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Optimization of Ram Semen Cryopreservation Using a Chemically Defined Soybean Lecithin-Based Extender

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Contents

The purpose of the present study was to investigate the effects of a chemically defined soybean lecithin-based semen extender as a substitute for egg yolk-based extenders in ram semen cryopreservation. In this study, 28 ejaculates were collected from four Zandi rams in the breeding season and then pooled together. The pooled semen was divided into six equal aliquots and diluted with six different extenders: (i) Tris-based extender (TE) containing 0.5% (w/v) soybean lecithin (SL0.5), (ii) TE containing 1% (w/v) soybean lecithin (SL1), (iii) TE containing 1.5% (w/v) soybean lecithin (SL1.5), (iv) TE containing 2% (w/v) soybean lecithin (SL2), (v) TE containing 2.5% (w/v) soybean lecithin (SL2.5) and (vi) TE containing 20% (v/v) egg yolk (EYT). After thawing, sperm motility and motion parameters, plasma membrane and acrosome integrity, apoptosis status and mitochondrial activity were evaluated. The results shown that total and progressive motility (54.43 ± 1.33% and 25.43 ± 0.96%, respectively) were significantly higher in SL1.5 when compared to other semen extenders. Sperm motion parameters (VAP, VSL, VCL, ALH and STR) were significantly higher in SL1.5 compared to other extender, with the exception of SL1 extender. Plasma membrane integrity (48.86 ± 1.38%) was significantly higher in SL1.5 when compared to other semen extenders. Also, percentage of spermatozoa with intact acrosome in SL1.5 (85.35 ± 2.19%) extender was significantly higher than that in SL0.5, SL2.5 and EYT extenders. The results showed that the proportion of live post-thawed sperm was significantly increased in SL1.5 extender compared to SL0.5, SL2 and EYT extenders. In addition, SL1, SL1.5 and SL2.5 extenders resulted in significantly lower percentage of early-apoptotic sperm than that in EYT extender. There were no significant differences in different semen extenders for percentage of post-thawed necrotic and late-apoptotic spermatozoa. Also, the results indicated that there are slight differences for percentage of live spermatozoa with active mitochondria between extenders. In conclusion, SL1.5 extender was better than other extenders in most *in vitro* evaluated sperm parameters.

Introduction

Abundant studies have been conducted to modify semen extenders for protecting sperm during freeze-thawing process (Gil et al. 2003; Fukui et al. 2008; Forouzanfar et al. 2010). In the discussion of modified semen extender for efficient prevention of sperm quality during freezing, different semen extenders have been used for ram sperm cryopreservation in the past decades (Watson 2000). Post-thaw sperm quality is reduced due to the occurrence of cold shock and osmotic stress during the freeze-thawing process (Salamon and Maxwell 2000). Most of this damage can be prevented by suitable extenders and cryoprotectant additives (Gil et al. 2003; Barbas and Mascarenhas 2009). Animal products such as egg yolk are a normal component in semen extenders to protect the sperm against cold shock and the cell membrane during freezing and thawing. It has been found that lecithin and low-density lipoproteins (LDL) are effective components of egg yolk for freezing (Medeiros et al. 2002; Moussa et al. 2002). On the other hand, egg yolk has many problems (for example, variability in egg yolk composition, risk of microbial contamination because of its animal origin). In the last few years, new extenders containing soybean lecithin as egg yolk alternative have been widely used to freeze semen (Gil et al. 2003; Fukui et al. 2008; Forouzanfar et al. 2010; Vidal et al. 2013). In most of the previous studies, commercial lecithin-based extenders with unknown formulation were used and a few studies (Sharafi et al. 2009; De Paz et al. 2010; Forouzanfar et al. 2010) have used a home-made lecithin-based extender with known formulation. In one previous study on ram semen cryopreservation with known formulation of lecithin-based extender, a narrow range of lecithin levels in extender were tested and limited evaluations on post-thawed sperm were performed (Forouzanfar et al. 2010). Thus, the present study was conducted to test a wide range of known soybean lecithin concentrations in ram semen extender by evaluating motility and motion parameters, plasma membrane and acrosome integrity, apoptosis status and mitochondrial activity of post-thawed ram sperm.

Material and Methods

Chemicals

Soybean lecithin (L-α-phosphatidylcholine (product number: P3644)) and fructose used in this study were purchased from Sigma (St. Louis, MO, USA), and other chemicals were purchased from Merck (Darmstadt, Germany).

