CryoLetters **35** (6), 473-481 (2014)

CAN LINOLEIC ACID IMPROVE THE QUALITY OF FROZEN THAWED BULL SPERM?

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Abstract

BACKGROUND: Cryopreservation is known to have a detrimental effect on the motility, viability and membrane integrity of sperm cells. **OBJECTIVE:** The aim of this study was to investigate the effect of various amount of linoleic acid supplementation to the Tris extender, on bull sperm parameters, DNA integrity and oxidative stress after freeze-thawing. **METHODS:** Ejaculates were split into five aliquots and extended to a final concentration of $18x10^6$ spermatozoa/ml with the base extender containing different doses of linoleic acid 0.125 ml, (L125); 0.250 ml (L250); 0.5 ml (L500), 1 ml (L1000) and no additive (control; L0). The extended samples were equilibrated slowly to 4°C for 4 h and then froze using a digital freezing machine. Frozen straws were thawed individually in water bath at 37°C for 30 s to analyse progressive motility and sperm motion characteristics as well as membrane integrity. Biochemical assays were performed in a spectrophotometer using commercial kits. DNA damage was evaluated by Comet Assay. **RESULT:** The addition of various linoleic acid did not improve the sperm subjective, CASA and progressive motilities, sperm motility characteristics and DNA integrity ($P>0.05$). L500 exhibited the greatest values for membrane integrity than that of the other groups ($P<0.001$). All supplementation groups led to lower percentages of tail abnormalities in comparison to the control ($P<0.001$). L500 and L1000 significantly decreased total abnormalities. In conclusion, our findings showed that L500 linoleic acid supplementation in semen extender was of great beneficial effect on frozen-thawed bull semen in terms of morphology and plasma membrane integrity.

Keywords: Bull sperm, cryopreservation, DNA integrity, linoleic acid, oxidative stress

INTRODUCTION

Sperm cell contains a high content of polyunsaturated fatty acid (PUFA) (4). This PUFA provide fluidity that is necessary for sperm motility and membrane fusion events, such as the acrosome reaction and sperm ovum

interaction, which are both necessary for fertilization (1). The range of PUFA is considered as a critical feature for capacitation and is associated with sperm motility (37). Cryopreservation is known to have a detrimental effect on the motility, viability and membrane integrity of sperm cell (36). Semen cryopreservation process includes temperature reduction, cellular dehydration, freezing and thawing (15). The processes of cooling, freezing and thawing produce osmotic and chemical stresses on the sperm membrane that decreases sperm viability and fertilizing ability. Both cold shock and freezing damages are associated with reactive oxygen species (ROS) and oxidative stress (35). Among the ROS-induced damages on sperm there are those mediated by oxidative reaction of sperm phospholipid-bound PUFA, leading to lipid peroxidation, it is therefore important to search tool which can minimize the extent of sperm damage and scavenge ROS (3). The phospholipid fraction of the sperm is characterized by a high concentration of linoleic acid (16). Linoleic acid and other PUFA are known to change the lipid membrane composition in many cells. They can be incorporated by the plasma membrane of the cells (21) by provoking modification in its structure and function (38) and increase the proportion of sperm displaying progressive motility and decrease the percentage of sperm abnormality (22).

Limited number of studies on the supplementation of unsaturated fatty acids for sperm production or cryopreservation produced positive findings. There are conflicting results on the effect of that on fresh and frozen-thawed sperm quality. Some authors suggest that supplementation with fatty acids on the maintenance of sperm function after long-term liquid storage or freezing needs to be addressed (9). Hence, the aim of this study was to investigate the effect various amount of linoleic acid supplementation to the Tris extender, on bull sperm parameters, DNA integrity and oxidative stress after freeze-thawing.

MATERIALS AND METHODS

Animals and semen collection

In this study, three Brown Swiss bulls aging 2-3 years and had good quality semen characteristics were selected to be the source of semen. The bulls were clinically proven to be free from any general and genital diseases and maintained at the Livestock Central Research Institute (Ankara, Turkey). Ejaculates were collected from the bulls via an artificial vagina at twice a week. The ejaculates were pooled in order to increase semen volume for a replicate and to eliminate variability among the evaluated samples. The heterospermic semen sample was immersed in a water bath at 34°C until their evaluation in the laboratory. The evaluation was performed within 15 min immediately after collection; each ejaculate was evaluated to determine percentages of total and progressive motility as well as concentration. The ejaculates containing spermatozoa with >80% forward progressive motility and concentrations higher than $1.0x10^9$ spermatozoa/ml were used in this study. This study was replicated eight times for each group. The experimental procedures were approved by the Animal Care Committee of Istanbul University, Faculty of Veterinary Medicine.

