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RESEARCH ARTICLE

Comprehensive Effects of Fetal Calf Serum in Soybean-Lecithin Based Goat Semen Cryopreservation Extenders and Impacts on Incubation Resilience

Mehmed Berk TOKER [1,a (*)] Selim ALCAY [1,b]

[1] Bursa Uludag University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, TR-16059 Bursa - TÜRKİYE
ORCIDs: [a] 0000-0003-4033-9749; [b] 0000-0002-2472-8157

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Abstract: The aim of this study was to investigate the effects of various fetal calf serum (FCS) doses on %1 soybean lecithin-based semen extenders for goat semen cryopreservation and the impacts on spermatological parameters over post-thaw and post-incubation (6 h) time periods. Sperm samples collected via electro-ejaculation method were pooled to overcome the individual differences and were used in the study. The samples then were split into four equal aliquots to create study groups as; 0.25%, 0.50%, 0.75% FCS supplemented and a sample of FCS-free control group. Each sample group was diluted to approximately 150 x 10^6 spermatozoon/mL final concentration and two step dilution method was used for cryopreservation. Study groups were examined for sperm motility, plasma membrane functional integrity with hypoosmotic swelling test (HOST), acrosome integrity by FITC-*Pisum sativum* agglutinin (PSA-FITC) and DNA damage by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (TUNEL). All samples were incubated for further 6 h in a humidified air chamber with 5% CO_2 at 39°C. The results indicated that FCS supplementation in soybean lecithin-based extenders for goat sperm cryopreservation had significant effects on post-thawing time point motility (P<0.05), plasma membrane integrity (P<0.05) and acrosomal integrity (P<0.05) parameters. Subsequently to 6 h of incubation period, DNA integrity results yielded better scores comparing to control group in addition with other spermatological parameters (P<0.05).

Keywords: *Cryopreservation, Fetal calf serum, Goat semen, Incubation resilience, Soybean lecithin*

Lesitin Bazlı Teke Sperma Sulandırıcılarına Eklenen Fötal Buzağı Serumunun Spermanın Dondurulma ve İnkübasyon Direnci Üzerine Etkileri

Öz: Bu çalışmanın amacı teke spermasının dondurulmasında kullanılacak %1 soya lesitini bazlı sulandırıcıya eklenen çeşitli dozlarda fötal buzağı serumunun (FBS) dondurma-çözdürme ve 6 saatlik inkübasyon süreci sonrası dönemde spermatolojik parametrelere etkilerini araştırmaktır. Sperma örnekleri elektro-ejakülatör ile alınmış ve bireysel farklılıkların üstesinden gelinmesi amacıyla birleştirilmiştir (pooling). Birleştirilen örnekler dört eşit gruba bölünmüş ve %0.25, %0.50, %0.75 FBS eklenmiş ve FBS eklenmemiş kontrol olacak şekilde çalışma grupları oluşturulmuştur. Her çalışma grubu dondurulma amacıyla 150 x 10^6 spermatozoon/mL final konsantrasyon olacak şekilde iki aşamalı olarak sulandırılmıştır. Çalışma grupları sperma motilitesi, hipo-ozmotik şişme testi ile ölçülen plazma membran fonksiyonel bütünlüğü, FITC-*Pisum sativum* agglutinin (FITC-PSA) ile ölçülen akrozomal bütünlük ve terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (TUNEL) testi ile ölçülen DNA bütünlüğü açısından incelenmiştir. Tüm gruplar çözdürme sonrası dönemde inkübasyon direncinin ölçülmesi amacıyla 6 saat boyunca %5 CO_2 ve 39°C sıcaklıklığa ayarlanmış bir ortamda inkübe edilmiştir. Çalışmanın sonuçları soya lesitini bazlı sperma dondurma sulandırıcılarına FBS eklenmesinin çözdürme sonrası motilite (P<0.05), plazma membran bütünlüğü (P<0.05) ve akrozomal bütünlük üzerine istatistiki anlam düzeyinde fark yarattığını göstermiştir. Buna ek olarak, gerçekleştirilen 6 saatlik inkübasyon sürecinin sonucunda FBS'nin adı geçen spermatolojik parametrelere ek olarak DNA bütünlüğü sonuçlarında da pozitif etki yarattığı tespit edilmiştir (P<0.05).

