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The Effects of Thawing Procedure and Supplementation on the Motility and Viability of Frozen-thawed Boar Semen

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ABSTRACT

The effect of two thawing procedures on frozen boar semen and supplementations to the fertilization media were studied. Frozen boar semen was thawed using either Percoll gradient or phosphate buffered saline (PBS) procedure. Supplementations were 1.0 mM L-glutamate, 1.0 mM N-acetyl-cysteine (NAC), and 1.0 mM NAC-amide (NACA). Spermatozoa were analyzed for forward progressive motility (FPM) and viability every 0.5 h for 3.0 h post-thawing. There were significantly ($P < 0.05$) higher numbers of viable (76.0 ± 5.1%) and FPM (30.0 ± 2.4%) spermatozoa at 3.0 h post-thawing using the PBS procedure compared to the Percoll gradient thawed spermatozoa (65.0 ± 3.9%; 10.0 ± 4.5%, respectively). Supplementation of 1.0 mM L-glutamate, 1.0 mM NAC, or 1.0 mM NACA had no significant effect on spermatozoa viability regardless of the time post-thaw. Supplementation of 1.0 mM L-glutamate, 1.0 mM NAC, or 1.0 mM NACA had no significant effect on FPM up to 1.0 h post-thaw. Spermatozoa with no supplementation or 1.0 mM L-glutamate had significantly higher ($P < 0.05$) FPM compared to the 1.0 mM NAC and 1.0 mM NACA supplemented groups at 1.5, 2.0, 2.5, and 3.0 h post-thaw. There was no significant difference between no supplementation or 1.0 mM L-glutamate on FPM regardless of the time post-thaw. There was no significant difference between 1.0 mM NAC or 1.0 mM NACA on FPM regardless of the time post-thaw. These results indicate that thawing procedure has an effect on spermatozoa viability and FPM but supplementation does not have an effect on the overall viability of spermatozoa during thawing, but may reduce FPM.

INTRODUCTION

Swine are valuable to science because they serve as a comparable model for human anatomy and physiology research. Research using swine as the experimental model often utilizes *in vitro* techniques to limit the experimental variability observed *in vivo*. The *in vitro* production of embryos is not as efficient as its natural counterpart, as a result of a decrease in the fertilization success rate during *in vitro* fertilization (IVF) and a reduction in viable IVF-derived embryos (Abeydeera 2002).

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Inefficiencies associated with *in vitro* production can be divided into two categories, the technique used to thaw frozen semen (Henkel et al. 2003, Chou et al. 2005) and the resulting media in which the semen is extended and cultured (Armstrong et al. 1999). Semen can be thawed using a saline wash procedure (Whitaker and Knight 2004, Chou et al. 2005) or Percoll gradient separation (Henkel et al. 2003). Both methods have variable results between replications and neither is close to the results obtained from fresh semen (Henkel et al. 2003). Current speculation is that oxidative stress during the freezing and thawing of semen produces large amounts of reactive oxygen species (ROS) that effects spermatozoa motility (Armstrong et al. 1999) and nuclear DNA (Fraser and Strzezek 2005).

Antioxidants supplemented to the semen extender have been shown to improve the viability of the spermatozoa (Funahashi et al. 2005) and perhaps lessen the harmful effects of the free radicals on the spermatozoa (Blount et al. 2001). One antioxidant of interest is N-acetyl-cysteine (NAC) because it reduces cystine to cysteine, thus modulating glutathione (GSH) biosynthesis (Nakata et al. 1996, Issels et al. 1998); and has the ability to supply its sulfhydryl group to enhance glutathione-S-transferase activity (Nakata et al. 1996), which attaches glutathione to various compounds. N-acetyl-cysteine has been implicated in protecting against oxidative stress as seen when supplemented during the later stages of embryo development by decreasing the incidence of early fetal death (Xu et al. 2005). A derivative of NAC, NAC-amide (NACA) is of interest because the amide addition neutralizes the otherwise negatively charged carboxyl group, allowing it to permeate most physiological membranes. Research has shown that NACA is able to replenish intracellular GSH and defend cells against oxidation (Grinberg et al. 2005, Wu et al. 2006).

Therefore, the objectives of this study were to, 1) determine the effects of two different thawing procedures on spermatozoa viability and forward progressive motility (FPM), and 2) determine the effects of 1.0 mM L-glutamate, NAC, and NACA supplementation to the thawing media on spermatozoa viability and FPM.

MATERIALS AND METHODS

Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The Modena medium (152.6 mM glucose, 23.46 mM Na-citrate, 11.9 mM Na-carbonate, 6.99 mM EDTA, 26.66 mM Tris, and 15.1 mM citric acid) was adjusted to pH 7.3. The PBS thawing medium was Dulbecco's phosphate buffered saline (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). The NACA was supplied by Novia Pharmaceuticals Ltd. (Haifa Bay, Israel).

