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Decrease in glutathione content in boar sperm after cryopreservation Effect of the addition of reduced glutathione to the freezing and thawing extenders

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Abstract

Although glutathione content in boar spermatozoa has been previously reported, the effect of reduced glutathione (GSH) on semen parameters and the fertilizing ability of boar spermatozoa after cryopreservation has never been evaluated. In this study, GSH content was determined in ejaculated boar spermatozoa before and after cryopreservation. Semen samples were centrifuged and GSH content in the resulting pellet monitored spectrophotometrically. The fertilizing ability of frozen–thawed boar sperm was also tested in vitro by incubating sperm with in vitro matured oocytes obtained from gilts. GSH content in fresh semen was 3.84 ± 0.21 nM GSH/10^8 sperm. Following semen cryopreservation, there was a 32% decrease in GSH content ($P < 0.0001$). There were significant differences in sperm GSH content between different boars and after various preservation protocols ($P = 0.0102$). The effect of addition of GSH to the freezing and thawing extenders was also evaluated. Addition of 5 mM GSH to the freezing extender did not have a significant effect on standard semen parameters or sperm fertilizing ability after thawing. In contrast, when GSH was added to the thawing extender, a dose-dependent tendency to increase in sperm fertilizing ability was observed, although no differences were observed in standard semen parameters. In summary, (i) there was a loss in GSH content after cryopreservation of boar semen; (ii) addition of GSH to the freezing extender did not result in any improvement in either standard semen parameters or sperm fertilizing ability; and (iii) addition of GSH to the thawing extender resulted in a significant increase in sperm fertilizing ability. Nevertheless, future studies must conclude if this is the case for all boars. Furthermore, since addition of GSH to the thawing extender did not result in an improvement in standard semen parameters, this suggests that during the thawing process, GSH prevents damage of a

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sperm property that is critical in the fertilization process but that is not measured in the routine semen analysis.

Keywords: Pig spermatozoa; Glutathione; Freezing; IVF

1. Introduction

The process of cooling and freeze–thaw produces physical and chemical stress on the sperm membrane that reduces sperm viability and fertilizing ability. Cold shock of spermatozoa is associated with oxidative stress and reactive oxygen species (ROS) generation [1].

ROS-induced damage to sperm is mediated by oxidative attack of bis-allylic methylene groups of sperm phospholipid-bound polyunsaturated fatty acids, leading to lipid peroxidation [2]. Since boar sperm have a high polyunsaturated fatty acid content they are very susceptible to lipid peroxidation [3,4]. The effects of lipid peroxidation include irreversible loss of motility, inhibition of respiration, leakage of intracellular enzymes, damage to sperm DNA [5], or deficiencies in oocyte penetration and sperm–oocyte fusion [6]. Semen represents a complex redox system that combines the antioxidant potential of seminal plasma and spermatozoa with the pro-oxidant potential of sperm through the production of ROS. Enzymatic antioxidant defense mechanisms in seminal plasma and spermatozoa include superoxide dismutase, glutathione reductase, gluthathione peroxidase and catalase. Non-enzymatic antioxidants include reduced glutathione (GSH), urate, ascorbic acid, Vitamin E, taurine, hypotaurine, carotenoids, and ubiquinones. The interplay of antioxidant and pro-oxidant mechanisms in semen determines the overall rate of lipid peroxidation in sperm.

Glutahione (L-γ-glutamyl-L-cysteinylglycine) is a tripeptide ubiquitously distributed in living cells. It plays an important role in the intracellular defense mechanism against oxidative stress [2]. Glutathione peroxidase uses GSH as the reducing equivalent to reduce hydrogen peroxide to H_2O and lipoperoxides to alkyl alcohols. The resulting oxidized glutathione (GSSG) is reduced to GSH by glutathione reductase using NADPH as the co-factor.

GSH content has been reported in mammalian sperm [7,8], including boar sperm [9]. However, GSH content in boar sperm before and after cryopreservation has never been evaluated.

