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Research Article

Long Term Incubation Resilience of Post-Thaw Ram Semen Diluted with Lecithin-Based Extender Supplemented with Bovine Serum Albumin

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Abstract

The objective of the study was to determine the optimum concentration of BSA in lecithin-based extender for post-thawing quality and incubation resilience (0 h, 6 h and 10 h) of ram sperm. Ejaculates were collected from five rams via electro ejaculation. Ejaculates were mixed to obtain pooled semen. Then, pooled semen was diluted with soybean lecithin-based extender without BSA (control) or supplemented with different concentrations of BSA (2.5 mg/mL, 5 mg/mL, 7.5 mg/mL and 10 mg/mL), at a final concentration of 150x106 spermatozoon/mL. Sperm motility, plasma membrane functional integrity (HOST), mitochondrial activity (rhodamine123), capacitation status (CTC), and DNA integrity (TUNEL) were evaluated. At the 10 h incubation, motility, plasma membrane functional integrity and mitochondrial function were better preserved in the BSA5 group compared to the control group. It was determined that high doses of BSA (5 mg/mL, 7.5 mg/mL and 10 mg/mL) affected acrosome reaction. The highest acrosome reaction rates were obtained in BSA10 groups in 6 h and 10 h incubation ($P<0.05$). TUNEL assay demonstrated that there were no differences among groups for DNA fragmentation at post-thaw and during incubation periods. The study shows that BSA supplemented extenders may have beneficial effect on ram semen parameters at 0 h, 6 h and 10 h of incubation. The results of the present study demonstrated a remarkable advantage of using 5 mg/mL of BSA in 1% lecithin-based extender.

Keywords: *Bovine serum albumin, Cryopreservation, Incubation resilience, Ram spermatozoa*

Sığır Serum Albumini İlave Edilmiş Lesitin Bazlı Sulandırıcı İle Sulandırılan Koç Spermasının Dondurma-Çözdürme Sonrası Uzun Süreli İnkübasyon Direnci

Öz

Bu çalışmanın amacı koç spermasının dondurma çözdürme sonrası ve inkübasyon direnci göz önüne alınarak (0, 6 ve 10. saatler) lesitin bazlı sulandırıcı için uygun Sığır Serum Albumini BSA konsantrasyonunun belirlenmesidir. Bu amaçla 5 koçtan sperma örnekleri alınmıştır. Alınan spermalar bir araya getirildikten sonra final konsantrasyonu 150x106 spermatozoon/mL olacak şekilde farklı dozlarda BSA içeren (2.5 mg/mL, 5 mg/mL, 7.5 mg/mL and 10 mg/mL) ve içermeyen (kontrol) soya fasülyesi lesitini bazlı sulandırıcılarla sulandırılmıştır. Spermanın değerlendirilmesi amacıyla motilite, plazma membran fonksiyonel bütünlüğü (HOST), mitokondriyal aktivite (Rhodamine123), kapasitasyon statüsü (CTC) ve DNA bütünlüğü değerlerine bakılmıştır. İnkübasyonun sonunda (10. saat) BSA5 grubunun motiliteyi, plazma membran fonksiyonel bütünlüğü ve mitokondriyal fonksiyonu diğer gruplara göre daha fazla koruduğu görülmüştür. BSA'nın yükselen dozları (5 mg/mL, 7.5 mg/mL and 10 mg/mL) akrozom reaksiyonunu etkilemektedir. İnkubasyonun 6 ve 10. saatlerinde en yüksek akrozom reaksiyonu oranları BSA10 grubunda gözlemlenmiştir ($P<0.05$). TUNEL sonuçları göz önüne alındığında; çözdürme ve inkübasyon sonrası gruplar arası DNA bütünlüğü bakımından bir fark olmadığı görülmektedir. Çalışma sonucunda; sulandırıcıya BSA ilave edilmesinin inkubasyonun 0, 6 ve 10. saatlerinde koç sperm parametreleri üzerinde yararlı etkisi olduğu görülmektedir. Çalışma sonucu elde edilen veriler göz önüne alındığında %1 lesitin bazlı sulandırıcıya 5 mg/mL BSA ilavesinin önemli bir avantaj oluşturduğu görülmektedir.

