Comparison of two different antioxidants in a nano lecithin-based extender for bull sperm cryopreservation

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ABSTRACT

The objective of the present study was to assess the effect of two different antioxidants, enzymatic compared with non-enzymatic, in a nano lecithin-based extender on post-thaw bull sperm quality. Semen samples ($n = 36$) were collected from six bulls. In the first experiment, 11 different extenders were prepared by adding five quantities of vitamin E (α-tocopherol) as a non-enzymatic antioxidant (VE: 0.1, 0.2, 0.4, 0.6 and 1.0 mM), or four quantities of glutathione peroxidase (GPx) as an enzymatic antioxidant (GPx: 0.5, 1, 2 and 3 mM) to the extender. Other extenders were a Control 1 (C1: Extender with ethanol) and Control 2 (C2: Extender without ethanol). Sperm motility (CASA), plasma membrane functionality test (HOST) and lipid peroxidation (MDA) were assessed to determine the optimal treatment in the first experiment. In the second experiment, the optimally supplemented group from the first experiment (GPx-1) was compared to C2 group. Apoptotic-like changes (Annexin staining), mitochondrial activity (Rhodamine-123 staining), acrosome integrity (PSA staining), DNA fragmentation (SCSA test) and *in vitro* embryo production capacity were evaluated. In the first experiment, there were the greatest percentages of plasma membrane functionality and least MDA ($P \leq 0.05$) in sperm diluted GPx-1 group. In the second experiment, percentage of live sperm, blastocyst formation and hatching rate were greater ($P \leq 0.05$) in the GPx-1 group compared with C2 group. In conclusion, data indicate adding 1.0 mM GPx as an enzymatic antioxidant to the nano lecithin-based extender can improve post-thaw quality and *in vitro* fertility of bull sperm.

1. Introduction

Membranes of bull sperm have an large amount of poly-unsaturated fatty acid content (Saraswat et al., 2012) and are vulnerable to peroxidative damage caused by reactive oxygen species (ROS). Furthermore, sperm lack cytoplasmic components including antioxidants which protect against ROS damage (Aitken and Fisher, 1994). Sperm survival, plasma membrane integrity (Alvarez and Storey, 1984; Bucak and Tekin, 2007), fertility (Aitken and Baker, 2004), enzyme activity (Baumber et al., 2000), motility (Alvarez

and Storey, 1984; Bucak and Tekin, 2007) and DNA integrity are reduced by ROS.

Vitamin E, a non-enzymatic antioxidant, is a major antioxidant agent of sperm (Surai et al., 1998), which has a marked scavenger function for controlling free radicals (Akiyama, 1999), and it is considered as a second-line defense antioxidant (Ighodaro and Akinloye, 2018). Furthermore, glutathione peroxidase can protect mammalian sperm against oxidative damage (Alvarez and Storey, 1989). Glutathione peroxidase is an enzymatic antioxidant, as well as catalase and superoxide dismutase. Because enzymatic antioxidants can inhibit the formation of free radicals or reactive oxygen species, these compounds are known as first line defense antioxidants (Ighodaro and Akinloye, 2018). Although there are beneficial effects of antioxidants on post thawed sperm quality (Bilodeau et al., 2001), antioxidant type and concentration must be optimized for extender type and composition. Furthermore, researchers have reported that different reactive oxygen species can affect some specific sperm quality variables, and there have been suggestions to use various antioxidants (Enzymatic or non-enzymatic) to assess the capacity against different reactive oxygen species (Vieira et al., 2018)..

In recent years, concerns have arisen about conventional semen extenders due to the composition variability and the risk related to both microbial contaminants and contamination with exotic disease agents in egg yolk and milk based extenders (Bousseau et al., 1998). Soy lecithin (SL) is an extract of soybeans that contains a natural mixture of phospholipids and fatty acids. As with egg yolk, the phospholipid fraction can protect the sperm plasma membrane during the cooling and freezing process (Amirat et al., 2004; Moussa et al., 2002). Advantages of SL-based extenders have been reported based on results from several studies with humans (Jeyendran et al., 2008), cattle (Aires et al., 2003; Arifiantini and Yusuf, 2010; Dodaran et al., 2015; Towhidi and Parks, 2012), horses (Papa et al., 2011), sheep (Crespilho et al., 2012; Forouzanfar et al., 2010; Sharafi et al., 2009), buffalo (Akhter et al., 2012) and goat semen (Salmani et al., 2013). Because interaction between conventional semen extenders and antioxidants significantly improved values for sperm variables post-thawing (Mata-Campuzano et al., 2015), there are potential benefits of different antioxidants (enzymatic or non-enzymatic) when added to nano lecithin-based extender and there is need for further evaluation and optimization of antioxidant concentrations in this regard.

