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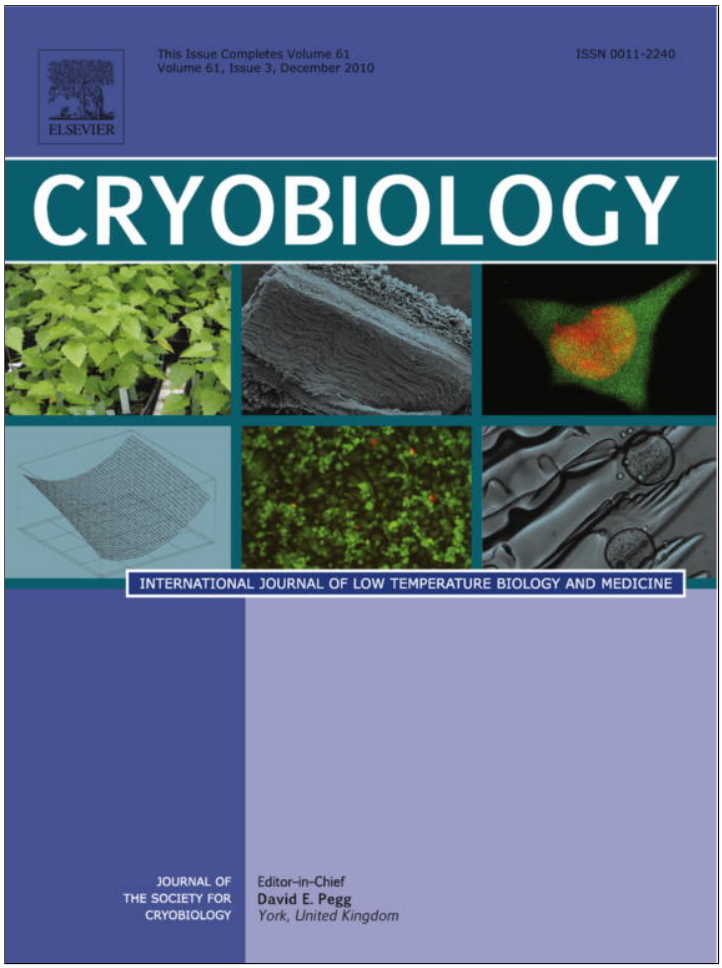

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Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: Antioxidants protect DNA integrity against cryodamage ☆

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ABSTRACT

This study was conducted to determine the effects of methionine, inositol and carnitine on sperm (motility, abnormality, DNA integrity and in vivo fertility) and oxidative stress parameters (lipid peroxidation, total glutathione and antioxidant potential levels) of bovine semen after the freeze–thawing process. Nine ejaculates, collected with the aid of an artificial vagina twice a week from each Simmental bovine, were included in the study. Each ejaculate, splitted into seven equal groups and diluted in Tris-based extender containing methionine (2.5 and 7.5 mM), carnitine (2.5 and 7.5 mM), inositol (2.5 and 7.5 mM) and no additive (control), was cooled to 5 °C and then frozen in 0.25 ml straws. Frozen straws were then thawed individually at 37 °C for 20 s in a water bath for the evaluation.

The extender supplemented with 7.5 mM doses of carnitine and inositol led to higher subjective motility percentages (61.9 ± 1.3% and 51.3 ± 1.6%) compared to the other groups. The addition of methionine and carnitine at doses of 2.5 and 7.5 mM and inositol at doses of 7.5 mM provided a greater protective effect in the percentages of total abnormality in comparison to the control and inositol 2.5 mM ($P < 0.001$). As regards CASA motility, 7.5 mM carnitine (41.6 ± 2.9% and 54.2 ± 4.9%) and inositol (34.9 ± 2.0% and 47.3 ± 2.2%) caused insignificant increases in CASA and total motility in comparison to the other groups. All of the antioxidants at 2.5 and 7.5 mM resulted in lower sperm with damaged DNA than that of control, thus reducing the DNA damage ($P < 0.05$). No significant differences were observed in CASA progressive motility and sperm motion characteristics among the groups. In fertility results based on 59-day non-returns, no significant differences were observed in non-return rates among groups. As regards biochemical parameters, supplementation with antioxidants did not significantly affect LPO and total GSH levels in comparison to the control group ($P > 0.05$). The maintenance of AOP level in methionine 2.5 mM was demonstrated to be higher (5.06 ± 0.38 mM) than that of control (0.96 ± 0.29 mM) following the freeze–thawing ($P < 0.001$). Supplementation with these antioxidants prior to the cryopreservation process protected the DNA integrity against the cryodamage. Furthermore, future research should focus on the molecular mechanisms of the antioxidative effects of the antioxidants methionine, carnitine and inositol during cryopreservation.