Animal and semen collection

Twenty-eight ejaculates (seven ejaculates for each ram) were collected from four sexually mature Zandi rams (3–5 years old) using artificial vagina twice a week during the breeding season. The rams were kept at the Center of Zandi Sheep Breeding (Varamin, Tehran, Iran) under uniform conditions. In each ejaculate, volume, concentration, sperm motility and morphology were assessed immediately after semen collection. Ejaculates that contain volume of 0.5–2 ml; minimum semen concentration of 3×10^9 spermatozoa/ml; total motility higher than 80%; <10% abnormal sperm were selected

and pooled together to avoid individual variability of rams and then were used for the experiment.

Preparation of extenders

A TE [(2.71 g Tris, 1.0 g fructose, 1.40 g citric acid in 100 ml distilled water, pH 7.0 and osmolarity 320 mOsm/Kg) with glycerol 7% (v/v)] was used as the basic semen diluent. Six different extenders were prepared by the addition of different levels of soybean lecithin (SL) and egg yolk (EY) to Tris-based extender. Extenders were designated as follows: (i) SL0.5: TE containing 0.5% (w/v) SL, (ii) SL1: TE containing 1% (w/v) SL, (iii) SL1.5: TE containing 1.5% (w/v) SL, (iv) SL2: TE containing 2% (w/v) SL, (v) SL2.5: TE containing 2.5% (w/v) SL and 6) EYT: TE containing 20% (v/v) EY.

Semen dilution, cryopreservation and thawing

The pooled semen was split into six equal aliquots and diluted to a final concentration of 350×10^6 spermatozoa/mL with the six extenders. The diluted semen was gradually cooled to 4°C for 2 h. The cooled semen was aspirated into 0.25-ml French straws (IMV, L'Agile France) and sealed with haematocrite pulp. The straws were placed in liquid nitrogen (LN) vapour (5 cm above the LN for 12 min) and then were plunged into the LN and stored until thawing. For thawing, straws were placed in a water bath at 37°C for 30 s.

Evaluation of post-thawed sperm quality

Sperm motility and motion parameters

The sperm motility and motion parameters were evaluated using a computer-assisted sperm analysis system (CASA; CEROS version 12.3; Hamilton-Thorne Biosciences, Beverly, MA, USA). Semen sample was placed in a chamber and the loaded chamber placed on the thermal plate of the microscope (37°C). Three randomly selected microscopic fields were scanned five times each. The mean of these 15 scans was used for statistical analysis. The thawed semen samples were analysed for the following nine motility parameters. Total motility (TM, %); progressive motility (PM, %); average path velocity (VAP, μm/s); straight line velocity (VSL, μm/s); curvilinear velocity (VCL, μm/s); amplitude of lateral head displacement (ALH, μm); beat cross frequency (BCF, Hz); straightness (STR, %); linearity (LIN, %).

Plasma membrane integrity

Plasma membrane integrity was assessed by means of the hypo-osmotic swelling (HOS) test as described by Revell and Marode (1994) with some modifications. Briefly, 10 μl of semen was mixed with 100 μl of a hypo-osmotic solution [9 g fructose, 4.9 g sodium citrate in 1000 ml distilled water (osmolarity: 100 mOsm/kg)] in a 1.5-ml test tube and incubated at 37°C for 30 min. After incubation, smear was prepared and 300 spermatozoa were counted by phase-contrast microscope (CKX41; Olympus, Tokyo, Japan) at 400× magnification. Sperm with swollen and coiled tails were recorded as intact plasma membrane integrity.

Acrosome integrity

A procedure described by Thys et al. (2009) with some modification was used for evaluation of acrosome integrity. Five hundred microlitres of sperm suspension (1×10^6 spermatozoa/ml) was added to the 1.5-ml microtube and centrifuged (600 g, 10 min). Supernatant was removed, and the sperm pellet dissolved in 50 μl 96% ethanol (in the same microtube). The sperm were fixed for 30 min (4°C) and thoroughly mixed the sperm by pipetting. Twenty microlitres of sperm suspension was put on a glass slide (in a thin line, not a droplet), and the ethanol allowed to evaporate. Then, 20 μl of PSA (50 μl/ml) was put on top of the sperm on the glass slide. The glass slides were incubated (10–15 min at 4°C), and the glass slides were dipped 15 times in distilled water. Then, the glass slides were allowed to dry and mounted with glycerol and covered with a 24 × 24 mm coverslip. Two hundred sperm per slide were assessed by a fluorescent microscope (BX51; Olympus) at 400 × magnification. Sperm with green fluorescence the in head, no fluorescence in head or green fluorescent band at the equatorial level were recorded as intact and damaged/disruption acrosome, respectively.