Semen processing

The volume was read from the graded collection tube soon after collection, concentration was determined using Accucell photometer (IMV Technologie, L'Aigle, France), progressive motility was evaluated subjectively using a phase contrast microscopy (200x, Olympus BX43, Tokyo, Japan) at 37°C. A Tris-based

extender (T) (Tris 30.7 g, citric acid 16.4 g, fructose 12.6 g, egg yolk 20% (v/v), glycerol 6% (v/v), 1000 ml distilled water, pH 6.8) was used as the base extender. Pooled ejaculates were split into five aliquots and diluted to a final concentration of $18x10^6$/ml spermatozoa with the base extender containing different doses linoleic acid (BioReagent, Sigma-Aldrich Chemical Co., USA); 0.125 ml, (L125); 0.250 ml (L250); 0.5 ml (L500), 1 ml (L1000) and no additive (control; L0), respectively. Then cooled slowly to 4°C equilibrated for 4 h. Diluted semen samples were loaded into 0.25 ml French straws after equilibrium and frozen in a programmable digital freezing machine (Digitcool 5300 ZB 250, IMV, France). Thereafter, the straws were plunged into liquid nitrogen at -196°C.

Assessment of in vitro sperm quality

Subjective motility was assessed using a phase-contrast microscope (100x, Olympus BX43, Tokyo, Japan). A drop of semen was placed on a pre-warmed microscope slide and subjectively assessed at 37°C for per cent progressive motility. In addition for estimating subjective sperm motility, a computer-assisted sperm motility analysis (CASA; IVOS version 12; Hamilton-Thorne Biosciences, MA, USA) was also used to analyse sperm motion characteristics. CASA was set up as pre-adjusted for bovine sperm analysis. A semen sample was diluted 1:4 in lactate ringer and then diluted semen sample was put onto a pre-warmed chamber slide (20 mm; Leja 4; Leja Products BV, The Netherlands), and sperm motility characteristics were determined with a 10x objective at 37°C. The following motility values were recorded: motility (%), progressive motility (%), average path velocity, µm/s (VAP), straight linear velocity, µm/s (VSL), curvilinear velocity, µm/s (VCL), amplitude of lateral head displacement, µm/s (ALH), beat cross frequency, Hz (BCF). A minimum of 10 microscopic fields were analysed which include at least 300 cells, for each assessment.

The hypo-osmotic swelling test (HOS test) was used to assess the functional integrity of the spermatozoa membranes. HOS test was performed by incubating 30 µl of semen with 300 µl of a 100 mOsm hypo-osmotic solution at 37°C for 60 min After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide and then was examined under a phase-contrast microscopy (400x, Olympus BX43, Tokyo, Japan) (20). Two hundred spermatozoa were counted for their swelling, which is characterized by coiled tail, meaning intact plasma membrane.

For the evaluation of sperm abnormalities, two drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock solution (24). One drop of this mixture was put on a slide and covered with a cover slip. The percentages of acrosome, head, tail and total abnormalities were determined by counting a total of 200 spermatozoa under phase-contrast microscopy (1000x, Olympus BX43, Tokyo, Japan) under oil immersion.

Assessment of DNA damage

Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay, which was performed at high alkaline conditions. Semen samples were immersed in 37°C water for 30 sec and then were centrifuged at 600g for 10 min at room temperature. Seminal plasma was removed and remaining sperm cells were washed with PBS (Ca^{2+} and Mg^{2+} free) two times to yield a concentration of $1x10^5$ spermatozoa/cm3. Each microscope slide was pre-coated with a layer of 0.65% high melting point agarose in distilled water and thoroughly dried at room temperature. 75 µl of 0.65% low melting point agarose at 50°C