The size of lecithin particles in extender is often thought to be an important factor. A small droplet size has a large surface area, thus, exerting a greater contact between diffusing oxygen, semen-soluble free radicals and antioxidant. Decreasing the size of the oil droplets led to a greater oxidation capacity (Lethuaut et al., 2002). By decreasing the size of lecithin particles, the number of phospholipids molecules in surface-active particles decreases, and the amount of surface-active component, such as antioxidants adsorbed at the interface, increases. Furthermore, there is an increase in the ratio of oxidizable fatty acids located near the interface to fatty acids embedded in the hydrophobic core of the droplets (Coupland et al., 1996). There is an apparent interaction between antioxidants and size of lecithin particles in the freezing media.

Hence, the objective of the present study was to compare the effects of supplementing nano lecithin-based extender with an enzymatic and a non-enzymatic antioxidant on Holstein bull sperm quality following cryopreservation and thawing.

2. Material and methods

2.1. Animals and semen processing

Semen samples were collected from Holstein bulls ($n = 6$; 3–5 years of age) maintained at the Iranian Nahadehaye Dami Jahed (NDJ) Company (Karaj, Iran). A total of 36 ejaculates (six ejaculates/bull) were collected from bulls using an artificial vagina twice a week for three successive weeks (two ejaculates/week/bull = six replicates/bull). Semen samples were immediately placed in a water bath (35 °C). Semen volume (by graduated tubes) and concentration (were determined using a calibrated photometer, IMV, L'Aigle, France) were recorded. Only semen samples with acceptable characteristics ($> 70\%$ progressive motility; $> 1.0 \times 10^9$ spermatozoa/mL and $< 10\%$ abnormal sperm forms) were selected for this study and samples that had a lesser quality were removed. After initial evaluation, semen samples (in each replicate) were pooled and divided into 11 equal aliquots. Each aliquot was diluted with one of the following 11 extenders to a final concentration of 40×10^6 spermatozoa/mL. The base extender was supplemented with different concentrations of vitamin E (0.1, 0.2, 0.4, 0.6 and 1.0 mM) and glutathione peroxidase (0.5, 1, 2 and 3 mM). Also, unsupplemented (Control 2, C2) and ethanol-supplemented basic extender (control 1, C1) were used as control extenders. Extended semen was loaded into 0.5 mL straws (IMV® Technologies, L'Aigle Cedex, France), equilibrated for 4 h at 4 °C and then frozen using a programmable freezer (Digit Cool®, IMV® Technologies, L'Aigle Cedex, France) (4 to −10 °C at 5 °C/min; -10 to -100 °C at 40 °C/min; and -100 to -140 °C at 20 °C/min). Straws were subsequently plunged into liquid nitrogen and stored until thawing. Frozen semen straws were thawed in a water bath at 37 °C for 45 s. After freeze-thawing process, two experiments were conducted. In the first experiments, sperm motility (CASA), plasma membrane functionality test (HOST) and lipid peroxidation (MDA) were assessed to ascertain the optimal treatment. In the second experiment, the group with optimal supplementations from Experiment 1 was compared with the samples for the C2 group. In Experiment 2, apoptotic-like changes (Annexin staining), mitochondrial activity (Rhodamine-123 staining), acrosome integrity (PSA staining), DNA fragmentation (SCSA test) and *in vitro* embryo production capacity were evaluated.

