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Supplemental Glutathione Improves Post-Thaw Quality of Holstein Bulls Sperm in a Nanomicelle based Extender

Research Article

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ABSTRACT

Adding antioxidants to semen extenders could reduce post-thaw sperm damages. Glutathione is an important intracellular antioxidant that has free radicals scavenging properties and multiple effects on DNA and protein synthesis. In the present study, effects different levels of reduced glutathione (GSH) containing 0 (control), 1.0, 2.5, or 5.0 mM in a nanomicelle-based extender on post-thaw quality of bovine sperm were investigated. Initially, lecithin was added to a tris-based extender containing GSH. The mixture was then emulsified by slowly shaking and sonification so that nano size of particles was obtained. Thirty-six ejaculates from six Holstein bulls were used. The post-thaw quality parameters including computer-assisted sperm analysis of motility, plasma membrane functionality, membrane integrity, acrosome integrity, morphology and level of apoptosis were measured. Compared to the control medium, the total motility and membrane integrity were significantly better in samples containing 2.5 mM GSH (67.3±1.2% *vs.* 73±1.2% and 71.3±1.5% *vs.* 77.6±1.5%, respectively). The extender supplemented with 2.5 mM GSH had the highest percentage of viable sperm during apoptotic test. In conclusion, supplementation of lecithin nanomicelle-based extender with 2.5 mM GSH reduced damages to bovine sperm during cryopreservation.

KEY WORDS antioxidant, bovine, cryopreservation, nanomicelle, sperm.

INTRODUCTION

Oxidative stress during freezing and thawing causes an imbalance between pro-oxidants and anti-oxidants whereby free radicals cause lipoperoxidation (Gadea *et al.* 2011). Antioxidant defense mechanisms in sperm strongly depend on glutathione peroxidase/reductase system, catalase, and superoxide dismutase. Reduced glutathione (GSH) is a ubiquitous non-enzymatic antioxidant of mammalian cells. It plays an essential role in intracellular defense mechanisms against free radicals (ROS) (Gadea *et al.* 2011) and is a critical substance for many cellular processes (Ballatori *et al.* 2009). GSH is the most abundant thiol with small molecular weight that presents in mammalian cells, and it is synthesized in the cytoplasm (Wachter *et al.* 2005) by the sequential action of two ATP-dependent enzymes (Bachhawat *et al.* 2013). Intracellular GSH levels are regulated by adjusting the rates of synthesis and export from cells (Ballatori *et al.* 2009). The interaction with enzymes, such as glutathione reductase and glutathione peroxidase (GPx), is the basis of the glutathione protection against ROS (De Oliveira *et al.* 2013). The freezing-thawing process of damages their antioxidant system decreases GSH content (Gadea *et al.* 2004; Gadea *et al.* 2011), and an increased ROS levels (Alvarez and Storey, 1992; Wang *et al.* 1997) which jeopardizes survivability of sperm.

The use of antioxidants is a strategy to overcome cryo-damage related to oxidative stress (Gadea *et al.* 2011). The addition of GSH to the sperm extender of stallions (De Oliveira *et al.* 2013), boars (Estrada *et al.* 2017), dogs (Ogata *et al.* 2015) and bull (Triwulanningsih *et al.* 2010). Reduced the level of ROS during the incubation period improved the motility parameters and viability of cryopreserved sperm (Gadea *et al.* 2011). Furthermore, adding lecithin (Salmani *et al.* 2014) and nanomicelles of lecithin (Nadri *et al.* 2019) to the semen extender improves the cryoprotection of goat semen. Since antioxidants improve post-thaw quality of bovine sperm an even better effect can be expected by combining antioxidants and lecithin nanoparticles in the freezing media, therefore, the objective of the present study was to evaluate the effect on sperm quality of various concentrations of glutathione added to a nanomicelle-based extender for bull sperm cryopreservation.

MATERIALS AND METHODS

All experimental procedures were approved by the Animal Welfare and Ethics Committee of the University of Tehran. Soybean lecithin (L-a-phosphatidylcholine, cat no.P3644) for the current research was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), whereas all other chemicals used in Tris buffer were bought from Merck (Darmstadt, Germany).

