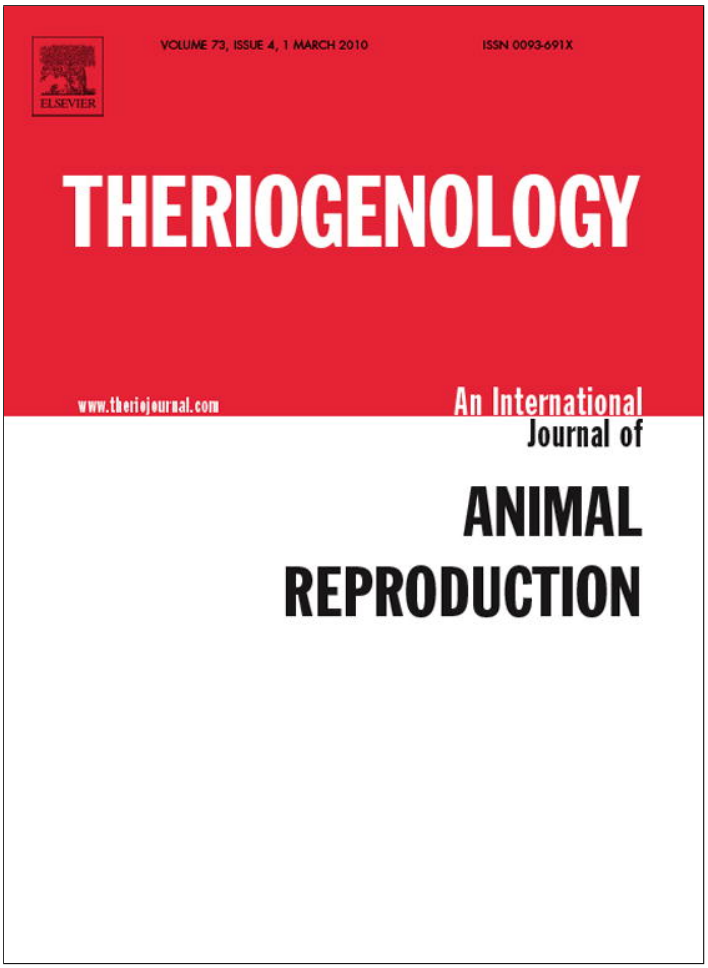

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In vitro comparison of egg yolk–based and soybean lecithin–based extenders for cryopreservation of ram semen

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Abstract

Substitution of egg yolk with soybean lecithin may reduce hygienic risks in extenders. Though a few studies have been performed on the effect of soybean lecithin in bull, to date evaluation of ram semen in vitro fertility after cryopreservation with use of soybean lecithin has not been studied. This study assessed the effect of 1% or 2% (wt/vol) soybean lecithin (L1 or L2) or 15% or 20% (vol/vol) egg yolk (E15 or E20) supplemented with 5% or 7% glycerol (G5 or G7) in a Tris-based medium for cryopreservation of ram (Oviss arries) semen. Although no significant difference was observed in pattern of capacitation, the best results in terms of sperm motility, viability postthaw, and cleavage rates were observed with L1G7 (51.9 ± 4.8%, 48.1 ± 3.5%, and 79.6 ± 3.9%, respectively) and E20G7 (51.8 ± 2.9%, 46.7 ± 4.0%, and 72.9 ± 6.4%, respectively). Our results also showed that 1% lecithin and 20% egg yolk was superior to 2% lecithin and 15% egg yolk. In terms of cleavage rate, 7% glycerol was superior to 5% glycerol. No significant difference was obtained between groups in terms of blastocysts rate per cleaved embryo. Therefore, we concluded that the optimal concentration of lecithin and egg yolk is 1% and 20%, respectively, along with 7% glycerol. In addition, our results suggest that lecithin can be used as a substitute for egg yolk.