The main objectives of this study were (i) to determine GSH content in boar sperm before and after cryopreservation; and (ii) to assess the effect of GSH supplementation of freezing and thawing extenders on standard semen parameters and sperm fertilizing ability in IVF.

2. Material and methods

Semen was routinely collected from mature fertile boars using the manual method and a dummy. The sperm-rich fraction was collected in a pre-warmed thermo flask and the gel-fraction was held on a gauze tissue covering the thermo opening. The semen was then diluted with isothermal Beltsville thawing solution (BTS) extender at a ratio of 1:1 (v/v).

2.1. Freezing and thawing protocol

Semen samples were processed using the straw freezing procedure described by Westendorf et al. [10] with minor modifications indicated in the following. Diluted semen was placed at 15 °C for 2 h and later centrifuged at 800 × *g* for 10 min. The supernatant was discarded and the semen pellet was re-suspended with lactose–egg yolk extender (LEY, 80 ml of 11% lactose and 20 ml egg yolk) to provide 1.5×10^9 spermatozoa/ml. After further cooling to 5 °C over a 90-min period, two parts of LEY–extender semen were mixed with LEY extender with 1.5% Orvus Es Paste (Equex-Paste, Minitüb, Tiefenbach, Germany) and 9% glycerol. The final concentration of semen to be frozen was 1×10^9 spermatozoa/ml and 3% glycerol. The diluted and cooled semen was loaded into 0.5 ml straws (Minitüb) and placed in liquid nitrogen vapor approximately 3 cm above the level of the liquid nitrogen for 20 min. The straws were then stored in liquid nitrogen until thawing.

Thawing was achieved by immersing the straws in a circulating water bath at 50 °C for 12 s [11]. Immediately after thawing, the semen was diluted in BTS.

2.2. Determination of GSH content in spermatozoa

Semen samples were centrifuged at 1000 × *g* for 5 min at room temperature and the resulting pellet resuspended in BTS and centrifuged again. The supernatant was discarded, the pellet resuspended in BTS, and sperm concentration adjusted to $1–5 \times 10^8$ sperm/ml. To release intracellular GSH, the sperm cells were lysed following three cycles of rapid cooling in liquid nitrogen and thawing at 37 °C. The resulting suspensions were centrifuged at 7000 × *g* for 10 min in order to remove membrane fragments.

Glutathione content was determined using a modified coupled optical test system [12]. In this system glutathione is oxidized by 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and then reduced by glutathione reductase with NADPH as hydrogen donor. During the oxidation of glutathione by DTNB, 2-nitro-5 thiobenzoeic acid is formed, which can be detected photometrically by a change of absorption at 412 nm. The total glutathione content (oxidized glutathione (GSSG) and reduced glutathione (GSH)) is calculated according to a standard curve.

2.3. Analysis of standard semen parameters

Percent motility and progression were determined by placing two sample aliquots on warm glass slides (39 °C) and examined under light microscopy (magnification 100×). The percentage of motile sperm was estimated to the nearest 5% and the forward progressive motility (FPM) using an arbitrary scale from 0 to 5.

The proportion of spermatozoa with a normal apical ridge (NAR) was evaluated after fixation in a buffered 2% glutaraldehyde solution and examined under phase-contrast microscopy (magnification 1000×) to analyse acrosomes [13]. NAR was determined on two slides per sample and a total of 200 spermatozoa per sample.

Eosin–nigrosin viability staining of sperm was also performed (EN). Semen was diluted 1:1 (v/v), with the staining solution (5% yellow eosin, 10% nigrosin in a citrate solution,

pH 7.4) and smeared onto slides. After being air-fixed, the stained spermatozoa were observed under brightfield microscopy and 200 sperms per sample were evaluated [14].

Sperm membrane integrity was evaluated applying a combination of the fluorophores carboxyfluorescein diacetate (DCF) and propidium iodide [15] on at least 200 cells per sample using an epifluorescence microscope.

