Antioxidant supplementation ameliorates bull sperm parameters and fertilizing ability following the freeze-thaw process

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Abstract: This study's goal was to reveal the effects of antioxidant supplementation on motility, motion characters, morphology, DNA integrity, and fertilizing potential of cryopreserved bovine sperm. The collection of ejaculates was performed from three Holstein bulls. At least ten ejaculates were collected per bull. The ejaculates were immediately separated into five aliquots, diluted in the Cryobos® commercial extender, including 2.5 mM taurine, 2.5 mM cysteine, 2.5 mM methionine, 2.5 mM glutamine, and no additives. Afterward, they were frozen in 0.25 ml French straws, and liquid nitrogen was used for the storage of semen. The supplementation of methionine resulted

in a higher subjective motility rate in comparison with the other groups. Taurine led to the lowest post-thawed CASA motility rate ($P < 0.05$). The addition of antioxidants did not cause any improvements in sperm motion characteristics when compared to the controls ($P > 0.05$). Cysteine led to a higher protection of acrosome integrity, in comparison with the other groups ($P < 0.05$). For the comet test, the minimum percentage of sperm with damaged DNA was obtained in the groups with cysteine and glutamine ($P < 0.05$). There was not any significant difference among the groups in terms of pregnancy rates ($P > 0.05$). In conclusion, cysteine supplementation to the semen extender prior to freezing ameliorated the post-thawed semen quality.

Keywords: Antioxidants; bull sperm parameter; Cryobos®; fertilizing ability; sperm freezing

1. Introduction

The first successful preservation of sperm was reported in 1940, and to date, many studies have been performed to achieve successful cryopreservation, and now a routine procedure is applied [1]. Despite successful results, cryopreservation can damage spermatozoa due to the loss of sperm motility, viability, and fertilizing potential [2]. Damage can occur with cryopreservation due to some causes, such as cold shock, oxidative stress, changes in osmotic pressure, membrane permeability and Ca efflux, and eventually, the lipid-protein organization is disrupted within cell membranes [1,3]. The membrane structure of sperm comprises 70% of phospholipids, 25% of natural lipids, and 5% of glycolipids, while the phospholipid membrane layer consists of 70% of poly-unsaturated fatty acids (PUFAs). These acids are highly affected by free radicals. Therefore, it leads to the functional impairment of the plasma membrane [4,5].

During cryopreservation, sperm membrane lipids can undergo peroxidation. Therefore, some free radicals can be generated during peroxidation, which results in poor sperm quality [6]. If free radicals are produced in a high amount, this also affects the DNA integrity of spermatozoa. Thus, it leads to disrupted acrosome reaction and capacitation besides membrane lipid peroxidation [3,7]. However, some scientists report that physiological amounts of free radicals are needed for sperm-oocyte fusion [3].

Due to the small cytoplasmic component of sperm, spermatozoa restrict the antioxidant capacity for scavenging oxidants. Therefore, during the sperm cryopreservation, the sperm antioxidant defense system may be inadequate to prevent free radicals from damaging the lipid membrane [8].

For the purpose of protecting sperm cells from oxidative stress and cryodamage during cryopreservation, different extenders were tried. Researchers report that the addition of different antioxidants into sperm extenders reduces the harmful effects of oxidants that emerge in the course of the sperm storage [9,10].

In addition to having antioxidant properties, glutamine is also used as a cryoprotective agent [11,12]. Mammalian cells are capable of using only thiol compounds, including glutathione and cysteine that penetrate the cell membrane easily, for the intracellular glutathione biosynthesis *in vitro* and *in vivo*. Cysteine was demonstrated to enhance the post-thawed sperm motility and morphology in bulls [10] and small ruminants [13,14].

It has been reported in some studies that methionine functions as a precursor amino acid of glutathione in the minimization of oxidative stress caused by free radicals [15]. Moreover, it has been shown that M protects the normal morphological status and the sperm membrane integrity of bull spermatozoa [9].

Taurine is also one of the amino acids used as an antioxidant to prevent the excessive free radical formation in spermatozoa [6]. The antioxidative and cryoprotective effects of T when supplemented to the semen freezing extender have been demonstrated in many studies on bull [10], ram [16], and rabbit spermatozoa [6], in case of its addition to the freezing extender. In this study, we aimed to analyze the effects of adding antioxidants to Cryobos® prior to freezing on motility, motion characters, sperm morphology, DNA integrity, and *in vivo* fertility of the post-thawed bovine sperm.

2. Materials and methods

2.1. Chemicals

The required chemicals were acquired from a local representative (Ankara, Turkey) of Sigma-Aldrich Co. USA unless otherwise indicated.