was mixed with 25 µl of the cell suspension then dropped on top of the first layer, and covered with coverslips. Slides were allowed to solidify for 10 min at 4°C in a moist box. The coverslips were removed and the slides were immersed in freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na^2–EDTA, 10 mM Tris, 1% Triton X-100 and 40 mM dithiothreitol (pH 10) for 1 h at 4°C. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA, (pH 13), for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at room temperature at 25 V and was adjusted to 300 mA. Subsequently, the slides were washed with a neutralizing solution of 0.4 M Tris, pH (7.5), in order to remove alkali and detergents. After neutralization, the slides were stained with 65µl of 20µg/ml ethidium bromide and covered with a coverslip. All these steps were conducted under dimmed light to prevent the occurrence of additional DNA damage. The images of 100 randomly chosen nuclei were analysed using a fluorescent microscope at a magnification of 400x (Zeiss, Germany). Nucleotide DNA extends under electrophoresis to form "comet tails," and the relative intensity of DNA in the tail reflects DNA break frequency. The percentage of the total DNA in the comet tail was taken as a measure of DNA break frequency. Tail DNA (%) was assessed in 100 cells by using Comet Assay III image analysis system (Perceptive Instruments, UK). Analysis was performed blindly by one slide reader (28)

Biochemical assays

Semen samples were centrifuged at 4°C at 1000g for 15 min in order to separate spermatozoa. Pellet was washed 3 times with a 0.5 ml of PBS. This final solution was homogenized 5 times by sonication in cold for 15 sec For the Lipid Peroxidation Analysis (LPO), 120µl of homogenate was mixed with 10µl 0.5mM butyl hydroxyl toluene (BHT) and kept in -80°C until the analysis. The rest of the homogenate was centrifuged at 8000g for 15 min and the supernatant was separated and kept in -80°C for the other enzyme analysis.

Enzyme analysis:

The levels of lipid peroxidase (LPO) were assessed with the commercial LPO-586TM Oxis research kit, Glutathione peroxidase (Gpx) levels with Gpx-340TM Oxis research kit, superoxide dismutase with Sigma–Aldrich Fluka FL 19160 kit, catalase (CAT) with OxisresearchTM Catalase520TM kit, GSH with Oxis researh-420TM kit and antioxidant capacity with Sigma-Aldrich Antioxidant assay CS 0790 kit with spectrophotometric analysis.

Statistical analysis

Data set is normally distributed using the Shapiro Wilk normality test. Homogeneity of variances with Levene's test groups was compared. The test revealed that the variances were homogeneous. After that, comparisons between the groups were made using analysis of variance with Duncan post hoc test. -, not significant and * $P <$ 0.001.

RESULTS

As set out in table 1, when compared to control different doses linoleic acid supplementation no significantly increased the percentages of post-thaw sperm subjective, CASA, progressive motilities and sperm motility characteristics such as VAP, VSL, VCL, ALH and BCF (P>0.05). L500 exhibited the greatest values for membrane integrity than that of the other groups (P<0.001). All supplementation groups

led to lower percentages of tail abnormalities in comparison to the control (P<0.001). L500 and L1000 significantly decreased total abnormalities, besides L500 led to the lowest percentages of the acrosome abnormalities (P<0.001).

As set out in table 2, There were no significance differences in DNA damage among treatment groups (P>0.05).

As set out in table 3, as regards to antioxidant activity CAT and total antioxidant activity were affected by supplementation of linoleic acid, notably group L500 yielded the greatest results in comparison to the other groups (P<0.001).

