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Effects of curcumin and dithioerythritol on frozen-thawed bovine semen

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Summary

The aim of this study was to determine the effects of curcumin and dithioerythritol added into bull semen extender on sperm parameters, lipid peroxidation, total glutathione and antioxidant potential levels of bull spermatozoa following the freeze/thawing process. Twenty-seven ejaculates obtained from three bulls were included in the study. Each ejaculate that was splitted into five equal groups and diluted in a Tris-based extender containing curcumin (0.5 and 2 mM), dithioerythritol (0.5 and 2 mM) and no additive (control) was cooled to 5 °C and frozen in 0.25-ml French straws. The extender supplemented with 0.5 mM dose of curcumin led to lower percentage of total abnormality (20.40 ± 2.36%) when compared to the control (30.60 ± 1.47%, $P < 0.05$). Curcumin and dithioerythritol at 0.5 mM provided a greater protective effect in the membrane functional integrity (54.40 ± 2.09% and 50.00 ± 2.68%), in comparison with control (37.20 ± 1.77%, $P < 0.001$). Supplementation with antioxidants did not significantly affect the lipid peroxidation and antioxidant potential levels, while the maintenance of total glutathione levels in curcumin 0.5 mM was demonstrated to be higher than that of control, following the freeze/thawing ($P < 0.05$). Supplementation with these antioxidants prior to the cryopreservation process may be recommended to facilitate the enhancement of sperm cryopreservation techniques.

Introduction

Semen cryopreservation has become important in artificial insemination (AI), in conjunction with the dissemination of genetic material, limiting the spread of sexually transmitted diseases and the banking of genome resources to preserve valuable transgenic lines. But cryopreservation produces sublethal damage that leads to cold shock, ice crystal formation, oxidative stress, osmotic changes and lipid–protein reorganisations within the cell membranes, resulting in the loss of motility, viability, *in vivo* fertilising capacity, deterioration of acrosomal and plasma membrane integrity, deoxyribonucleic acid (DNA) damage (Watson, 1995; Aitken *et al.*, 1998; Bailey *et al.*, 2000; Vishwanath & Shannon, 2000; Medeiros *et al.*, 2002). Cold shock in spermatozoa is generally associated with oxidative stress and dead spermatozoa and atmospheric/molecular oxygen in the environment are suggested to be responsible for the generation of reactive oxygen species (ROS). Oxidative stress is a cellular condition characterised by an imbalance between the production of ROS and the scavenging capacity of the antioxidants. When the production of ROS exceeds the available antioxidant defence system, significant oxidative damage occurs to the sperm organelles through the damage of lipids, proteins and DNA and thus ultimately leads to sperm death. ROS-induced damage to spermatozoa is mediated by the oxidative attack of sperm phospholipid-bound polyunsaturated

fatty acids, resulting in lipid peroxidation (LPO) (De Lamirande & Gagnon, 1992; Gomez *et al.*, 1996; Irvine, 1996). Reactive oxygen species, generated during freezing/thawing process, are eliminated by antioxidant system existing in semen/seminal plasma. Total glutathione (tGSH) and antioxidant potential (AOP) have been described as defence mechanisms against the ROS and LPO (Bilodeau *et al.*, 2001; Aitken & Baker, 2004; Gadea *et al.*,2004). However, this antioxidant capacity in sperm cells, due to the small cytoplasmic component that contains antioxidants to scavenge oxidants, is limited. Thus, mammalian spermatozoa may be insufficient in preventing LPO during the freeze/thawing process (Aurich *et al.*, 1997; Storey, 1997). In recent years, the addition of antioxidants to the freezing extender (Chen *et al.*, 1993; Bilodeau *et al.*, 2001 Uysal *et al.*, 2007; Sarıözkan *et al.*, 2009) has been shown to protect spermatozoids against the harmful effects of ROS and to improve post-thaw sperm functions such as motility, viability and fertility.

