Kafkas Univ Vet Fak Derg
20 (3): 393-398, 2014
DOI: 10.9775/kvfd.2013.10248

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effect of Egg Yolk and Soybean Lecithin on Tris-Based Extender in Post-Thaw Ram Semen Quality and *in vitro* Fertility [1]

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[1] *This study presented 6th National Congress on Animal Reproduction and Artificial Insemination, 18-22 May 2011, p.86-87, Belek, Antalya - TURKEY*

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Makale Kodu (Article Code): KVFD-2013-10248

Summary

The aim of the current study was to evaluate the effect of egg yolk and different soybean lecithin concentrations on the efficiency of ram semen cryopreservation and to test the fertilizing ability of frozen-thawed ram semen. Ejaculates with a thick consistency, rapid wave motion (3-5), and >70% initial motility were pooled. Pooled semen were then divided into four groups and diluted at 1/5 (semen/extender) with 1%, 3%, 6% lecithin (L1, L3 and L6) or 20% egg yolk (EY20) using the two-step dilution method. As expected, the results of the current study showed that both motility and the rates of defective acrosomes in sperm were negatively affected by the cryopreservation procedure (P<0.001). The motility values of at 5°C and post-thawed semen in the EY20 group were significantly higher than those in the L1, L3 and L6 groups (P<0.05). There were no differences in motility rates among the lecithin groups at the dilution, cooling, equilibration or post thawing stages (P>0.05). The results of in vitro fertilization, as assessed by the rate of blastocyst formation, were more successful in the EY20 group than those noted in different lecithin groups. In conclusion, freezing ram semen with an extender containing egg yolk could yield better post-thaw sperm parameters and embryonic development compared to lecithin containing extenders.

Keywords: *Cryopreservation, Lecithin, Egg yolk, Ram semen, IVF*

Tris-Bazlı Sulandırıcılarda Yumurta Sarısı ve Soya Lesitininin Eritme Sonrası Koç Spermasının Kalitesi ve *in vitro* Fertilite Üzerine Etkisi

Özet

Bu çalışmanın amacı, yumurta sarısı ve farklı lesitin konsantrasyonlarının koç spermasının dondurulabilirliği üzerine etkisini değerlendirmek ve dondurup çözdürülen koç spermasının fertilizasyon yeteneğini tespit etmektir. Kitle hareketi (3-5) ve >%70 motiliteye sahip ejakülatlar birleştirildi (pooling). Pooling yapılan sperma dört gruba bölündü ve %1, %3, %6 (L1, L3 ve L6) lesitin veya %20 yumurta sarısı (EY20) içeren sulandırıcılar ile 1/5 (sperma/sulandırıcı) oranında iki aşamalı sulandırma yöntemi kullanılarak sulandırıldı. Bu çalışmanın sonucunda; motilite ve akrozomal bozukluk oranlarının dondurma prosedüründen olumsuz yönde etkilendiği tespit edildi (P<0.001). EY20 grubunun 5°C'de ve eritme sonrası motilite değerleri L1, L3 ve L6 gruplarına göre yüksek bulundu (P<0.05). Sulandırma, soğutma, ekilibrasyon ve eritme sonrası aşamalarda motilite oranları bakımından lesitin grupları arasında farklılık saptanmadı (P>0.05). Blastosist oranları bakımından değerlendirilen in vitro fertilizasyon sonuçlarına göre, EY20 grubunun farklı lesitin gruplarından daha başarılı olduğu tespit edildi. Sonuç olarak, yumurta sarısı içeren sulandırıcı ile dondurulan koç spermasının lesitin gruplarına göre eritme sonrası spermatolojik özellikler ve embriyonik gelişim bakımından daha üstün olduğu tespit edildi.