Anahtar sözcükler: *Sperma dondurma, Fötal buzağı serumu, Teke sperması, İnkübasyon direnci, Soya lesitini*

INTRODUCTION

Cryopreservation of domestic ruminants' semen is an excellent method to procure better and desired gene merits to obtain greater yield numbers within next generations [1]. To achieve the aim of enhanced success rates in terms of

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(*) Corresponding Author
Tel: +90 224 2941350 Cellular phone: +90 530 038 8394
E-mail: berktoker@uludag.edu.tr (M.B. Toker)

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cryopreservation, various ways and methods have been investigated in different species throughout years [2-4]. Despite goat semen can be freezable like other ruminant semen, some of its' physiologic specialities need to be taken into consideration to reach the best out of it [5]. The interaction between egg-yolk in freezing media and egg-yolk coagulating enzyme (EYCE) produced in bulbourethral gland of bucks' as an enzyme is one of the biggest challenges for cryopreservation of semen [6]. Sperm cells are exposed to various effects within cryopreservation process that hampers the well-being of the cells [7,8]. Soybean lecithin contains low-density lipoprotein fractions as it is in the composition of egg-yolk [9]. Phosohatidylcholines have a great role in the defense systems of sperm membrane throughout the freeze-thawing process and soybean lecithin can be used as an extender component in regards of this matter [4]. Since there have been researches done and published about using soybean lecithin, it can be a better choice instead of egg-yolk in goat semen cryopreservation extenders [10-12].

Cold shock and ice crystal formations that happen in the process of cryopreservation of semen affect spermatological parameters and functions, and hampers the success of fertility [13]. Various substances have been used in freeze-thawing processes to protect sperm cells and prevent the effects of physical and chemical stress sources to increase the achievement of the method [14]. Through freezing and thawing process, fetal calf serum (FCS) acts as a buffer to osmotic shock, protects membranes and reduces the impacts of ice formation [15]. FCS contains many elements that cells need such as hormones, growth factors, sugars, amino acids, lipids and trace elements to contribute continuation of a tissue [16]. FCS has also been used widely as a protein to stabilize the cell culture mediums for a while [17,18].

This study aimed to supplement vital low-density lipoprotein (LDL) resources by adding soybean lecithin as the main component of the sperm cryopreservation extender. In addition to that, supplementing the extender with 0.25%, 0.50% and 0.75% FCS to overcome the negative effects of cryopreservation process and to find out the effects over spermatological parameters on post-thaw and 6 h of incubation time periods is one of the main aims of the research. To carry out the study, Saanen bucks were chosen as the sperm donors and the incubation resillience of the sperm samples assessed for 6 h. To evaluate the sperm samples; percentage of motility, sperm membrane funcitonal integrity with hypo-osmotic swelling test (HOST), FITC conjugated *Pisum sativum* agglutinin (PSA FITC) for acrosomal integrity and Terminal Deoxynucleotidyl Transferase-Mediated Fluorescein (TUNEL assay for DNA damage were investigated in the study at two time points (post-thaw 0 h and 6 h).

Material and Methods

Ethical Statement

Experimental designs and studies have been approved by Bursa Uludağ University Scientific Ethical Committee (Approval no: 2016-13/03).

Chemicals

The chemicals that have been used throughout the study were purchased from Merck KGaA (Darmstadt, Germany) unless otherwise indicated.

Animals, Collection and Processing of Semen

Study was conducted with five Saanen bucks (3 to 5 years old) during non-breeding season that were housed at a farm in Bursa (latitude 40°11'N, longitude 29°04'E, altitude 155 m above sea level), Türkiye. Animals were maintained with similar feeding and housing conditions and water consumption was up to will. Semen samples were gathered from animals by electro-ejaculation method (Minitüb GmbH, Germany) for five times with at least three-day intervals [15]. After collection, gathered samples were immersed to a 32-34°C water bath in sterile glass tubes for evaluation of spermatological parameters. Collected semen samples were evaluated for volume (measured with conical tubes that graduated at 0.1 mL intervals), consistency, wave motion (3-5 on a 0-5 scale), concentration (at least 1 x 10^9 spermatozoa/mL, measured with haemocytometer) and motility (at least 75%) and pooled afterwards [10].