Anahtar sözcükler: *Sığır serum albumini, Kriyoprezervasyon, İnkübasyon drenci, Koç sperması*

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INTRODUCTION

Semen cryopreservation is the pillar of the reproductive biotechnology [1]. This reversible process brings semen metabolism to a standstill, in this way it is possible to storage genetic material throughout long time [2]. Even though cryopreservation is a reversible operation, there are some detrimental effects (cold shock, ice crystallization, lipid peroxidation etc.) of this process [2,3]. These adverse effects may cause irreversible decrease on motility, viability and fertilization ability of spermatozoa [4,5]. Therefore, the success of semen cryopreservation depends on minimizing the adverse effects and maintaining the post-thaw semen quality [1,5]. There are a lot of research, aim of which was to increase success of ram semen cryopreservation with different procedures and additives [6-10].

Lecithin is a low-density lipoprotein fraction that is mainly found in plants and plays an important role in the regulation of an animal cells' bio-membrane [11]. In addition, it is used for semen cryopreservation either as an ingredient of egg yolk or as an extracted substance [11,12]. Many researchers have obtained acceptable post-thaw semen parameters with using lecithin-based extender [11-16] but upgrading these results is possible with optimized lecithin base extenders.

Bovine Serum Albumine (BSA) has multifunctional effect on sperm with its macromolecular structure and antioxidant capacity. Therefore, BSA increases the post-thaw motility [17,18], protects the membrane against cold shock [17] maintains DNA integrity during freezing-thawing and incubation periods [18]. In addition, it induces the capacitation [19,20] and acrosome reaction [21]. In brief; BSA not only protect the spermatozoa against freezing-thawing process but also stimulates the capacitation and acrosome reaction. Besides, BSA increase the possibility of sperm-zona pellucida interactions and fertility results. As a consequence of these, BSA supplemented extenders are used for cryopreservation or liquid storage of bull [18], ram [22], goat [23,24], stallion [25], buffalo [26], rabbit [27] and turkey semen [28].

Many researchers used lecithin or BSA based extenders to improve post-thaw quality and fertilization ability of spermatozoa [16-18,20,21,28]. However, collaborate using of these ingredients for ram semen cryopreservation has not been performed yet. The aim of this study was determining the appropriate dose of BSA in lecithin-based extenders for improving motility, viability, DNA integrity in 0 h, 6 h and 10 h of incubation. Also, we aimed to recognize the capacitation and acrosome reaction of ram spermatozoa at the same time periods.

MATERIAL and METHODS

Scientific Ethical Committee (Uludag University, Bursa, Turkey) have approved all issues concerning the experimental setups and evaluation techniques (2015-07/03).

Chemicals

All chemicals used in the study were obtained from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA).

Experimental Design

This study was designed to evaluate the efficacy of BSA supplementation to the extender in ram sperm cryopreservation. Therefore, we used various concentrations of BSA (0 mg/mL, 2.5 mg/mL, 5 mg/mL, 7.5 mg/mL or 10 mg/mL) in lecithin-based extenders during non-breeding season.

Semen Extender Preparation

Extenders contained 223.7 mM Tris, 55.5 mM fructose, 66.6 mM citric acid, 100.4 mM Trehalose, 4.03 mM EDTA, 1 mM cysteine, 4 g/L penicillin G, 3 g/L dihydrostreptomycin, 1% lecithin in distilled water. Relevant concentrations of BSA according to experimental design added to each group of extenders, but the control.

Semen Collection and Dilution

Semen collection from Kivircik Rams, which were maintained with same conditions at Uludag University, Faculty of Veterinary Medicine in Bursa, Turkey, was performed by electrically stimulated ejaculation (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand) five times in every other day. After the collection, the ejaculates were transferred to a water bath (37°C). Rapid wave motion and motility evaluated by phase-contrast microscope (Olympus BX51, Olympus Optical Co., Tokyo, Japan) with a warm slide (37°C). Spermatozoa concentration on native semen was evaluated with hemocytometric method. For this purpose, semen was diluted with alcohol (1:200 semen/semen-alcohol dilution) then the solution was dropped to Thoma's Couting Slide and the concentration of spermatozoa were counted. Ejaculates have rapid wave (>+3 on 0-5 scale), >75% motility and >$1.0x10^9$ spermatozoon/mL were chosen for cryopreservation. In order to eliminate individual differences, ejaculates were pooled.