Curcumin, a polyphenolic compound that is richly found in the dietary spice turmeric which dates back over 5.000 years, is one of the natural antioxidant and acts as anti-apoptotic, anti-inflammatory, antitoxic and anticancer agent (Miller *et al.*, 2001; Surh *et al.*, 2001; Goa *et al.*, 2004). Curcumin has been subjected in many researches regarding various cell systems against ROS-induced damage and organ transplantation and storage due to its highly efficient cryoprotective and antioxidative properties against cold shock and oxidative damage (Kunchandy & Rao, 1990; Abuarqoub *et al.*, 2007; Kanitkar & Bhonde, 2008; Mathuria & Verma, 2008). The supplementation of post-thawed Angora goat spermatozoa by curcumin provided higher motility and superoxide dismutase (SOD) activity, compared to the control (Bucak *et al.*, 2010). Dithioerythritol, an antioxidant, is known as a protamine disulphide bond-reducing agent (Lindemann *et al.*, 1998; Watanabe & Fukui, 2006). It prevents the oxidation of sulphydryl groups, but also has a mucolytic effect on mucoprotein disulphide bonds, which might possibly damage the frozen membranes (Ollero *et al.*, 1998). It was stated that dithioerythritol provided a protective effect against toxin-induced apoptosis and oxidative damage in lymphocytes (Deshpande & Kehrer, 2006). The addition of dithioerythritol seemed to improve bull and human sperm motility during liquid storage or in the frozen state (Barmatz *et al.*, 1994; Lindemann *et al.*, 1998). No previous study was performed for investigating the roles of curcumin and dithioerythritol on sperm motility characteristics and antioxidant capacities of post-thawed bull spermatozoa. This research was therefore conducted to determine the effects of curcumin and dithioerythritol on sperm parameters, motion characteristics, LPO, tGSH and AOP levels following the freeze/thawing process.

Materials and methods

Chemicals

The chemicals used (butylated hydroxytoluene (BHT) B-1378, curcumin C1386, dithioerythritol D-9680, Trizma base T6791, citric acid monohydrate C0706 and fructose F2543) and biochemical assay kits (LPO-586™, GSH-420™ and AOP-490™) were obtained from Sigma-Aldrich Chemical Co. (Interlab, Ankara, Turkey) and OxisResearch™, Bioxytech, CA, (Genmar, Ankara, Turkey) respectively.

Animals and semen collection

Ejaculates from three Holstein bulls (3 and 4 years of age) were used in the study. The bulls, belonging to the Lalahan Livestock Central Research Institute (Ankara, Turkey), were maintained under uniform feeding and housing conditions. A total number of 30 ejaculates (10 ejaculates for each bull) were collected from the bulls with the aid of an artificial vagina twice a week, according to AI standard procedures. Only ejaculates containing spermatozoa with >80% forward progressive motility and concentrations higher than 1.0×10^9 spermatozoa ml^{-1} were used. Nine ejaculates for each bull 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 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 (Trizma base 254 mM, citric acid monohydrate 78 mM, D (−) fructose 70 mM, egg yolk 15% (v/v), glycerol 6% (v/v), pH 6.8) was used as the base extender (freezing extender). Each ejaculate was split into five equal parts and diluted to a final concentration of 60×10^6 ml^{-1} spermatozoa with the Tris base extender containing curcumin (0.5 and 2 mM) and dithioerythritol (0.5 and 2 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^{-1} from 4 to −10 °C; −40 °C min^{-1} from −10 to −100 °C; −20 °C min^{-1} from −100 to −140 °C in a digital freezing machine (Digitcool 5300 ZB 250; IMV). Thereafter, the straws were plunged into liquid nitrogen. 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 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 analyse 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 to 1.92; low and high elongation gates 7 to 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., the Netherlands), 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, average path velocity (VAP, $\mu m\ s^{-1}$), straight linear velocity (VSL, $\mu m\ s^{-1}$), curvilinear velocity (VCL, $\mu m\ s^{-1}$), amplitude of lateral head displacement (ALH, μm) and linearity index (LIN = VSL/VCL × 100). For each evaluation, 10 microscopic fields were analysed to include 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) (Schafer & Holzmann, 2000). One 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).

Hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling test was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails. This was performed by incubating 30 μl of semen with 300 μl of a 100 mOsm hypo-osmotic solution (9 g fructose + 4.9 g sodium citrate per litre of distilled water) at 37 °C for 60 min. After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide. Two hundred spermatozoa were evaluated (magnification 1000×) with bright-field microscopy. Spermatozoa with swollen or coiled tails were recorded (Revell & Mrode, 1994; Buckett *et al.*, 1997).

