Kafkas Univ Vet Fak Derg
21 (3): 307-311, 2015
DOI: 10.9775/kvfd.2014.12366

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Effect of α-Lipoic Acid Supplementation to Extender on Quality of Frozen-Thawed Bull Semen [1] [2]

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

[2] *This study was presented 6th Congress on Animal Reproduction and Artificial Insemination with International Participation, 18-22 May 2011, Antalya, Turkey*

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KVFD-2014-12366 Received: 29.09.2014 Accepted: 26.11.2014 Published Online: 11.01.2015

Abstract

This study was conducted to investigate the possible protective effect of α-lipoic acid (ALA) supplementation to extender on damages in the quality of bull semen elicited by freze-thawing process-induced oxidative stres. Ejaculates were collected via an artificial vagina from the bulls at once a week. Then they were split into five aliquots and extended with the Tris base extender containing different doses of ALA, except control. The extended samples were equilibrated slowly to 4°C for 4 h and then frozen using a digital freezing machine. Frozen straws were thawed to analyse progressive motility and sperm motility characteristics as well as plasma membrane integrity. Biochemical assays were performed in a spectrophotometer using commercial kits. Treatment of extender with ALA groups caused a significant decrease in percentages of post-thaw sperm CASA, progressive motilities and sperm motility characteristics such as VAP, VSL and VCL except ALA0.5. Besides ALA0.5 showed lower percentages of acrosome and total abnormalities in comparison to the control. In conclusion, findings generated here showed that ALA0.5 supplementation in Tris based semen extender was of great beneficial effect on frozen-thawed bull semen in terms of morphology and plasma membrane integrity.

Keywords: *Bull sperm, Cryopreservation, α-lipoic acid, Oxidative stress*

Sulandırıcıya Katılan α-Lipoik Asidin Dondurulmuş-Çözdürülmüş Boğa Spermasının Kalitesi Üzerine Etkisi

Özet

Bu çalışma dondurma-çözündürme işleminin neden olduğu oksidadif stresden dolayı boğa spermasında meydane gelen hasarlar üzerine sulandırıcıya katılan α-lipoik asidin (ALA) muhtemel koruyucu etkisini araştırmak amacı ile yapıldı. Ejakülatler haftada bir kez olmak üzere suni vajen yardımı ile toplandı, sonrasında beş eşit parçaya ayrıldı ve kontrol grubu dışında diğerleri farklı dozlarda ALA içeren Tris bazlı sperma sulandırıcısı ile sulandırıldı. Sulandırılan spermalar 4°C'de 4 saat süre ile ekülibre edildi ve otomatik sperma dondurma cihazı kullanılarak dondurulduu. Dondurulan spermalar çözündürülerek plazma membran bütünlüğünün yanı sıra spermanın ileri yönlü hareketi ve sperma hareket özellikleri değerlendirildi. Biyokimyasal analizler ticari kit kullanılarak spektrofotometre de yapıldı. ALA0.5 grubu dışında ALA ilave edilmiş sulandırıcı ile sulandırılan gruplarda dondurma çözündürme sonrası CASA ve ileri yönlü spermatozoa hareketi ile VAP, VSL, VCL (spermatozoonun tüm hareketlerinin ortalaması, spermatozonun ilk harekete başladığı ve hareketini sonlandırdığı yer arasındaki en kısa mesafe, gerçek eğrisel yolda kat ettiği mesafe) gibi spermatozoa hareket özellikleri üzerine olumsuz etki gösterdi. Bunun yanında, kontrol grubu ile karşılaştırıldığında ALA0.5 akrozom ve total anormalitenin daha düşük oranlarda olmasını sağladı. Sonuç olarak, bu çalışmada elde edilen bulgular Tris bazlı sperma sulandırıcısına ilave edilen ALA0.5'in morfoloji ve plazma membran bütünlüğü üzerine olumlu etkilerinin olduğunu gösterdi.