Mitochondrial activity

The percentage of sperm with functional mitochondria was evaluated by Rhodamine-123 (R123; Invitrogen™, Eugene, OR, USA) and PI. Ten microlitres of Rhodamine-123 solution (0.01 mg/ml) was added to 500 μl of diluted semen samples by Tris buffer (50×10^6 spermatozoa/ml) and incubated for 20 min in the dark. Samples were then centrifuged at 500 × g (3 min), and sperm pellet was resuspended in 500 μl Tris buffer. Then, 10 μl of PI (1 mg/ml) was added to sperm suspension. The sperm were analysed with a FACSCalibur (Becton Dickinson, San Khosoz, CA, USA) flow cytometer. For each sample, 10 000 events were collected.

Phosphatidylserine (PS) translocation

Annexin-V is a calcium-dependent probe that has being used for tracking externalization of PS in the sperm membrane. A commercial PS Detection Kit (IQP, Groningen, the Netherlands) was used according to the manufacturer's instructions. Briefly, sperm were washed in calcium buffer and re-adjusted the concentration to 1.0×10^6 spermatozoa/ml in calcium buffer. Then, 10 μl Annexin V-FITC was added to 100 μl sperm suspension and incubated for 20 min on ice. Afterwards, 10 μl PI was added to sperm suspension and incubated for at least 10 min on ice. Following flow cytometry, sperm subpopulations were determined as: (i) live spermatozoa ($Annexin^-/PI^-$), (ii) early-apoptotic spermatozoa ($Annexin^+/PI^-$), (iii) late-necrotic spermatozoa ($Annexin^+/PI^+$) and (iv) necrotic spermatozoa ($Annexin^-/PI^+$). The sperm were analysed with a

FACSCalibur (Becton Dickinson) flow cytometer. For each sample, 10 000 events were collected.

Statistical analysis

All data were analysed using Proc GLM of SAS 9.1 (version 9.1; SAS Institute, 2002, Cary, NC, USA). The results were expressed as Lsmean ± SEM. The Tukey's test was used to compare Least squares means. All data were checked for normal distribution by PROC UNIVARIATE and Shapiro–Wilk test. The GLM model used in the present study was as follow:

$$Y_i = \mu + T_i + e_i$$

where Y_i is the observed dependent variables (including motility and motion parameters, plasma membrane integrity, acrosome integrity, mitochondrial activity and apoptosis status), μ is mean of population, T_i is the effect of ith treatment (i = 1, 2, 3, 4, 5, 6 and 7) and e_i is random residual error.

Results

In the present study, a significant effect was found between the different extenders for sperm motility and motion parameters (Table 1). The most motility and motion parameters of sperm were significantly higher in SL1.5 extender compared to other extenders.

Plasma membrane integrity (Fig. 1) was significantly higher only in SL2 (56.43 ± 4.62) compared to SL0.5 (41.7 ± 4.62). Other extenders had no significant difference in this parameter.

The evaluations showed that acrosome integrity in post-thawed spermatozoa was affected by different extenders (Fig. 2), and SL1.5 extender resulted in significantly higher percentage (85.35 ± 2.19) of post-thawed acrosome intact spermatozoa compared to SL0.5 (78.06 ± 2.19), SL2.5 (74.55 ± 2.19) and EYT (76 ± 2.19) extenders.

In our study, flow cytometry analysis was used to evaluate mitochondrial activity and apoptosis status of spermatozoa. The effect of different extenders on the percentage of live spermatozoa with active mitochondria is shown in Fig. 3. The results showed that SL2 extender (56.43 ± 4.62) resulted in significantly higher percentage of live spermatozoa with active mitochondria compared to SL0.5 extender (41.7 ± 4.62). However, there were no significant differences between other groups.

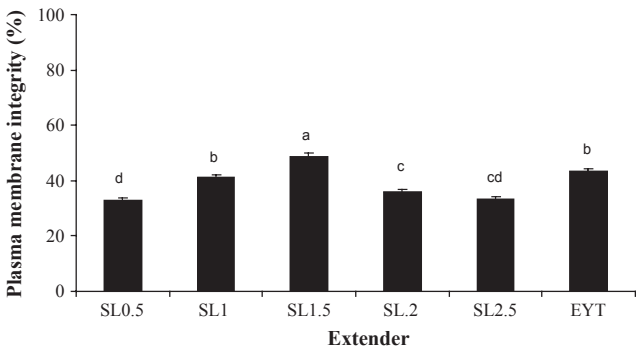

Fig. 1. The effect of different extenders on plasma membrane integrity of post-thawed spermatozoa (Lsmean ± SEM). [a,b,c,d]indicate differences ($p < 0.05$)