Semen

Frozen semen was obtained from Swine Genetics International Ltd. (Cambridge, IA, USA). The semen was extended using a yolk-based media and frozen in 5.0 mL straws in a Cryo-Med chamber to control the freezing rate. Upon arrival in the laboratory, straws were cut into 1.0 cm long pellets and stored in liquid nitrogen until use. All semen used was from the same boar but not from a single collection.

Semen thawing using Percoll gradient

A frozen semen pellet was thawed in 2.0 mL Modena and then overlain on a Percoll gradient (2.0 mL 90% Percoll medium (v:v, Modena) overlain with 2.0 mL 45% Percoll medium (v:v, Modena)). The gradient was centrifuged at 700 x *g* for 20 min. The semen was then washed by centrifugation at 500 x *g* for 5 min in mTBM. After washing, the spermatozoa pellet was re-suspended with mTBM to a concentration of 4×10^5 spermatozoa/mL and incubated at 39°C in 5% CO_2 until analysis.

Semen thawing using PBS wash

A frozen semen pellet was thawed as previously described (Whitaker and Knight 2004). Briefly, the semen pellet was thawed in PBS at 39°C and centrifuged at 36.3 x *g* for 5 min. The semen was then washed by centrifugation twice at 553 x *g* for 5 min. After washing, the spermatozoa pellet was re-suspended with mTBM to a concentration of 4×10^5 spermatozoa/mL and incubated at 39°C in 5% CO_2 until analysis.

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 and stained pink, whereas spermatozoa that had degraded membranes incorporated the dye and stained purple (Colenbrander et al. 2002). A total of 100 cells were counted for each pellet analyzed using a phase-contrast microscope at 400X magnification.

Motility

Forward progressive motility was analyzed by placing 20 µL of spermatozoa in 0.1 M sodium citrate buffer (v:v) on a 38°C glass slide. A total of 100 cells were either classified as either FPM or non-FPM for each pellet analyzed using a phase-contrast microscope at 400X magnification.

Experiment 1: Comparison of semen thawing methods on spermatozoa viability and FPM

A frozen semen pellet was thawed using either the PBS wash or the Percoll gradient procedure and then analyzed for viability and FPM at 3.0 h post-thawing. For this experiment a total of 60 semen pellets were used, by thawing and analyzing 30 pellets for each procedure.

Experiment 2: Comparison of fertilization media supplements on spermatozoa viability and FPM

Based on the results from Experiment 1, a frozen semen pellet was thawed using the PBS wash procedure and incubated in mTBM supplemented with either 1.0 mM L-glutamate, NAC, or NACA for 3.0 h. Spermatozoa were analyzed for viability and FPM every 0.5 h for 3.0 h post-thaw. For this experiment a total of 90 semen pellets used, by thawing and analyzing 30 pellets for each supplement.

Statistical Analysis

Data in both experiments were analyzed by one-way ANOVA using the PROC ANOVA procedures of SAS (SAS Institute, Cary, NC) because the data were balanced in all cases. When there was a significant effect of treatment, significant differences were determined using LSMEANS statement and Tukey adjustment for multiple comparisons. The effects included in the model were treatment and technician. Technician effects were not significant ($P < 0.05$) and deleted from the final model. A probability of less than 0.05 ($P < 0.05$) was considered significant.