Biochemical assays

Briefly, diluted semen samples were centrifuged at 800 ***g*** for 20 min at 4 °C 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 (Cassani *et al.*, 2005). Then, the sperm suspension was transferred into a 2-ml beaker in ice water and sonicated with a probe (Bandelin Sonopuls, UW 2070, Pro-Nr. 51900037369.004, Bandelin Electronic, Berlin, Germany) for 10 s on ice repeated six times at intervals of 30 s. This treatment rendered all spermatozoa that had their tails destroyed. For LPO analysis, 10 μl, 0.5 mM BHT 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 AOC measurement.

Determination of LPO levels

Lipid peroxidation levels were determined using commercial kits by LPO-586™ Oxis Research (OxisResearch™) by spectrophotometry (UV 2100 UV-VIS Recording Spectrophotometer; Shimadzu, Tokyo, Japan). The assay is based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole with malondialdehyde (MDA) and 4-hydroxyalkenals (LPO) at 45 °C. One molecule of either MDA or 4-hydroxyalkenal reacts with two 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^{-1}.

Determination of tGSH levels

Total glutathione levels were determined with GSH-420™ OxisResearch (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 oxidised glutathione (GSSG) to the reduced state (GSH). The chromogen, 4-chloro-1-methyl-7-trifluoromethylquinolinium methylsulphate, is added forming thioethers with all thiols present in the sample. Upon addition of base to raise the pH greater than 13, a β-elimination specific to the GSH thioether results in the chromophoric thione. The results are expressed as μmol for 10^9 cells ml^{-1}.

Determination of AOP levels

Antioxidant potential levels were determined with AOP-490™ Oxis Research (OxisResearch™) kit by spectrophotometry. Assay was based upon the reduction of Cu++ 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^{-1}.

Statistical analysis

Twenty-seven repetitions were conducted for the experiment. Results were expressed as the mean ± SEM. Sperm motility, motion characteristics, abnormalities and biochemical data were analysed by analysis of variance, followed by Tukey's *post hoc* test to determine significant differences between groups. Differences with values of $P < 0.05$ were considered to be statistically significant. Statistical analyses were performed by using the SPSS/PC computer programme (SPSS, version 12.0, Chicago, IL, USA).

Results

Sperm parameters (percentages of motility, sperm motion characteristics, acrosome and total abnormalities and HOST)

The effects of curcumin and dithioerythritol on the sperm parameters of frozen bull semen are presented in Table 1. Curcumin 2 mM gave the highest LIN percentage (49.80 ± 2.24%) among the groups. The extender supplemented with 0.5 mM dose of curcumin led to lower percentage of total abnormality (20.40 ± 2.36%), when compared to the control (30.60 ± 1.47%, $P < 0.05$). While curcumin and dithioerythritol at 0.5 mM led to higher percentages of post-thaw motility when compared to the control groups, these increases seemed to be nonsignificant ($P < 0.05$). Curcumin and dithioerythritol at 0.5 mM provided a greater protective effect in the membrane functional integrity (54.40 ± 2.09% and 50.00 ± 2.68%) in comparison with control (37.20 ± 1.77%, $P < 0.001$). No significant differences were observed in sperm acrosome abnormalities among the groups ($P > 0.05$).

LPO, tGSH and AOP levels

The influence of antioxidants on LPO, tGSH and AOP levels in thawed bull spermatozoa is set out in Table 2. Supplementation with antioxidants did not significantly

Table 1 Mean (±SE) sperm parameters in pooled semen supplemented with different antioxidants of bovine semen following freeze/thawing