Anahtar sözcükler: *Boğa sperması, Dondurarak koruma, α-lipoik asit, Oksidatif stress*

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INTRODUCTION

Cryopreservation is known to have a harmful effect on sperm motility [1,2]. The processes of cooling, freezing and thawing cause osmotic and chemical stresses on the sperm membrane that decreases sperm viability and fertilizing ability. This cold shock and freezing damages are associated with increased reactive oxygen species (ROS) and oxidative stress [3]. ROS stimulates a loss of sperm function associated with peroxidative damage to the mitochondria and plasma membrane, when produced in excessive amounts. Further, spermatozoa are more susceptible to peroxidative damage because of high concentration of polyunsaturated fatty acids and low antioxidant capacity [4]. Depletion of antioxidant defences or rise in free radical production can affect antioxidant balance and cause oxidative stress leading to cell death [5,6]. Enzymatic and non-enzymatic antioxidants play an important role in scavenging free radicals [7]. Lipoic acid is a disulfide compound that is found naturally in mitochondria as coenzyme for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase [8]. There is general agreement about the antioxidant properties of α-lipoic acid (ALA). It scavenges hydroxyl radicals [9] and singlet oxygen [10]. Besides ALA crosses biological membranes easily and suppresses free radicals, because of its small size and high lipophilicity [11]. Exogenous supplementation with this substance has been reported to increase unbound lipoic acid levels, which can act as a potent antioxidant and reduce oxidative stress both in vitro and in vivo [12]. Because of this antioxidant attributes, a number of experimental and clinical studies have been carried [8]. However, few studies were made on the efficacy of ALA suplementation during freze process of semen. It is hypothesized that in order to protect sperm against cryopreservation-induced oxidative stress, a successful antioxidant treatment should be implemented. The combination of extender with ALA may be appropriate approach to reduce the side effects of oxidative stress. Therefore, this study was conducted to investigate the possible protective effect of ALA supplementation to extender at various amounts on damages in the quality of bull semen elicited by freze-thawing process-induced oxidative stress.

MATERIAL and METHODS

Animals and Semen Collection

In this study, three Holstein bulls with clinically healthy, fertility proven and 2-3 years old which they were raised and maintained at the Livestock Central Research Institute (Ankara, Turkey), were used for semen collection. Ejaculates were collected via an artificial vagina from the bulls at once a week. The ejaculates containing spermatozoa with >80% forward progressive motility and concentrations higher than $1.0x10^9$ spermatozoa/ml were used in this study. The ejaculates were pooled in order to increase semen volume for making replication and to eliminate variability among the samples. This study was replicated eight times for each group. The semen sample was immersed in a water bath at 34°C until their evaluation; each ejaculate was evaluated to determine percentages of CASA progressive and total motilities as well as concentration. The experimental procedures were approved by the Animal Care Committee of Istanbul University, Faculty of Veterinary Medicine (number and date: 2006/172 and September 27, 2006).

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). 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), 1.000 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 ALA (Sigma-Aldrich Chemical Co., USA); 0.5 mM, (ALA0.5); 1 mM (ALA1); 2 mM (ALA2), 4 mM (ALA4) and no additive (control), respectively. Then all aliquouts were slowly cooled to 4°C equilibrated for 4 h. Diluted semen samples were loaded into 0.25 ml French straws after equilibration 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 Sperm Quality

Progressive and total motilities were evaluated using a computer-assisted sperm motility analysis (CASA; IVOS version 12; Hamilton-Thorne Biosciences, MA, USA). In addition CASA was also used to analyse sperm motility 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) [13]. 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 [14]. One drop of this mixture was put on a slide and covered with a cover slip. The percentages of acrosome and total abnormalities were determined by counting a total of 200 spermatozoa under phase-contrast microscopy (1000x, Olympus BX43, Tokyo, Japan) under oil immersion.

Biochemical Assays

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 Catalase-520TM kit, GSH with Oxis researh-420TM kit and antioxidant capacity with Sigma-Aldrich Antioxidant assay CS 0790 kit. The assesment of sperm biochemical assays was investigated using spectrophotometric analysis by the method of previously described by Tasdemir et al.[15].

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 variance with Duncan post hoc test using the SPSS/PC computer programme (version 14.1, Chicago, IL). The $P<0.05$ value was considered as significant.

RESULTS

As seen in *Table 1*, treatment of extender with ALA groups caused a significant decrease in percentages of post-thaw sperm CASA, progressive motilities and sperm motility characteristics such as VAP, VSL and VCL as compared to the control except ALA0.5 ($P<0.05$). ALA0.5 and ALA1 exhibited the greatest values for membrane integrity than that of the other groups ($P<0.01$). Besides ALA0.5 showed lower percentages of acrosome and total abnormalities in comparison to the control ($P<0.05$, $P<0.01$) *(Table 1)*.