Keywords: Capacitation status; Cryopreservation; Extenders; IVF; Ram semen

1. Introduction

The role of sperm cryopreservation for in vivo and in vitro production of human and animal embryos is evident [1,2]. Literature studies reveal that sudden temperature changes such as cold and warm shocks, as well as ice formation and dissolution during the freezing-thawing process, affects the integrity of cells at both the structural and substructural levels [3–6]. Therefore, different cryoprotectants or extenders have been used to prevent cryoinjuries. The most common cryoprotectant in use for sperm cryopreservation is glycerol [7–9]. Glycerol is a permeating cryoprotectant able to cross the cell membrane, but the basis of its cryoprotective properties is not completely understood [10]. Also, an extender should contain an energy source substrate (glucose or fructose), a source of lipoprotein or high-molecular-weight material to prevent cold shock (such as egg yolk, milk, or soybean lecithin), ionic or nonionic substances to maintain a suitable osmotic pressure and pH, and other additives, such as enzymes and antibiotics [10,11].

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The main effective component of egg yolk is the low-density lipoprotein fraction like lecithin, which protects the membrane phospholipid integrity during cryopreservation [12,13]. However, in recent years, there has been frequent opinion against the use of egg yolk due to the wide variability of its constituents, which makes evaluation of its beneficial component complex [12–14]. Furthermore, egg yolk increases the risk of microbial contamination and thereby allows subsequent production of endotoxin, which may reduce the potential fertilizing capacity of spermatozoa [10,15]. This has led to the replacement of egg yolk with alternative cryoprotectants such as plant-derived lecithin from soybeans for both animal and human sperm cryopreservation.

Different concentrations of egg yolk ranging from 15% to 40% and optimal glycerol ranging from 4% to 8% have been used in basic extenders for ram and bovine sperm freezing. Thus, the aim of the current study was to compare the effect of two egg yolk concentrations with two soybean lecithin concentrations on ram semen during in vitro fertility after the freezing-thawing process by using two concentrations of glycerol.

2. Materials and methods

All chemical reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise indicated.

2.1. Extender preparation

The basic extender used in this study was composed of 2.7 g Tris (Merck, Darmstadt, Germany), 1.0 g fructose (Merck), 1.4 g citric acid (Merck), 100 IU penicillin and 1 mg streptomycin, respectively. The osmolarity and pH were set at 320 mOsm and 7.2, respectively. Eight different extenders were prepared by the addition of different concentrations of glycerol (Merck), soybean lecithin, and egg yolk. Extenders were designated as follows: L1G5, 1% (wt/vol) lecithin and 5% glycerol; L1G7, 1% (wt/vol) lecithin and 7% glycerol; L2G5, 2% (wt/vol) lecithin and 5% glycerol; L2G7, 2% (wt/vol) lecithin and 7% glycerol; E15G5, 15% (vol/vol) egg yolk and 5% glycerol; E15G7, 15% (vol/vol) egg yolk and 7% glycerol; E20G5, 20% (vol/vol) egg yolk and 5% glycerol; E20G7, 20% (vol/vol) egg yolk and 7% glycerol (Fig. 1). Concentration of glycerol and the procedure for freezing was based on our previous study [16].

2.2. Semen collection and processing

For this study, six ejaculates from each ram were collected by artificial vagina twice a week during the breeding season from three mature Bakhtiari rams (Oviss arries) (3 and 4 yr of age) known to have good fertility. The ejaculates were evaluated and accepted for evaluation if the following criteria were met:

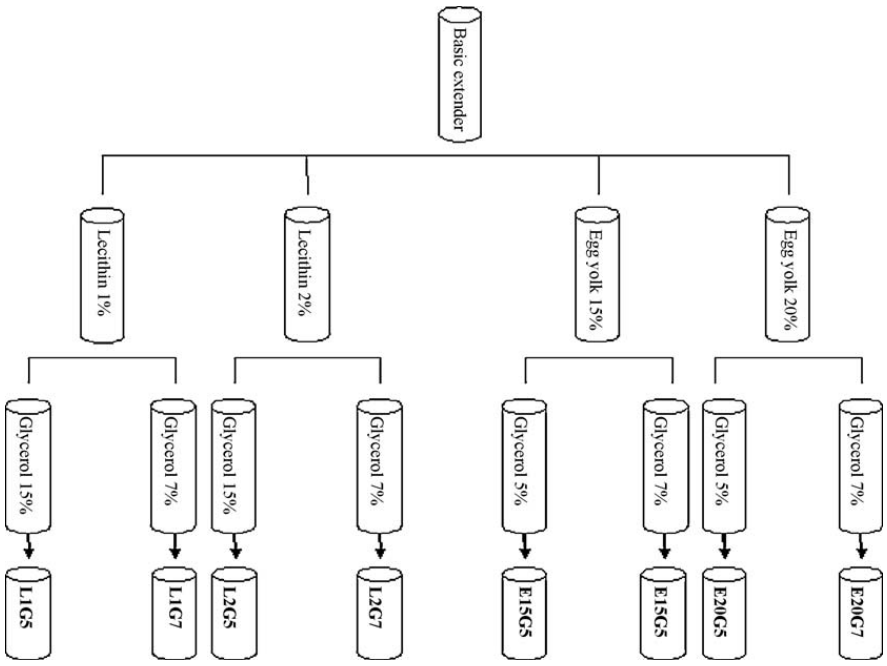

Fig. 1. Experimental design for preparation of extenders. Eight extenders were prepared from soybean lecithin (1% and 2%, wt/vol) or egg yolk (15% and 20%, vol/vol) in combination with glycerol (5% and 7%) in a Tris-based medium.

volume varying between 0.75 and 2 mL, sperm concentration of 3×10^9 sperm/mL, motile sperm percentage higher than 70%, and less than 10% abnormal sperm. To eliminate individual differences, semen samples from the three rams were pooled. Each pooled sample was split into eight equal aliquots and diluted with eight extenders. The diluted semen was gradually cooled to 4 °C for 2 to 3 h. The cooled semen was loaded into 0.25-mL French straws (Biovet, L'Agile France), at concentration of 1×10^9 sperm/mL, according to the method described by Matsuoka et al. [17] with a slight modification. In brief, they were exposed to liquid nitrogen vapor for 12 min, plunged into liquid nitrogen (LN_2), and stored in LN_2 until thawed and used for evaluation of sperm parameters and in vitro fertilization.

2.3. Evaluation of sperm after freezing-thawing

2.3.1. Sperm motility

Sperm motility was carried out according to Gil et al. [18]. Briefly, three straws from different freezing treatments were thawed at 37 °C for 10 sec and pooled in a test tube. Percentage of sperm motility was assessed using a phase-contrast inverted light microscope (CKX41; Olympus, Tokyo, Japan) with a warm stage maintained at 37 °C. Semen samples were diluted with fertilization medium and loaded onto a sperm counting chamber (Sperm Processor, Aurangabad, India), three different microscopic fields for each semen sample were assessed, and the mean of the three successive estimations were recorded as the final motility.

2.3.2. Sperm viability

For viability, the hypo-osmotic swelling test (HOST) was used according to Revell and Marode [19]. In brief, 25 μL semen was added to 200 μL hypo-osmotic solution (100 mOsm/L, 57.6 mM fructose and 19.2 mM sodium citrate). After 30 min incubation at room temperature, the mixtures were homogenized and evaluated under an inverted light microscope. A total of 200 spermatozoa were counted in at least five different microscopic fields. The percentage of sperm with swollen and curved tails was recorded.

2.3.3. Acrosomal status

Acrosome reaction was assessed with chlortetracycline (CTC) staining as described by Perez et al. [20] with little modification [18]. A CTC working solution (750 mM) was freshly prepared in a buffer containing 20 mM Tris, 130 mM NaCl, and 5 mM D,L-cysteine at a pH of 7.8. Five microliters of semen was mixed with 20 μL CTC working solution. After 20 sec, the reaction was stopped by the addition of 5 μL 1% (vol/vol) glutaraldehyde in 1 M Tris-HCl, pH 7.8. Smears were prepared on a clean microscope slide and examined under an epifluorescent microscope (BX51; Olympus).