Anahtar sözcükler: *Dondurma, Lesitin, Yumurta sarısı, Koç sperması, IVF*

INTRODUCTION

Cryopreservation impacts lipid composition and the organization of the sperm plasma membrane [1]. In addition, sudden temperature changes, such as cold shocks, ice formation and dissolution during the freezing-

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thawing process, affect the integrity and function of the acrosome, nucleus, mitochondria, axonema and plasma membrane [2-4].

Extenders with different cryoprotectants such as glycerol have been used to protect various cell compartments [5,6]. Semen extenders generally contain simple carbohydrates (such as glucose) as an energy source, a high-molecular-weight material to prevent cold shock (such as egg yolk, milk, or soybean lecithin), ionic or non-ionic substances to maintain a suitable osmotic pressure and pH, and antibiotics [7,8].

Egg yolk and skim milk are the most common additives of animal origin that are used for sperm cryopreservation. The egg yolk's main effective component is the lipoprotein fraction, e.g., lecithin, which protects the membrane's phospholipid integrity during cryopreservation [5,9,10]. However, various experts have recommended against the use of egg yolk due to the wide variability of its constituents [9,10]. In addition, some researchers have shown that products of animal origin could increase the risk of microbial contamination during the AI procedure of domestic animals [11] and that microbial contamination could result in the subsequent production of endotoxin, which could reduce the potential fertilizing capacity of spermatozoa [5]. Egg yolk has been shown to be antigenic and to induce antibodies in both systemic circulation and the reproductive tract [12]. Lecithin (or phosphatidylcholine) is a phospholipid that is distributed widely in plants and plays an important role in the regulation of an animal cells' bio-membrane [13].

This has resulted in the replacement of egg yolk with alternative cryoprotectants such as soybean-derived lecithin for both animal and human sperm cryopreservation. The aim of the current study was to evaluate the effects of egg yolk and three different soybean lecithin concentrations (1%, 3% or 6%) on the freezability and post-thaw fertilizing ability of ram semen.

MATERIAL and METHODS

Semen Extender Preparation

Tris based four different extenders were prepared by the addition of egg yolk and different concentrations of soybean lecithin (L-α-Phosphatidyl choline from Soybean Type II-S Sigma, P5638) [14]. Extenders (Extender A and B) were designated as follows: EY20, 20% (v/v) egg yolk, L1, 1% (w/v) lecithin, L3, 3% (w/v) lecithin and L6, 6% (w/v) lecithin. Each supernatant was obtained after centrifugation (1.000 g, 20 min). These supernatants were filtered through a 0.22 µm- filter (Millex-GV, 0.22 µm). While the Extender A did not contain glycerol, the freezing extender (Extender B) was composed of the cooling extenders (88%, v/v) and glycerol (12%, v/v).

Semen Collection, Evaluation and Dilution

Six Kivircik rams aged 3-5 yrs old and maintained at Uludag University, Faculty of Veterinary Medicine in Bursa, Turkey, were used as the material during a non-breeding season. Semen was collected by electrically stimulated ejaculation (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand) [2,15]. Ram semen was collected five times in every other day.

Collected semen was placed in a warm water bath (30°C) and immediately evaluated for consistency, wave motion (0-5 scale), and percentage of motile spermatozoa [15]. Ejaculates with a thick consistency, rapid wave motion (3-5 on a 0-5 scale), and >70% initial motility were pooled.

Briefly, pooled semen was divided into four groups. Each of the groups was diluted to a ratio of 1:1 (semen/extender) with extender A, which was one of four soybean-derived lecithin 1%, 3%, 6% (L1, L3 or L6) or 20% egg yolk (EY20) and cooled to 5°C within 1 h. The cooled sperm groups were then diluted to a ratio of 1/1 (semen/extender) with extender B (previously cooled at 5°C), which was one of four soybean-derived lecithin 1%, 3%, 6% (L1, L3 or L6) or 20% egg yolk (EY20) (6% glycerol). Extender B was added in five steps at 5 min intervals and equilibrated at 5°C for 2 h.