Preparation and evaluation of nanomicelles

The extender stock solution contained 249.29 mM Tris, 69.38 mM fructose, 88.48 mM citric acid (pH=7.2) and 5% glycerol 5% (v/v). The nanomicelle stock was prepared by adding 3% (w/v) soy lecithin to a tris buffer at 15 ˚C (Zieliński *et al.* 1989), which was subsequently shaken slowly for15 min. Finally, to prepare micelles with particle size of < 100 nm, the emulsion was sonicated (mechanical probe sonicator, Ultrasonic technology development TOPSONICS, Tehran, Iran) for 45 min in an ice water bath. In the last step remaining titanium was removed from the medium by using a 0.22 µpore syringe. The cleaned medium was then stored at 4 ˚C until incorporated into semen extender. The mean diameter of the lecithin nanomicelle particles was measured by dynamic light scattering (DLS) using a Zetasizer system (Zeta Plus, Brookhaven Instruments Corp., Holtsville, NY, USA). Further characteristics of the nanomicelles, including size distribution, shape, and particle aggregation, were assessed using a transmission electron microscope (TEM; Zeiss Em10C, Oberkochen, Germany). A 20 µL drop of the sample was placed on a Formvar Carbon film coated on 300 mesh copper grid (EMS for 2 min). Excess liquid was absorbed with filter paper then negatively stained with a 20 µL drop of 2% uranyl acetate for 1-2 min and excess liquid was absorbed with filter paper and the grid was allowed to air dry. Grid was examined either on a Zeiss EM10C transmission electron microscope operating at an accelerating voltage of 100 kV and was assessed.

Animals and semen collection

Six healthy Holstein bulls owned by the NDJ Company in Karaj, Iran were used. From each animal, 6 ejaculates were collected from each animal, using an artificial vagina twice a week, for 5 consecutive weeks (totally 60 ejaculates). Ejaculates were transferred to a water bath (34 ˚C) and subsequently evaluated for volume, motility, morphology, and sperm concentration. Samples were frozen following these standards were met: total semen motility ≥ 70%, semen concentration of $\geq 1.0 \times 10^9$ sperm/mL and abnormal sperm count ≤ 15%. In order to compensate for individual differences, the ejaculates were pooled and split into four equal aliquots. These aliquots were diluted at room temperature with extenders containing different concentrations of GSH (0 or control, 1, 2.5 and 5 mM). The final sperm concentration before freezing was 20×10^6 sperm/mL.

Extender preparation

Base semen extender contained Tris 249.29 mM, fructose 69.38 mM and citric acid 88.48 mM (pH, 7.2) and glycerol 5% (v/v). The nanomicelle suspension was split into four aliquots and the final experimental extenders contained no glutathione (control, L-0) or 1 (L-1), 2.5 (L-2.5) or 5 mM (L-5) GSH. The freezing process was performed according to a standard procedure.

Briefly, semen was extended at room temperature (~25 ˚C) for 5 minutes, and then, slowly cooled to 4 ˚C and maintained for a minimum of 4 h. The cooled semen was put into a protective 0.25 mL straws (Biovet, L'Agile France) at 20×10^6 sperm/mL, then sealed with polyvinyl alcohol powder. The straws were exposed to liquid nitrogen vapor, 4 cm above the liquid nitrogen for 12 min. Afterward, the straws were plunged into liquid nitrogen for storage. After a minimum of 8 weeks, the straws were thawed individually at 37 ˚C for 30 s in a water bath and evaluated.

Post-thaw sperm evaluation

Assessment of motility characteristics

Motion characteristics of sperm was measured according to Gil *et al.* (2003). Three straws from independent replicates were thawed at 37 ˚C for 30 s and fluid was pooled in a microtube. Sperm motility was assessed using a computer-assisted sperm analysis system (CASA, Version12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA). Prior to counting all semen samples were diluted 1:1 with Tris-

buffered medium and incubated at 37 ˚C for 5 min. A 5 µL drop of the semen sample was placed on a Makler chamber slide to evaluate sperm motility parameters (beat cross frequency (BCF), average lateral head displacement (ALH), curvilinear velocity (VCL), straight linear velocity (VSL), average path velocity (VAP), straightness (STR), linearity (LIN)). Table 1 shows the setup for computer-assisted analysis (CASA) of bovine sperm kinetics.