2.4. In vitro fertilization

2.4.1. Oocyte in vitro maturation

Ovaries were collected from slaughtered ewes (during the breeding season) in normal saline at approximately 30 °C and transported to the laboratory within 2 to 3 h from collection. The conditions for maturation were a slightly modified version of those described by Shirazi et al. [21]. After washing with fresh normal saline, cumulus-oocyte complexes (COCs) were recovered by aspiration. Cumulus-oocyte complexes were collected under a stereomicroscope and washed in the passed-through washed aspiration medium (Hepes-Tissue Culture Media (H-TCM) + 10% Fetal Calf Serum (FCS) + 100 IU/mL heparin), and groups of 8 to 10 COCs were delivered into plates containing 200 μL maturation medium (TCM-199 + 10% FCS + 5 μg/mL follicle-stimulating hormone [FSH] + 5 μg/mL luteinizing hormone [LH] + 0.1 mM cysteamin) and cultured for 24 h in 5% CO_2 at 39 °C and maximum humidity.

2.4.2. In vitro fertilization

Matured COCs were washed in fertilization medium (Fert-TALP (Tyrode's albumin, lactate and pyruvate)) [22] several times, then they were transferred in groups of 40 to 50 to a 200-μL drop of fertilization medium under oil. For insemination, three straws of frozen sperm representing one treatment were thawed (37 °C, 40 sec) and pooled. Motile spermatozoa were obtained by centrifugation at 700 × *g*, on Pure Sperm layers of 80 and 40 (Nidacon; Gothenburg, Sweden) for 15 min at room temperature. A total 2×10^6 spermatozoa/mL was added to each group of matured COCs and incubated for 24 h in 5% CO_2 in humidified air at 39 °C.

2.4.3. In vitro culture

Embryo culture was carried out in modified Synthetic Oviduct Fluid (SOF) medium [23] under mineral oil in a humidified atmosphere of 5% CO_2 and 90% N_2 at 39 °C. Twenty-four hours after insemination, presumptive zygotes were denuded by repeated pipetting and were transferred in groups of five to six into 20-μL droplet of culture SOF without serum and glucose. On Day 3 postinsemination (Day 0 = day of insemination), embryos were transferred to SOF medium supplemented

with 10% charcoal stripped serum and 1.5 mM glucose. Cleavage and blastocyst rates were assessed on Days 3 and 8 postinsemination, respectively.

2.5. Statistical analysis

Each treatment was replicated six times. For each replicate, three straws were thawed and pooled for evaluation of sperm parameters.

For assessment of fertilization and embryo development, each treatment consisted of at least three replicates. Analysis of variance (ANOVA) was used for comparisons of means. When the ANOVA test showed statistical differences, the mean of the treatments were compared using Duncan's multiple range test (DMRT), and a confidence level of $P < 0.05$ was considered to be significant. Statistical evaluations were carried out using the Statistical Package for Social Studies software (Version 16.0; SPSS, Chicago, IL). Information on CTC staining was analyzed using the GENMOD procedure of SAS (Version 9.0, SAS Institute Inc., USA) to fit a generalized linear model with a logit link function.

3. Results

The results of sperm motility and viability and of acrosome status after the freezing-thawing process are presented in Tables 1 and 2, respectively.

Fig. 2 shows three types of spermatozoa after CTC staining: (1) uniform fluorescence head, or uncapacitated sperm (F pattern); (2) post-acrosomal region without fluorescence, or capacitated sperm (B pattern); and (3) fluorescent-free head or a thin fluorescent band on the equatorial segment (sperm that underwent an acrosome reaction [AR pattern]).

Table 1
Mean percentage of motility and of viability of ram spermatozoa after freeze-thawing in eight different extenders composed of different concentrations of lecithin, egg yolk, and glycerol.