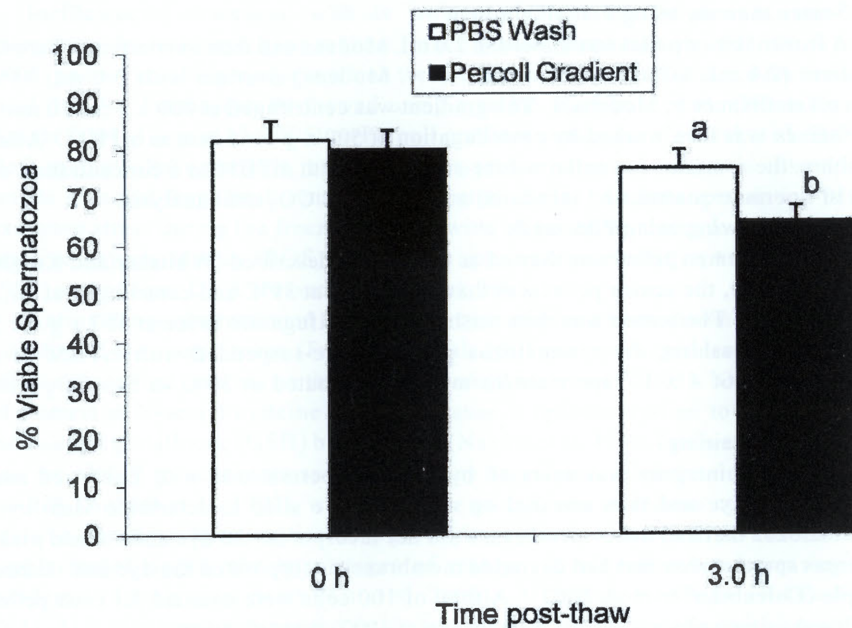

FIGURE 1. Effect of thawing procedure on spermatozoa viability after thawing (0 h) and 3.0 h post-thawing. PBS Wash, phosphate buffered saline wash procedure; Percoll gradient, Percoll gradient wash procedure. Data are expressed as mean ± SE. [a,b] Means with different superscripts at a given time differ at least P < 0.05.

RESULTS

Experiment 1: Comparison of semen thawing methods on spermatozoa viability and FPM

There were no significant differences in viability or FPM between the PBS wash and Percoll gradient thawed semen immediately after thawing. At 3.0 h post-thaw there were significantly ($P < 0.05$) higher numbers of viable spermatozoa from the PBS wash thawing procedure (76.0 ± 5.1%) compared to the Percoll gradient thawing procedure (65.0 ± 3.9%) (Figure 1). At 3.0 h post-thaw there were significantly ($P < 0.05$) higher numbers of FPM spermatozoa from the PBS wash thawing procedure (30.0 ± 2.4%) compared to the Percoll gradient thawing procedure (10.0 ± 4.5%) (Figure 2).

Experiment 2: Comparison of fertilization media supplements on spermatozoa viability and FPM

There was no significant effect of 1.0 mM L-glutamate, 1.0 mM NAC, or 1.0 mM NACA supplementation on viability at any time h post-thaw. There was no significant effect of 1.0 mM L-glutamate, 1.0 mM NAC, or 1.0 mM NACA supplementation on

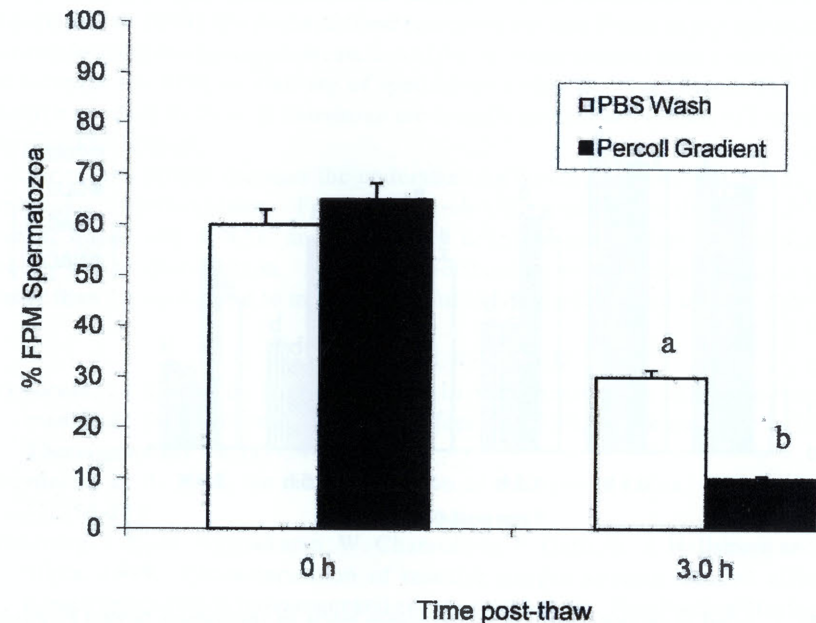

FIGURE 2. Effect of thawing procedure on spermatozoa forward progressive motility (FPM) after thawing (0 h) and 3.0 h post-thawing. PBS Wash, phosphate buffered saline wash procedure; Percoll gradient, Percoll gradient wash procedure. Data are expressed as mean ± SE. [a,b] Means with different superscripts at a given time differ at least P < 0.05.

FPM at 0, 0.5 and 1.0 h post-thaw. Supplementation of 1.0 mM L-glutamate elicited significantly higher ($P < 0.05$) FPM compared to other supplementations at 1.5, 2.0, 2.5, and 3.0 h post-thaw (Figure 3). There was no significant difference between the 1.0 mM NAC and 1.0 mM NACA supplemented groups.