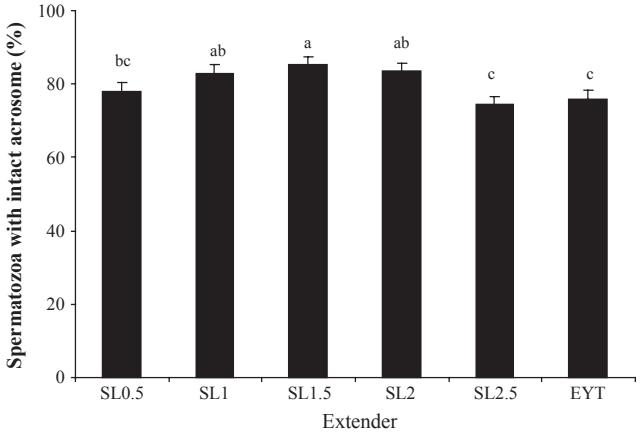

Fig. 2. The effect of different extenders on intact acrosome of post-thawed spermatozoa (Lsmean ± SEM). [a,b,c]indicate differences ($p < 0.05$)

Table 1. The effect of different extenders on post-thawed sperm motility and motion parameters (Lsmean ± SEM)

	Extender						
Parameter	SL0.5	SL1	SL1.5	SL2	SL2.5	EYT	SEM
TM (%)	34.86[d]	47.14[b]	54.43[a]	39.57[c]	35.43[d]	48.43[b]	1.33
PM (%)	17.00[c]	21.71[b]	25.43[a]	19.86[b]	16.29[c]	20.86[b]	0.96
VAP(μm/s)	83.49[b]	97.54[a]	97.97[a]	84.7[b]	80.34[b]	82.47[b]	1.44
VSL (μm/s)	63.71[b]	71.64[a]	74.97[a]	58.56[c]	58.27[c]	55.63[c]	1.22
VCL (μm/s)	152.83[c]	170.74[b]	176.70[a]	143.11[d]	141.39[d]	140.77[d]	1.38
ALH (μm)	7.96[bc]	8.67[ab]	8.87[a]	7.89[c]	7.83[c]	7.79[c]	0.25
BCF (Hz)	27.99[b]	27.83[b]	29.97[a]	24.57[c]	25.06[c]	27.26[b]	0.58
STR (%)	76.14[a]	73.43[ab]	74.71[ab]	69.14[dc]	72.14[bc]	67.57[d]	1.32
LIN (%)	41.57	41.86	42.14	40.71	41.29	39.57	0.8

TM, total motility; PM, progressive motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity; SL0.5, Tris-based extender (TE) containing 0.5% (w/v) soybean lecithin; SL1, TE containing 1 (w/v) soybean lecithin; SL1.5, TE containing 1.5% (w/v) soybean lecithin; SL2, TE containing 2% (w/v) soybean lecithin; SL2.5, TE containing 2.5% (w/v) soybean lecithin; EYT, TE containing 20% egg yolk.
[a,b,c,d]Different superscripts within rows are significantly differences ($p < 0.05$).

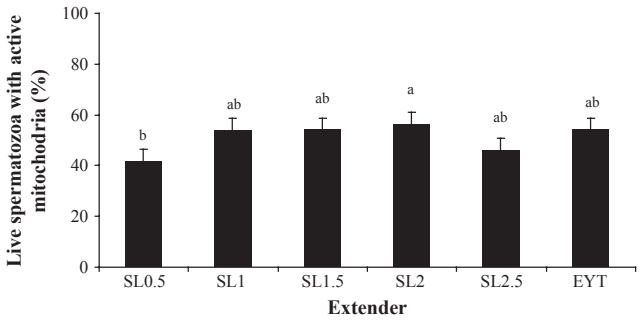

Fig. 3. The effect of different extenders on post-thawed live spermatozoa with active mitochondria (Lsmean ± SEM). [a,b] indicate differences ($p < 0.05$)

As shown in Table 2, the percentage of live spermatozoa in SL1 and SL1.5 extenders was significantly higher compared to SL0.5, SL2 and EYT extenders. Also, the percentage of early-apoptotic spermatozoa in SL1, SL1.5 and SL2.5 extenders was significantly lower than that in EYT extender. There were no significant differences in different semen extenders for percentage of late-apoptotic and necrotic spermatozoa.

Discussion

In the present study, it has been investigated the effect of a new semen extender containing different levels of soybean lecithin to preserve sperm quality during freeze-thawing process and sperm motility and motion parameters, plasma membrane and acrosome integrity, apoptosis status and mitochondrial activity were evaluated as predictors for *in vitro* quality of post-thawed ram spermatozoa.