Extender	Percentage motility ± SD, %	Percentage viability ± SD, %
L1G5	50.1 ± 2.1^{a}	47.2 ± 3.8^{a}
L1G7	51.9 ± 4.8^{a}	48.1 ± 3.5^{a}
L2G5	38.6 ± 3.6^{b}	31.2 ± 4.1^{b}
L2G7	39.5 ± 3.9^{b}	32.7 ± 3.9^{b}
E15G5	41.2 ± 4.2^{bc}	36.6 ± 4.1^{bc}
E15G7	42.0 ± 5.7^{bc}	39.2 ± 3.7^{bc}
E20G5	48.6 ± 4.2^{ad}	44.8 ± 4.2^{ad}
E20G7	51.8 ± 2.9^{ad}	46.7 ± 4.0^{ad}

[a–d]Different letters within the same column showed significant differences. Viability was assessed by HOST.

Table 2
Mean percentage of uncapacitated (F), capacitated (B), and acrosome-reacted (AR) ram spermatozoa after freeze-thawing in eight different extenders composed of different concentrations of lecithin, egg yolk, and glycerol.*

Treatments	Capacitation status, %		
	F pattern	B pattern	AR pattern
L1G5	10.10	63.63	26.26
L1G7	8.08	60.60	31.31
L2G5	5.20	57.29	37.5
L2G7	6.06	61.61	32.32
E15G5	6.06	58.58	35.35
E15G7	9.09	57.57	33.33
E20G5	9.09	60.60	30.30
E20G7	9.00	57.00	34.00

* Percentage of uncapacitated, capacitated, and acrosome-reacted after freezing-thawing analysis using CTC staining. No significant differences.

A comparison between extenders composed of 1% soybean and different concentrations of glycerol (L1G5 vs. L1G7) showed no significant difference in terms of sperm motility or viability between the two groups. Similar results were observed for extenders composed of 2% lecithin (L2G5 vs. L2G7). However, a comparison of percentage motility and viability between 1% and 2% lecithin (L1G5 vs. L2G5 or L1G7 vs. L2G7) revealed a significant difference between the two groups. Similar results were obtained when egg yolks were used instead of lecithin.

Comparisons of sperm motility and viability between extenders containing similar concentrations of glycerol but composed of either 1% lecithin or 15% egg yolk showed significantly higher motility and viability in 1% lecithin (L1G5 vs. E15G5 or L1G7 vs. E15G7). However, by increasing the concentration of

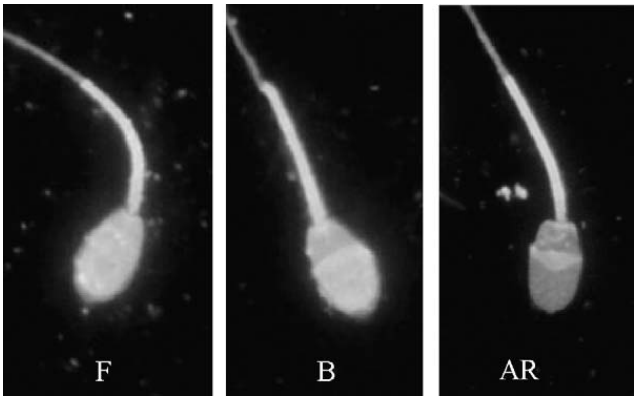

Fig. 2. Fluorescent patterns of spermatozoa stained with CTC. Uniform fluorescence head, or uncapacitated sperm (F pattern); post-acrosomal region without fluorescence, or capacitated sperm (B pattern); and fluorescent-free head or a thin fluorescent band on the equatorial segment, or acrosome-reacted sperm (AR pattern).

Table 3
Number of in vitro–matured oocytes and their cleavage and blastocyst rates after insemination with fresh semen or post–frozen-thawed spermatozoa using different extenders.*