Groups	CASA motility %	CASA progressive motility %	CASA total motility %	VAP ($\mu m\ s^{-1}$)	VSL ($\mu m\ s^{-1}$)	VCL ($\mu m\ s^{-1}$)	ALH (μm)	LIN %	Acrosome abnormality %	Total abnormality %	HOST %
Control	42.20 ± 0.01^{ab}	13.20 ± 1.20^{ab}	50.00 ± 6.20^{abc}	123.66 ± 3.97^{ab}	79.90 ± 4.22^{ab}	241.48 ± 7.48^{a}	9.82 ± 0.29	35.40 ± 2.25^{b}	9.60 ± 1.08	30.60 ± 1.47^{a}	37.20 ± 1.77^{c}
Curcumin 0.5 mm	46.20 ± 1.80^{a}	20.60 ± 1.81^{a}	57.00 ± 2.45^{a}	130.12 ± 7.35^{a}	94.02 ± 6.50^{ab}	242.38 ± 12.36^{a}	9.64 ± 0.26	40.60 ± 1.43^{ab}	5.40 ± 1.03	20.40 ± 2.36^{b}	54.40 ± 2.09^{a}
Curcumin 2 mm	30.40 ± 3.59^{bc}	17.40 ± 2.97^{a}	40.00 ± 4.21^{bc}	123.96 ± 3.99^{ab}	103.76 ± 3.88^{a}	215.28 ± 7.39^{ab}	9.10 ± 0.59	49.80 ± 2.24^{a}	6.20 ± 0.86	24.00 ± 2.19^{ab}	42.20 ± 2.44^{bc}
Dithioerythritol 0.5 mm	44.60 ± 2.82^{ab}	14.60 ± 1.99^{ab}	54.80 ± 2.11^{ab}	130.22 ± 4.85^{a}	83.06 ± 3.48^{ab}	240.92 ± 11.54^{a}	9.12 ± 0.34	36.40 ± 1.83^{b}	7.20 ± 1.36	24.60 ± 2.86^{ab}	50.00 ± 2.68^{ab}
Dithioerythritol 2 mm	25.20 ± 1.62^{c}	7.20 ± 2.01^{b}	35.40 ± 2.29^{c}	99.38 ± 8.83^{b}	69.94 ± 11.69^{b}	189.32 ± 13.90^{b}	8.52 ± 0.66	37.60 ± 3.27^{b}	8.40 ± 1.08	27.00 ± 0.89^{ab}	26.20 ± 1.88^{d}
P	*	*	*	*	*	*	–	*	–	*	**

a, b, c, d: Different superscripts within the same column demonstrate significant differences (*$P < 0.05$, **$P < 0.001$).
CASA, computer-assisted sperm motility analysis; ALH, amplitude of lateral head; HOST, Hypo-osmotic swelling test; LIN, linearity index; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight linear velocity.

Table 2 Mean (±SE) lipid peroxidation (LPO) levels, total GSH and antioxidant potential (AOP) of bovine semen supplemented with different antioxidants of bovine semen following freeze/thawing

Groups	LPO μm (10^9 cell ml^{-1})	Total GSH μm (10^9 cell ml^{-1})	AOP mm (10^9 cell ml^{-1})
Control	11.05 ± 1.90	480.56 ± 17.39^{b}	1.63 ± 0.72
Curcumin 0.5 mm	23.08 ± 6.10	556.50 ± 7.31^{a}	1.18 ± 0.14
Curcumin 2 mm	17.96 ± 4.21	511.16 ± 24.09^{ab}	1.56 ± 0.55
Dithioerythritol 0.5 mm	5.02 ± 1.57	506.82 ± 22.37^{ab}	0.91 ± 0.21
Dithioerythritol 2 mm	6.98 ± 0.46	462.18 ± 0.00^{ab}	2.08 ± 0.36
P	–	*	–

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

affect LPO and AOP levels in comparison with the control group ($P > 0.05$). The maintenance of tGSH levels in curcumin 0.5 mM group was demonstrated to be higher than that of control, following the freeze/thawing ($P < 0.05$).

Discussion

Reporting the useful effects of curcumin (Bucak *et al.*, 2010) on post-thawed sperm quality (motility and morphology) and dithioerythritol (Coyan *et al.*, 2010) on tGSH levels and GPx activities of cooled ram spermatozoa has given rise to its use as an antioxidant additive and has been shown to have cryoprotective effects when added to the freezing extender.

Sperm motility per se becomes crucial in facilitating the passage through cervix and uterotubal junction; even more important, it makes possible the actual penetration of the cumulus cells and zona pellucida of the ovum (Garner & Hafez, 1993). In the present investigation, supplementation of antioxidants medium led to nonsignificant differences in the sperm motilities and motion characteristics, except for LIN value in 2 mM curcumin. The result related to CASA motility is in contrast to our previous findings (Bucak *et al.*, 2010) which demonstrated a marked improvement in CASA motility of post-thawed ram spermatozoa supplemented with the curcumin 2.5 mM, whereas results of sperm motion characteristics, except for LIN value in 2 mM, and acrosome abnormality are in agreement with report obtained from ram spermatozoa (Bucak *et al.*, 2010) in which a difference was not recorded in the supplementation with curcumin for the frozen state of ram spermatozoa. The HOST test assesses the resistance of the sperm plasma membrane to damage induced by the loss in permeability under the stress of swelling driven by the hypo-osmotic treatment. Thus, this provides a form of a membrane stress test, which is particularly useful when testing the membrane-stabilising action of antioxidants (Bucak & Tekin, 2007). In this study, the highest HOST rates were obtained when sperm samples were cryopreserved with curcumin and dithioerythritol at 0.5 mM.

