Lipid Peroxidation During the Cryopreservation Process of Porcine Spermatozoa

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Abstract

The potential advantages of sperm cryopreservation have not been fully accomplished due to the limiting detrimental effects the freezing process has on sperm structure and composition. Previous studies have suggested that cells suffer lipid peroxidation damage during the cryopreservation process, specifically indicating the damage results from mechanical stress during the preparatory and freezing processes. In this present study, sperm samples were analyzed for lipid stability throughout sample processing through evaluations for lipid peroxidation and lipid free radical concentration. Our analysis was completed in three experiments. In Exp. 1, lipid stability levels were evaluated from five separate boar ejaculates frozen using three different freezing methods to compare cryopreservation techniques. In Exp. 2, lipid peroxidation amounts for fresh post-ejaculate and albumin extended boar samples were compared. Experiment 3 involved evaluations of the semen processing to examine sample and seminal fluid alterations. Samples tested from the freezing protocol included fresh, extended, addition of a wash buffer, cooling to 17 ºC, centrifugation, addition of two egg-yolk extenders, cooling to 5 ºC and postthaw values. Though there was no difference between the three freezing treatments, significant differences were noted between the fresh and extended samples ($P < 0.001$). These findings were exemplified by the step by step analysis of the processing and freezing protocol. The lipid peroxidation amounts accumulated after each the procedural step ($P < 0.001$). Significant differences were also observed in the lipid radical levels ($P < 0.001$). The results of the pre-freezing protocol, alterations in lipid stability do not appear to be due to thermal or mechanical stress. The largest gains of both lipid parameters developed after the addition of an egg-yolk freezing extender. The results suggest further studies in alterative extenders are needed.

Index terms— lipid peroxidation, sperm, cryopreservation, porcine.

1 Introduction

everal articles have been published based on lipid peroxidation and oxygen free radicals in human and murine semen samples.

Negative correlations have between associated between increased levels of malondialdehyde (MDA) and reduced motility [1][2][3] and poor fertilization capability 4 in human sperm. Though general conclusions may result from trials based on other species, species differences in composition have demonstrated the presence of varying protective enzymatic mechanisms against peroxidation . [5][6][7][8] Very few articles have been published on lipid radicals in relation to boar semen. However, the studies published suggested lipid radicals were more detrimental than the radical hydroxyls. [9][10][11] MDA and reactive oxygen species levels have been identified in samples

incubated at 37 ºC 9,10 , cooled liquid storage 11,12 and post-thaw. 13 Though peroxidation levels have been analyzed in previous reports specifically studying the postcollection and post-thawed samples, the possibility of the interaction of the cells and cryoprotectant creating radicals during pre-freezing procedures has not been investigated. Given the minimal success of freezing methodologies for porcine semen and the high susceptibility of these cells to free radical damage, the objective of this study was to investigate the possible source of damage on a molecular level evaluating the lipid stability of the phospholipid membrane during the freezing and pre-freezing process.

2 II.

3 Materials and Methods

4 a) Semen Preparation

Semen specimens were collected artificially from boars at the Texas Tech University New Deal Farm by trained staff. During the post-collection evaluation, standard measurements including total gel-free volume, concentration, and motility were obtained. To fulfill standard breeding practices, all samples used possessed a minimum motility rate of 60 % postcollection and met the breeding requirements of the swine unit. From the filtered ejaculate, a 30 mL sample was retrieved and extended with 60 mL of a pre-heated X-Cell extender (IVM; Maple Grove, MN). The sample was then packaged for transport to one of two possible laboratories.