As seen in *Table 2*, there were no significance differences in GPx and LPO activity among treatment groups ($P>0.05$). Conversely GSH, CAT and total antioxidant activity were affected by supplementation of ALA, notably group ALA1 yielded the greatest results in comparison to the other groups ($P<0.05$).

Table 1. *Mean (±SE) sperm values in frozen thawed bull semen*
Tablo 1. *Boğa spermasının dondurma çözündürme sonrası ortalama spermatolojik değerleri*

Analysis	Control	Lipoic acid (0.5 mM)	Lipoic acid (1 mM)	Lipoic acid (2 mM)	Lipoic acid (4 mM)	P
CASA motility (%)	39.50±3.33^{a}	43.25±1.87^{a}	24.63±3.06^{bc}	29.63±4.75^{b}	18.13±2.39^{c}	*
Progressive motility (%)	20.63±1.65^{a}	23.63±2.67^{a}	8.75±1.70^{b}	11.25±1.95^{b}	7.63±1.49^{b}	*
VAP (µm/s)	90.88±0.93^{a}	91.00±1.96^{a}	75.75±4.17^{b}	78.38±4.26^{b}	64.25±2.96^{c}	*
VSL (µm/s)	73.13±0.93^{a}	70.00±1.69^{a}	60.50±2.99^{b}	61.00±3.05^{b}	48.75±2.43^{c}	*
VCL (µm/s)	148.25±1.83^{ab}	156.63±3.36^{a}	127.38±6.81^{bc}	135.13±7.72^{b}	111.25±5.45^{d}	**
ALH (µm/s)	6.50±0.14^{bc}	7.75±0.31^{a}	6.25±0.25^{bc}	6.75±0.41^{ab}	6.63±0.50^{bc}	*
BCF (Hz)	14.50±0.71^{abc}	13.63±0.57^{abc}	15.75±0.80^{a}	12.38±0.63^{c}	14.50±1.41^{abc}	*
HOS T (%)	36.63±0.84^{b}	42.75±0.41^{a}	41.50±0.42^{a}	37.00±0.82^{b}	33.63±0.37^{c}	**
Acrosome abnormalities (%)	5.75±0.49^{ab}	4.38±0.53^{c}	4.63±0.32^{bc}	4.63±0.57^{bc}	5.00±0.39^{bc}	*
Total abnormalities (%)	14.25±0.62^{b}	10.50±0.98^{c}	11.88±0.99^{bc}	13.88±0.74^{b}	18.25±0.96^{a}	**

*^{a,b,c} Different superscripts within the same row demonstrate significant differences (** P<0.01, * P<0.05)*

Table 2. *Mean (±SE) glutathione peroxidase (GPx), lipid peroxidase (LPO), reduced glutathione (GSH), catalase (CAT) and total antioxidant activities in frozen thawed bull semen*
Tablo 2. *Boğa spermasında dondurma çözündürme sonrası ortalama glutatyon peroksidaz (GPx), lipit peroksidaz (LPO), redükte glutatyon (GSH), katalaz (CAT) ve total antioksidan değerleri*

Analysis	Control	Lipoic acid (0.5 mM)	Lipoic acid (1 mM)	Lipoic acid (2 mM)	Lipoic acid (4 mM)	P
GPx (mU/ml-10^9 cell/ml)	15.00±0.38	15.88±0.79	14.88±0.13	15.00±0.27	14.88±0.13	-
LPO (µm/ml-10^9 cell/ml)	0.63±0.32	0.25±0.25	0.13±0.13	0.38±0.26	0.38±0.18	-
GSH (µm/ml-10^9 cell/ml)	18.25±1.05^{c}	27.38±8.86^{bc}	54.13±11.71^{a}	30.00±8.94^{b}	32.13±9.50^{b}	*
CAT (µm/ml-10^9 cell/ml)	11.63±2.76^{b}	6.25±2.13b^{c}	24.00±5.25^{a}	8.25±2.34^{bc}	17.13±4.08^{ab}	*
Total antioksidant activities (mmol/trilox/ml-10^9 cell/ml)	10.25±1.74^{bc}	6.63±1.41^{bc}	18.50±3.52^{a}	8.13±1.54^{bc}	13.88±2.75^{ab}	*