Extenders and Dilution of Semen

Extenders were freshly prepared prior to the study day. Extenders consisted of 223.7 mmol/L Tris, 55.5 mmol/L fructose, 66.6 mmol/L citric acid, 100.4 mmol/L Trehalose, 4.03 mmol/L EDTA, 4 g/L penicillin G, 3 g/L dihydrostreptomycin and 1% lecithin in distilled water and relevent concentrations of FCS were supplemented to the groups. Lecithin was dissolved by a magnetic stirrer in room temperature to create a homogeneous extender solution beforehand adding FCS to the relevant groups. Experiment groups were arranged as; control (without FCS supplementation), FCS0.25 (with 0.25% FCS added), FCS0.50 (with 0.50% FCS added) and FCS0.75 (with 0.75% FCS added) [10]. Pooled samples were divided into four equal aliquots in every repetition of the study and diluted with the relevant extender to approximately 150 x 10^6 spermatozoon/mL final concentration.

Semen Cooling, Freezing, Thawing and Incubation

Diluted semen samples were gradually cooled in a water bath by the aid of ice cubes within an h to 4°C and equilibrated for another two h at the same temperature [19]. Upon equilibration, diluted samples were loaded into 0.25 mL French straws. Freezing procedure was conducted

as; 3°C/min from +4°C to -8°C and 15°C/min from -8°C to -120°C in liquid nitrogen vapor, by Nicool Plus PC gamete freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France). After -120°C point, straws plunged into -196°C liquid nitrogen for storage [10]. Three sperm straws from each investigation group were thawed at 37°C for 30 sec in a water bath for spermatological parameter evaluations. Thawed samples were then incubated for 6 h in a humidified air chamber with 5% CO_2 at 39°C to examine the impacts of incubation effects over semen characteristics [20].

Evaluation of Semen

Spermatological parameters were analysed by a person throughout the study at two different time points; post thawing (0 h) and post-incubation (6 h). Motility and HOST assessments were conducted with a phase-contrast microscope (Olympus BX51-TF - Olympus Optical Co., Ltd., Japan) over a warm slide of 37°C. Fluorescent spermatological parameters (PSA-FITC and TUNEL) were evaluated by a fluorescent attachment on the same microscope. Spermatological motility parameter was subjectively assessed at 400x magnification with a warm slide (37°C).

Hypo-osmotic swelling test (HOST) was conducted to analyse the functional integrity of sperm membrane. The method based on curled and swollen tails of sperm cells. 10 µL of semen sample were incubated for 60 min at 37°C with 100 µL HOST solution (9 g fructose and 4.9 g sodium citrate per liter of distilled water - 100 mOsm). After 60 min of incubation, 20 µL of mixture was examined over a warm slide that was covered with a slip. At least 200 cells were evaluated under 1000x magnification and the recorded results were presented as percentiles [21].

Acrosomal integrity of sperm cells were evaluated by FITC conjugated *Pisum sativum* agglutinin (PSA-FITC). The method was carried out with slight modifications of Toker et al.[20]. Basic changes of the method from the indicated citation are; 20 µL of semen sample was resuspended in 500 µL of PBS and centrifuged at 100 g for 10 min. After discarding the supernatant, sperm pellet was suspended in 250 µL PBS for the second time and the method was applied as the same herehence.

DNA fragmentation rates were obtained by using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. In Situ Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Germany) was used as Ustuner et al.[19] indicated.

Data of the examinations were analyzed using SPSS (SPSS 20.0 for Windows; SPSS, Chicago, IL, USA). Normality of the distribution was tested by using Shapiro Wilk. Semen parameters were analyzed according to distribution and Kruskal Wallis test then Mann Whitney U test used to determine the significances between study groups.

Results

The impacts of FCS on post-thaw and post-incubation semen parameters throughout the study were analysed by 4 spermatological evaluation methods. The results of sperm motility percentages, status of plasma membrane functional integrity, acrosomal integrity and sperm DNA damage were presented in *Table 1* for both time periods (post-thaw and 6 h of incubation).

Motility

Sperm motility was affected and decreased as expected throughout the thawing and incubation procedures. Despite presenting a higher value than the control, 0.25% group didn't differentiate significantly on post-thaw. But, 0.50% and 0.75% FCS groups were better with a statistically significant value on post-thawing time point (P<0.05). After incubation period, status did not change in terms of motility parameter. 0.50% and 0.75% FCS groups were presented significantly better results for motility (P<0.05).