Assessment of cell membrane functionality

The hypo-osmotic swelling (Richens *et al.* 2017) test based on the presence of curled and swollen tails of sperm after a hypoosmotic treatment was used. The assay was performed by incubating 10 µL of semen with 1 mL of a 150 mOsm/L hypoosmotic solution (57.6 mM fructose and 19.2 mM sodium citrate) at 37 ˚C for 60 min at room temperature (Schäfer and Holzmann, 2000). The mixtures were homogenized and evaluated under an inverted light microscope. 200 sperms were counted in five different microscopic fields for estimating the coiled and swollen sperms.

Assessment of live and dead sperms or sperm viability

Membrane integrity of sperm was assessed by eosin-nigrosin staining method. The stain contained 1.67 g eosin-Y, 10 g nigrosin, and 2.9 g sodium citrate dissolved in 100 mL distilled water. Sperm smears were prepared by mixing a drop of sperm sample with two drops of stain and spreading it with a coverslip. Viability was measured by counting 400 sperm under a phase-contrast inverted light microscope (CKX41; Olympus, Tokyo, Japan). Sperm (any part from it) stained in pink color was counted as dead sperm (Bucak *et al.* 2007), while live sperm is in shining state.

Assessment of acrosome integrity

The integrity of acrosome was assessed using Pisumsativum agglutinin (PSA) (Thys *et al.* 2009). As described by report of Sharafi *et al.* (2015), 5 µL of sperm suspension was added to 100 µL ethanol (purity, 96%) and 15 min later, 10 µL of the sperm suspension was mixed with 30 µL of PSA on a glass slide. 200 sperm per slide were assessed at 400x with a fluorescent microscope (BX51, Olympus) equipped with fluorescence illumination and an FITC filter (excitation at 455-500 nm and emission at 560-570 nm). Sperm heads stained in green color were counted as intact acrosome, whereas those with no fluorescence color were recorded as damaged or disrupted acrosome.

Assessment of sperm morphology

An aliquot (10 µL) of semen was pipetted into 1.5 mL tubes, containing 1 mL Hancock's solution (Najafi *et al.* 2013b).

The Hancock's solution was prepared by mixing 62.5 mL formalin (37% formaldehyde), 150 mL sodium saline solution, 150 mL buffer solution, and 500 mL double-distilled water. In total, 200 sperm were assessed at 1000× under phase-contrast microscope (CKX41; Olympus, Tokyo, Japan). Readers want to know types of abnormal sperms

Phosphatidylserine translocation assay

Apoptotic sperms were assessed by flow cytometry (FACSCalibur flowcytometer; Becton Dickinson, San Jose, CA, USA) (Sharafi *et al.* 2009). For apoptosis status, Annexin-V was used to show apoptosis by phosphatidylserine translocation in the plasma membrane of sperm.

A commercial PS Detection Kit (IQP, Groningen, the Netherlands) was used according to the manufacturer's instructions. Briefly, sperms were washed in calcium buffer then centrifuged and 10 µL annexin V-FITC was added to 100 µL semen suspension, incubated for 15 min at room temperature. Then, 10 µL of propidium iodide (Gadea *et al.* 2011) were added to the suspension prior to analyzing the sample in the flow cytometer. Sperms were categorized into four classes: live sperm (An-/PI-), early-apoptotic sperm (An+/PI-), late-apoptotic sperm (An+/PI+) and necrotic sperm (An-/PI+).