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Introduction

Cryopreservation of bovine semen has been widely used as a vital tool of livestock industry, particularly in conjunction with the dissemination of genetic material and the banking of genome resources to preserve valuable transgenic lines. However, cryopreservation also induces sublethal damage to the spermatozoa, which may result

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in loss of motility, viability, in vivo fertilizing capacity, deterioration of acrosomal and plasma membrane integrity, and damage of deoxyribonucleic acid (DNA) [34,54,5]. The damage taking place during cryopreservation has been attributed to cold shock, ice crystal formation, oxidative stress, osmotic changes and lipid–protein reorganizations within the cell membranes [10,53].

The characteristic feature of biological membrane is the asymmetrical arrangement of lipids within the bilayer. The lipid composition of the plasma membrane of mammalian sperm cells is markedly different from those of mammalian somatic cells. The fact which sperm cells contain a high content of polyunsaturated fatty acids, makes the membranes more susceptible to peroxidative damage. It is suggested that membrane is thought to be a primary target of freezing or cold shock damage in cells [3,7]. The presence of high concentrations of long chain polyunsaturated fatty acids (PUFA) within the lipid structure of cell membranes requires efficient antioxidant systems to defend against peroxidative damage and associated sperm dysfunction [4,6]. The protective antioxidant systems in spermatozoa are primarily of cytoplasmic origin. Spermatozoa discard most of their cytoplasm during the terminal stages of differentiation, thus lack the significant cytoplasmic component containing antioxidants that counteract the damaging effects of reactive oxygen species (ROS) and lipid peroxidation (LPO). Correspondingly, spermatozoa are susceptible to LPO during cryopreservation and thawing, leading to subsequent sperm dysfunctions [4,6,48]. In bovine semen, ROS are generated primarily by dead spermatozoa via an aromatic amino acid oxidase catalyzed reaction [51]. Low concentrations of ROS are physiologically involved in the maintenance of the fertilizing ability and capacitation/acrosome reaction of spermatozoa. However, excessive ROS impair sperm function and enzymatic activity [11,29]. Spermatozoa and seminal plasma possess an antioxidant system comprising taurine, reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) to prevent oxidative damage. However, this antioxidant capacity in sperm cells, due to the small cytoplasmic component containing antioxidants to scavenge oxidants, is limited. Thus, mammalian spermatozoa may, however, be insufficient in preventing formation of LPO during the freeze–thawing process [9,48].

In recent years, the addition of antioxidants such as GSH and ascorbic acid to equine sperm [9,11], taurine and cysteine to bovine sperm [13,18,26,44,52] has been shown to protect sperm against the harmful effects of ROS and to improve post-thaw sperm motility, viability and fertility. Methionine acts as a precursor amino acid of glutathione for protecting cells from oxidative damage, and plays a vital role in detoxification [40,41]. In addition, the thiol group of methionine was shown to chelate lead and remove it from tissues [37]. As related to carnitine, a vitamin-like compound, is biosynthesized from two essential aminoacids, lysine and methionine, in liver, kidney and brain. L-carnitine, is highly found in mammalian epididymis and spermatozoa. It plays a role in generating metabolic energy by facilitating transportation of fatty acid into the mitochondria. Epididymal cells and spermatozoa derive energy from carnitine that is present in epididymal fluid [27]. The close correlations between seminal carnitine and semen quality were demonstrated in various models (ram, human, stallion and rat) [14,43,49]. Among compounds of the epididymal fluid, inositol exists in high concentrations, and plays an important role in maintaining viability of epithelial and sperm cells in the epididymis as an essential growth factor [20]. It was stated that the motility of frozen–thawed bull sperm can be improved using inositol in the extender [42].

To our knowledge, there is not any study which investigated the roles of the antioxidants methionine, carnitine and inositol in the cryopreservation extender, against cryodamage of Simmental bovine semen. This research was therefore conducted to determine the effects of supplementation of the freezing extender with the antioxidants methionine, inositol or carnitine on sperm (sperm motility, abnormality, DNA integrity and in vivo fertility) and oxidative stress parameters (lipid peroxidation (LPO), total glutathione (tGSH) and antioxidant potential (AOP) levels) of bovine semen after the freeze–thawing process.