2.2. Materials (reagents or chemicals)

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

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

Parameters	Set value
Classifier	WHO 2010
Calibration	20 x
Frame Rate	50
Chamber Depth	20 μm
Magnification	200 X
Threshold	89
Minimum contrast	60 Pixels
Minimum VCl mean	15 μm/s
Minimum STR mean	0.7 μm/s
Minimum ALH mean	0.3 μm/s

2.3. Preparation of the base extender and lecithin nanoparticles

A soy lecithin extender [tris (3.025% w/v), citric acid (1.7% w/v), fructose (1.25% w/v), lecithin (Sigma P3644, 3% w/v in distilled water) and glycerol (7% (v/v)] with pH = 6.8 and osmolarity = 300 mOsm/Kg was used as the base extender. Results from a previous study (Patent No-87523, Patent Office of the Islamic Republic of Iran) in which the 3% of nano-lecithin (w/v) was used were important in determining methods used in the present study. Briefly, Lecithin nanoparticles were prepared with sonication (by an S-4000 Misonix sonicator QSONICA, Newtown, CA, USA) at 60% amplitude in ice bath to prevent heating for 30 min (5 s sonication and 10 s rest intermittently to allow cooling of the sample). The supernatant was collected and centrifuged (Sigma 1–14) at $10,000 \times g$ for 15 min at 4 °C. Samples were subsequently filtered using 0.22 μm syringe filters (Jet-Biofil, Canada). The particle size of the nano-particles was assessed using dynamic light scattering ((Brookhaven Instruments, Holtsville, NY, USA). Average diameter of particle size was 94.6 nm. Samples were stored at 4 °C until use.

2.4. Methods to evaluate semen

2.4.1. Sperm motility and velocity variables

A computer assisted sperm motility analysis program (CASA, Video Test-Sperm 2.1, St. Petersburg, Russia) was used to analyze sperm motion characteristics (Table 1). A drop (10 μL) of semen was placed in both the chambers of a glass slide (Microscopic Slides, ISOLAB, 20 μm), covered with cover slide and loaded in CASA for analysis. Motility and velocity variables [total motility (TM, %), progressive motility (PM, %), linearity (LIN, %), curvilinear velocity (VCL, μm/s), straight linear velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm), straightness (STR, %), average path velocity (VAP, μm/s) and beat cross frequency (BCF, Hz)] were assessed in this study. A minimum of seven microscopic fields and 400 spermatozoa were analyzed for each sample.

2.4.2. Plasma membrane functionality

The hypo-osmotic swelling test (HOST) was used to evaluate sperm plasma membrane functionality post-thaw (Revell and Mrode, 1994). Briefly, 30 μL of semen and 300 μL of hypo-osmotic solution [fructose (0.05 M + sodium citrate (0.023 M) in distilled water, osmolality = 100 mOsm/kg] were mixed. Osmolality of medium was evaluated by osmometer (Osmomat 030; Gonotec. GmbH, Germany). This mixture was incubated for 60 min at 37 °C and then 0.2 mL of the mixture was placed on a warm slide and mounted with a cover slip. Sperm ($n = 200$) were evaluated using a phase-contrast microscope and sperm with coiled tails were recorded.

2.4.3. Lipid peroxidation

The malondialdehyde (MDA) concentrations, as indices of lipid peroxidation, were quantified using the thiobarbituric acid (TBA) reaction (Esterbauer and Cheeseman, 1990). Briefly, 1 mL of the diluted semen (250×10^6 spermatozoa/mL) was mixed with 1 mL of cold trichloroacetic acid (20% w/v) to precipitate protein. The precipitate was pelleted by centrifuging ($960 \times g$ for 15 min at 4 °C). Supernatant (one mL) was subsequently incubated with 1 mL of 0.67% (w/v) TBA in a boiling water bath (100 °C, 10 min). After cooling, the absorbance was determined using a spectrophotometer (Shimadzu®, UV-2100) at 532 nm. MDA was expressed as nM/mL.

2.4.4. Acrosomal integrity

Acrosomal integrity of sperm was evaluated using FITC-conjugated *Pisum sativum agglutinin* (PSA-FITC) (Emamverdi et al., 2013). Briefly, 500 μL of tris buffer-diluted semen (1×10^6 spermatozoa/mL) was added to a micro centrifuge tube and centrifuged ($600 \times g$, 10 min). Supernatant was removed and sperm pellets were re-suspended and fixed in 50 μL ethanol (96%) for 30 min (4 °C), after which 20 μL of fixed sample was mounted on microscopic slide and ethanol was allowed to evaporate. Then, 20 μL of PSA (50 μL/mL) was placed on the evaporated sample. Glass slides were incubated (10–15 min at 4 °C), then rinsed by immersing 15 times in distilled water. Afterward, prepared slides were allowed to dry, samples were covered with glycerol, and a coverslip was added. Sperm ($n = 200$) were assessed using fluorescence microscopy (BX51; Olympus, Japan) equipped with fluorescence illumination and a FITC filter (excitation at 455–500 nm and emission at 560–570 nm) at 400× magnification. Sperm with and without green fluorescence in

head, or a green fluorescent band at the equatorial location were scored as intact and damaged acrosomes, respectively.