Experiment #1. Five semen samples from five boars were collected and processed individually for this experiment. Upon arrival, the extended sample from each boar was placed in a refrigeration unit (17 °C) for 1 h. At the end of the refrigeration, 210 mL of a wash buffer maintained at 17 ºC was added before continuing the storage in refrigeration unit for another 1.5 h. The samples were then centrifuge for 20 min at 805 x g (Sorvall RT6000 Centrifuge, Kendro Lab Products; Asheville, NC) in a 15 ºC setting. The supernant fluid was removed and discarded. The remaining pellets were reconstituted in 30 mL of a 25 % egg-yolk extender, Boarciphos A (IVM; Maple Grove, MN), also maintained at 17 °C. The sample was allowed to equilibrate for 1.5 h in a secondary refrigeration unit set at 5 °C. With 10 min intervals between, a second 25 % egg-yolk extender with 6 % glycerol, Boarciphos B (IVM; Maple Grove, MN), cooled to 5 ºC, was added three times in 10 mL increments. After the entire 30 mL was added, the sample was maintained at 5 ºC for 1 h. One ml aliquots of the extended sample were placed in 1.8 mL labeled cryo-vials (Nalge Nunc International; Denmark) before being cryopreserved. Through labeling, specimens were separated into three freezing treatment groups: Control: Liquid nitrogen mist for 10 min A: 5 min held in a fluid vat at -10 ºC followed by 4 min in a fluid vat at -25 ºC (average cooling rate -4.3 o C/min) B: 5 min held in a fluid vat at -10 ºC followed by 4 min in a fluid vat at -25 ºC, both vats equipped with a proprietary filter to slow cooling rates (average cooling rate -2.8 o C/min) Freezing treatments ”A” and ”B” were two optimal freezing treatments of a unique freezing technology (UFT: Supachill USA; Lubbock, TX) developed in previous trials within our laboratory (references). Once frozen, samples were plunged into liquid nitrogen and stored until testing began for lipid peroxidation rates and lipid free radical amounts. Experiment #2. For Exp. #2, we used fresh post-ejaculate boar samples (N=5) and extended postejaculate samples (N=5) for lipid stability comparisons from five boars. The above protocol discussed in semen processing was utilized and completed until arrival at the off-site lab. One mL aliquots were extracted from all 10 samples and placed in 1.8 mL cryo-tubes (Nalge Nunc International; Denmark). Immediately afterwards, each vial was plunged in liquid nitrogen for freezing and storage. Experiment #3. The third phase of this trial utilized the same protocol discussed above in Exp. #1 on three semen samples from separate boars. However, the proportions of original semen sample and the various extenders used were increased. This ensured sufficient amounts for three 1 mL aliquots of semen samples and fluid to be extracted during each major step of the protocol without influencing final ratios. The semen samples represent the whole fractions available at that step. Fluid samples were created by centrifuging addition samples during each step specified below. The procedural steps represented include:

1. Ejaculation samples post-transport 2. Extended samples post-transport 3. Extended samples cooled to 17 ºC for 1 h 4. Samples equilibrated with a wash buffer at 17 ºC for 1.5 h 5. Samples after centrifugation for 20 min at 805 x g 6. Samples equilibrated with the first freezing extenders at 5 ºC for 1.5 h 7. Samples equilibrated with the second freezing extenders at 5 ºC for 1.5 h 8. Post-thaw values of optimal freezing treatment.

Due to the density of the semen pellet after centrifugation and concerns of altering final concentrations, a semen sample was not extracted for this step. While thawing the samples, fluid specimens were not removed for analysis. After extraction during each step, the aliquots were plunged and stored in liquid nitrogen.

5 b) Quantification of Lipid Peroxidation

After thawing at room temperature and then being vortexed, a 200 ?L sample was extracted from each aliquot and utilized for this test. The semen samples were mixed with 200 ?L of SDS solution, 1.5 mL of 20% AA solution (at 3.5 pH), 1.5 mL TBAR solution (1.6 g 2-thiobaritric acid with 200 mL of deionized water) and 600 ?L of deionized water. After vortexing each sample, the samples were incubated in a 100 ºC water bath for 2 h. The samples were then cooled for 10 min at room temperature before centrifuged at 1006 x g (CRU-500 Centrifuge, Damon/IEC; Needham Heights, MA) for 10 min. A 0.5 mL sample of fluid was again extracted from each tube and mixed with 2 mL of butanol. After a second centrifugation at 1200 x g (Sorvall RC5C Centrifuge,

Kendro Lab Products; Asheville, NC) for 10 min, each fluid sample was transferred into a glass absorbance box individually for analysis at 532 nm wavelength in a spectrophometer (Coleman 575, Perkin-Elmer; Oak Brook, IL).

6 c) Quantification of Free Lipid Radicals

A 250 ?L sample was extracted from each specimen and placed into a pre-labeled 12 x 75 mm glass tube. After diluting each sample in 2 mL of a 2:1 chloroform:heptane mixture, each tube was thoroughly vortexed. The samples were then centrifuged for 10 min at 1572 x g (CRU-500 Centrifuge, Damon/IEC; Needham Heights, MA). Fluid in the amount of 700 ?L was extracted from each specimen and transfer to a new set of glass tubes. Samples were dried under a flow of normal air at ambient temperatures for 15-20 min. The dried extracts were reconstituted in 2 mL of heptane before performing a spectrophotometer (Coleman 575, Perkin-Elmer; Oak Brook, IL) analysis at a 233 nm wavelength. The lipid radical control, required for proper calculations, was processed the same as a sample except the control contained 250 ?L of water instead of a sample.