Material and methods

Chemicals

The antioxidants used (butylated hydroxy toluene (BHT) B-1378, methionine M-5308, carnitine 544361 and inositol I7508) and biochemical assay kits (LPO-586™, GSH-420™ and AOP-490™) were obtained from Sigma–Aldrich Chemical Co., USA and OxisResearch™ Bioxytech, USA, respectively.

Animals and semen collection

Three Simmental bulls (3–4 years of age) were housed at the Lalahan Livestock Central Research Institute (Ankara, Turkey), and maintained under uniform feeding and housing conditions. A total number of 30 ejaculates (10 ejaculates for each Simmental bovine) were collected from the bulls with the aid of an artificial vagina twice a week, according to AI standard procedures. The ejaculates containing spermatozoa with >80% forward progressive motility and concentrations higher than 1.0×10^9 spermatozoa/ml were used in the study. Nine ejaculates for each bovine were included in the study. Immediately after collection, the ejaculates were immersed in a warm water bath at 34 °C until their assessment in the laboratory. Semen assessment was performed within approximately 20 min.

Semen processing

The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals and sperm concentration was determined by means of an Accucell photometer (IMV, L'Aigle, France). Sperm motility was estimated using phase-contrast microscopy (200×). A Tris-based extender (Tris 254 mM, citric acid 78 mM, fructose 70 mM, egg yolk 15% (v/v), glycerol 6% (v/v), pH 6.8) was used as the base extender (cryopreservation diluent). Each ejaculate was split into seven equal experimental groups and diluted to a final concentration of 60×10^6/ml spermatozoa with the base extender containing methionine (2.5 and 7.5 mM), carnitine (2.5 and 7.5 mM), inositol (2.5 and 7.5 mM) and no additive (control). Diluted semen samples were loaded into 0.25-ml French straws and cooled down to 4 °C in 2 h, frozen at a programmed rate of −3 °C/min from +4 to −10 °C; −40 °C/min from −10 to −100 °C; −20 °C/min from −100 to −140 °C in a digital freezing machine (Digitcool 5300 ZB 250, IMV, France). Thereafter, the straws were plunged into liquid nitrogen. The study was replicated nine times. At least after 24 h, frozen straws were thawed in a 37 °C water bath for 20 s immediately before use.

Semen evaluation

Evaluation of sperm parameters

Analysis of subjective and CASA motilities. Subjective motility was assessed using a phase-contrast microscope (100× magnification), with a warm stage maintained at 37 °C. A wet mount was made using a 5-μl drop of semen placed directly on a microscope slide

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and covered by a cover slip. Sperm motility estimations were performed in three different microscopic fields for each semen sample. The mean of the three successive estimations was recorded as the final motility score. Besides estimating subjective sperm motility, a computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was also used to analyze sperm motion characteristics. CASA was set up as follows: phase contrast; frame rate – 60 Hz; minimum contrast – 70; low and high static size gates −0.6 to 4.32; low and high intensity gates – 0.20–1.92; low and high elongation gates 7–91; default cell size – 10 pixels; default cell intensity – 80. Thawed semen was diluted (5 μL semen + 95 μL extender) in a Tris-based extender (without egg yolk and glycerol) and evaluated immediately after dilution. A 4-μL sample of diluted semen was put onto a pre-warmed chamber slide (Leja 4, Leja Products, Luzernestraat B.V., Holland) and sperm motility characteristics were determined with a 10× objective at 37 °C. The following motility values were recorded: motility (%), progressive motility (%), total sperm motility, VAP (average path velocity, μm/s), VSL (straight linear velocity, μm/s), VCL (curvilinear velocity, μm/s), ALH (amplitude of lateral head displacement, μm) and LIN (linearity index (LIN = (VSL/VCL) × 100). For each evaluation, 10 microscopic fields were analyzed including at least 300 cells.

Assessment of sperm abnormalities. For the assessment of sperm abnormalities, at least three drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock solution (62.5 ml formalin (37%), 150 ml sodium saline solution, 150 ml buffer solution and 500 ml double-distilled water) [45] drop of this mixture was put on a slide and covered with a cover slip. The percentages of sperm acrosome and total abnormalities were determined by counting a total of 200 spermatozoa under phase-contrast microscopy (magnification 1000×, oil immersion).