2.4.5. Mitochondrial activity

Rhodamine-123 (R123; Invitrogen TM, Eugene, OR, USA) and propidium iodide (PI) were used to evaluate mitochondrial activity (Emamverdi et al., 2013). Briefly, 10 μL of Rhodamine-123 solution (0.01 mg/mL) was added to 500 μL of tris buffer-diluted semen (50×10^6 sperm/mL) and incubated (30 min, 25 °C) in a darkened area. Samples were subsequently centrifuged ($500 \times g$, 3 min) and sperm pellets were re-suspended in 500 μL tris buffer. Afterward, 10 μL of PI (1 mg/mL) was added to sperm suspension. The samples were analyzed using a FACS Caliber (Becton Dickinson, San Khosoz, CA, USA) flow cytometer. For each sample, 10,000 events were counted to determine percentage of live sperm with functional mitochondria. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates.

2.4.6. Phosphatidylserine (PS) translocation

A commercial PS Detection Kit (IQP, Groningen, the Netherlands) was used to determine viable, apoptotic-like and dead sperm according to the manufacturer's instructions (Dodaran et al., 2015). Briefly, semen samples were washed in calcium buffer solution and re-adjusted to a concentration of 1.0×10^6 spermatozoa/mL in calcium buffer. Then, 10 μL of Annexin V-FITC was added to 100 μL sperm suspension and incubated for 20 min at the room temperature. Afterward, 5 μL of PI was added to the sperm suspension and incubated for 15 min at room temperature in the dark. Each tube was subsequently analyzed using flow cytometry (Becton Dickinson, San Khosoz, CA, USA). For each sample, 10,000 events were counted. Sperm were classified into three groups: live (A^-/ PI^-), apoptotic-like (A^+/PI^-) and dead (A^-/PI^+ or A^+/PI^+). Green fluorescence was detected in FL1 with a 530/30 nm band-pass filter and red fluorescence (propidium) was detected in FL2 with a 585/42 nm band-pass filter.

2.4.7. Sperm chromatin structure assay (SCSA)

The SCSA measures the susceptibility of sperm chromatin to DNA denaturation *in situ* induced by low pH treatment. The assay was conducted using the protocol described by Evenson and Jost (Evenson and Jost, 2000). The assay is based on the metachromic properties of a DNA binding fluorescent dye, acridine orange (AO). The AO intercalates with DNA and emits green fluorescence when bound to intact, double strand DNA and red fluorescence when bound to single stranded fragmented DNA. These properties were utilized in a fluorescent assay using flow cytometry. Upon excitation by laser light, the emitted red and green fluorescent signals from individual cells are detected by photomultiplier tubes. Routinely, 5000 individual sperm cells are analyzed in a few minutes (Evenson and Jost, 2000). An aliquot of washed sperm in PBS was diluted to a concentration of 1×10^6 spermatozoa/mL. This cell suspension was treated with an acid detergent solution that contained 0.1% Triton X-100, 0.15 M NaCl, and 0.08 M HCl for 40 s, and then stained with 6 mg/L purified acridine orange in a phosphate-citrate buffer (Evenson and Wixon, 2005). The sperm DNA fragmentation index (DFI) was calculated as the percentage of sperm with fragmented DNA divided by the total number of sperm counted.

2.4.8. In vitro embryo production (IVEP) capacity

The IVEP was conducted using the method described previously (Mohammadi-Sangcheshmeh et al., 2014). Briefly, Ovaries were collected from cows at a local abattoir and cumulus-oocyte complexes (COCs) were isolated from follicles using the aspiration method. COCs were washed in maturation medium. The maturation medium consisted of TCM 199 with Earle's salts served as basic medium and was modified with 100 μg/mL l-glutamine, 250 μg/mL pyruvate, 600 μg/mL hemicalcium lactate, 800 μg/mL NaHCO₃, 1.4 mg/mL HEPES, 50 μg/mL gentamicin, 12% heat-inactivated estrus cow serum, and 10 μg/mL FSH (Follitropin, Vetrepharm, Ireland). *In vitro* maturation (IVM) was performed by culturing ten COCs for 24 h in a 50 μL maturation medium droplet under mineral oil at 39 °C, 5% CO_2 in a humidified atmosphere.