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. Supplementation of antioxidants can prevent this process (Aitken, 1984). Some authors (Aitken, 1984; Alvarez & Storey, 1989; Aitken *et al.*, 1993) stated that spontaneous LPO of the membranes of mammalian spermatozoa destroyed the structure of the lipid matrix, as a result of the invasion by ROS. These attacks ultimately led to the impairment of sperm functions (sperm motility, functional membrane integrity and fertility), through oxidative stress and the production of cytotoxic aldehydes. For this study, LPO releasing could not be prevented when the samples were cryopreserved with curcumin and dithioerythritol. The current findings relating to LPO are also in agreement with the results performed on ram and goat spermatozoa in which a decrease was not recorded in the level of MDA in the presence of curcumin, cystein and hyaluronan for the frozen state (Bucak *et al.*, 2007, 2008, 2010) or in methionine and dithioerythritol for sperm liquid storage (Coyan *et al.*, 2010). It can be hypothesised that antioxidants may facilitate the maintaining of post-thawed sperm membrane stability, evaluated by HOST, against oxidative stress, without eliminating the production of LPO. In addition to this view, we can state that LPO release does not seem an influential factor on the sperm quality following freeze/thawing process.

Antioxidant mechanisms comprising tGSH, GSH-Px and SOD exist as a defence mechanism against LPO in the semen and are important in maintaining sperm functions (O *et al.*, 2006). This antioxidant capacity in the sperm cell may, however, be insufficient in preventing LPO during the freeze/thawing process (Storey, 1997). 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 fertilisation of gametes (Perreault *et al.*, 1984; Nasr-Esfahani & Johnson, 1992; Irvine, 1996). In the present study, supplementation with curcumin 0.5 mM did elevate tGSH levels after thawing. This finding is in agreement with studies of ram spermatozoa, in which an increase in tGSH level was recorded in the presence of GSH, curcumin and dithioerythritol for the frozen state or during storage

(Bucak *et al.*, 2008, 2010; Coyan *et al.*, 2010), and is also in contrast to studies in goat (Ateşşahin *et al.*, 2008) and ram (Bucak *et al.*, 2007) spermatozoa, where increase in tGSH level was not observed when cryopreserved or stored with taurine, cysteine, cysteamine and hyaluronan. As related to the AOP, it is of seminal plasma because of the sum of enzymatic (e.g. SOD, catalase and glutathione peroxidase) and nonenzymatic (e.g. ascorbate, urate, vitamin E, pyruvate, glutathione, taurine and hypotaurine) antioxidants (Alvarez & Storey, 1989; Benzie & Strain, 1996; Donnelly *et al.*, 1999, Siciliano *et al.*, 2001). Low levels of seminal AOP have a key role in male infertility (Lewis *et al.*, 1997, Fingerova *et al.*, 2007). In a few studies, oral antioxidant application such as cysteine to infertile male (Ciftci *et al.*, 2009) and ginger rhizome to rat (Khakil *et al.*, 2009) had significant improving effects on semen AOP. Additionally, a study performed in buffalo showed that total antioxidant status of seminal plasma was significantly higher in presence of taurine, compared to the control and trehalose group (Shiva Shankar Reddy *et al.*, 2010). But in the present study, antioxidants did not increase the AOP levels.

Conclusions

Curcumin and dithioerythritol at 0.5 mM resulted in higher HOST values while only curcumin 0.5 mM provided cryoprotective effect on total sperm abnormality. Supplementation with antioxidants did not significantly affect LPO and AOP levels in comparison with the control groups. The maintenance of tGSH levels in curcumin 0.5 mM group was demonstrated to be higher than that of control, following the freeze/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 curcumin and dithioerythritol during cryopreservation.

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