Assessment of sperm DNA damage. Sperm DNA damage was investigated using the single cell gel electrophoresis (COMET) assay that was generally performed at neutral conditions. Our method was similar to several protocols with few modifications in previous studies [33,24]. The straws were thawed by gently shaking in a 37 °C water bath for 10s, and centrifuged at 600*g* for 10 min at 4 °C. The remaining sperm cells were washed with PBS (Ca^{2+} and Mg^{2+} free).

Each precleaned slide was pre-coated with a layer of 1% normal melting point agarose in PBS (Ca^{2+} and Mg^{2+} free) and then dried at room temperature. Approximately, 100,000 sperm cells (18 μl) were mixed with 0.75% low melting point agarose (50 μl) at 37 °C, and this suspension was dropped onto the first agarose layer. Slides were allowed to solidify for 20 min at 4 °C. The coverslips were removed and the slides were immersed in freshly prepared cold lysis buffer. The slides were then incubated at 37 °C in lysis buffer with 20 μg/ml of proteinase for 2 h. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh neutral electrophoresis buffer at 4 °C for 20 min incubation to allow the DNA to unwind. Electrophoresis was performed at room temperature, at 25 V for 20 min. Following electrophoresis, the slides were air-dried and subsequently stained with 50 μl of 8 μl/ml ethidium bromide and covered with a coverslip.

The images of 200 randomly chosen nuclei were analyzed visually. Observations were made at a magnification of 400× using a fluorescent microscope (Olympus, Japan). Each image was classified according to the intensity of the fluorescence in the comet tail, and given a value of 0, 1, 2, 3 or 4 (from undamaged class 0 to maximally damaged class 4). Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a "comet" pattern, whereas whole sperm heads, without a comet, were not considered as damaged.

Evaluation of in vivo fertility. In fertility trials, the fertilizing ability of spermatozoa was calculated based on the non-return rates at 59 days. A total of 272 cows were artificially inseminated with one insemination dose (15×10^6) of frozen samples with antioxidants and control samples, each comprising the semen of one bull. The effective non-return rates (NNR) were determined at 59 days post-insemination by palpation per rectum.

Oxidative stress parameters. Briefly, diluted semen samples were centrifuged at 800*g* for 20 min at 4 °C in order to separate the cells from the diluted seminal plasma, and spermatozoa were washed twice with PBS at 800*g* for 20 min. After centrifugation, supernatant was discharged and pellet was completed to 500 μL with PBS. Then the sperm suspension was transferred into a 2-ml beaker in ice water and sonicated with a probe (Bandelin Sonopuls, Bandelın Electronic HeinrichstraBe, D-12207, Gerate-Typ:UW 2070, Pro-Nr. 51900037369.004, Berlin) for 10 s on ice repeated 6 times at intervals of 30 s to separate sperm head and tail. For LPO analysis, 10 μL of 0, 5 mM BHT (butyl-hydroxitoluen) was added into 120 μL homogenate samples and stored at −86 °C until analysis. The remaining homogenate was centrifuged at 8000*g* for 15 min at +4 °C and supernatant was collected and stored at −86 °C for tGSH and AOP analysis.

Determination of LPO levels. LPO levels were determined using commercial kits by LPO-586™ Oxis Research by spectrophotometry (UV 2100 UV–vis Recording Spectrophotometer Shimadzu, Japan). The assay is based on the reaction of a chromogenic reagent, *N*-methyl–2-phenylindole with MDA and 4-hydroxyalkenals (LPO) at 45 °C. One molecule of either MDA or 4-hydroxyalkenal reacts with 2 molecules of *N*-methyl–2-phenylindole in acetonitrile, to yield a stable chromophore with maximal absorbance at 586 nm. The results are expressed as μmol for 10^9 cells/ml.

Determination of tGSH levels. Total GSH levels were determined with GSH-420™ OxisResearch kit by spectrophotometry. The method is based on the formation of a chromophoric thione. The sample is buffered and the tris (2-carboxyethyl) phosphine is added to reduce any oxidized glutathione (GSSG) to the reduced state (GSH). The chromogen, 4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate, is added forming thioethers with all thiols present in the sample. Upon addition of base to raise the pH greater than 13, β-elimination specific to the GSH-thioether results in the chromophoric thione. The results are expressed as μmol for 10^9 cells/ml.