7 d) Statistical Analysis

The analysis of the lipid peroxidation and lipid free radical amounts for the three freezing treatments was analyzed using analysis of variance (ANOVA). Boar and freezing treatment were both used as part of the statistical model. The means were separated through Fisher's Least Squared Differences (LSD).

Results of the fresh and extended values for lipid peroxidation were compared using. Though the results were significant, the sample size was too small to complete a LSD analysis.

On the procedural analysis in Exp. #3, the lipid peroxidation and lipid free radical amounts were also analyzed using the ANOVA model. Since these values represent accumulations of lipid instability over time, a linear regression model function was used to analyze each parameter.

All statistical analyses were performed using the SPSS Version 8.0 Software (SPSS, Inc.; Chicago, IL).

8 III.

9 Results

10 a) Experiment #1

When comparing the three freezing treatments using five separate semen samples, the differences between the treatments were not significant for either lipid evaluations. However, there was an interaction between the boar and lipid peroxidation amounts ($P < 0.005$). These differences, shown in Table **??**, concur with the variability of semen quality between boars. When comparing membrane lipid stability between fresh, post-ejaculation samples and extended post-ejaculation samples there was a significant difference. Lipid peroxidation rates were much higher for the fresh samples as compared to the extended samples ($P < 0.001$; Table **??**I). Different letter superscripts indicate significant differences between the lipid peroxidation levels of the sample types ($P < 0.001$)
c) Experiment #3

Referring to Table **??**II, lipid peroxidation levels accumulated during the handling procedure in both specimen types was evaluated. The levels of peroxidation were significantly different within the semen samples ($P < 0.001$) and fluid samples ($P < 0.001$). Using the LSD procedure, significant differences were observed at various transition points of the procedure including steps 1 to 2, steps 4 to 6, and steps 7 to 8 in the sample ($P < 0.05$) and fluid ($P < 0.001$). Furthermore, increasing amounts of MDA were correlated between the semen and fluid levels ($r = 0.96$). These increases in peroxidation rates are emphasized in Figure I. **??**,d) indicate significant differences between lipid peroxidation levels of the procedural steps in the samples ($P < 0.05$). Different letter superscripts (w,x,y,z) indicate significant differences between lipid peroxidation levels of the procedural steps in the fluid extracted ($P < 0.001$).

Referring to Table **??**V, lipid free radical levels also showed significant variations during the procedure for the samples ($P < 0.001$) and fluids ($P < 0.001$) evaluated. The amounts found from the samples and fluid extracted were again correlated ($r = 0.985$). Using the LSD procedure, a significant increase was found after the addition of the egg-yolk freezing extender in the sample ($P < 0.001$) and fluid ($P < 0.001$). The lipid free radical accumulation is presented in Figure II. In lieu of the accumulation trends of lipid instability, the reduction of motility is correlated with higher levels of both lipid peroxidation ($r = -0.913$, $P < 0.001$) and free radicals ($r= -0.940$, $P < 0.001$) as demonstrated in Table **??**. Different letter superscripts (a,b) indicate significant differences between lipid free radical levels of the procedural steps in the samples ($P < 0.001$). Different letter superscripts (y,z) indicate significant differences between lipid free radical levels of the procedural steps in the fluid extracted ($P < 0.001$).

IV.

11 Discussion

High lipid peroxidation levels have been associated in several articles with reduced sperm functionality. [1][2][3][4] Similar results were found in the present study as well (refer to Table **??**). However, it has been suggested in other

articles that the cause of this membrane degradation was due to the mechanical stresses of cryopreservation. Our data suggests that the changes observed are due to the pre-freezing procedures, especially the addition of egg-yolk extenders at a cooled state (refer to Tables **??**II and IV). Different number superscripts indicate significant differences between motility percents ($P < 0.001$). Different letter superscripts (a,b) indicate significant differences between lipid peroxidation levels ($P < 0.001$). Different letter superscripts (y, z) indicate significant differences between lipid free radical amounts ($P < 0.001$). Negative correlations were found between motility percentages and lipid peroxidation (r = -0.913) amounts and between motility percentages and lipid free radical (r = -0.940) amounts.