Briefly, pooled ejaculate was split into five equal aliquots and diluted (37°C) to final concentration of approximately $150x10^6$ (spermatozoon/mL) with BSA supplemented extenders and control extender. Within 1 h, diluted semen was gradually cooled to 4°C and then equilibrated for 2 h at 4°C.

Semen Freezing and Thawing

After the equilibration, semen was loaded into 0.25 mL French straws. Semen was frozen at 3°C/min from +5°C to -8°C and at 15°C/min from -8°C to -120°C in liquid nitrogen vapor using the Nicool Plus PC freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France). The straws were then plunged into liquid nitrogen at -196°C where

they were stored. Three straws from each group were thawed at 37°C for 30 s in a water bath and incubated in humidified air chamber with 5% CO_2 for 10 h at 39°C to evaluate post-thaw semen characteristics.

Semen Evaluation

We evaluated sperm motility, plasma membrane integrity (hypoosmotic swelling test [HOST]), mitochondrial activity, (R123; Invitrogen TM, Eugene, OR, USA), capacitation status (Chlortetra cycline [CTC] staining) and DNA integrity (using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling [TUNEL]) at three-time points (post-thaw at 0 h, 6 h and 10 h). All processes and measurements were conducted by the same person during the study. Sperm motility was assessed subjectively using a phase-contrast microscope (400×) with a warm slide (37°C).

Assessment of plasma membrane functional integrity was performed with HOST test described by Leboef et al.[29] with little modification [30]. Mitochondrial activity was evaluated using a combination of fluorescent stains, Rhodamine 123 (R123) and PI methodology of Fraser et al. was used to performe [31]. Capacitation status evaluation was performed as described by Pérez et al.[32]. For the TUNEL technique, we used the In Situ Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with slight modifications [33].

Statistical Analysis

All data obtained from study were analyzed using SPSS (SPSS 20.0 for Windows; SPSS, Chicago, IL, USA). Data were represented as mean ± standard deviation. Shapiro Wilk test was used as normality test. Means of obtained semen parameters were analyzed using Kruskal Wallis test. Statistical differences between the various treatment group means were determined by Mann Whitney U test. Differences with values of $P<0.05$ were considered to be statistically significant.

RESULTS

The standard semen parameters of the BSA were evaluated in 5 independent experiments by the same person. Percentages of sperm motility, plasma membrane functional integrity, mitochondrial activity and DNA fragmentation were indicated in *Table 1*. Capacitation status are showed in *Table 2*.

Sperm motility was progressively reduced after incubation *(Fig. 1)* ($P<0.001$). Post-thaw sperm motility rates in BSA7.5 group was higher than the control and BSA2.5 groups at 0 h ($P<0.05$). After 6 h incubation, BSA2.5 group had higher motility rate than the control group ($P<0.05$). At the end of the 10 h incubation, mean motility rates better preserved in the BSA2.5, BSA7.5 and BSA10 groups than the control groups ($P<0.05$). The highest percentage of motility was observed in BSA5 group at post-thaw and incubation periods ($P<0.05$).

Plasma membrane functional integrity deteriorated after post-thaw and incubation ($P<0.001$). Membrane functional integrity was better preserved in the BSA7.5 and BSA10 groups than the control group at 0 h ($P<0.05$). The BSA2.5 group had higher membrane integrity percentage than the control group at 6 h ($P<0.05$). After 10 h incubation, the similar result was obtained in the BSA2.5, BSA7.5 and BSA10 groups ($P>0.05$). Membrane integrity was protected in BSA5 group compared to the other groups ($P<0.05$).