2.2. Semen collection and cryopreservation process

The collection of ejaculates was performed twice a week from three Holstein bulls (aged 3 and 4 years), kept at Lalahan Livestock Central Research Institute (Ankara, Turkey). The bulls were on a regular semen collection schedule, and a standard breeding soundness assessment was applied to them. An artificial vagina in the presence of a mounted animal was utilized for semen collection. At least ten ejaculates were collected per bull. Only ejaculates having > 70% of progressively motile sperm, estimated on a subjective basis by a phase-contrast microscope, and having concentrations above $1.0x10^9$ spermatozoa/ml were utilized. After the ejaculates were collected from bulls, they were taken to a water bath at a temperature of 37 °C for evaluation. An Accucell photometer (IMV, L'Aigle, France) was used for determining sperm concentration. Phase-contrast microscopy (200x) was employed for assessing sperm motility. Cryobos®, which is a commercial extender, was utilized as the base

extender. The dilution of a portion of every ejaculate to a concentration of $60x10^6$ sperm cells/mL was performed using the pre-warmed (37 °C) Cryobos® extender (Magapor Co. Ltd., Zaragoza, Spain), including cysteine (C) (2.5 mM), taurine (T) (2.5 mM), methionine (M) (2.5 mM), and glutamine (G) (2.5 mM) and not containing any additives (control). The loading of the extended semen samples into 0.25 ml French straws was performed, and they were cooled to a temperature of 4 °C in a period of 2 h and frozen in a freezer (Digitcool 5300 ZB 250, IMV, France). Afterward, the straws were placed in liquid nitrogen (-196 °C) and kept for at least 24 h prior to analysis. The frozen samples were thawed in a hot water bath (37 °C for at least 30 s) for evaluation [9,10].

2.3. Computer-assisted semen analysis (CASA)

Prior to CASA evaluation (Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA), the pre-warmed Tris buffer was used for diluting the semen samples to acquire a sperm density of approximately $10 – 20 \times 10^6$ sperm/mL. The examination of the samples was carried out using a phase-contrast microscope with a warmed stage (37 °C). CASA was set up as follows: phase contrast; frame rate – 60 Hz; minimum contrast – 70; low and high static size gates – 0.6–4.32; 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. Sperm total motility (TM, %), average path velocity (VAP, μm/s), progressive motility (PM, %), curvilinear velocity (VCL, μm/s), linearity (LIN, %), straight-line velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm), straightness (STR, %) and beat/cross frequency (BCF, Hz) were among the variables analyzed. For all assessments, the analysis of five microscopic fields was conducted to include a minimum of 200 cells [9].

2.4. Evaluation of sperm abnormalities

To evaluate acrosomal abnormalities in the semen samples, 7.5 μL of the semen at a minimum was pipetted into 2 mL tubes that included 0.5 mL Hancock's solution [17]. For the purpose of identifying the ratio of sperm acrosome abnormalities, 200 spermatozoa in total were examined using a phase-contrast microscope (1,000 x magnification, oil immersion).

2.5. Evaluation of sperm DNA damage

The centrifugation of the extended semen samples was performed at 300 g for 10 min at a temperature of 4 °C. The removal of the seminal plasma was carried out, and (Ca2+ and Mg2+ free) PBS was utilized for washing the remaining sperm cells to acquire a concentration of $1x10^5$ spermatozoa/cm^3 [18]. The COMET assay, usually applied under high alkaline conditions, was used for examining the damaged sperm DNA [19]. The analysis of the images of 50 nuclei selected randomly was conducted using the comet assay software program (CASP). A fluorescent microscope (Olympus, BX51, Japan) was utilized for performing observations at a magnification of 400x. The damage was assigned by a tail of fragmented DNA that migrated from the sperm head, leading to a 'comet' pattern, while the whole sperm heads, without a comet, were not regarded as damaged.

2.6. Fertility trials

Artificial the insemination of 246 cows in total was carried out using the frozen samples with antioxidants and the control samples, all of them contained the sperm of one bull. The determination of effective non-return rates (NNRs) was performed at 59 days post-insemination by rectal palpation.

2.7. Statistical analysis

The experimental design included five replications. The results of the study were presented in tables. The comparison of the groups in terms of motion properties, sperm motility, acrosome abnormalities, and DNA damage was made by employing the analysis of variance and Tukey's post-hoc test to assign significant differences. Pearson's chi-square test was carried out to compare the groups in terms of the gestation rate. For these tests, the SPSS/PC software package (version 14.01; SPSS Inc., Chicago, IL, USA) was used. Statistical significance was set at $P < 0.05$.