2.4. In vitro fertilization protocol

Ovaries from prepuberal gilts were obtained at a local slaughterhouse and transported to the laboratory in saline solution (0.9%, w/v, NaCl) with 100 mg/l kanamicyn at 35 °C. Oocytes surrounded by cumulus cells, were obtained from 3 to 6 mm diameter follicles and washed twice in 35 mm plastic Petri-dishes containing modified Dulbecco's phosphate buffered saline (mPBSD) supplemented with 1 mg/ml polyvinyl alcohol and 0.005 mg/ml phenol red. They were washed twice again in maturation medium previously equilibrated for a minimum of 3 h at 38.5 °C under 5% CO_2 in 95% humidified air.

The culture media used for oocyte maturation was Waymouth medium supplemented with 10 UI/ml PMSG, 10 UI/ml hCG, 1 μg/ml estradiol-17b, 10% (v/v) foetal calf serum and 10% porcine follicular fluid (v/v), as previously described by Coy et al. [16]. The maturation medium was added to the Petri-dish in 3×100 μl droplets covered with paraffin oil and 20 oocytes introduced in each droplet and incubated at 38 °C under 5% CO_2 in air.

The in vitro fertilisation medium was TCM199 supplemented with 12% heat inactivated foetal calf serum, 0.9 mM sodium pyruvate, 3.05 mM D-glucose, 8.75 mM calcium lactate, 0.68 mM penicillin G, 3.6 mM caffeine and 0.068 mM streptomicyn sulphate at pH 7.4, as previously described [16].

After thawing, the sperm samples were centrifuged at $50 \times g$ for 3 min and the supernatants centrifuged at $1200 \times g$ for 3 min. The resulting pellets were diluted in supplemented TCM199 without calcium lactate and caffeine. Aliquots of 100 μl of semen were placed on Petri-dishes containing 2 ml of fertilization medium (final concentration of 1×10^6 spermatozoa/ml) and 20 in vitro matured oocytes previously washed twice in equilibrated fertilization medium. After 18 h, the cultured oocytes were fixed in 3:1 (v/v) ethanol:acetic acid for 24 h, stained with 1% lacmoid and examined under a phase contrast microscope to assess penetration rate (PEN), mean number of sperm per penetrated oocyte (S/O), monospermy rate (MON) and rate of male pronuclear formation (MPF).

2.5. Experimental design

2.5.1. Experiment 1: evaluation of GSH content in ejaculated boar spermatozoa

Semen parameters and GSH content in boar ejaculated spermatozoa were determined in 44 ejaculates from 27 boars.

2.5.2. Experiment 2: determination of GSH content in fresh, refrigerated or cryopreserved boar spermatozoa

Semen parameters and GSH content were determined in boar spermatozoa from 25 ejaculates from 10 boars, after refrigeration for 24 h at 15 °C and after cryopreservation.

2.5.3. Experiment 3: effect of GSH supplementation of the freezing extender on the viability and in vitro fertilizing ability of cryopreserved boar spermatozoa

Ejaculates from four boars (four per boar) were processed with or without addition of 5 mM GSH to the freezing extender. Standard semen parameters and in vitro sperm fertilizing ability were evaluated.

2.5.4. Experiment 4: effect of GSH supplementation of the thawing extender on the viability and in vitro fertilizing ability of cryopreserved boar spermatozoa

To examine the effect of GSH supplementation during the thawing process, frozen straws from two different boars (four ejaculates per boar) were diluted immediately after thawing in BTS (control), BTS + 1 mM GSH and BTS + 5 mM of GSH. After 30 min of equilibration, standard semen parameters were evaluated and the sperm fertilizing ability tested in the IVF system.

2.6. Statistical analysis

Data are expressed as the mean ± S.E.M. and analysed by two-way ANOVA, considering the specific sperm treatment and boar samples as the main variables in experiments 2–4. IVF data (experiments 3 and 4) were modelled according to the binomial model of parameters and were analysed by two-way ANOVA. When ANOVA revealed a significant effect, values were compared by the Tukey test. Differences were considered statistically significant at $P < 0.05$.