Semen Freezing and Thawing

Equilibrated semen was placed into 0.25 ml straws and frozen at 3°C/min from +5°C to -8°C and at 25°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) [2]. The straws were then plunged into liquid nitrogen at -196°C where they were stored for at least one month. At least three straws from each group of pooled ejaculates were thawed at 37°C for 30 sec in a water bath to evaluate post-thaw semen characteristics.

Semen Evaluation

Motility and acrosomal and other morphological defects (OMD) were assessed at the following four time points: after dilution with extender A, at 5°C, after equilibration, and post-thaw. All semen samples studied were frozen by the same person, and each of the semen parameters was evaluated by the same person on each occasion throughout the study. Sperm motility was assessed subjectively using a phase-contrast microscope (Olympus BX 51) (400x) with a warm slide (38°C) [15]. Defected acrosome and OMDs (head, midpiece and tail defects) were assessed using the Giemsa staining method. At least 200 spermatozoa per smear were evaluated for morphological defects [14].

In vitro fertilization (IVF)

Oocyte in vitro maturation: All chemicals were purchased from Sigma. Ovaries were collected from slaughtered ewes (during the non-breeding season) and placed in normal

saline at approximately 30°C. The ovaries were transported to the laboratory within 2 to 3 h of collection. The conditions for maturation, fertilization and culture were a slightly modified version of those described by Gómez et al.[16]. After washing the ovaries with fresh normal saline, cumulus-oocyte complexes were recovered by slicing. Cumulus-oocyte complexes were collected into tissue culture medium 199, which was supplemented with Hepes (free acid) 15 mM, sodium Hepes 15 mM, 0.33 mg/ml sodium bicarbonate, 0.01 mg/ml heparin sodium salt, 0.075 mg/ml penicillin G-potassium salt, 0.05 mg/ml streptomycin sulfate, 0.08 mg/ml kanamycin monosulfate and 10% fetal bovine serum (FBS) (F9665). After rinsing 3 times in this medium, oocytes with a homogeneous ooplasm surrounded by several layers of cumulus cells were matured in multi-well dishes (Nunc™, 176740). Each well contained 50 cumulus-oocyte complexes (COC) and 500 µl of maturation medium-199 containing 10% v/v FBS, 10 µg/ml FSH and 10 µg/ml LH. The wells were covered with mineral oil (M8410), and the oocytes were cultured for 24 h in 5% CO_2 in humidified air at 39°C.

In vitro fertilization and culture: Matured COCs were denuded of their cumulus and corona cells by aspiration through a narrow hand-drawn pipette. The cumulus-free oocytes were washed and transferred into multi-well dishes. Each well contained 500 µl of bicarbonate-buffered synthetic oviduct fluid (BSOF) medium, supplemented with 2% sheep serum, 0.1 mg pyruvic acid, 0.15 mg/ml L-glutamine, 0.08 mg/ml kanamycin monosulfate, 0.075 mg/ml penicillin G- potassium salt and 0.05 mg/ml streptomycin sulfate. For insemination, three straws of frozen sperm were thawed (37°C 30 s) and pooled. Two layer Percoll gradients (90 to 45%) were prepared in 10 ml centrifuge tubes, 2 ml of 90% Percoll was placed at the bottom of the tube, and 2 ml of 45% Percoll was carefully layered on the top. Care was taken to avoid mixing the 2 layers. Then, 200 µl of the semen samples were placed at the top of the Percoll gradient. The samples were centrifuged at 3.000 g for 15 min. The top layer of Percoll was discarded, and the sperm pellet was resuspended in 3 ml of Hepes buffered modified SOF (HSOF) and centrifuged again at 600 *g* for 6 min. The supernatant was then discarded. Oocytes were co-incubated with 0.6-1.0x10^6 sperm/ml in groups of 50 in 500 µl of BSOF medium covered with mineral oil in 5% CO_2 in humidified air at 39°C for 24 h. *IVF* experiments were repeated four times with straws of different groups (L1, L3, L6 or EY20). Oocytes were washed in SOF medium to remove the spermatozoa and were then cultured in SOF medium without serum and glucose. On day 3 post-insemination (Day 0= day of insemination), embryos were transferred into SOF medium supplemented with FBS and glucose. Cleavage and blastocyst rates were assessed on Days 2 and 8 post-insemination, respectively.