Table 1. Mean (±SE) sperm values in frozen thawed bull semen

Analysis	Control	Linoleic acid (0.125 ml)	Linoleic acid (0.250 ml)	Linoleic acid (0.5 ml)	Linoleic acid (1 ml)	F
Subjective motility (%)	53.8±3.63	58.8±4.30	59.4±3.46	56.9±4.11	60.0±4.23	1,89^{-}
CASA motility (%)	48.5±4.57	49.9±4.19	51.3±4.83	50.5±3.70	46.9±3.94	1,76^{-}
Progressive motility (%)	31.0±3.80	32.8±3.73	33.6±4.13	33.3±3.58	29.1±4.00	2.04^{-}
VAP (µm/s)	100.4±3.26	100.3±1.93	102.7±2.58	99.8±3.19	98.4±3.05	1,87^{-}
VSL (µm/s)	78.6±2.05	78.4±1.18	80.1±1.41	78.3±2.26	77.9±1.91	1,66^{-}
VCL (µm/s)	162.5±6.02	163.5±4.11	167.1±5.98	162.3±6.32	158.3±5.64	2,12^{-}
ALH (µm/s)	6.7±0.18	6.9±0.20	6.9±0.25	6.9±0.24	6.8±0.15	2,05^{-}
BCF (Hz)	12.3±0.59	13.2±0.40	13.2±0.51	13.3±0.37	12.9±0.54	1,96^{-}
HOS T (%)	40.3±1.31^{ab}	39.5±0.68^{a}	42.1±0.52^{b}	45.6±0.10^{c}	38.9±0.52^{a}	8,27*
Acrosome abnormalities (%)	5.4±0.46^{b}	7.8±0.31^{c}	5.6±0.50^{b}	3.9±0.40^{a}	4.9±0.52^{ab}	9,11*
Tail abnormalities (%)	3.5±0.50^{b}	1.88±0.40^{a}	2.13±0.40^{a}	1.13±0.13^{a}	1.4±0.18^{a}	7,67*
Total abnormalities (%)	19.3±0.73^{c}	17.4±0.99^{bc}	15.1±1.00^{ab}	12.5±0.93^{a}	13.0±0.76^{a}	8,84*

^{a,b,c} Different superscripts within the same row demonstrate significant differences ($^{*}P$<0.001)

$^{-}$ No significant difference (P>0.05)

Table 2. Mean (±SE) DNA damage values in frozen thawed bull semen

Analysis	Control	Linoleic acid (0.125 ml)	Linoleic acid (0.250 ml)	Linoleic acid (0.5 ml)	Linoleic acid (1 ml)	F
Tail Lengh (μm/s)	99.0±5.34	95.4±8.89	94.8±9.56	95.5±6.02	89.0±8.48	2,03[-]
Tail intensity (%)	30.6±4.28	26.0±3.36	23.3±3.84	25.4±2.94	22.6±2.81	1,87[-]
Tail moment (μm/s)	20.2±2.90	17.9±2.72	14.5±2.28	16.7±1.94	15.6±2.93	1,79[-]

[-] No significant difference (P>0.05)

Table 3. Mean (±SE) glutathione peroxidase (GPx), lipid peroxidase (LPO), reduced glutathione (GSH), catalase (CAT) and total antioksidant activities in frozen thawed bull semen

Analysis	Control	Linoleic acid (0.125 ml)	Linoleic acid (0.250 ml)	Linoleic acid (0.5 ml)	Linoleic acid (1 ml)	F
GPx (mU/ml-10^9cell/ml)	15.2±0.28	14.7±0.35	15.1±0.27	15.8±0.88	15.6±0.82	1,68[-]
LPO (μm/ml-10^9cell/ml)	0.9±0.27	0.49±0.28	0.50±0.25	0.61±0.31	0.57±0.30	2,14[-]
GSH (μm/ml-10^9cell/ml)	29.3±8.70	23.1±2.53	20.7±1.20	18.6±1.01	17.6±1.48	1,98[-]
CAT (μm/ml-10^9cell/ml)	8.5±1.85[b]	2.4±0.58[a]	10.0±3.06[b]	20.8±3.82[c]	2.8±0.48[a]	10,25[*]
Total antioksidant activities (mmol/trilox/ml-10^9 cell/ml)	8.2±1.23[b]	4.2±0.39[a]	9.2±2.04[b]	16.5±2.54[c]	4.5±0.32[a]	11,54[*]

[a , b, c] Different superscripts within the same row demonstrate significant differences (*P<0.001)

[-] No significant difference (P>0.05)

DISCUSSION

Cryopreservation imposes irreversible damage to sperm, including swelling and disruption of sperm plasma membrane (39). Phospholipids, which are cell membrane components, have ensured membrane fluidity, in the event they have protected bull spermatozoa from cold shock (12). Unsaturated fatty acid such as linoleic acid is high level participation in phospholipid of cell membrane and the supplementation of linoleic acid to the extender for sperm freezing is enhanced the sperm cryopreservation (13). This study showed that addition of linoleic acid did not improve the sperm subjective, CASA and progressive motilities as well as sperm motility patterns (VAP, VSL, VCL, ALH and BCF) with respect to the control. Similar to our findings, it has been reported that the supplementation of conjugated linoleic acid does not improved the motility parameters in Jersey bull (25) however, in the latter study, progressive and CASA motilities are greater than our results. Recent study in bull (7) concluded that were no improvement of sperm motility during cryopreservation when extender was supplemented with different antioxidants.