Linear regression analysis was used to further investigate relationships between semen parameters (Pearson correlation) in experiment 2.

3. Results

3.1. Experiment 1: evaluation of GSH content in ejaculated boar spermatozoa

GSH content in ejaculated boar spermatozoa was determined in 44 ejaculates from 27 boars. The mean value was 3.84 ± 0.21 nM GSH/10^8 cells. There were significant differences in GSH content between different boars ($P < 0.001$) ranging from 1.05 to 6.16 nM GSH/10^8 cells.

3.2. Experiment 2: determination of GSH content in fresh, refrigerated or cryopreserved boar spermatozoa

Standard semen parameters and GSH content were determined in freshly ejaculated spermatozoa, refrigerated sperm suspensions for 24 h at 15 °C, and frozen–thawed spermatozoa. GSH content was significantly lower in cryopreserved sperm as compared to fresh ejaculated spermatozoa ($P < 0.0001$, Table 1), with a 32% decrease after cryopreservation. Significant differences were also observed between boars, and preservation protocols (interaction $P = 0.0102$).

Table 1
Glutathione content (nM GSH/10^8 cells) and sperm quality parameters in fresh, stored and cryopreserved spermatozoa (ejaculates = 25) (mean ± S.E.M.)

	GSH nM/10^8 cells	Motility (%)	FPM (0–5)	EN (%)	NAR (%)	DCF (%)
Treatment						
Fresh	4.99 ± 0.23[a]	74.61 ± 1.27[a]	3.21 ± 0.07[a]	84.42 ± 1.39[a]	92.53 ± 0.59[a]	90.42 ± 1.31[a]
Stored for 24 h at 15 °C	4.72 ± 0.18[a]	69.03 ± 1.59[a]	3.08 ± 0.04[a]	83.15 ± 1.99[a]	91.23 ± 1.08[a]	84.08 ± 1.68[a]
Frozen–thawed	3.36 ± 0.34[b]	50.00 ± 2.31[b]	3.74 ± 0.07[b]	56.68 ± 2.98[b]	33.24 ± 2.13[b]	43.36 ± 3.47[b]
Source of variability						
Boar	0.0319	0.4351	0.0157	0.1041	0.0399	0.0095
Treatment	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Boar × treatment	0.0102	0.2339	0.1552	0.7794	0.1059	0.3911

Numbers within columns with different superscripts (a and b) differ ($P < 0.05$). FPM: forward progressive motility (0–5). EN: eosine–nigrosine stain. NAR: normal apical ridge. DCF: sperm membrane integrity assessed with carboxyfluorescein diacetate. ANOVA, *P*-values.

All semen parameters were affected by preservation method with significant decrease in frozen–thawed semen, except forward motility, which showed an increased value in frozen semen. Although there were differences in semen parameters between different boars (FPM, NAR and DCF), the interaction value was not significant (Table 1).

No significant correlation was observed between semen parameters and GSH content after different preservation treatments. Pearson correlation coefficient analysis indicated an inverse correlation between GSH and NAR in ejaculated spermatozoa ($r = -0.4263$, $P = 0.0002$) and a direct correlation between GSH and forward progressive motility in cryopreserved sperm ($r = 0.2960$, $P = 0.0411$, Table 2).

3.3. Experiment 3: effect of GSH supplementation to the freezing extender on the viability and in vitro fertilizing ability of cryopreserved boar spermatozoa

Addition of 5 mM GSH to the freezing extender had no a significant effect on semen parameters (data not shown) or sperm fertilizing ability (Table 3). However, significant

Table 2
Relationships between seminal parameters and GSH content in fresh, stored and cryopreserved spermatozoa (*r*: Pearson correlation coefficient; *P*: significance)

	Fresh		Stored for 24 h at 15 °C		Frozen–thawed	
	r	*P*	*r*	*P*	*r*	*P*
Motility	0.2006	0.0911	0.0949	0.5121	−0.1135	0.4425
FPM	0.0923	0.4404	−0.1284	0.3741	0.2960	0.0411
EN	0.1457	0.2221	0.2124	0.1386	−0.1439	0.3293
NAR	−0.4263	0.0002	0.1807	0.2091	−0.1197	0.4179
DCF	0.0031	0.9792	0.2842	0.0502	0.1006	0.4962
Morphology	0.0784	0.5124				

FPM: forward progressive motility (0–5). EN: eosine–nigrosine stain. NAR: normal apical ridge. DCF: sperm membrane integrity assessed with carboxyfluorescein diacetate.