Statistical Analysis

Sperm-related data were analyzed by analysis of variance (ANOVA) using the General Linear Model (GLM) procedure. When the ANOVA test showed statistical differences, the mean of the treatments were compared using the Tukey's test. Repeated measures ANOVA (using GLM procedures) were conducted to compare the results at different stages of the cryopreservation process. *In vitro* fertilization-related statistical computations were performed using Chi-square analysis (SPSS 10.0 for Windows; SPSS, Chicago, IL, USA). P-values less than 0.05 were considered to be statistically significant.

RESULTS

Percentages of motility, defected acrosomes and OMD (other morphological defects) of the diluted, cooled at 5°C, equilibrated and post thawed ram semen from different extenders presented in *Table 1*.

Sperm motility was progressively reduced by the cooling and freeze-thawing processes (P<0.001). The motility values of semen cooled at 5°C and post-thaw in the EY20 group were significantly higher than those in the L1, L3 and L6 groups (P<0.05). There were no differences in motility rates between lecithin groups at the stages of dilution, cooling, equilibration or post-thaw (P>0.05).

Acrosome integrity was negatively affected by the freeze-thawing process (P<0.001). The percentage of defective acrosome and OMD rates were not affected by the extender components (L1, L3, L6 and EY20) during the freeze-thawing stages (P>0.05).

The cleavage and blactocyst formation rates after insemination with frozen-thawed spermatozoa using L1, L3, L6 and EY20 presented in *Table 2*. The highest cleavage rates were observed with the EY20 treatment group (85.50%) as compared with the L1 (28.6%), L3 (41.76%) or L6 (37.4%) groups (P<0.05). In addition, there were significant differences with respect to the number of 8 cell embryos and morulae among the EY20 group and other lecithin extender groups (P<0.05).

The number of blastocysts in fertilized oocytes in the EY20 group was 5 (4.03%). However, no blastocyst was obtained in the L1, L3 and L6 groups.

DISCUSSION

This study compared the effectiveness of soybean lecithin and egg yolk containing Tris-based extenders on post-thaw sperm parameters and *in vitro* fertilizing ability. Most semen extenders contain egg yolk and skim milk as sources of lipoprotein that protects sperm cells from cold shock and other damage [9,10]. However, the possible disadvantages of using egg yolk, including its potential to be a cause of allergic reactions, the risk of bacterial contamination and its variable effect on semen have been

Table 1. *The mean percentage of motility and rates of defective acrosomes and other morphological defects (OMD) after dilution, at 5°C, equilibration and post-thawing using different extenders composed of different concentrations of lecithin (1%, 3% and 6%) and egg yolk (20%)*

Tablo 1. *Farklı lesitin konsantrasyonları (%1, %3 ve %6) ve yumurta sarısı (%20) kullanarak dondurulan sperma gruplarının, sulandırma sonrası, 5°C, ekilibrasyon ve eritme sonrası aşamalarda ortalama motilite, akrozomal ve diğer morfolojik bozukluk oranları*