Following IVM, COCs were washed twice in HEPES-buffered synthetic oviductal fluid (HSOF) and groups of ten oocytes were transferred to 44 μL fertilization drops overlaid with equilibrated mineral oil. The fertilization medium was SOF supplemented with 2% (v/v) estrous sheep serum. A straw of frozen sperm was thawed at 37 °C for 1 min, and then carefully layered on top of the Percoll gradient system. Sperm was diluted to 50×10^6 spermatozoa/mL in HSOF, which would produce a 2×10^6 spermatozoa/mL in fertilization drops. There was fertilization of matured oocytes by adding 2 μL diluted sperm, 4 IU/mL heparin, PHE (20 μM peni-cillamine, 10 μM hypotaurine, 1 μM epinephrine) to the 44 μL fertilization drops. Oocytes and sperm were co-cultured for 18 h in an incubator using the same conditions as described for IVM.

At 18 h post-insemination, cumulus cells were removed and presumptive zygotes were washed three times in HSOF. Thereafter, 15–20 putative zygotes placed in 30 μL culture drops consisting of SOFaa (2% BME-essential amino acids, 1% MEM-nonessential amino acids) supplemented with 8 mg/mL bovine serum albumin, 1 mM glutamine, 0.34 mM tri-sodium citrate and 2.77 mM myo-inositol under equilibrated mineral oil. Culture was performed at 38.5 °C, under 5% CO_2 5% O_2, 90% N_2 in a humidified atmosphere. Developmental data were recorded for cleavage- and blastocyst-stage embryos at days 3 and 9 post-insemination, respectively. The day of insemination was considered as Day 0.

2.5. Statistical analysis

All data were assessed for normal distribution by PROC UNI-VARIATE and Shapiro–Wilk test. The sperm variable values that had non-normal distributions, were arcsine-transformed before analysis. The results with use of the Levene test indicated the variances were homogeneous. Data were subsequently analyzed using the GLM procedure of SAS 9.1 (SAS Institute, version 9.1, 2002, Cary, NC, USA). The results were expressed as LSMean ± SEM. The Tukey's test was used to compare least squares means. Differences

Table 2

Effect of different extenders on post-thaw sperm motion variables.

Variable	VE-0.1	VE-0.2	VE-0.4	VE-0.6	VE-1	GPx-0.5	GPx-1	GPx-2	GPx-3	C1	C2	P-value	SEM
TM (%)	68.88	68.11	70.66	69.22	69.88	69.22	68.66	69.88	68.11	70.66	71.66	0.54	1.36
PM (%)	47.33[d]	48.11[bcd]	50.77[abc]	49.29[abcd]	46.11[d]	47.87[dc]	49.11[abcd]	49.11[abcd]	47.66[dc]	50.00[ab]	51.77[ab]	0.05	1.19
Lin (%)	31.82	30.57	32.50	31.34	30.86	32.05	32.31	32.99	31.30	31.28	32.76	0.63	0.88
VSL (μm/s)	47.45	45.98	50.02	45.02	38.07	45.48	42.55	44.55	41.13	46.15	47.47	0.95	5.43
VCL (μm/s)	118.25	118.72	127.01	114.89	96.71	114.09	108.38	110.70	100.12	119.93	119.23	0.95	14.58
VAP (μm/s)	63.71	61.07	67.09	60.07	50.48	60.86	57.55	59.16	54.48	62.28	62.99	0.95	7.64
ALH (μm/s)	2.36	2.46	2.75	2.50	2.03	2.53	2.34	2.32	1.98	2.68	2.64	0.84	0.33
STR (μm/s)	61.60	61.27	63.10	61.86	61.67	61.77	62.35	62.59	59.62	62.65	63.64	0.24	0.94
BCF (Hz)	12.67[abc]	13.13[a]	12.94[ab]	12.20[abc]	11.21[c]	11.41[bc]	11.55[abc]	12.25[abc]	12.17[abc]	12.65[abc]	12.72[abc]	0.04	0.52

Different superscripts within rows indicate differences (P ≤ 0.05).

were considered significant at $P \leq 0.05$. The model used in the present study was as follows:

$$y_{ijk} = \upsilon + a_i + bj + e_{ijk},$$

Where y_{ijk} stand for observed dependent variables, υ is the mean of the population, a_i is the effect of first main factor (Glutathione), bj is the effect of second main factor (α-tocopherol) and e_{ijk} is the random residual error. The cleavage and blastocyst rates and hatching rate in IVF were compared using the chi-square test.