Statistical analyses

The normality of data distributions were evaluated with the Shapiro-Wilk test and all endpoints were confirmed to have a normal distribution. The effects of GSH concentrations on frozen-thawed sperm quality was inferred by a general linear model (Proc general linear models (GLM) of SAS 9.1 (SAS, 2002)) and pairwise comparisons were performed by Tukey's tests. Results were expressed as mean ± SEM. The level of significance was set on $P < 0.05$.

RESULTS AND DISCUSSION

The nanomicelles had an average size of 73.7 ± 2.3 nm, zeta potential of -19 mv and polydispersity index of 0.29 ± 0.006. Table 2 shows the result of sperm kinetics and motility. Both extenders L-2.5 and L-5 significantly ($P<0.05$) improved survival and motility compared to the control L-0 and L-1($P<0.05$). Other parameters were not significantly different.

In Table 3 the results of the viability and functionality characteristic, as well as the normality of sperm, are given. There were no significant differences between the 4 extenders treatments for cell membrane functionality (HOST), acrosomal integrity or morphology. However, extender L-2.5 significantly better-protected sperm membrane integrity than did the control.

Table 1 Setup for computer-assisted analysis (CASA) of bovine sperm kinetics

Parameters	Set value
Calibration	20 x
Frame rate	50 frame/sec
Chamber depth	20 µm
Magnification	200 X
Minimum contrast	60 pixels
Minimum VCL mean	15 µm/s
Minimum STR mean	0.7 µm/s
Minimum ALH mean	0.3 µm/s

Table 2 Effect of different concentration of glutathione (GSH) on motility and velocity parameters of post-thawed bull sperm motility and further motion parameters measured by computer-assisted analysis (CASA) (Mean±Standard error of the means)

Variable (unit)[1]	Extenders*			
	L-0	L-1	L-2.5	L-5
Total motility (%)	67.3±1.2[b]	70.0±1.2[ab]	73.3±1.2[a]	71.6±1.2[ab]
Progressive motility (%)	43.6±0.9	43.7±0.9	46.5±0.9	46.2±0.9
VSL (µm/s)	26.5±2.6	21.3±2.6	21.2±2.6	22.9±2.6
VCL (µm/s)	106.2±7.2	85.8±7.2	82.1±7.2	91.4±7.2
VAP (µm/s)	41.6±3.9	33.0±3.9	32.3±3.9	34.1±3.9
STR (%)	59.6±0.9	61.3±0.9	60.6±0.9	62.5±0.9
LIN (%)	23.3±1.2	24.5±1.2	23.8±1.2	23.8±1.2
ALH (µm)	3.7±0.3	2.6±0.3	2.4±0.3	2.8±0.3
BCF (Hz)	13.0±0.4	14.5±0.4	15.0±0.4	14.6±0.4

L-0: control (nanomicelle-based extender without GSH); L-1, L- 2.5 and L-5: nanomicelle-based extender with 1, 2.5 and 5 mM of GSH respectively.
VSL: straight linear velocity; VCL: curvilinear velocity; VAP: average path velocity; LIN: linearity; STR: straightness; ALH: average lateral head displacement and BCF: beat cross frequency.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Table 3 Effects of different concentrations of GSH added to Lecithin on cell membrane functionality (%), cell membrane integrity (%), acrosome integrity (%) and sperm normality (%) of post-thawed bull sperm (Mean±Standard error of the means)

Variable (unit)	Extenders*			
	L-0	L-1	L-2.5	L-5
Cell membrane functionality (%)	73.5±2.4	73.7±2.4	77.4±2.4	73.7±2.4
Sperm viability (%)	71.3±1.5[b]	73.4±1.5[ab]	77.6±1.5[a]	75.0±1.5[ab]
Acrosome integrity (%)	66.2±2.1	68.5±2.1	74±2.1	72.4±2.1
Normal morphology (%)	82.2±0.5	85.3±0.5	87.3±0.5	82.9±0.5

L-0: control (nanomicelle-based extender without GSH); L-1, L- 2.5 and L-5: nanomicelle-based extender with 1, 2.5 and 5 mM of GSH respectively.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Table 4 shows that sperm viability was significantly (P<0.05 *vs*. the L-0 and L-1) more reduced by the L-2.5 extender. The mean count for late apoptotic and necrotic sperm was lower for the L-2.5 extender but statistically not significantly different from the other extenders.