Stages	Groups	n	Motility (%)	Defective Acrosome (%)	OMD (%)
After dilution	Lecithin 1%	5	72.0±1.2	13.5±3.0	1.9±0.4
	Lecithin 3%	5	72.0±1.2	10.5±2.5	1.5±0.7
	Lecithin 6%	5	71.0±1.0	8.6±2.2	1.4±0.6
	Eggyolk20%	5	75.0±2.7	7.5±1.6	1.7±0.4
	General Mean	20	72.5±1.5^{x}	10.0±2.3^{x}	1.6±0.6
At 5°C	Lecithin 1%	5	62.0±2.6^{b}	11.9±1.0	1.8±0.8
	Lecithin 3%	5	65.0±1.6^{b}	14.5±1.7	1.3±0.7
	Lecithin 6%	5	65.0±0.1^{b}	13.7±2.9	1.9±0.6
	Eggyolk20%	5	71.0±1.0^{a}	14.8±1.9	2.0±0.5
	General Mean	20	65.8±4.7^{y}	13.7±1.9^{y}	1.8±0.6
Equilibration	Lecithin 1%	5	50.0±5.2	22.1±2.6	1.5±0.5
	Lecithin 3%	5	53.0±4.6	19.2±1.6	1.4±0.6
	Lecithin 6%	5	53.0±3.4	20.9±2.4	1.6±0.2
	Eggyolk20%	5	61.0±1.9	18.6±1.4	2.5±0.7
	General Mean	20	54.3±9.2^{z}	20.2±2.0^{z}	1.7±0.5
Post-thaw	Lecithin 1%	15	31.0±1.8^{b}	31.7±2.6	1.9±0.4
	Lecithin 3%	15	28.7±1.6^{b}	36.2±4.1	2.3±0.6
	Lecithin 6%	15	30.0±1.4^{b}	36.3±4.3	1.5±0.3
	Eggyolk20%	15	45.7±1.4^{a}	27.4±1.6	2.5±0.4
	General Mean	60	33.8±1.5^{t}	32.9±3.1^{t}	2.1±0.4

a,b: *The mean values having different letters within the same column showed significant differences (P<0.05),* ***x,y,z,t:*** *The general mean values having different letters within same column for the different stages showed significant differences (P<0.001),* ***OMD:*** *Other morphological defects*

Table 2. *The number of in vitro-matured oocytes and their number of 2-cell, 8-cell, morulae and blactocyst rates after insemination with post frozen-thawed spermatozoa using different extenders*

Tablo 2. *Farklı sulandırıcılar kullanılarak dondurulan spermalarla yapılan fertilizasyon sonrası in vitro olgunlaştırılmış oosit sayıları ile 2-hücreli, 8-hücreli, morula ve blastosist oranları*

Groups	Number of Fertilized Oocytes	Number of 2-Cell Embryos (%)	Number of 8-Cell Embryos (%)	Number of Morulae (%)	Number of Blastocyst (%)
L1	140	40 (28.60)^{a}	16 (11.43)^{a}	5 (3.60)^{a}	0^{a}
L3	91	38 (41.76)^{b}	15 (16.50)^{a}	5 (5.50)^{a}	0^{a}
L6	123	46 (37.40)^{ab}	18 (14.63)^{a}	3 (2.44)^{a}	0^{a}
EY20	124	106 (85.50)^{c}	86 (69.35)^{b}	17 (14.01)^{b}	5 (4.03)^{b}

a-c: *The mean values having different letters within the same column showed significant differences (P<0.05)*

reported [7,9,11,17]. On the other hand, soybeans contain a high component of low-density lipoprotein, e.g., lecithin or egg yolk-like lecithin [5]. The use of animal-free culture medium (defined component of medium) is a popular choice in assisted reproductive technology.

Extender composition assists with the stabilization of cells during the freezing and thawing processes [2,14]. Extenders containing soybean lecithin could be an alternative to the conventional extenders that include egg yolk [5,18]. De Leeuw et al. has noted that bull sperm survive freezing more effectively in egg yolk-containing diluents than in soybean lecithin [19]. In this study, the freeze-thaw process negatively affected sperm motility and acrosome integrity (P<0.001). Post-thaw sperm recovery was significantly greater when sperm was frozen in egg yolk containing extenders as compared to lecithin-containing groups. The beneficial effect of egg yolk may be due to its cryoprotective abilities and nutritive properties [14].