Determination of AOP levels. AOP levels were determined with AOP-490™ Oxis Research kit by spectrophotometry. Assay was based upon the reduction of Cu^{2+} to Cu^{+} by the combined action of all antioxidants present in the sample. A chromogenic reagent, Bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), selectively forms a 2:1 complex with Cu^{+} which has a maximum absorbance at 490 nm. A standard of known uric acid (a water soluble antioxidant) concentration is used to create a calibration curve. The results are expressed as mmol, 10^9 Cells/ml.

Statistical analysis

The study was replicated nine times. Results were expressed as the mean ± SEM. Sperm motility, motion characteristics and abnormality were analyzed by analysis of variance, followed by Tukey's post hoc test to determine significant differences between groups. Sperm with damaged DNA and fertility results based on 59-day non-returns were evaluated by the chi-square test and *Z*-test, respectively. Differences with values of $P < 0.05$ were considered

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to be statistically significant. Statistical analyses were performed by using SPSS 11.5 package program.

Results

Sperm parameters (percentages of motility, motion characteristics, abnormalities, damaged DNA and in vivo fertility)

The effects of methionine, carnitine and inositol on the sperm parameters of frozen bovine semen are presented in Table 1. Samples cryopreserved in 7.5 mM carnitine and inositol had higher percent motility (61.9 ± 1.3% and 51.3 ± 1.6%) compared to other groups ($P < 0.05$). The addition of methionine and carnitine (2.5 and 7.5 mM) and inositol 7.5 mM provided a greater protective effect in the percentages of total abnormality in comparison to the control and inositol 2.5 mM ($P < 0.001$). As regards CASA motility, 7.5 mM carnitine (41.6 ± 2.9% and 54.2 ± 4.9%) and inositol (34.9 ± 2.0% and 47.3 ± 2.2%) caused insignificant increases in CASA motility and total motility in comparison to the other groups. In the comet test, all the antioxidants at 2.5 and 7.5 mM resulted in lower sperm with damaged DNA than that of control, reducing the DNA damage ($P < 0.05$). No significant differences were observed in CASA progressive motility and sperm motion characteristics among the groups ($P > 0.05$). No significant differences were observed in non-return rates among groups. Fertility results based on 59-day non-returns after rectovaginal insemination are shown in Table 2.

Analysis of LPO, tGSH and AOP levels

The influence of antioxidants on LPO and antioxidant activities in thawed bovine sperm are shown in Table 3. Supplementation with antioxidants did not significantly affect LPO and tGSH levels in comparison to the control group ($P > 0.05$). The maintenance of AOP in methionine 2.5 mM was demonstrated to be higher (5.06 ± 0.38 mM) than that of control (0.96 ± 0.29 mM) following the freeze–thawing ($P < 0.001$).

Table 2
Fertility results based on 59-day non-returns after rectovaginal insemination with frozen–thawed bovine semen.

Group	Total inseminated cows	Non-return rates (%)
Control	25	22/25 (88.0)
Methionine 2.5 mM	39	34/39 (87.18)
Methionine 7.5 mM	48	43/48 (89.58)
Carnitine 2.5 mM	39	29/39 (74.36)
Carnitine 7.5 mM	40	34/40 (85.00)
Inositol 2.5 mM	44	41/44 (93.18)
Inositol 7.5 mM	37	34/37 (91.89)

The same column shows no significant differences among proportions ($P > 0.05$).