3. Results

3.1. Sperm motility and velocity variables

The results indicated that there was no difference between extenders for total motility, LIN, VSL, VCL, VAP and ALH and STR (Table 2). There was the greatest percentage of post-thaw sperm progressive motility in the C2 group while the least percentage of progressive motility in VE-1 group. It is noteworthy that there was no difference among C2, VE-0.4, VE-0.6, GPx-1, GPx-2 and C1 groups in terms of progressive motility (Table 2). The value for the BCF variable was greater in VE-0.2 when compared to VE-1 and GPx-0.5 groups (Table 2).

3.2. Sperm plasma membrane functionality and lipid peroxidation

The plasma membrane functionality in post-thaw sperm was greater in GPx-1 compared to VE-0.2, VE-0.4, VE-0.6, VE-1 and GPx-2 groups (Table 3). Also, there was the least percentage of MDA concentration for the GPx-1 group, while there was the greatest percentage of MDA in VE-1 group (Table 3). Based on the results (HOST and MDA) the GPx-1 group in Experiment 1 was selected to compare with the C2 group in Experiment 2.

3.3. Acrosome integrity, mitochondrial activity, PS translocation and DNA integrity

Based on the acrosome integrity, mitochondrial activity, PS translocation and DNA integrity variables (especially plasma membrane functionality and MDA concentration), the GPx-1 group was selected as the optimal home-made extender for complementary assessment of acrosome integrity, PS translocation, mitochondrial activity and DNA integrity. For these complementary assessments, GPx-1 was compared with the C2 group.

In terms of acrosome integrity, there were no differences between the GPx-1 and C2 group (Table 4). Similarly, there was no difference between groups for values of the mitochondrial activity variable (Table 4). The percentage of live sperm was greater in the GPx-1 compared to that in C2 group, while there was no difference in percentage of apoptotic-like sperm between groups (Table 4). Also, there was no difference among groups for the percentage of dead sperm (Table 4). There was also no difference between C2 and GPx-1 groups for the DFI variable (Table 4).

Table 3

Effect of different extenders on plasma membrane functionality and MDA concentration of post-thawed sperm.

Variable	Extender											P-value	SEM
	VE-0.1	VE-0.2	VE-0.4	VE-0.6	VE-1	GPx- 0.5	GPx-1	GPx- 2	GPx- 3	C1	C2		
HOST (%)	46.04[ab]	39.23[bc]	39.24[bc]	39.91[bc]	32.81[c]	44.65[ab]	51.17[a]	39.81[bc]	45.30[ab]	45.13[ab]	43.33[abc]		3.04
MDA (nM/mL)	2.87[ab]	1.73[ab]	1.95[ab]	2.80[ab]	4.84[a]	1.87[ab]	0.69[b]	2.21[ab]	2.74[ab]	3.13[ab]	2.31[ab]		0.77

Different superscripts within rows indicate differences (P ≤ 0.05).

Table 4

Effect of different extenders on dead, apoptotic-like and live (by phosphatidylserine translocation test), mitochondrial activity (RH$^+$, Rhodamine-123 staining), acrosome integrity (PSA staining), and DNA fragmentation index (DFI) of sperm after cryopreservation.

Extender	Variable (unit)					
	Live (%)	Apoptotic (%)	Dead (%)	RH$^+$ (%)	PSA$^+$ (%)	DFI (%)
C2	67.90 [b]	14.38	17.72	63.31	76.23	4.96
GPx-1	73.94 [a]	14.65	11.4	64.87	70.23	3.63
SEM	1.20	2.01	1.55	2.46	1.42	1.38

Different superscripts indicate differences (P $\leq$ 0.05).

3.4. In vitro embryo production (IVEP) capacity

The IVEP results indicated that, for the GPx-1 group, there were more desirable results compared to C2 group in terms of blastocyst formation and hatching rates (82.01% and 52.08%, respectively). Percentage cleavage rate in the GPx-1 group was, however, less than that in C2 group (Table 5).