Mitochondrial function decreased with the freeze-thaw process ($P<0.001$). While, post-thaw mitochondrial function

Table 1. *The mean of studied sperm resilience parameters on different extender groups*

Incubation Period (h)	Group	Motility (%)	HOST (%)	Mitochondrial Function (%)	DNA Fragmentation (%)
0	Control	53.00±2.54^{a}	63.40±3.25^{a}	58.0±1.46^{a}	4.75±0.96
	BSA 2.5	54.00±2.07^{a}	65.40±2.13^{ab}	59.4±2.13^{a}	5.00±0.82
	BSA 5	61.00±2.07^{c}	71.20±3.88^{c}	68.0±2.36^{c}	4.60±0.55
	BSA 7.5	57.00±2.54^{b}	67.00±1.46^{b}	63.6±4.22^{b}	5.00±1.15
	BSA 10	56.00±2.07^{ab}	67.40±2.75^{b}	63.6±3.50^{b}	5.75±1.26
6	Control	22.00±4.14^{a}	32.20±3.17^{a}	26.8±1.78^{a}	9.75±2.50
	BSA 2.5	26.00±3.87^{b}	36.40±1.92^{b}	30.20±2.57^{b}	9.60±1.14
	BSA 5	35.00±3.27^{c}	43.40±4.56^{c}	38.60±2.50^{c}	8.40±1.14
	BSA 7.5	25.00±3.27^{ab}	34.60±3.85^{ab}	29.40±4.37^{ab}	9.00±0.82
	BSA 10	24.00±3.87^{ab}	35.20±4.16^{ab}	30.80±4.00^{b}	10.20±0.84
10	Control	2.00±2.54^{a}	10.40±3.50^{a}	7.20±2.01^{a}	19.67±1.15
	BSA 2.5	6.00±2.07^{b}	13.40±2.41^{b}	11.40±2.32^{b}	18.25±2.22
	BSA 5	12.00±2.54^{c}	21.00±2.17^{c}	20.40±3.62^{c}	18.00±1.41
	BSA 7.5	8.00±2.54^{b}	12.20±2.57^{ab}	14.20±2.88^{b}	19.50±3.32
	BSA 10	7.00±2.54^{b}	13.40±1.80^{b}	13.60±3.96^{b}	19.50±4.20

$^{a, b, c}$ Values with different superscripts in the same column for each of incubation time are significantly different ($P<0.05$)
Data is presented in Mean± S.D.

Table 2. *Effect of BSA on capacitation status of frozen-thawed sperm*

Incubation Period (h)	Group	Uncapacitated (%)	Capacitated (%)	Acrosome Reacted (%)
0	Control	24.40±1.82^{a}	58.20±0.84^{a}	17.80±2.68
	BSA 2.5	22.20±1.30^{a}	58.80±1.48^{a}	19.00±1.41
	BSA 5	22.60±3.05^{a}	59.40±2.30^{a}	18.00±4.47
	BSA 7.5	17.80±1.30^{b}	64.00±2.34^{b}	18.20±1.79
	BSA 10	15.20±2.28^{b}	67.40±2.41^{b}	17.40±1.14
6	Control	14.40±2.61^{a}	66.40±1.52^{a}	19.20±1.92^{a}
	BSA 2.5	15.20±1.10^{ab}	65.40±0.89^{ab}	19.40±1.34^{a}
	BSA 5	17.80±1.30^{b}	61.40±2.07^{b}	20.60±1.52^{a}
	BSA 7.5	11.00±1.41^{c}	63.00±3.08^{ab}	26.00±3.81^{b}
	BSA 10	9.60±0.55^{c}	63.40±2.41^{ab}	27.00±2.12^{b}
10	Control	6.80±0.84^{a}	58.80±1.79	34.40±1.52^{a}
	BSA 2.5	6.60±1.52^{a}	58.60±2.07	34.80±1.10^{a}
	BSA 5	6.00±0.71^{ab}	57.60±1.34	36.40±1.14^{ab}
	BSA 7.5	4.40±1.14^{bc}	56.20±2.59	39.40±1.82^{bc}
	BSA 10	3.60±0.89^{c}	54.60±4.39	41.80±4.09^{c}

^{a,b,c} *Values with different superscripts in the same column for each of incubation time are significantly different (P<0.05)*
Data is presented in Mean± S.D.