Discussion

This study investigated the effects of the antioxidants methionine, carnitine and inositol on sperm and oxidative stress parameters following freeze–thawing of bovine semen. Mammalian spermatozoa are highly sensitive to LPO, which occurs as a result of the oxidation of membrane lipids by partially reduced oxygen molecules, e.g., superoxide, hydrogen peroxide and hydroxyl radicals. Spontaneous peroxidation of the membranes of mammalian spermatozoa destroys the structure of the lipid matrix, due to the attacks of ROS, formed through the univalent reduction of oxygen, during cryopreservation. The damage ultimately leads to loss of membrane integrity, membrane deterioration, decreased sperm motility, loss in fertility, leakage of intracellular enzymes and damage of the sperm DNA, through the oxidative stress and the production of cytotoxic aldehydes [1,2,6]. Supplementation with antioxidants can prevent this process [47]. In the current study, used antioxidants did not achieve any effectiveness in the prevention of LPO formation, when compared to the control. The current findings relating to LPO are also in agreement with the results performed on ram and goat sperm in which a decrease was not recorded in the level of MDA in the presence of hyaluronan and thiols for the frozen state [15,16] or in methionine and dithioerythritol for sperm liquid storage [21]. Sperm motility per se becomes crucial in facilitating the passage through cervix and uterotubal junction. It is also important for the actual penetration of the cumulus cells and zona pellucida of the ovum [28]. In this study, 7.5 mM doses of carnitine and inositol led to higher motility percentages, compared to the other groups. This result was contrast to that previously reported for frozen–thawed sperm of Angora goat, where carnitine and inositol addition resulted in insignificant increases in subjective and CASA sperm motilities [17]. As related to morphology, all of the antioxidants, except for inositol 2.5 mM, provided cryoprotective effect on the sperm morphology which is in agreement with previous study of Bucak et al. [17]. The axoneme and associated dense fibers of the mid-pieces in sperm, which are responsible for the sperm motility, are covered by mitochondria that generate energy by oxidative phosphorylation. Large amounts of ROS can impair the sperm motility, producing axonemal damage as a result of ATP depletion [22,28]. In contrast to this effect of ROS, it is hypothesized that 5 mM doses of carnitine and inositol can have a cryoprotective influence on the functional integrity of the axosome and mitochondria-improving post-thawed sperm motility without preventing LPO. The integrity of sperm DNA has vital importance to the sperm cell. Some authors suggest that sperm DNA integrity is a more objective marker of sperm function as opposed to the sperm parameters such as motility. Although DNA damage could be result of free radical induced damage in previous studies [39,50], used antioxidants

Table 1
Mean (±S.E.) sperm parameters in semen supplemented with different antioxidants of Simmental bovine semen following freeze–thawing.

Groups	Subjective motility (%)	CASA motility (%)	CASA progressive motility (%)	CASA total motility (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	LIN%	Total abnormality %	Damaged DNA%
Control	39.4 ± 2.2^{c}	27.3 ± 5.4^{ab}	9.0 ± 3.1	42.2 ± 4.9^{ab}	114.6 ± 6.9	79.4 ± 5.2	226.9 ± 13.3	9.7 ± 0.3^{a}	37.3 ± 1.0	26.3 ± 1.5^{a}	20.2^{a}
Methionine 2.5 mM	41.3 ± 1.8^{c}	28.9 ± 3.5^{ab}	11.7 ± 2.4	39.4 ± 3.6^{ab}	119.7 ± 5.3	87.6 ± 4.7	224.2 ± 7.6	9.23 ± 0.3^{a}	40.9 ± 1.5	18.4 ± 1.4^{b}	7.8^{c}
Methionine 7.5 mM	45.6 ± 2.4^{bc}	25.9 ± 6.1^{ab}	7.6 ± 1.7	36.4 ± 6.8^{ab}	116.1 ± 7.0	79.9 ± 3.3	226.0 ± 15.6	9.51 ± 0.3^{a}	37.9 ± 2.2	20.4 ± 1.1^{b}	16.6^{b}
Carnitine 2.5 mM	29.4 ± 1.5^{d}	25.4 ± 5.2^{ab}	8.6 ± 2.2	36.4 ± 6.3^{ab}	103.9 ± 3.5	73.9 ± 2.8	193.1 ± 6.2	7.78 ± 0.2^{b}	40.8 ± 1.8	20.4 ± 0.6^{b}	9.3^{c}
Carnitine 7.5 mM	61.9 ± 1.3^{a}	41.6 ± 2.9^{a}	12.8 ± 1.4	54.2 ± 4.9^{a}	127.2 ± 9.0	87.4 ± 3.5	233.2 ± 14.3	9.08 ± 0.3^{ab}	39.8 ± 2.2	16.8 ± 0.7^{b}	8.2^{c}
Inositol 2.5 mM	24.4 ± 1.5^{d}	19.3 ± 2.7^{b}	6.9 ± 1.2	32.1 ± 3.4^{b}	108.2 ± 3.0	77.0 ± 2.3	183.2 ± 28.5	9.37 ± 0.40^{a}	38.6 ± 1.2	21.4 ± 1.2^{ab}	10.1^{c}
Inositol 7.5 mM	51.3 ± 1.6^{b}	34.9 ± 2.0^{ab}	13.3 ± 1.5	47.3 ± 2.2^{ab}	119.1 ± 4.0	80.8 ± 3.5	227.3 ± 8.6	9.36 ± 0.3^{a}	38.4 ± 1.8	17.2 ± 1.0^{b}	8.6^{c}
P	**	*	–	*	–	–	–	*	–	**	*

a, b, c, d: Different superscripts within the same column demonstrate significant differences (*$P < 0.05$, **$P < 0.001$).