Table 3
IVF results for mature oocytes fertilized with frozen–thawed boar sperm in two different treatments, with (GSH) or without (control) addition of GSH to the freezing extender: rate of penetration (%PEN), mean number of spermatozoa per penetrated oocyte (S/O), monospermy rate (%MON) and rate of male pronuclear formation (%MPN)

Boar	Treatment	Number of oocytes	%PEN	S/O[a]	%MON[a]	%MPN[a]
67	Control	91	87.91 ± 3.44[a]	2.41 ± 0.17[a]	36.25 ± 5.41[a]	72.50 ± 5.02
67	GSH	97	69.07 ± 4.72[a,b]	1.83 ± 0.12[a,b]	43.28 ± 6.10[a,b]	73.13 ± 5.46
A	Control	87	56.32 ± 5.35[b]	1.81 ± 0.15[a,b]	53.06 ± 7.20[a,b]	83.67 ± 5.33
A	GSH	96	66.67 ± 4.84[b]	2.25 ± 0.21[a,b]	43.75 ± 6.25[a,b]	79.69 ± 5.07
BB	Control	117	75.94 ± 3.72[a]	2.08 ± 0.17[a,b]	52.48 ± 4.99[a,b]	71.29 ± 4.52
BB	GSH	115	72.06 ± 3.86[a]	2.25 ± 0.17[a]	47.96 ± 5.07[a,b]	73.40 ± 4.58
PI774	Control	124	33.06 ± 4.24[c]	1.75 ± 0.16[a,b]	56.10 ± 7.85[a,b]	65.85 ± 7.50
PI774	GSH	123	31.71 ± 4.21[c]	1.36 ± 0.09[b]	69.23 ± 7.49[b]	74.36 ± 7.08
Source of variability						
Treatment			0.264	0.479	0.727	0.743
Boar			<0.001	0.012	0.011	0.190
Interaction			0.020	0.017	0.320	0.815

Numbers within columns with different superscripts (a–c) differ ($P < 0.05$).
[a] Based on penetrated oocytes.

differences between boars were observed for each IVF parameter ($P < 0.015$, Table 3), except for %MPN. Concerning penetration rate and sperm number per penetrated oocyte, ANOVA indicated that there were significant differences between boars, as also shown by the significant value obtained for the interaction of preservation treatment and individual boars ($P = 0.020$ and $P = 0.017$, respectively).

Table 4
IVF results for mature oocytes fertilized with frozen–thawed boar sperm in three different treatments, with (1 and 5 mM GSH) or without (control) addition of GSH to the thawing extender: rate of penetration (%PEN), mean number of spermatozoa per penetrated oocyte (S/O), monospermy rate (%MON) and rate of male pronuclear formation (%MPN)

Boar	Treatment	Number of oocytes	%PEN	S/O[a]	%MON[a]	%MPN[a]
PI774	Control	135	18.52 ± 3.36[a]	1.20 ± 0.08[a]	80.00 ± 8.16[a]	48.00 ± 10.20[a]
PI774	1 mM GSH	154	25.97 ± 3.54[a,b]	1.32 ± 0.10[a]	77.50 ± 6.69[a]	60.00 ± 7.84[a,b]
PI774	5 mM GSH	148	31.76 ± 3.84[b]	1.36 ± 0.10[a]	74.47 ± 6.43[a]	72.34 ± 6.60[a,c]
BB	Control	96	83.33 ± 3.82[c]	3.16 ± 0.29[b]	31.25 ± 5.21[b]	81.25 ± 4.39[b,c]
BB	1 mM GSH	107	85.05 ± 3.46[c]	3.44 ± 0.17[b]	14.29 ± 3.69[b]	84.62 ± 3.80[c]
BB	5 mM GSH	118	97.46 ± 1.46[c]	4.01 ± 0.28[b]	23.48 ± 3.97[b]	84.35 ± 3.40[c]
Source of variability						
Treatment			<0.001	0.254	0.292	0.062
Boar			<0.001	<0.001	<0.001	<0.001
Interaction			0.578	0.480	0.409	0.167

