January 14, 2008
Accepted: June 3, 2008

buffered saline (D-PBS; Invitrogen, Carlsbad, CA, USA) containing 75 µg/ml potassium penicillin, 50 µg/ml streptomycin sulfate, and 0.1% BSA (fraction V; 43H1097, initial fraction by heat shock). The fertilization medium was a modified Tris-buffered medium (mTBM) formulated by Abeydeera and Day (1997).

Spermatozoa preparation

A commercially obtained frozen semen pellet (Swine Genetics International Ltd., Cambridge, IA, USA) was thawed as previously described (Whitaker and Knight, 2004). Briefly, the semen pellet was thawed in D-PBS at 39ºC and centrifuged at 36.3 x *g* for 5 min. The semen was then washed twice at 553 x *g* for 5 min. After washing, the spermatozoa pellet was re-suspended with mTBM to a concentration of 4 x 10^5 spermatozoa/ml and incubated at 39ºC in 5% CO_2 until analysis.

Spermatozoa assessments

Viability staining

Membrane integrity was assessed by staining spermatozoa with Eosin red and Aniline blue dye and then smeared on a microscope slide to determine viability. Spermatozoa that had intact membranes did not incorporate the dye stained pink, whereas spermatozoa that had degraded membranes incorporated the dye and stained purple (Colenbrander *et al*., 2002).

Motility

Forward progressive motility was analyzed by placing 20 µl of spermatozoa in 0.1 M sodium citrate buffer (v:v) on a 38ºC glass slide under a stereomicroscope. Spermatozoa were either classified as having forward progressive motility or non-motile.

DNA integrity

Sperm DNA fragmentation was analyzed using the single cell gel electrophoresis or "Comet" assay (Tatemoto *et al*., 2000). Sperm were mixed with 2% low-melting agarose at 40°C on a glass slide and covered. The slides were kept at 4°C for 10 min to allow solidification of the agarose. The coverslip was removed and the slide was immersed in a lysing solution (1% N-lauroyl-sarcosine, 2.5 M NaCl, 10 mM EDTA, 10 mM Tris, pH 10, and 1% Triton X-100) for 1 h to lyse the cells and permit DNA unfolding. The slides were placed on a horizontal gel electrophoresis unit and equilibrated for 20 min in Tris-Borate-EDTA (TBE) electrophoresis buffer (pH 8.2). Electrophoresis was conducted for 20 min at 50 V and then the slides were stained with 10 µg/ml 4, 6-diamidino-2-phenylindole (DAPI) for 10 min, washed with distilled water, and covered with a cover-slip. The cover-slips were sealed with clear nail polish and examined through a fluorescent microscope using a fluoroisothiocyanate filter set (FITC); wavelength of excitation, 470 ± 20 nm (470/40 nm); wavelength of emission, 515 nm (long pass). The length of migrated DNA or comet tail of 20-25 oocytes in each experimental group was individually measured using a micrometer.

Experimental design

Frozen-thawed semen pellets were incubated in 5.0 mM GSH supplementation or control. At 0.5 h and 6.0 h after thawing, a sample from each treatment was analyzed for forward progressive motility, viability, and DNA fragmentation. The procedure was repeated 3 times. Data were analyzed by one-way ANOVA using the PROC ANOVA procedures of SAS (SAS Institute, Cary, NC) because the data were balanced in all replicates. When there was a significant effect of treatment, significant differences were determined using LSMEANS statement and Tukey adjustment for multiple comparisons. A probability of less than 0.05 was considered significant.

Results

Results indicate that supplementing 5.0 mM GSH to the thawing media and mTBM did not affect forward progressive motility (80 ± 5%) compared to the control (75 ± 5%) at 0.5 h post-thawing (Fig. 1). At 6.0 h post-thawing, the 5.0 mM GSH supplemented spermatozoa had significantly ($P < 0.05$) less forward progressive motile (10 ± 5%) compared to the control (30 ± 5%; Fig. 1).

Supplementing 5.0 mM GSH to the thawing media and mTBM did not have a significant effect on spermatozoa viability (74.7 ± 2.3%) compared to the control (72.1 ± 2.3%) at 0.5 h post-thawing. There was also no significant difference at 6.0 h post-thawing between the 5.0 mM GSH supplemented spermatozoa (62.7 ± 2.3%) and the control (70.7 ± 2.3%).

Observations from the Comet assay indicate that supplementing 5.0 mM GSH to the thawing media and mTBM did not have a significant effect on the length of DNA migration (36.7 ± 2.7 µm) compared to the control (32.4 ± 2.7 µm) at 0.5 h post-thawing. The length of DNA migration of the 5.0 mM GSH supplemented spermatozoa was significantly ($P < 0.05$) longer (74.6 ± 2.7 µm) compared to the control (40.1 ± 2.7 µm) at 6.0 h post-thawing (Fig. 2).