Treatment	Number of Oocytes	Percentages of embryos developed to		
		Cleavage	Blastocyst	
			Based no. fertilized oocytes	Based no. cleaved embryos
Fresh	181	90.6 ± 1.4^{a}	35.9 ± 2.9^{a}	41.7 ± 3.8
L1G5	340	68.4 ± 5.5^{b}	31.1 ± 3.5^{a}	46.5 ± 5.1
L1G7	380	79.6 ± 3.9^{c}	38.3 ± 4.7^{a}	45.6 ± 4.6
L2G5	290	60.5 ± 1.7^{bd}	13.4 ± 2.5^{b}	38.6 ± 3.5
L2G7	285	61.1 ± 5.3^{bd}	27.4 ± 4.3^{c}	44.9 ± 8.4
E15G5	174	56.3 ± 4.3^{d}	26.3 ± 6.3^{c}	44.8 ± 7.6
E15G7	227	66.8 ± 6.1^{eb}	17.2 ± 5.9^{b}	41.2 ± 5.9
E20G5	158	67.4 ± 5.7^{eb}	25.4 ± 2.9^{c}	37.6 ± 7.5
E20G7	191	72.9 ± 6.4^{c}	27.2 ± 3.4^{c}	38.3 ± 4.9

[a–d]Different letters within the same column show significant differences among the groups.
* Blastocysts rate is expressed in terms of number of inseminated oocytes or cleaved embryos.

egg yolk from 15% to 20%, no such difference was observed (L1G5 vs. E20G5 or L1G7 vs. E20G7).

Unlike motility and viability, the above differences were not observed for different patterns of capacitation and acrosome (Table 2).

Table 3 shows the cleavage rate and blastocyst formation rates in terms of the number of oocytes inseminated, or cleaved embryos. Higher cleavage rates were observed with fresh semen compared with that for frozen-thawed sperm obtained from eight different extenders. Among different extenders, the highest cleavage rate was obtained for the extender L1G7 (79.6%) which was not significantly different from the extender E20G7 (72.9%). The lowest cleavage rate was observed for the extender E15G5 (56.3%).

The highest blastocyst rate in terms of inseminated oocytes was obtained in the fresh (35.9%), L1G5 (31.1%), and L1G7 (38.3%) groups, which were significantly different than those of the other six groups. The blastocyst rate in terms of inseminated oocytes was similar between L2G7 (27.4%), E15G5 (26.3%), and E20G5 (25.4%) but significantly lower than those of the other groups. The lowest blastocyst rates were obtained in the L2G5 (13.4%) and E15G7 (17.2%) groups, which were significantly lower than those of the other seven groups.

Unlike the blastocyst rate in terms of inseminated oocytes, the rate of blastocyst formation in terms of cleaved oocytes was not significant between the eight extenders and fresh semen.

4. Discussion

The use of non animal origin chemically defined medium is the method of choice in assisted reproductive technology [24–26]. Egg yolk is a main component in extenders for storage and cryopreservation of semen in most mammalian species including bull, ram, goat, pigs, and even humans. The cryoprotectant fraction of egg yolk is related to the low-density lipoprotein [12,26–28]. However, possible disadvantages of using egg yolk, including bacterial contamination and variability, have been reported [10,13,15,24]. Also, it has been suggested that egg yolk could reduce postthawing viability and acrosome integrity of spermatozoa in some species such as ram [27], goat [29,30], and buffalo [31]. On the other hand, soybean contains a high component of low-density lipoprotein like lecithin or egg yolk–like lecithin. The current study was an attempt to compare sperm parameters and in vitro fertility of freeze-thawed ram semen using soybean lecithin or egg yolk Tris-based extenders.

As expected, the results of the current study showed that sperm motility and viability decreased after freezing-thawing in comparison with that of the prefreezing state [32,33]. Percentage of motility and of viability were highest in the L1G7, L1G5, E20G7, and E20G5 groups, which were significantly greater than those of the L2G7, L2G5, E15G5, and E15G7 groups (Table 1) and suggested that a high concentration of soybean lecithin was toxic for sperm motility and viability, whereas higher concentrations of egg yolk were required to preserve motility and viability. The reduction in motility and viability was likely due to the high viscosity of soybean lecithin. Indeed, van Wagtendonk-de Leeuw et al. reported that high concentrations of lecithin increased viscosity of extenders and suggested that particular debris in the extenders could reduce fertility [34]. We also observed more particle debris in the extender based on 2%

lecithin. It has been suggested that lecithin in soybean and egg yolk protects sperm membrane phospholipids and increases tolerance to the freezing process [12,27,28]. Therefore, possibly an optimal amount of lecithin could be obtained from 1% soybean lecithin and 20% egg yolk, respectively.