The objective of this study was to show the effect of enriching lecithin nanomicelles containing semen extenders with GSH on the quality of frozen-thawed bull sperms. The second objective was to find the optimal concentration of GSH concentration for enrichment of the lecithin nanomicelle fractions. The data showed that supplementation of lecithin- nanomicelles with 2.5 mM GSH improved motility rate, membrane integrity, acrosome integrity, and sperm viability after thawing procedure.

Using a flowcytometric assay, we clearly showed that this concentration of GSH better preserved viable sperm counts and likely decreased late apoptotic and necrotic sperm concentration caused by cryopreservation and thawing.

Furthermore, 2.5 mM GSH addition to the extender improved the motility parameters of bull sperm, whereas a higher concentration of GSH seems to be suboptimal for sperm quality. To the best knowledge of the authors, there is no study published t on the cryoprotective efficacy of GSH enriched nanomicelles in bull semen.

For the commonly used egg yolk based extenders Triwulanningsih *et al*. (2010) reported that adding 0.5 mM glutathione improved bovine sperm survival.

Table 4 Effect of the different concentration of glutathione (GSH) on the percent of viable, apoptotic-like features and necrotic sperm according to the Annexin-V/PI staining of post-thaw bull sperm (Mean±Standard error of the means)

Variable (unit)	Extenders*			
	L-0	L-1	L-2.5	L-5
Viable sperms (% An-/PI-)	61.2±2.5[b]	58.9±2.5[b]	73.6±2.5[a]	63.9±2.5[ab]
Early apoptotic sperms (% An+/PI-)	11.8±0.9	10.5±0.9	12.6±0.9	12.5±0.9
Late apoptotic sperms (An+/PI+)	16.6±1.5	14.3±1.5	9.6±1.5	14.6±1.5
Necrotic sperms (An-/PI+)	10.3±2.8	16.2±2.8	3.9±2.8	8.8±2.8

L-0: control (nanomicelle-based extender without GSH); L-1, L- 2.5 and L-5: nanomicelle-based extender with 1, 2.5 and 5 mM of GSH respectively.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

This concentration is 5-fold lower than we found but is caused by the character of the extender (Mata-Campuzano *et al.* 2015; Najafi *et al.* 2018). Studies on semen of other species are difficult to compare due to different properties of the semen itself and the extenders used for cryopreservation. Probably, the optimal concentration of GSH in different extenders is different. In equine, the addition of 2.5 mM GSH to the egg yolk semen extender increased progressive motility compared to the control (De Oliveira *et al.* 2013). In boar sperm, supplementing the freezing media with 2 mM GSH had a positive impact on motility rates, nuclear stability and membrane integrity (Estrada *et al.* 2017), whereas use of 5 mM GSH in egg yolk or lecithin based extender did not show a significant effect on boar semen parameters or sperm fertility after thawing process (Gadea *et al.* 2004). Adding 1 or 5 mM GSH to the thawing media increased the motility of human sperm, but no effect on viability was detected (Gadea *et al.* 2011). However, our results were confirmed with other reports that glutathione increased sperm motility (Sinha *et al.* 1996; Munsi *et al.* 2007; De Oliveira *et al.* 2013).

Our current study shows that the addition of 2.5 mM GSH to lecithin-nanomicelles extender resulted in increased cell membrane integrity and viable sperms in phosphatidylserine translocation assay in bull. GSH might be reduced lipid peroxidation, although it had not been assessed in this study.

De Oliveira *et al.* (2013) reported that using 2.5 mM GSH to the egg yolk extender increased sperm viability and plasmatic membrane integrity and the concentrations above 2.5 mM resulted in deterioration in sperms (De Oliveira *et al.* 2013). Similarly, it has been reported that the addition of GSH up to 2.0 mM to egg yolk extender improved buffalo bull sperm viability (Ansari *et al.* 2010). In bulls, the addition of 0.5 mM GSH to the egg yolk diluent improved the viability and intact plasma membrane of the chilled sperm (Triwulanningsih *et al.* 2010). This discrepancy might be due to different protocols (chilling versus freezing) different concentrations of added glutathione and different extenders (egg yolk-based versus lecithin-based extenders). In the present research, we added the negative charged nanomicelles with GSH.