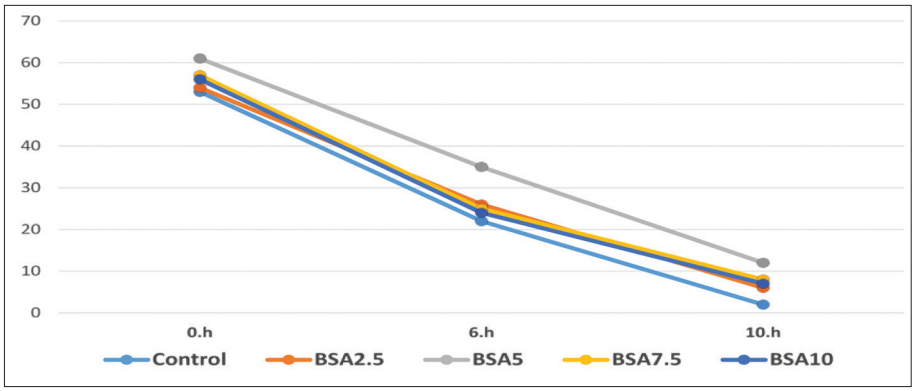

Fig 1. The motility results of the experiment groups during incubation

values at 0 h in BSA7.5 and BSA10 groups were higher than the BSA2.5 and control groups. After 6 h incubation, BSA2.5 and BSA10 group had higher mitochondrial function rate than the control group (P<0.05). At the end of the 10 h incubation, mitochondrial function was successfully preserved in BSA2.5, BSA7.5 and BSA10 groups compared to the control group (P<0.05). Similar to motility and plasma membrane integrity, the best mitochondrial function was obtained for the BSA5 group compared to the other groups (P<0.05).

The post-thaw and after incubation DNA damaged spermatozoa percentages were not statistically significant in all groups (P>0.05).

Post-thaw uncapacitated and capacitated status in control, BSA2.5 and BSA5 groups were higher than the other groups (P<0.05) In addition, acrosome reacted (AR) values were not significant among groups. After 6 h incubation, uncapacitated rates in BSA7.5 and BSA10 groups were lower than the compared to the other groups (P<0.05). Capacitation rates in control group were higher than the BSA5 group (P<0.05). AR rates in BSA7.5 and BSA10 groups were higher than the other groups (P<0.05). At the end of the 10 h incubation, uncapacitated rates in control and BSA2.5 groups were higher than the BSA7.5 and BSA10 groups (P<0.05). Capacitated rates were not significant between groups. AR rates in BSA10 groups were higher than the control, BSA2.5 and BSA5 groups (P<0.05).

DISCUSSION

Cryopreservation process have a detrimental effect on spermatozoa because of temperature change, cold shock, ice crystallization and lipid peroxidation. These restrictive effects provoke to decrease of motility, viability, mitochondrial membrane function, DNA integrity and fertilizing ability of spermatozoa [11,12,16]. Various supplements were tested to minimize the adverse effect of cryopreservation [5-12,14,16,28].

Bovine serum albumin is one of the attempted supplements, because of a good amino acid profile and protective functions [17,21]. In the present study, we compared the effect of exogenous addition of BSA in lecithin-based extender on ram semen quality at post-thaw and during the incubation periods.

Motility is one of the essential semen quality parameters as an indicator of attaining to oocyte [33]. The post-thaw motility values of ram semen cryopreserved with lecithin-based extender ranged between 31.0% - 48.1% [5,11,14,34]. Our post-thaw motility results clearly higher than these studies. Good interaction between lecithin-BSA could be the reason of these results. BSA stimulates sperm motility [17,34] but there is no enlightening explanation about mechanism of its stimulative effect [17]. In our study; although BSA supplementation prompted to clear increase on motility, BSA2.5 group had not sufficient effect to make statistical difference at all incubation times. BSA5 group had better motility values than other groups at post thaw and after incubation ($P<0.05$). When sufficient BSA doses (BSA5, BSA7.5, BSA10) were compared among each other, it was shown that increasing doses of BSA caused gradually decrease on motility.