However, several studies performed in different breeds have reported results inconsistent with our findings. The addition of linoleic acid albumin to the cryopreservation medium improved motility after freezing–thawing of sperm in Japanese Black bull (27). Conjuge linoleic acid supplementation to embryo culture media can improve post-thawing embryo survival without affecting embryo production rates (17). It shows that fatty acids are important compounds to prolong the viability of spermatozoa (34). Based on our results, we can hypothesize that these discrepancies between the studies may be due to the density of other substances used extenders as well as different freezing protocols.

In point of post thaw sperm motility patterns (VAP, VSL, VCL, ALH and BCF), current findings agree with those reported by Soares et al. (25) who demonstrated that no improvement supplementation of oleic and linoleic acids are deleterious to the quality of non-sorted, frozen-thawed ram sperm and do not produce a beneficial effect to sex-sorted sperm. Changes in the extender and its composition, supplementation with various linoleic acids at different concentrations and antioxidant capacities, may explain why linoleic acid supplementation does not improve sperm motion characteristics

DNA integrity is more important device of sperm function to compare with the sperm parameters such as motility (19). DNA damage has a significant negative effect on the developing embryo quality (2) and reportedly leads to early embryonic death (10). However, the linoleic acid that we have used in our study did not provide any clear improvement at DNA integrity as our previous reports which used various antioxidants (29) and at different concentrations of trehalose (31). Besides, previous research on bovine sperm, the antioxidants carnitine and methionine did not maintain DNA integrity (23) In contrast to the results obtained, it has been shown that antioxidants added to sperm extender, reduce the DNA damage (30, 32). These contradictory results can be hypothesized that DNA damage may be associated not only with oxidative damages but also with osmotic damages.

However, all supplementation groups led to lower percentages of tail abnormalities with respect to the control. L500 and L1000 significantly decreased total abnormalities, while L500 led to the lowest percentages of the acrosome abnormalities. The reduced levels of polyunsaturated arachidonic and linoleic acids present in bull semen were associated with the reduced sperm quality (6). In harmony with our findings, it has been reported that antioxidants supplementation do not improve the sperm motility while some of them have effects on abnormalities and plasma membrane integrity positively (29) In addition, in ovine semen, the addition of oleic-linoleic acids to the semen extender resulted a beneficial effect in the preservation of sperm cell viability (18). Lipids play an important role in the maturation, viability and function of sperm cell (26). The integrity and functional activity of sperm membrane is major importance in the fertilization process and assessment of membrane function is useful indicator of fertilizing ability of sperm cell (33). It is indicated that α-lipoic acid had a protective action against to oxidative damage and abnormal sperm motility (14). But, according to study performed in bull, supplementation of conjugated linoleic acid did not show any improvement for sperm integrity (25). Elsewhere Estienne et al. (9) stated that supplementation with omega-3 fatty acids had no effect on the characteristics of sperm morphology. Based on the current results, this situation may be due to its protective effect on the integrity of sperm cells

which are associated with supplement behaviour.

Oxidative stress reflects an imbalance between production of ROS and cellular antioxidant defence mechanisms (11). It has been suggested that the shift of the lipid composition of mammalian spermatozoa is a key factor of membrane stabilisation (5) Catalase activity within the cell is largely located in the peroxisomes, it catalyses the reduction of H_2O_2 to O_2 and H_2O. It also supervises oxidative the oxidative stress in cells (8). In our study, the CAT and total antioxidant activities were the greatest in the L500 group. Although this group did not exhibit any positive effects on motilities, it showed a clear effect on sperm morphology and the plasma membrane integrity. This suggests that CAT and total antioxidant activities may have an influential factor on the sperm morphology and membrane integrity following freeze–thawing process.

In conclusion, the data generated here showed that L500 linoleic acid supplementation in semen extender was of great beneficial effect on frozen-thawed bull semen in terms of morphology and plasma membrane integrity

Acknowledgement: This study was financed under a project supported by the Scientific and Technological Research Council of Turkey (TUBITAK) (Project No: 106G005, TURKHAYGEN-1).

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