Glycerol has remained the cryoprotectant of choice for spermatozoa from all species [7–9,11] and is commonly used at concentrations of 4% to 8%. The toxicity of glycerol limits the use of high concentration of glycerol in cryoprotective media [35–37]. The results of this study suggest that there is no statistical significant difference between 5% and 7% glycerol on sperm motility and viability. However, the optimum level of glycerol concentration depends on numerous factors such as components of the extenders, cooling rate, and freezing-thawing method [37–39].

The results of this study also revealed that, unlike sperm motility and viability, which were affected by the concentration of soybean lecithin and egg yolk, the capacitation status was not affected by any of these factors. Another difference observed between these two additives was increased agglutination in egg yolk–based extenders, which has been well documented in the literature by Aires et al. [10].

To evaluate fertilization and developmental potential of the cryopreserved sample, each semen sample was used for insemination of oocytes. Our results revealed that the constituent of the extenders effected fertilization but not the developmental potential. As shown in Table 3, the highest cleavage rate was obtained with the fresh semen sample (90.6%), which was significantly greater than those of all the cryopreserved groups. The results of this study also revealed that among eight different extenders, the highest cleavage rates belonged to L1G7 (79.6%) and E20G7 (72.9%), which were significantly different from those of the other six groups (Table 3). In addition, the cleavage rates obtained in this study were acceptable when compared with those of other literature reports for the ram [1,40] and similar to those reported for cattle in our laboratory [22,41]. This suggested that the procedure used in this study may be considered as an ideal freezing procedure for ram sperm cryopreservation or for future research in this field.

In addition, the overall results for cleavage rate followed the pattern of sperm motility and viability, suggesting that optimal amounts of lecithin are obtained from 1% soybean lecithin and 20% egg yolk, respectively. In addition, a comparison of results between L1G5 and L1G7 groups reveals that the cleavage rate is significantly higher in the L1G7 group. Although no significant difference is observed between these two groups in terms of viability and motility, the difference is due to a higher concentration of glycerol. Similar results have been obtained between E15G5 and E15G7 or E20G5 and E20G7, further confirming that 7% glycerol might be the more ideal concentration for cryopreservation. Although the results of this study are not comparable with those for bovine and commercial extenders, Aries et al. showed in a literature study that there is no difference in terms of sperm oocyte interaction in the hemi-zona assay for commercial lecithin-based or egg yolk–based extenders [10].

Similar to cleavage rate, the blastocyst yield per oocyte also followed the pattern of cleavage rate. However, no significant difference was obtained in terms of blastocyst rate per cleaved embryo, which suggested that the type of extender affects fertilization potential while it has no effect on developmental potential up to the blastocyst stage. These results, in agreement with those reported by Byrne et al., have shown that once cleaved, oocytes were equally competent to develop to the blastocyst stage [1]. Further assessment of developmental potential of these embryos could require field trials. In this regard, a literature study has revealed no significant difference between soybean and other extenders in bovines [42], unlike the latter report by Wagtendonk-de Leeuw et al. that showed a significant reduction of 56-d nonreturn rates [34] while Aries et al. concluded that chemically defined soybean lecithin was superior in the field trials and functional sperm parameters. However, to our knowledge, this is the first report on use of soybean lecithin in rams.

In this study, we have demonstrated that some of the sperm function tests can be used as a criterion for the prediction of ram semen after freezing-thawing, as the in vitro tests yielded good results. After sperm freezing in soybean lecithin containing a Tris-based extender that consisted of 1% lecithin and 7% (L1G7) glycerol, a higher percentage of motile and viable spermatozoa and higher percentage of cleavage rates were obtained. Also, egg yolk–containing Tris-based extenders that consisted of 20% egg yolk and 7% glycerol had the same effect as L1G7. Thus, freezing ram semen in these two types of extenders offers a high number of functional spermatozoa available for in vitro fertilization and artificial insemination. We conclude that the animal protein–free extenders based on lecithin as considered here are replaceable by animal-based, egg yolk–based extender.

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