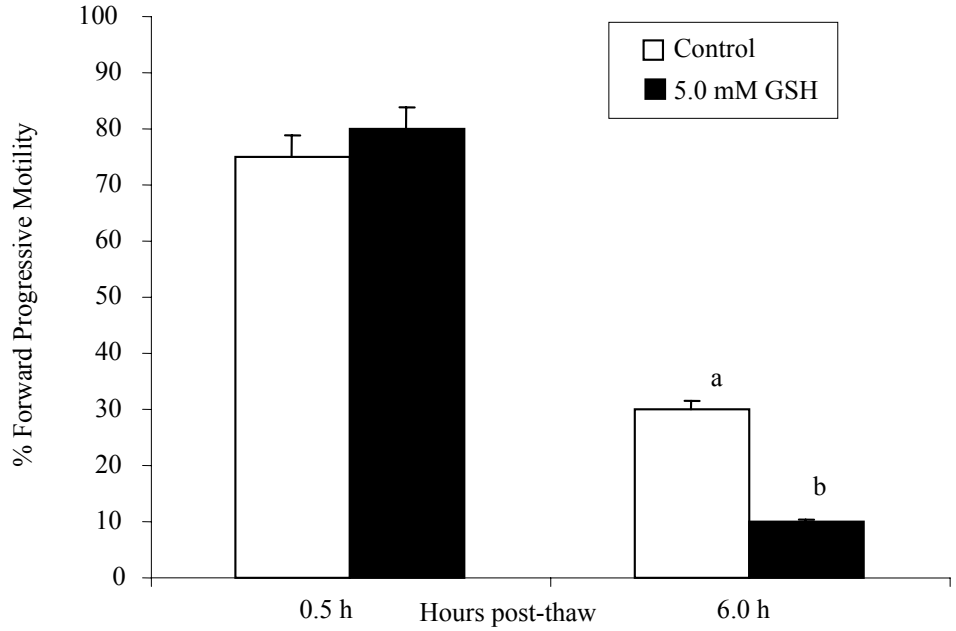

Figure 1. Effect of 5.0 mM Glutathione (GSH) supplementation on spermatozoa forward progressive motility at 0.5 h and 6 h post-thawing. Control, no GSH supplemented; 5.0 mM GSH, media supplemented with 5.0 mM GSH. Data are expressed as mean ± SEM. [a,b]Means with different superscripts at a given time differ (P < 0.05).

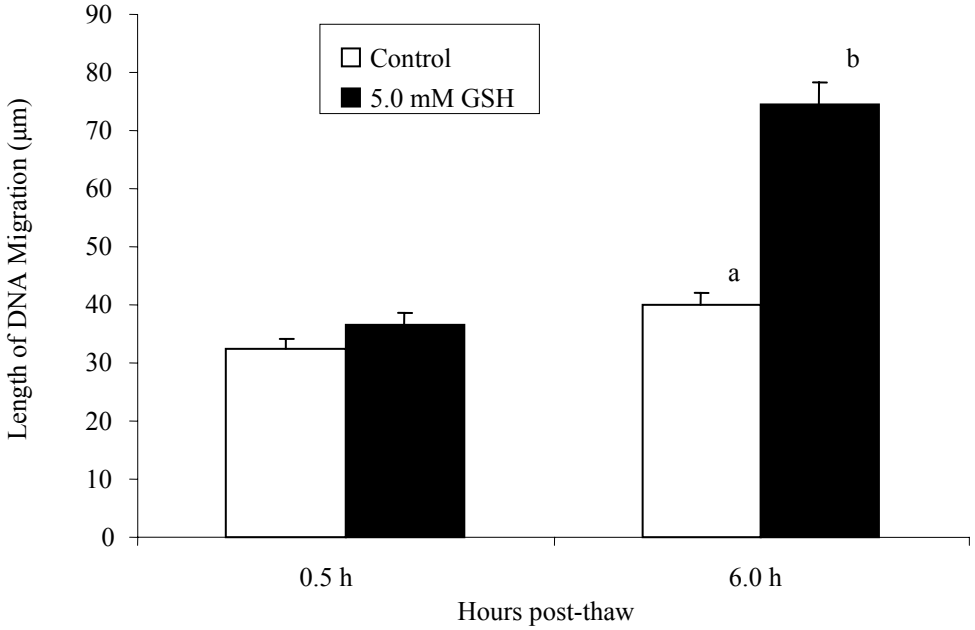

Figure 2. The effect of 5.0 mM GSH supplementation on sperm DNA migration at 0.5 h and 6 h post-thawing. Control, no GSH supplemented; 5.0 mM GSH, media supplemented with 5.0 mM GSH. Data are expressed as mean ± SEM. [a,b]Means with different superscripts at a given time differ (P < 0.05).

Discussion

Supplementation of 5.0 mM GSH to the thawing media and mTBM generated no significant differences compared to the control with respect to forward progressive motility, viability, or DNA fragmentation at 0.5 h post-thawing. These results indicate that 0.5 h is not adequate time for 5.0 mM GSH to affect the spermatozoa.