Centrifugation is a common step to concentrate the specimen in the cryopreservation preparation process. Yet it has been suggested that centrifugation may cause undue stress. 14 It has been observed in dog semen, that lower centrifugal speeds resulted in a higher amount of sperm lost in the supernate while viability losses were higher at increased centrifugational velocities. Yet, membrane integrity was maintained at the various speeds. 15 Our evaluation of the seminal fluid extracted before and after centrifugation, show very little difference in lipid stability (refer to Table **??**II and IV).

Neild and colleagues reported a capacitation-like phenomenon after centrifugation of stallion sperm. 16 Thus, permeability alterations may be due to other sources than lipid peroxidation.

Protein degradation due to peroxidation has been observed. 8 Baumber et al. correlated high peroxidation levels in post-thawed semen with DNA fragmentation. 17 This may actually be the cause for the reduced fertilization capability recognized in sperm after cryopreservation . 18 As observed in bovine semen, our results showed higher levels of peroxidation in frozen-thawed semen versus fresh or cooled (refer to Tables **??**II and IV). However, due to the trends at various points in the procedural analysis, our results do not demonstrate the stress in relation to cooling and thawing as suggested by Chatterjee and Gagnon. 19 Instead, the largest accumulation of both lipid peroxides and lipid radicals was observed during the addition of the freezing egg yolk extender (refer to Tables **??**II and IV). Egg yolk contains phosphatidylcholine, similar to that already present in the membrane of sperm. Though studies have demonstrated the protective effects of egg yolk, 20 our results indicate a negative side. Misik and colleagues 21 demonstrated the amplification in MDA levels in egg yolk held at 22 ^{o}C compared to 50 ^{o}C. The introduction of a diluent with ongoing lipid peroxidation or the addition of a product, specifically lipid peroxides (LOOH), will enhance lipid peroxidation. 22 Thus, alternative extenders need to be investigated. Braun and colleagues observed an increase in the membrane integrity and motility of frozen stallion semen with the exclusion of egg-yolk. 23 Dacaranhe and Terao observed reduced iron-peroxidation levels with the presence of phosphatidylserine. 24 A trial on frozen stallion semen has already verified an improvement in motility with the addition of phosphatidylserine . 25 The addition of antioxidants, such as Vitamin E, may need to be incorporated into breeding schemes to reduce lipid peroxidation rates. Increases in semen quality have been observed in 26 turkey, boar, 12,27 ram, 28 and horse samples 17 with antioxidant supple-mentation. Dietary antioxidant supplementation has also increased semen quality in boars 27 and roosters . 29 In the swine industry, semen cryopreservation is not frequently used due to the low number of piglets commonly produced. Previous studies have suggested that the structure of the sperm cells is damaged by the mechanical stress of freezing, thus reducing function . One example is the difference observed in the membrane lipid stability before and after freezing. In the present study, we attempted to find when these changes occur by comparing semen samples from various procedural steps in the freezing process. The most significant lipid stability change was observed after an egg-yolk extender was added to the sample. Our results suggest the lipid alterations may not be due to the mechanical stress of freezing but rather by the extenders used. Though many freezing protocols use egg-yolk as a protectant and nutrition source for sperm, new extenders that stabilize lipids may need to be investigated. Also, dietary vitamin supplements may also improve outcomes. Further research is needed.

1

	Lipid Peroxidation		Radicals	
Boar	(nM/mL)	SEM	(A233/mL)	SEM
Yellow (n = 3)	62.3 b	2.79	5.7	0.28
Pink (n = 3)	58.6 b	2.19	5.5	0.24
White (n = 3)	58.2 b	2.36	5.7	0.18
Green (n = 3)	52.2 a	2.19	5.9	0.10
Orange (n = 3)	50.8 a	2.76	5.8	0.25

Different letter superscripts indicate significant differences between the lipid peroxidation of five boars ($P <$ b) Experiment #2

Figure 1: Table 1 :

2

Sample	n	Lipid Peroxidation (nM/mL)	SEM
Fresh 5		14.3 a	2.24
Extended	5	1.6 b	0.36

Figure 2: Table 2 :

3

Malondialdehyde Amounts (nM/mL)

[Note: Different letter superscripts (a,b,]

Figure 3: Table 3 :

4

Free Radical Amounts (nM/mL)

Figure 4: Table 4 :

5

Lipid Peroxidation Radicals

Figure 5: Table 5 :

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