Numbers within columns with different superscripts (a–c) differ ($P < 0.05$). ANOVA, P-values.
[a] Based on penetrated oocytes.

3.4. Experiment 4: effect of GSH supplementation to the thawing extender on the viability and in vitro fertilizing ability of cryopreserved boar spermatozoa

No significant differences in semen parameters were found between control and GSH-supplemented thawing extender (data not shown). In contrast, GSH addition to the thawing extender resulted in an significant increase in sperm in %PEN ($P < 0.001$) and also an increase in %MPN, although it did not reach statistical significance ($P = 0.062$) (Table 4). For all the studied IVF variables a significant boar effect was detected ($P < 0.001$).

4. Discussion

The main findings emerging from this study are the decrease in GSH content observed in boar sperm after cryopreservation and a tendency to increase in boar sperm fertilizing ability after addition of GSH to the thawing extender.

Glutathione is the major non-protein thiol compound in mammalian cells. GSH participates in a number of cell functions including amino acid transport, DNA and protein synthesis, reduction of disulfide bonds and protection against oxidative stress. The sulphydryl groups of GSH have been shown to confer protection against cell damage by oxidants, electrophiles and free radicals (reviewed by Irvine [2]).

The results obtained for GSH content in ejaculated boar semen (3.84 ± 0.21 nM/10^8 cells) are in good agreement with previous reports (3 nM GSH/10^8 cells [9]) and with the results obtained in other mammalian species including human sperm (6.7 ± 0.4 nM GSH/10^8 cells [7]; 6.2 ± 0.6 nM GSH/10^8 cells [17]; 3.49 ± 0.87 nM GSH/10^8 cells [8]), and bull sperm (2.73 ± 0.42 nM GSH/10^8 cells [18]). As previously reported, GSH values in mouse sperm are significantly higher than in other mammalian species (90 nM GSH/10^8 cells [7]). It is noteworthy that comparisons between different studies are often difficult to interpret due to differences in the method of analysis [19] and sperm processing protocols [20].

The results of experiment 1 indicated significant differences in GSH content between different boars. These differences could be related to differences in the fertility potential of these boars [21]. A relationship between GSH content in sperm and seminal plasma and infertility has been previously found in human sperm [8,22] and also between GSH content in sperm and their ability to penetrate bovine cervical mucus [8].

The results also show that cryopreservation of boar sperm is associated with a significant decrease in GSH content (Table 1), as previously reported in bovine [20] and human sperm (Molla and Gadea, data not published). This would be expected to result in changes in the sulphydryl content of proteins from the sperm membranes and nucleus [23]. Similarly, cryopreservation of boar sperm is associated with a reduction in the sperm motility and viability. Interestingly, forward progressive motility was increased in cryopreserved spermatozoa. This could be related to changes brought about by the media used in boar sperm cryopreservation named capacitation like alterations [1,24]. However, these preliminary results must be confirmed in future studies where sperm motility will be monitored by computer-assisted semen analysis (CASA). In

contrast, preservation of boar spermatozoa at 15 °C did not result in a decrease in GSH content or sperm viability. This suggests that the effects of cryopreservation on GSH content and sperm motility and viability observed are specific to the process of freezing and thawing at low temperatures.