4. Discussion

There is an increased worldwide concern regarding the microbiological safety of animal-derived semen extenders. The present research and other studies, therefore, are in progress for developing chemically defined extenders free of compounds of animal origin (Crespilho et al., 2012). The efficacy of SL extenders, however, remains debatable. There has been development of a nano lecithin-based extender for bull sperm (Patent No-87523, Patent Office of the Islamic Republic of Iran) that increased sperm cryosurvival in comparison with conventional SL extender. There is an apparent interaction between antioxidants and size of lecithin particles in the freezing media. To improve efficiency of the nano lecithin-based extender, the present study was designed to investigate the effect of adding two different antioxidants (glutathione peroxidase as an enzymatic antioxidant, and α-tocopherol as a non-enzymatic antioxidant) on bull sperm quality after cryopreservation.

In Experiment 1, supplementing nano lecithin-based extender with α-tocopherol or GPx did not affect the percentage of total motility, LIN, VSL, VCL, VAP and ALH and STR of post-thaw sperm. The SL extenders have an antioxidative property which is attributed to the amino-alcohol groups of the phospholipids of this compound (Judde et al., 2003). Also, there have been reports that there is no synergistic effect between the SL extenders and alpha tocopherol. Accordingly, the lack of a difference between the extender supplemented with alpha tocopherol and C2 might be due to lack of synergistic effect between nano lecithin and alpha tocopherol.

The findings of current study are inconsistent with those of two previous studies (Nasiri et al., 2012; Towhidi and Parks, 2012) where supplementing extender with α-tocopherol improved freezability of bull sperm. Saraswat et al. (2012), however, reported there was a lesser quality of post-thawed goat sperm when the citrate-egg yolk-based extender was supplemented with vitamin E. These inconsistent results may be due to the differences in size of lecithin in the extender, animal species and antioxidant concentration used.

The greatest percentage of progressive motility was in the C2 group and the least percentage in VE-1 group in the present study. The reason for lesser progressive sperm in VE-1 group might be due to the greater amounts of antioxidants added to the extender which can have negative effect on some sperm characteristics through reducing the physiological concentrations of reactive oxygen species (Hussaini et al., 2017).

Plasma membrane functionality of post-thawed sperm was greater in the GPx-1 compared with VE-0.2, VE-0.4, VE-0.6, VE-1 and GPx-2 groups (Table 3). Also, the percentage of live sperm was greater in the GPx-1 compared with C2 group (Fig. 1), possibly due to glutamine regulation function on cell integrity and redox potential (Curi et al., 2005). Furthermore, hydrogen peroxide (H$_2$O$_2$) is the most deleterious ROS in sperm which can affect sperm plasma membrane negatively, and it has been suggested that enzymatic antioxidants such as GPx can protect sperm against H$_2$O$_2$ (Vieira et al., 2018). It, therefore, seems that improvement in plasma membrane functionality in GPx-1 is due to the protective effect of GPx against H$_2$O$_2$.

In the present study, adding 1.0 mM GPx was effective in preventing MDA production and preserving plasma membrane

Table 5

Effect of different extenders on the development rates of *in vitro* cattle embryos.

Extender	Variable			
	Oocytes (n)	Cleavage rate % (n)	Blastocysts rate % (n)	Hatching rate % (n)
C2	150	70.46 (106/150)	16.37 [b] (18/106)	52.08 [b] (11/18)
GPx-1	154	66.34 (104/154)	29.15 [a] (30/104)	82.01 [a] (25/30)
SEM		3.21	2.25	13.15

Different superscripts indicate differences (P ≤ 0.05).

functionality compared to other media. These results are consistent with those of other studies where there was inhibition of LPO by antioxidants during liquid storage or cryopreservation of sperm (Salmani et al., 2013). The GPx promotes maintenance of sperm quality by preventing loss of plasma membrane integrity of post-thaw sperm (Bilodeau et al., 2001; Bucak and Tekin, 2007). Based on results from Experiment 1 of the present study, 1.0 mM glutathione peroxidase was used in Experiment 2.

Results from Experiment 2 indicate 1 mM GPx there was a greater percentage of live sperm compared to that of the C2 group. This finding is consistent with that of Hu et al (2016) where glutathione pretreatment of bull sperm before freeze-thawing process improved post thaw sperm quality. Results of the present study, therefore, confirm that supplementation of the semen extenders with glutathione can enhance enzymatic antioxidant capacity (Hu et al., 2016). Also, it is well established that GPx can regulate oxidative stress in sperm (Alvarez and Storey, 1989). Thus, perhaps in the present study, the supplementation with GPx had a positive effect on sperm tolerance against oxidative stress which ultimately led to a greater percentage of live sperm.