The control group having significantly higher (P < 0.05) percentages of motile sperm at 6 h post-thawing compared to the 5.0 mM GSH supplemented

treatment indicates that GSH may reduce the motility of the sperm. The decrease in motility could be related to the effect of GSH on the function of the sperm tail. There was no significant difference in viability between the GSH treatment and the control at 6 h post-thawing, indicating that there was no membrane damage associated with the supplementation of 5.0 mM GSH. A spermatozoon is propelled by a flagellum to reach and fertilize the oocyte. Energy required for this action is obtained in part from the large numbers of mitochondria found near the base of the tail (Eddy and O'Brien, 1994). The GSH may specifically cause a detrimental effect to the mitochondria, including decondensation and degradation of the DNA contained inside the mitochondria, reducing the motility of the GSH supplemented spermatozoa. Our findings are contrary to other findings that report GSH increased motility and viability when supplemented to the media (Munsi *et al*., 2007). The discrepancy might be due to different experimental models (bull versus boar) and perhaps the concentration of antioxidants added. Munsi *et al.* (2007) found the ideal concentration was 0.5 mM GSH, whereas we tested only 5.0 mM GSH. Further research on GSH concentrations needs to be done using boar semen.

Our results also indicate that supplementing 5.0 mM GSH significantly increased ($P < 0.05$) the length of DNA migration at 6 h post-thawing compared to the control. Based on the Comet assay, this indicates greater DNA degradation in the 5.0 mM GSH supplemented spermatozoa compared to the control. Since there was no significant difference at 0.5 h post-thawing, the prolonged exposure to 5.0 GSH degraded the nuclear DNA significantly ($P < 0.05$) more than the control environment (Fig. 2).

Faser and Strzezek (2007a) concluded that the damage associated with thawing frozen boar semen could be a result of ROS and oxidative damage. They also reported that a decrease in motility, viability, and increase in DNA damage are highly correlated when semen is thawed. This is in agreement with our findings that a decrease in motility was seen with an increase in DNA degradation. We observed this when comparing GSH supplemented media to the control. In order to obtain a better understanding of the mechanisms, further research should be done to compare fresh semen and frozen-thawed semen from an individual boar.

To the best of our understanding, this is the first study looking at the effect of antioxidants during semen thawing on DNA degradation specifically in boars. The Comet assay is an under-utilized technique that allows researchers to observe what is happening inside the sperm nucleus. We observed that supplementation of 5.0 mM GSH is detrimental to the sperm DNA and motility but does not affect the membrane integrity. It is unclear as to the direct action of GSH in the thawing media. Further research needs to be done to determine if GSH is acting as an antioxidant in the environment or directly on the sperm. A better understanding of the actions of antioxidants during thawing could lead to more successful IVF techniques and methodology.

References

Abeydeera LR, Day BN. 1997. *In vitro* penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. *Theriogenology*, 48:537-544.

Abeydeera LR. 2002. *In vitro* production of embryos in swine. *Theriogenology*, 57:257-273.

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

Colenbrander B, Fazeli AR, van Buiten A, Parlevliet J, Gadella BM. 1992. Assessment of sperm cell membrane integrity in the horse. *Acta Vet Scand Suppl*, 88:49-58.

Donnelly ET, McClure N, Lewis SE. 1999. Antioxidant supplementation *in vitro* does not improve human sperm motility. *Fertil Steril*, 72:484-495.

Eddy EM, O'Brien D. 1994. The spermatozoon. *In*: Knobil E, Neill JD (Eds.). *The Physiology of Reproduction*. 2nd Ed. New York, USA: Raven Press. pp. 29-77.

Fraser L, Strzezek J. 2005. Effects of freezing-thawing on DNA integrity of boar spermatozoa assessed by neutral comet assay. *Reprod Domest Anim*, 40:530-536.

Fraser L, Strzezek J. 2007a. Effect of different procedures of ejaculate collection, extenders and packages on DNA integrity of boar spermatozoa following freezing-thawing. *Anim Reprod Sci*, 99:317-329.

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

Funahashi T, Sano T. 2005. Select antioxidants improve the function of extended boar semen stored at 10ºC. *Theriogenology*, 63:1605-1616.

Gadea J, Gumbao D, Mata's C, Romar R. 2005. Supplementation of the thawing media with reduced glutathione improves function and the *in vitro* fertilizing ability of boar spermatozoa alter cryopreservation. *J Androl*, 26:749-756.

Jiang ZL, Li QW, Li WY, Hu JH, Zhao HW, Zhang SS. 2007. Effect of low density lipoprotein on DNA integrity of freezing-thawing boar sperm by neutral comet assay. *Anim Reprod Sci*, 99:401-407.

Li MW, Meyers S, Tollner TL, Overstreet JW. 2007. Damage to chromosomes and DNA of rhesus monkey sperm following cryopreservation. *J Androl*, 28:493-501.

Munsi MN, Bhuiyan MM, Majumder S, Alam MG. 2007. Effects of exogenous glutathione on the quality of

chilled bull semen. *Reprod Domest Anim*, 42:358-362.

Tatemoto H, Sakurai N, Muto N. 2000. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during *in vitro* maturation: role of cumulus cells. *Biol Reprod*, 63:805-810.

Whitaker BD, Knight JW. 2004. Exogenous γ-glutamyl cycle compounds supplemented to *in vitro* maturation medium influence *in vitro* fertilization, culture, and viability of porcine oocytes and embryos. *Theriogenology*, 62:311-322.