Plasma Membrane Functional Integrity (HOST)

Hypo-osmotic swelling test results presented similar statistical effects with motility throughout the study. At

Table 1. The mean (X±Sx) of evaluated sperm resilience parameters on different extender groups

Incubation Period (h)	Group	Motility (%)	Plasma Membrane Integrity (%)	Acrosome Integrity (%)	DNA Damage (%)
0 h	Control	48.67 ± 2.97^{a}	59.00 ± 2.55^{a}	69.20 ± 3.11^{a}	4.60±0.40
	0.25	50.33 ± 3.52^{a}	59.80 ± 2.68^{a}	73.40 ± 2.30^{a}	4.00±0.55
	0.50	53.67 ± 2.97^{b}	65.00 ± 1.22^{b}	74.40 ± 3.21^{a}	3.80±0.37
	0.75	58.00 ± 2.54^{c}	71.60 ± 2.51^{c}	78.40 ± 2.19^{b}	3.20±0.37
6 h	Control	4.00 ± 2.07^{x}	14.00 ± 1.58^{x}	56.60 ± 5.50^{x}	7.80 ± 0.37^{x}
	0.25	4.00 ± 2.07^{x}	14.40 ± 1.95^{x}	60.40 ± 4.67^{xz}	7.40 ± 0.25^{x}
	0.50	9.00 ± 3.87^{y}	22.40 ± 4.72^{y}	63.60 ± 5.13^{xy}	5.60 ± 0.40^{yz}
	0.75	17.00 ± 2.54^{z}	29.80 ± 2.39^{z}	67.20 ± 4.44^{yz}	5.20 ± 0.37^{yz}

^{a,b,c} and ^{x,y,z}: Values with different superscripts in the same column for each of incubation time are significantly different (P<0.05)

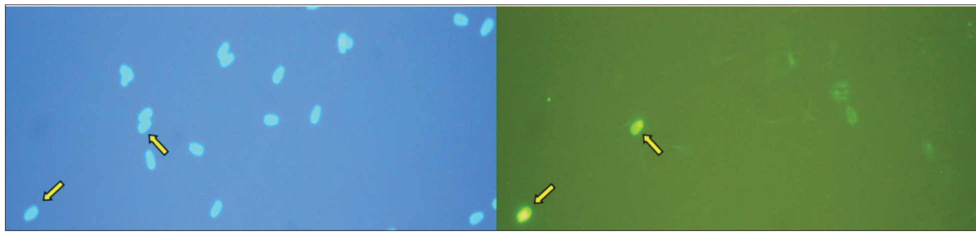

Fig 1. DNA fragmentation by TUNEL indicated with arrows

post-thaw and post-incubation time point, there was no statistically significant difference between the control and 0.25% FCS groups. Yet, the groups containing 0.50% and 0.75% FCS were yielded better and higher integrity scores than the control (P<0.05).

Acrosomal Integrity (PSA-FITC)

The effects of freezing on sperm acrosomal integrity presented itself by post-thaw and incubation time points inevitably. The group containing 0.75% FCS yielded the best and statistically significant result comparing to the control and the other two study groups on post-thaw (P<0.05). Though 0.75% FCS group kept presenting better results comparing to the control group after incubation (P<0.05), no significance amongst FCS groups were seen.

DNA Damage (TUNEL)

The results of TUNEL assay presented no significant difference amongst the study groups in post-thaw time point (P<0.05). After incubation period, 0.50% and 0.75% FCS containing groups yielded significantly better results (P<0.05) and showed the effects of FCS on DNA damage by the time *(Fig. 1)*.

Discussion

The hampering effect of cryopreservation over spermatological parameters and the preventive scientific experiments have been shown by many studies throughout the years [20,22]. Sperm motility, acrosome and plasma membrane integrities, as well as DNA integrity values are some of the values shown to be affected in previous studies [23-25]. The aim of this study was to inhibit these negative effects of cryopreservation process. For this purpose, soybean lecithin (1%, v/v) was added to the semen freezing extenders as the main component. Control (without FCS), FCS0.25 (with 0.25% FCS added), FCS0.50 (with 0.50% FCS added) and FCS0.75 (with 0.75% FCS added) groups were designed for investigating the effects of FCS throughout the cryopreservation process. These effects were analysed at two different time points; at post-thaw (0 h) and after 6 h of incubation to have a better understanding for the survivability of the cells.