High soybean lecithin concentrations have been noted to be toxic for sperm motility and viability [5]. The impact of lecithin on sperm motility results from the extender viscosity and the presence of particulate debris [20]. Herein, there were no significant differences between the L1, L3 and L6 groups in terms of post-thaw motility (P>0.05). The extenders were filtered in a 0.22 µm filter to remove debris. The similarity in the results obtained from the different lecithin concentrations was perhaps due to the filtration technique, with small micron membranes used.

The freeze-thawing process may have a detrimental effect on sperm morphology, specifically for acrosome integrity [2]. The post-thaw percentage of defective acrosomes was higher than those of diluted, cooled and equilibrated spermatozoa (P<0.001). It has been suggested that phospholipids from egg yolk or soybean lecithin might integrate with the sperm membrane to form a protective film against the formation of lethal intracellular

ice crystals and protect the sperm membrane from mechanical damage during the freeze-thawing process [4]. The percentage of post-thaw defective acrosomes and OMD rates were not affected by the extender groups used.

Although, several researchers have developed different extender compositions and protocols for freezing ram semen, in general, fertility results are not comparable to those obtained with fresh semen and natural mating. These reductions in fertilization capacity have typically been attributed to a reduced rate of sperm motility and freeze-thawing-induced morphological and genomic abnormalities [2,14,15]. Spermatozoa that are morphologically defective or have poor motility tend to have low success rate of oocyte fertilization. The decreased fertility rates of the L1, L3 and L6 groups compared to those observed in sperm that were frozen with egg yolk are consistent with findings from other authors [17,19-21]. Those authors have observed a deleterious effect or a reduction in the fertility of semen frozen in the presence of soybean lecithin.

This reduction may be the result of soybean lecithin adherence to the surface of the sperm plasma membrane [19], changes in the composition of the sperm membrane or a strong interaction between soybean lecithin and the lipids of the sperm membrane during freezing and thawing procedures [22]. In addition, the adherence of soybean lecithin to the sperm membrane results in the inhibition of sperm acrosome reaction [23]. However, these results are different from studies of semen frozen in diluents containing egg yolk or soybean lecithin, which were conducted by Aires et al.[7], Akhter et al.[24] and Forouzanfar et al.[5].

There are many reports concerning the presence of lecithin concentrations in freezing media. However, there are conflicting reports concerning the beneficial effect of lecithin [5,17-19,21]. Increased lecithin concentrations in freezing media positively affect bull [7,11,25] and stallion [21] sperm fertility rates. In the present study, reducing the soybean lecithin concentration from 6% to 1% did not negatively affect the survival rates of ram sperm following cryo preservation. In general, similar embryonic development was observed among the lecithin-containing groups, with the exception of the number of 2-cell embryos in the L1 group with the lowest number.

The process of fertilization involves complex bio-chemical and physiological events that cannot be explained only by semen or oocyte quality. For example, the media used during IVF and the breeding season during which oocytes are recovered both impact embryonic development [26]. In addition, herein, frozen ram semen was evaluated using *in vitro* fertilization. The cleavage rate in IVF studies using frozen ram semen varies between 13-88% [27,28]. Higher cleavage rates to 2-cell embryos were obtained in the EY20 group (85.50%) as compared to the L1, L3 and L6 groups (28.6%, 41.76%, 37.40%, respectively ($P<0.05$).

Poor oocyte quality is a common cause of infertility. The quality tends to be poor during the prepubertal stage and anestrous periods in seasonal breeders [29]. In our study, embryonic development related results, i.e., morulae and blastocyst rates, were lower than that noted in other studies [5,16,28]. This finding could be explained by the fact that the oocytes were collected during a non-breeding season.

In conclusion, freezing ram semen with an extender containing egg yolk could yield greater post-thaw sperm parameters and embryonic development compared to lecithin-containing groups. In addition, freeze-thawing processes had a detrimental effect on sperm motility and morphology. Post-thaw sperm quality and fertilizing ability were not affected by different lecithin concentrations. Further studies should be performed to determine the lecithin concentration and extender preparation technique that would properly improve post-thaw semen quality and fertilizing capability.

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