3. Results

3.1.Sperm characteristics (percentages of subjective and CASA sperm motility and motion properties, acrosome abnormalities, DNA damage, and *in vivo* fertility)

As is seen from Table 1, the addition of antioxidants did not cause any improvements in sperm motion characteristics such as VAP, VCL and ALH when compared to the controls ($P > 0.05$). The lowest value was found in the diluent with cysteine compared to the other antioxidant added diluent and control group in VSL, STR and LIN, which are among sperm motility properties ($P< 0.05$). According to Table 2, although M was determined to give the highest value in subjective motility ($P > 0.05$), the freezing extender supplemented with C yielded the best CASA motility rate ($P < 0.05$). Furthermore, in the comet test, the minimum percentage of sperm with damaged DNA obtained in the groups with C and G, when compared to the other experimental groups ($P < 0.05$). Besides, it found that the acrosome integrity preserved better in the C-added group in comparison with the other groups ($P < 0.05$). There was not any significant difference among the groups in terms of pregnancy rates (Table 3) ($P > 0.05$).

4. Discussion

In the present study, the effects of cryopreservation on *in vitro* sperm characteristics and *in vivo* fertility results after the semen was frozen with antioxidants in the presence of the Cryobos® extender were evaluated. The decreased motility rate [5] and damage to DNA integrity [20] that would ultimately induce cell death were observed in the cryopreserved spermatozoa. Besides, this leads to a poor fertility rate in case of the usage of the frozen-thawed semen for artificial insemination [21].

The seminal plasma that contains superoxide dismutase, albumin, catalase, and T protects spermatozoa from oxidative stress [22]. At the same time, freeze-thawing is known to decrease the sperm endogenous antioxidant levels and make sperm vulnerable to oxidative stress [23]. In the current study, the freeze-thawing process of bovine spermatozoa also led to a significant reduction in the subjective sperm motility of the control group, as expected. However, in the experimental groups, M led to higher subjective motilities than those of the control group. The best CASA motilities were obtained with the freezing extender supplemented with C. The lowest values of sperm motion characteristics of VSL, STR and LIN motion were obtained in the C-supplemented freezing extender compared to the controls.

In this study, the loss of sperm motility was determined in the control group, compared to other groups supplemented with some antioxidants. In the present research, the supplementation of C led to the significant cryoprotective activity on post-thawed CASA motility, motion characteristics such as VSL, STR and LIN, and acrosomal abnormalities. These results are consistent with those obtained in the studies performed on bull [22], ram [13], and goat semen [14]. Other studies demonstrated that C improved semen quality, such as chromatin integrity and pregnancy rate, in the case of its administration as adjunct therapy post-varicocelectomy in humans [24]. Furthermore,

adding C to the freezing extender caused a considerable increase in the post-thaw motility rate, fertilization success, and decreased DNA damage in common carp semen [25]. According to our hypothesis, C has a cryoprotective effect on the functional acrosome integrity, enhancing the post-thawed sperm quality.

In this study, adding T to the freezing extender did not lead to enhanced semen quality parameters, including sperm CASA motility and acrosomal abnormalities, when compared to the controls, and it bore similarities to the findings of the study carried out by Bucak and Uysal [13]. The results of the current study contradict the findings acquired by Ateşşahin et al. [26] and Alvarez and Storey [6], who indicated a remarkable enhancement in the motility of rabbit semen after the freeze-thawing procedure. This may originate from differences in the sperm extender composition, animal species, and T concentration utilized in the study.

In this study, adding G to the freezing medium did not result in enhanced sperm parameters, including CASA motility, CASA sperm motion characteristics, and acrosomal abnormalities, in comparison with the control groups. G and glycerol are reported to have a synergistic cryoprotective effect on sperm cryopreservation [12]. It has also been stated that adding G to the semen medium enhances the post-thawed sperm motility and fertilizing ability of different species [11,12,27] and ensures the protection of sperm against functional and structural damage caused by cool storage [28]. However, in the current study, it was observed that G did not create a difference compared to the control, T and M groups.

The mechanism that underlies the decrease in fertility of cryopreserved sperm has not been fully enlightened. However, the role of sperm DNA in sperm function and fertilization capacity during the cryopreservation process is of increasing interest.

Fertilization success can be attributed to sperm factors, such as vital, motile, and morphologically normal spermatozoa [29]. An increase in the assessment of genomic integrity has been observed in recent times because of studies associating the degree of DNA damage with different fertility results, involving fertilization rates, embryo cleavage, pregnancy, implantation, and live birth [30,31].