Once we detected a decrease in the GSH content in the boar frozen–thawed spermatozoa, we assessed the effect of GSH supplementation of freezing and thawing extenders on standard semen parameters and sperm fertilizing ability in IVF (experiments 3 and 4). One question that remains to be answered is why addition of GSH to the thawing extender and not to the freezing extender increases boar sperm fertilizing ability in vitro. A plausible explanation for this question would be that cryodamage of antioxidant enzymes during the freezing process results in an overall decrease of enzymatic antioxidant defenses in boar sperm. This damage could selectively affect superoxide dismutase (SOD), glutathione reductase (GRD) and glutathione peroxidase (GPX) to a lesser extent. During thawing, there is an increase in the production of ROS [1], with an increase in the superoxide anion production due to a decrease in SOD activity and a decrease in GSH content due to a decrease in GRD activity and to an increase in GSH oxidation by hydrogen peroxide. A decrease in intracellular GSH and an increase in ROS production during thawing would lead to an increase in lipid peroxidation. GSH provided exogenously after thawing (experiment 4) would increase the intracellular levels of GSH which will be used by GPX to prevent hydrogen and lipid peroxide-induced damage. Addition of GSH to the freezing extender, on the other hand, would result in a high rate of ROS-induced GSH oxidation during rewarming and thawing. This is supported by our results that show that there is a decrease in GSH content during cryopreservation. In contrast, addition of GSH after thawing would allow any residual antioxidant enzymatic activity still remaining in the sperm cell to recover, to decrease steady-state ROS levels, and, therefore, to decrease GSH oxidation. This hypothesis is supported by previous studies which show that there is a decrease in SOD activity during cryopreservation and an increase in lipid peroxidation of human and bovine spermatozoa after thawing [20,25]. Sadly, this information is not well known yet in the boar spermatozoa [26,27]. However, if GSH would be acting in concert with GPX to decrease the steady-state level of hydrogen peroxide and membrane lipid peroxides in boar sperm during thawing, an increase in sperm motility and viability should also be expected.

It has also been reported that damage to the plasma membrane overlaying the flagellum or head during cryopreservation of human sperm can occur independently [28] and Holt and North [29] reported that sperm head plasma membranes were preferentially damaged by freezing and thawing. Although acrosomes became swollen after freezing and thawing, the incidence of outer acrosomal membrane vesiculation remained at control (unfrozen) levels with all treatments. However, the results indicate that these semen parameters are not affected by GSH addition. The only effect that is observed is an increase in boar sperm fertilizing ability. Therefore, cryopreservation-induced oxidative stress in boar sperm affects a sperm property that is related to both sperm binding and penetration of the zona pellucida, sperm–oocyte membrane fusion, oocyte activation, or pronuclear formation. In this sense, the membrane fusion events involved in binding with the oolema and in the acrosome reaction appear to be more vulnerable to ROS-induced damage than is overall motility in human spermatozoa [30].

Also, we have previously demonstrated that the IVF is the most precise tool for evaluating boar semen capacities, in both refrigerated and frozen–thawed semen [11,31]. So the use of IVF systems could help us to detect differences that classical assays are not able to do [32].

Other reports indicate that cryopreservation also results in ROS-induced DNA fragmentation [33]. However, this would only partially explain the results since a decrease in DNA fragmentation by GSH supplementation during thawing would explain the observed increase in pronuclear formation rate, but it would not explain the increase in penetration rate. Nevertheless, it has been shown the relationship between the ability to form a male pronucleus in pig oocytes with intracellular concentration of GSH [34,35]. Also the addition of GSH to the insemination medium in an IVF system improves the developmental competence of in vitro matured pig oocytes in a dose-dependent manner [36].

In summary, there was a loss in GSH content after cryopreservation of boar semen. Addition of GSH to the freezing extender did not result in any improvement in either standard semen parameters or sperm fertilizing ability; while addition of GSH to the thawing extender resulted in a significant increase in sperm fertilizing ability. Nevertheless, future studies must conclude if this is the case for all boars. Furthermore, since addition of GSH to the thawing extender did not result in an improvement in standard semen parameters, this suggests that during the thawing process, GSH prevents damage of a sperm property that is critical in the fertilization process but that is not measured in the routine semen analysis.

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