The result showed that acrosome integrity and sperm viability were improved by lecitin-nanomicelles supplemented with 2.5 mM GSH. It seems that these results can be illustrated in two ways according to many previous reports. The first way, it could be due to the antioxidant effects of GSH against ROS production and decrease membrane lipid peroxidation during the freezing and thawing process (Rawash *et al.* 2018). During the freezing-thawing process, the production of reactive oxygen species (ROS) could induce changes in the function and structure of sperm membranes (Wang *et al.* 1997; Chatterjee and Gagnon, 2001). Lipid peroxidation triggers the loss of membrane integrity and led to the death of cell (Geva *et al.* 1998; Guthrie and Welch, 2012). The second way, it is possible interaction between nanomicelles as phospholipid donor membrane with the sperm cell membrane. It was reported that incubation of human sperm for with micelles made from glycerophospholipid mixtures increased sperm motility and resistance to oxidative stress (Ferreira *et al.* 2018). Therefore, it seems that micelles or nanomicelles with or without GSH can improve the quality of sperm. Transfer of negatively charged lipid more occurs between membrane and charged molecules. On the other hand, lipid monomers are transferred presumably through the aqueous solution phase from donor particles to cell membranes, resulting in a change in the membrane potential of them (Richens *et al.* 2017). Sperm membrane are subject to exchanging in the lipids and renewal by various mechanisms, such as endocytosis. Lipids move between different membranes and nonmembrane (Cohen *et al.* 2016). Probably, GSH concentration around 5.0 mM or higher can lead to an imbalance in redox regulation during bull sperm cryopreservation, as suggested in the case of red deer (Anel-López *et al.* 2012). Reactive oxygen species act as physiologically critical signaling messengers (Niki, 2014), and usually, there is a balance between concentrations of ROS and antioxidant scavenging systems (Agarwal *et al.* 2004). Effect of high GSH concentrations could not be due to excessive ROS scavenging, though, but rather the contrary. Anel-López *et al.* (2012) obtained results compatible with higher oxidative stress when using 5.0 mM GSH for freezing red deer spermatozoa.

Addition of 5.0 mM of GSH to a glycerol-based extender increased the DNA integrity of cryopreserved human spermatozoa, but could not increase motility or reduce lipid peroxidation (Varghese *et al.* 2005). Indeed, other studies have found paradoxical results with other antioxidants, with a good protective activity was accompanied by increasing oxidative activity (Domínguez-Rebolledo *et al.* 2010).

These contradictory results may be due to particle size, extenders and breed. For instance, GSH at 1 mM did not have a significant effect on DNA damage in red deer (Anel-López *et al.* 2012), but slightly damage was increased in ram semen (Mata-Campuzano *et al.* 2015). However, the extender could influence on the efficiency of the antioxidant, according to (Mata-Campuzano *et al.* 2015; Najafi *et al.* 2018).

Adding GSH above 2.5 mM had deleterious influence on stallion spermatozoa cryopreservation (De Oliveira *et al.* 2013), but supplementation of INRA82 extender with 5.0 mM glutathione improved miniature Caspian stallion spermatozoa quality during storage at 5 C (Zhandi and Ghadimi, 2014), A similar observation was reported in ram semen, with GSH at 5.0 mM having a positive impact in refrigeration up to 48 h (Mata-Campuzano *et al.* 2014). A detailed explanation of the mechanism of the cryoprotective effects is beyond the purpose of this study. An excellent review of the advances in cryopreservation of bull sperm is given by Ugur *et al.* (2019).

CONCLUSION

The 2.5mM GSH concentration in a lecithin nanomicelle-based semen extender could improve post-thaw bull sperms motility, motion characteristics parameters, sperm viability and normality, and acrosome integrity.

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