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Table 3
Mean (±}S.E.) LPO levels, total GSH and AOP of Simmental bovine semen supplemented with different antioxidants of Simmental bovine semen following freeze–thawing.

Groups	LPO μM (10^9 cell/ml)	Total GSH μM (10^9 cell/ml)	AOP mM (10^9 cell/ml)
Control	20.42 ± 1.92	584.09 ± 29.75	0.96 ± 0.29[b]
Methionine 2.5 mM	25.94 ± 4.87	517.33 ± 13.11	5.06 ± 0.38[a]
Methionine 7.5 mM	15.96 ± 4.92	537.02 ± 40.28	3.07 ± 0.97[ab]
Carnitine 2.5 mM	14.10 ± 2.25	568.80 ± 24.50	1.64 ± 0.20[b]
Carnitine 7.5 mM	23.73 ± 2.02	615.81 ± 39.19	1.75 ± 0.34[b]
Inositol 2.5 mM	15.05 ± 3.07	530.62 ± 27.65	2.08 ± 0.21[b]
Inositol 7.5 mM	20.94 ± 4.95	555.63 ± 49.01	3.12 ± 0.67[ab]
P	–	–	**

a,b: Different superscripts within the same column demonstrate significant differences (**$P < 0.001$).

provided cryoprotection against DNA and morphology damage of post-thawed sperm. We can state that LPO release does not seem an influential factor on the sperm parameters such as subjective motility, morphology and DNA damage following freeze–thawing process.

The antioxidant system comprising GSH, SOD, CAT and GPx has been described functioning as a defense mechanism to restrict harmful effects of LPO against sperm parameters in semen [36]. Glutathione, for example is a tripeptide thiol compound which has many important functions in the cellular physiology, including the protection of the cell from oxidative stress, synthesis of protein and DNA, and the fertilization of gametes [31,35,38]. In the present study, the supplementation with antioxidants did not elevate total GSH levels after thawing. These findings, contrary to studies of ram sperm in which an increase of total GSH level was recorded in the presence of GSH for the frozen state or during storage [16,21], were also agree with studies in goat [8] and ram [15] sperm, where elevating of total GSH level was not observed when cryopreserved or stored with taurine, cysteine, cysteamine and hyaluronan. As related to the total AOP, which is the sum of enzymatic (e.g., superoxide dismutase, catalase, and glutathione peroxidase) and nonenzymatic (e.g., ascorbate, urate, vitamin E, pyruvate, glutathione, taurine, and hypotaurine) antioxidants [6,12,23,46]. Low levels of seminal AOP has a key role in male infertility [25,32]. In a few studies, oral antioxidant application such as cysteine to infertile male [19] and ginger rhizome to rat [30] had significant improving effects on semen AOP. In the present study, methionine 7.5 mM increased the AOP level, compared to groups of control, inositol 2.5 mM and carnitine. It is noteworthy that the AOP increase with methionine 7.5 mM was not related to post-thaw sperm motility, fertilizing ability, levels of LPO and tGSH.

Conclusions

It can be stated that the addition of antioxidants (7.5 mM doses of carnitine and inositol) improve subjective sperm motility. Moreover, all the antioxidants at 2.5 and 7.5 mM resulted in lower proportions of spermatozoa with abnormal morphology and damaged DNA than those of controls, except for inositol 2.5 mM in sperm morphology. The effects of antioxidants was not associated with fertility, although the highest non-return rate was achieved by adding methionine 7.5 mM and inositol 2.5 and 7.5 mM. The use of antioxidants in the extender did not lead to significant reduction of LPO releasing and improve tGSH levels and AOP activities, except for AOP in the group with 2.5 mM methionine, following the thawing of semen. Supplementation with these antioxidants prior to the cryopreservation process may be recommended to facilitate the enhancement of sperm cryopreservation techniques. Furthermore, future research should focus on the molecular mechanisms of the antioxidative effects of the antioxidants methionine, carnitine and inositol during cryopreservation.

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