The sperm-environment interaction is performed with plasma membranes that have an essential role in sperm metabolism [26]. Therefore, plasma membrane integrity is important for capacitation, acrosome reaction and finally oocyte fusion of sperm [35]. However, plasma membrane permeability and integrity get harmed from cold shock and lipid peroxidation (LPO) [33]. Cold shock causes phase transition of membrane lipids and then plasma membrane could lose its selective permeability [36]. The protection against cold shock is possible with increasing the fluidity of membrane [37]. The protective effect of BSA against cold shock is based on this expected impact. BSA attaches to the sperm membrane then changes sperm membrane lipid composition and decreases to phospholipid concentration [19]. The proper protection against cold shock could be evaluated with assessment of plasma membrane functional integrity. HOST is the optimized test for detecting the subtle changes of sperm membrane functional integrity [35]. In the present study, the HOST values in BSA5 group were higher than in the other groups at post-thaw and after incubation ($P<0.05$). The HOST values are in a good agreement with the previous researches [5,11,12,28,33].

The negative effect of cold shock and LPO is not only limited to losing of plasma membrane integrity, but also the organelle membranes suffered from them as well. Membrane integrity loses lead to malfunction or dysfunction of organelles [38]. When reactive oxygen species (the result of cooling, cold shock or cryostress) induce mitochondrial membrane damage for this reason ATP synthesis is interrupted and consequently spermatozoon loss its motility and metabolism function [38]. Therefore, mitochondrial membrane function is important for sperm fertilization ability. In the current study, sperm mitochondrial function was assessed by R123 fluorescent staining. The BSA5 group protected mitochondrial activity properly and this protective effect proceeded reach up to 10 h incubation.

Capacitation and acrosome reaction are essential for the last journey of spermatozoon. It has been related with sperm penetration and fusion to zona pellucida [39]. The trigger of capacitation process is the increment of intracellular calcium concentration so it can be regarded as a key factor that regulating induction of sperm capacitation [40]. The BSA promotes Ca^{+2} to influx into spermatozoon and rises intracellular Ca^{+2} concentration. In this way it stimulates sperm membrane reorganization [41]. In the study, there was no statistical difference between low doses BSA and control for uncapacitated spermatozoa rates at 0 h. It is not an undesirable result because higher rate of capacitated spermatozoa at 0 h may be the indicator of precapacitation. Considering the progressive times of incubation, higher BSA doses decreased the capacitated spermatozoa rates. The decrease of capacitated sperm with higher dose BSA groups was relevant with the increase of acrosome reacted sperm. Briefly, higher doses of BSA was induce acrosome reaction and the highest results of 6 h and 10 h was obtained with BSA10 ($P<0.05$).

The other adverse effect of cryopreservation is the DNA damage [33,42]. On the contrary of the other assessment methods; DNA integrity is not the direct indicator of sperm fertilizing ability [42]. It is important for identification of seriously damaged spermatozoa and embryo development ability [42]. According to our data; there were no difference among DNA integrities of control or BSA groups. In many studies using ram spermatozoa, the post-thaw and after 6 h incubation results of DNA fragmentation rates are in a rate between 3.6%-11.9% [12,35,43] and 5.8%-12.4% [12,43] respectively. In our study, similar DNA fragmentation rates were obtained.

The results of the present study indicated that BSA5 group preserved sperm motility, plasma membrane functional integrity and sustained mitochondrial activity better than both BSA-free and higher doses of BSA supplemented extenders throughout freezing-thawing and incubation period. Considering to all sperm parameters mentioned above; BSA5 group was the optimum for ram semen preservation. Beneficial effect of BSA supplementation looked promising to increase the utility of lecithin-based extender for ram spermatozoa. Future studies should be aimed at confirming the usefulness of the supplementation with BSA5 group regarding field fertility.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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