^{a,b,c} Different superscripts within the same row demonstrate significant differences (P<0.05)*

DISCUSSION

Oxidative stress is a condition associated with an increased rate of cellular damage induced by ROS [16]. Unsaturated fatty acid is high level take part in phospholipid of cell membrane and the supplementation to the extender for sperm freezing is promoted the sperm cryopreservation efficiency [17]. ALA and its reduced form have been referred to as a universal antioxidant that functions in both membrane and aqueous environment [18]. It increases in semen quality and minimized to the oxidative stress [19] and puts down a variety of ROS [7]. These facts indicate ALA thus to has a protective action against the impairment of mitochondrial function, oxidative damage and sperm motility [20]. Current study showed that addition of ALA did not improve the CASA and progressive motilities as well as sperm motility characteristics with respect to the control. Earlier reports have shown when ROS increased during cryopreservation in bovine sperm, it impairs sperm function [21]. The results related to CASA and progressive motilities are in consistent with our previous findings Tasdemir et al.[15] and Büyükleblebici et al.[22] which demonstrated that supplementing various antioxidants to freeze the spermatozoa did not have any marked effects on the progressive and CASA motilities. This study also similar to recent studies in the bull [23], boar [24], rabbit [25] and stallion [26] concluded that were no improvement of sperm motility during cryopreservation when their diet was supplemented with fatty acids. However, current findings disagree with Japanese Black bull study, the addition of linoleic acid albumin to the extender marked improved motility parameter after freeze thawing [27]. In point of post thaw sperm motility patterns (VAP, VSL, VCL, ALH and BCF), current findings contrary to those reported by Soares et al.[21] who demonstrated that no improvement dietary supplements of oleic and linoleic acids were deleterious to the quality of ram sperm. Besides, VAP, VSL and VCL were lower than previous reports in which supplemented with the different doses sugars [28] as well as different doses curcumin and dithioerythritol [29]. Based on our results, after all we can hypothesize that these discrepancies between the studies may be due to the testing form.

Phospholipids, which are cell membrane components, have ensured membrane fluidity, in the event they have protected bull spermatozoa from cold shock [30]. It shows that fatty acids are important compounds to prolong the viability of spermatozoa [31]. Similar to our findings, Bucak et al.[29] showed that bull sperm samples in which 0.5 mM curcumin had been added, caused a decrease on the total abnormality (20.40±2.36%) and an increase membrane functional integrity (54.40±2.09%), but their membrane integrity and total abnormality results were greater than those obtained in our study in which supplemented with ALA0.5. In a dog breed sperm freezing study, Michael et al.[32] reported that after freezing and thawing, supplementing N-acetyl cysteine, taurine and catalase, to freeze the spermatozoa showed marked effects on the sperm morphology and plasma membrane integrity. It was indicated the ALA had a protective action against to oxidative damage and abnormal sperm motility [20]. Otherwise, in contrast with our findings, it has been proposed the adding antioxidants to freeze the spermatozoa do not have any marked effects on the acrosome and total abnormalities [27].

Thiols are the major components of cellular antioxidant system, which play an important role in detoxification of xenobiotic compounds and in the antioxidation of ROS and free radicals [33]. Consistent to the present investigation, ALA is also reported to accelerate the glutathione pool by reduction of oxidized glutathione [8]. Consistent to the present investigation Selvakumar et al.[19] who demonstrated a significant advance treatment with ALA increased in semen quality and minimized to the oxidative stress. Besides, Bucak et al.[34] reported antioxidants can improve sperm morphology and functional membrane integrity without influencing ROS formation in Angora goat. Otherwise, the addition of inositol do not improve sperm and biochemical parameters [35]. ALA0.5 showed a clear effect on sperm morphology and the plasma membrane integrity. However, this healing effect was not supported by antioxidant activities. This suggests that ALA0.5 might be due to the direct effect on the sperm morphology and membrane integrity or might be the antioxidant properties of egg yolk proteins, which are capable of preventing the oxidation of polyunsaturated fatty acids following freeze-thawing. Besides, it has dentrimental effect excess of 0.5 mM.

In conclusion, findings generated here showed that ALA0.5 supplementation in Tris based semen extender was of great beneficial effect on frozen-thawed bull semen in terms of morphology and plasma membrane integrity.

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