Soybean lecithin is a great source for lipoproteins that contains phosphatidylcholine and many fatty acids. These fatty acids offer a great amount of stabilty for mammalian cells [26,27]. Soybean lecithin is shown to be an alternative to egg-yolk for goat semen cryopreservation in previous studies [4,12,28]. Fetal calf serum (FCS) is a great source of mixed elements and nutrients, and has been used in cell cultures [29]. FCS is a mixture that consists of low and high molecular weight biomolecules, many factors regarding the subject of growth, hormones, amino acids, trace elements, vitamins and matters for antioxidant properties that are vital for cellular growth and survivability [30,31]. To our understanding, there is no report about cryopreservation goat semen with FCS in a soybean lecithin-based extender yet. Sandal et al.[32] reported a study about cryopreservation of goat semen with various methods containing FCS but used egg-yolk in semen cryopreservation extenders. Sariözkan et al.[17] investigated the effects of 10% FCS in long term (72 h) liquid storage (5°C) of rabbit semen and did not report positive effects in terms of motility and acrosomal integrity. Blank et al.[16] studied the influence of different concentrations of FCS in cryosurvival of chicken spermatozoa and found out that FCS may be a viable supplement for chicken semen during freezing and thawing process.

Motility values decreased as expected on post-thaw and post incubation time points, respectively. At post-thaw, 0.50% and 0.75% FCS added groups yielded better results comparing to the control (P<0.05). After incubation, the effects of time over motility were observed but the status of the results did not change and presented the same statistical difference as it is on post-thaw time point (P<0.05). Mata-Campuzano et al.[7] indicated the similar effects of incubation and the results on a study that was conducted on rams. Sandal et al.[32] reported less effective results in terms of motility by adding FCS to extenders comparing to the non-added groups. This result may be originating from adding a high dose of FCS (10%) comparing to our study.

Plasma membrane integrity and the functionality has vital impacts on maturation of cell and ability of penetration to ovum for semen [9]. It was naturally affected by cryopreservation process [10]. Our study showed that, as the amount of FCS increased more the integrity and the functionality of plasma membrane was protected. At post-thaw and post incubation time points 0.50% and 0.75%

FCS groups presented statistically significant results comparing to the control (P<0.05). Although it has been done in different topics, our findings were consistent in terms of post-incubation membrane integrity concept with Alcay et al.[9] and presented higher post-thaw values than the study Salmani et al.[1].

Acrosomal integrity of goat sperm cells carry of great importance in terms of penetration ability thereby the potential of fertility [33]. The protective effect of 0.75% FCS study group on both time points of the study indicated the success of the component with regards to acrosomal integrity (P<0.05). Despite, both 0.25% and 0.50% FCS groups yielded better scores comparing to the control, neither of it was statistically significant in two time points (P>0.05). Memon et al.[34] and Sun et al.[35] indicated the decreasing effect of cryopreservation process in terms of acrosomal integrity in their studies. Anakkul et al.[36] had investigated the effects of incubation on acrosomal integrity and found the same reducing effect of the process on goat semen.

Effects of FCS on DNA integrity presented the importance by incubation period. Although there is not a significant difference observed on post-thaw time point amongst groups (P>0.05), by the end of the 6 h incubation time, 0.50% and 0.75% FCS yielded statistically significant results comparing to the control (P<0.05). As DNA integrity is a great indicator of early embryo development maintenance for semen [10], presented results refer the importance of adding FCS to the goat semen cryopreservation extenders especially over time. Regarding the topic of DNA damage, Sandal et al.[32] published similar results with current study by adding FCS to the sperm extenders.

As a conclusion, supplementing various doses of FCS to soybean lecithin-based goat semen cryopreservation extenders indicated its importance within post-thaw and 6 h of incubation periods. FCS has long been a well-known additive for embryonic development, but the effects on sperm cryopreservation of it requires more attention with further evaluations.

Availability of Data and Materials

The dataset generated during the current study is available from the corresponding author (M.B. Toker) on reasonable request.

Ethical Statement

Experimental designs and studies have been approved by Bursa Uludağ University Scientific Ethical Committee (Approval no: 2016-13/03)

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Conflict of Interests

The authors declared that there is no conflict of interest.

Author's Contribution

MBT and SA designed and performed the experiments. MBT analyzed the data, made tables and wrote the paper. Both authors reviewed and approved the final manuscript.

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