DISCUSSION

Our results indicate that thawing frozen semen pellets using the PBS wash procedure produce greater numbers of viable and FPM spermatozoa by 3.0 h after thawing compared to the Percoll gradient thawed semen (Figures 1 and 2). Our rates of success are similar to previous research (Henkel et al. 2003, Chou et al. 2005) but neither of those studies directly compared thawing procedures. Greater emphasis is placed on studying different semen freezing protocols (Grossfeld et al. 2008) and thawing protocols based on temperature (Paulenz et al. 2007) rather than thawing techniques. The temperature used to thaw frozen boar semen is well documented (Abeydeera 2002) and our results strengthen this recommendation. Additionally, our results from experiment 1 provide evidence that there might not be a definitive technique to thaw frozen boar semen, but rather the technique that causes the highest

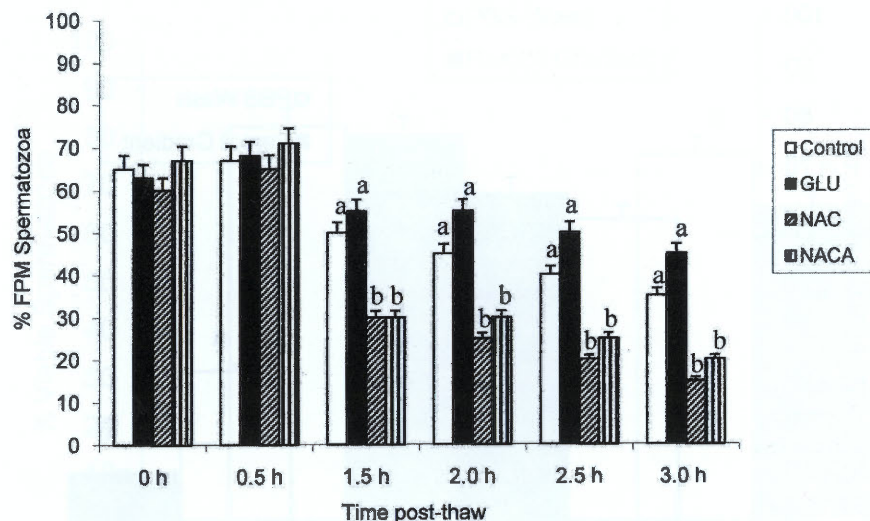

FIGURE 3. Effect of supplementation to the fertilization media on spermatozoa forward progressive motility (FPM) at 0, 0.5, 1.5, 2.0, 2.5, and 3.0 h post-thawing. Control, no supplementation to fertilization media; GLU, 1.0 mM L-glutamate supplementation; NAC, 1.0 mM N-acteyl-cysteine supplementation; NACA, 1.0 mM N-acetyl-cysteine-amide supplementation. Data are expressed as mean ± SE. [a,b] Means

viability and FPM for the researcher should be employed. This could be different between researchers and not allow for a standardized thawing technique. Our results (Figures 1 and 2) indicate that the PBS thawing procedure is best for our research.

Results of experiment 2 indicate that supplementing 1.0 mM NAC or NACA significantly ($P < 0.05$) decreased FPM 1.5 h after thawing but did not affect spermatozoa viability (Figure 3). The decrease in FBM could be related to the effect of antioxidants on the function of the spermatozoon tail. 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 supplementation of 1.0 mM NAC or NACA may specifically cause a detrimental effect to the mitochondria such as decondensation and degradation of mitochondrial DNA, thus reducing the motility of the spermatozoa. This explanation however is contrary to other findings that report antioxidants, specifically GSH, increased motility and viability when supplemented to the media (Munsi et al. 2007). The discrepancy could be due to different experimental models (bull versus boar) and perhaps the concentration of antioxidants supplemented. A study evaluating the effects of increasing doses of antioxidants on FPM and viability needs to be completed before further conclusions are made.

Fertilization media is routinely supplemented with caffeine to increase penetration rates of the oocyte (Abeydeera and Day 1997). However, supplementation of caffeine has been shown to increase the incidence in polyspermic penetration (Funahashi et al.

2000). Research has found glutamate receptors in the spermatozoa of humans and mice (Hu et al. 2004) and perhaps these receptors are also found in pig spermatozoa. Our results indicate that supplementation of the excitatory amino acid, L-glutamate did not increase the FPM or viability of spermatozoa after thawing (Figure 3). Further research needs to be done to determine the specific actions and effects of L-glutamate compared to caffeine.

Our results further increase the understanding of semen thawing technology and applicatios of antioxidants. Further research still needs to be done to determine specific doses, and if NAC and NACA are acting as antioxidants in the media or directly on the spermatozoa. A better understanding of the actions of antioxidants during thawing could lead to more successful IVF techniques and methodology.

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