In Experiment 2, there was no difference between C2 and GPx-1 groups in percentage of intact acrosomes (PSA$^+$) after cryopreservation, suggesting there is a stabilizing antioxidant effect with the C2 group on acrosomal membranes (Singh and Rajender, 2015). Presence of a normal, intact acrosome is essential for the sperm acrosomal reaction required for fertilization (Thomas et al., 1997). It has been suggested that lipids may help prevent sperm cold shock by chelating calcium ions in the medium, preventing calcium entry into sperm (Watson, 1981). In the present study, it seems that GPx was not effective in inducing a synergic effect with lecithin in extender to enhance acrosome integrity.

There was no difference in percentage of sperm with active mitochondria after freezing and thawing in the tested media of the present study. The loss of mitochondrial membrane potential is associated with a loss of sperm motility (Storey, 2004). The extent of this association is highly species dependent, however, because there is considerable interspecies variation in the extent to which the sperm are dependent on oxidative phosphorylation for ATP generation, with stallion, bull, and boar sperm being particularly susceptible to changes in mitochondrial function (Storey, 2004). The results of the present study indicate GPx is conducive to survival of bull sperm by interfering with apoptotic pathways but not by affecting mitochondrial activity.

The generation of lipid peroxides could alter plasma membrane fluidity (Aitken et al., 1993) which is necessary for sperm-oocyte fusion. Glutathione peroxidase is present in semen and provides an intracellular defense of sperm against oxidative stress. It is well established that the freeze-thawing process results in a significant reduction in the glutathione peroxidase content of the semen (Bilodeau et al., 2000) which can weaken the defense system of sperm against oxidative stress. In another study, it has been reported that a relatively greater concentration of GPx may induce degradation of mitochondrial DNA (Whitaker et al., 2008) and that glutathione peroxidase at lesser concentrations may be conducive for sperm viability *via* inhibiting the lipid peroxides (Salmani et al., 2013). The results of Brouwers and Gadella (2003) indicate the major phospholipids involved in the oxidative damages after freezing were those occurring in the inner portion of the cell membrane. It seems logical, therefore, to hypothesize that an intracellular antioxidant such as GPx could be more efficient than other molecules, such as vitamin E, for protection against such damage (Brouwers and Gadella, 2003).

In Experiment 2, there was no difference between extenders for DNA fragmentation rate (Fig. 1) indicating there was an equivalent protective effect of extenders. These results are consistent with those of Martin et al (2004) where DNA integrity was not affected by extender type after bull sperm cryopreservation. Celeghini et al. (2008), however, reported that cryopreservation of bull semen in Botu-Bov® extender led to further damage to the sperm chromatin structure compared to use of Bioxcell® as extender (Celeghini et al., 2008). Among other sperm quality assays, evaluation of DNA integrity has been considered important because early embryo development depends on the presence of normal DNA. After cryopreservation, sperm are particularly susceptible to DNA damage (Bilodeau et al., 2000). Bulls with a lesser sperm LPO had a greater calf-siring capacity.

In Experiment 2 of the present study, there was no difference between GPx-1 and C2 groups for mitochondrial activity. This result was consistent with that of Perumal et al (2011) where addition of reduced glutathione to bull semen extender led to improvement in the mitochondrial membrane potential. The reason for this outcome has, however, not been ascertained.

In the present study, insemination with sperm cryopreserved in the GPx-1 group resulted in a greater percentage blastocyst development rate compared with the C2 group. Also, in the GPx-1 group, the production of MDA was less than with the C2 group. The greater percentage blastocyst development rate might be due to a greater quality of sperm in the GPx-1 group.

In summary, supplementing nano lecithin-based extender with 1.0 mM GPx resulted in the greatest percentage of plasma membrane functionality, plasma membrane integrity and least amount of MDA production. Also, blastocyst development rate and hatching rate percentages with use of the glutathione peroxidase extender was greater than with the C2 group. These findings indicate that adding an enzymatic antioxidant to a nano lecithin-based extender could be suitable for cryopreservation of bull sperm. These findings also warrant further *in vivo* fertility tests.

Declaration of Competing Interest

The authors have no conflict of interest to disclose for this study.

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