The comet test was applied for evaluating DNA damage in this study. In the comet test, C and G led to a significant reduction in DNA strand breakage, in comparison with the other groups. Cryopreservation generally leads to a sublethal cryoinjury to spermatozoa, reducing cell viability after thawing [6]. It has been demonstrated that human [32] and bull [33] spermatozoa induce membrane phosphatidylserine translocation, therefore, showing cryopreservation as a reason for apoptosis after the freezing-thawing process [34]. Results similar to ours were obtained in studies using the comet assay and indicating that cryopreservation deteriorates sperm DNA integrity [9,25,35,36].

No significant differences were determined in any group in terms of pregnancy rates. Therefore, the antioxidants used in this study did not have a significant function in improving fertility results related to pregnancy rates following the freezing-thawing of bull semen. The finding mentioned above is consistent with the findings obtained in the studies conducted before and demonstrating no enhancement in the fertilizing capability of sperm with antioxidants supplemented to the freezing extender [9,10,36].

In conclusion, this study suggested that the cryopreservation process caused impairment in semen quality by reducing the percentages of sperm motility and by increasing the percentages of abnormal and DNA damaged sperm. These impairments in the frozen-thawed semen are potently related to cryopreservation-induced oxidative stress and a decrease in endogenous antioxidant enzyme activities. Therefore, adding cysteine to the

freezing medium played a protective role in sperm quality parameters against structural and functional damage of the freezing-thawing process.

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TABLES

Table 1. Mean (± SE) sperm motion characteristics in the frozen-thawed bull semen

Groups	VAP (n = 5)	VSL (n = 5)	VCL (n = 5)	ALH (n = 5)	STR (n = 5)	LIN (n = 5)
Taurine	121.4 ± 3.22	89.14 ± 1.42^{ab}	246.1 ± 8.19	9.96 ± 0.27	74.80 ± 1.46^{a}	39.0 ± 1.37^{ab}
Methionine	124.3 ± 2.33	97.0 ± 2.79^{a}	246.18 ± 5.23	10.0 ± 0.19	78.60 ± 1.86^{a}	41.40 ± 1.28^{a}
Cysteine	126.5 ± 5.39	83.82 ± 2.87^{b}	254.94 ± 13.47	9.84 ± 0.46	68.60 ± 2.99^{b}	36.20 ± 2.26^{b}
Glutamine	119.7 ± 2.03	95.04 ± 1.39^{a}	234.72 ± 5.28	9.36 ± 0.18	79.40 ± 1.24^{a}	42.80 ± 0.86^{a}
Control	119.5 ± 3.83	93.22 ± 3.23^{a}	231.24 ± 8.18	9.22 ± 0.14	78.40 ± 1.36^{a}	42.80 ± 0.96^{a}
P	-	*	-	-	*	*

^{a,b} : Different superscripts within the same column indicate significant differences (* P < 0.05).

- : No significant difference (P > 0.05)

VAP: Average path velocity (μm/s), VSL: Straight-line velocity (μm/s), VCL: Curvilinear velocity (μm/s), ALH: Amplitude of lateral head displacement (μm), STR: Straightness (%), BCF: Beat/cross frequency (Hz)

Table 2. Mean (± SE) subjective and CASA sperm motility, acrosome abnormality, and DNA damage values in the frozen-thawed bull semen

Groups	Subjective motility % (n = 5)	Total CASA motility % (n = 5)	Acrosome integrity % (n = 5)	DNA damage % (n = 5)
Taurine	47.0 ± 2.00	55.6 ± 4.92^{b}	7.8 ± 0.50^{a}	3.28 ± 0.19^{a}
Methionine	56.0 ± 9.66	66.8 ± 2.39^{ab}	7.6 ± 0.51^{a}	3.19 ± 0.11^{a}
Cysteine	54.0 ± 4.30	70.0 ± 3.17^{a}	5.4 ± 0.89^{b}	1.89 ± 0.22^{b}
Glutamine	55.0 ± 5.47	58.6 ± 5.59^{ab}	7.4 ± 0.65^{a}	2.09 ± 0.16^{b}
Control	47.0 ± 3.74	62.4 ± 4.22^{ab}	7.6 ± 0.61^{a}	3.61 ± 0.16^{a}
P	-	*	*	*

^{a,b}: Different superscripts within the same column indicate significant differences (* $P < 0.05$).

- : No significant difference ($P > 0.05$)

Table 3. Mean fertility results based on 59-day non-returns after artificial insemination with the frozen-thawed bull semen

Groups	Non-return rates %
Taurine	62.00
Methionine	61.41
Cysteine	54.16
Glutamine	52.00
Control	52.00
P	-

- : No significant difference ($P > 0.05$)