The results of this study showed that total and progressive motility and most motion parameters were superior in SL1.5 extender compared to other extenders. This result is similar to previous reports on the cryopreservation of sheep (Forouzanfar et al. 2010) and goat (Roof et al. 2012) semen. The reduction in motility and motion parameters in extenders containing lower (SL0.5 and SL1) and higher (SL2 and SL2.5) levels of lecithin compared to SL1.5 may be related to inadequate support to provide the necessary protection and high viscosity (van Wagtendonk-de Leeuw et al. 2000), respectively. Also, the spermatozoa might be able to swim more easily in semen extenders containing optimum levels of soybean lecithin than in other extenders, which would lead to better sperm motion parameters.

The plasma membrane integrity of spermatozoa is necessary to maintain sperm functionality during storage in the female's reproductive tract. The disruption of plasma membrane integrity caused by disarrangement of lipids within the membrane during cryopreservation may induce further cellular damage and consequently lead to sperm death (Holt and North 1984, 1994). In the present study, SL1.5 resulted in a higher percentage of sperm with functional membrane integrity compared to other SL-based and EY-based extenders. It has been reported that the functional membrane integrity of spermatozoa has direct relationships with sperm motility (Gil et al. 2003; Salmani et al. 2013). Then, it seems that a part of positive effect of SL1.5 on total and progressive motility, and some motion parameters of sperm may be related to plasma membrane integrity.

The results of this study showed that the percentage of spermatozoa with intact acrosome in SL1, SL1.5 and SL2 extender was significantly higher than SL2.5 and EYT extenders. Our result is in agreement with finding of Aboagla and Terada (2004) who reported that extenders containing no egg yolk had a higher proportion of intact acrosome than extenders containing egg yolk. On the other hand, Vidal et al. (2013) reported that no difference in acrosome integrity between SL-based extenders and milk-based extender. This discrepancy may be due to different species. Although the precise mechanism by which lecithin exerts its effects on plasma membrane of spermatozoa during freeze-thawing process is not clear, but it has been suggested that lecithin in soybean protects sperm membrane phospholipids by occupying sites on the plasma membrane and increases tolerance to the freezing process (Quinn and Chow 1980; Watson 1981; Graham and Foote 1987; Moussa et al. 2002).

It has been identified that mitochondria supply energy needed for sperm motility (Eddy and O'Brien 1994). Assessment of frozen-thawed spermatozoa with R123 fluorescent dye indicated that the percentage of live spermatozoa with active mitochondria was higher in SL2 compared to SL0.5 extender. The results of this study do not show a logical relationship between sperm motility parameters and mitochondrial activity. This result reveals that sperm motility may be relatively independent of mitochondrial activity as suggested by other researchers (Martinez-Pastor et al. 2008; Del Valle et al. 2012). Also, our result indicated that different

Table 2. The effect of different extenders on subpopulations of post-thawed sperm detected with the Annexin-V (A)/propidium iodide (PI) assay after flow cytometry analyses (Lsmean ± SEM)

	Extenders						
Sperm subpopulations	SL0.5	SL1	SL1.5	SL2	SL2.5	EYT	SEM
Live sperm (A^-/PI^-)	31.28^{bc}	41.94^{a}	40.97^{a}	35.24^{bc}	38.61^{ab}	29.35^{c}	2.41
Early apoptotic sperm (A^+/PI^-)	41.87^{ab}	37.86^{b}	38.93^{b}	40.62^{ab}	36.74^{b}	50.2^{a}	3.66
Late apoptotic sperm (A^+/PI^+)	25.95	18.86	17.88	21.69	23.81	19.3	2.82
Necrotic sperm (A^-/PI^+)	0.90	1.44	2.22	2.45	0.84	1.15	0.54

SL0.5, Tris-based extender (TE) containing 0.5% (w/v) soybean lecithin; SL1, TE containing 1 (w/v) soybean lecithin; SL1.5, TE containing 1.5% (w/v) soybean lecithin; SL2, TE containing 2% (w/v) soybean lecithin; SL2.5, TE containing 2.5% (w/v) soybean lecithin; EYT, TE containing 20% egg yolk.
[a,b,c]Different superscripts within rows are significantly different ($p < 0.05$).

extenders (SL-based and EY-based) had little effect on spermatozoa with functional mitochondria.

The results of this study indicated that the percentages of live spermatozoa after freeze-thawing process in SL1 and SL1.5 extenders were significantly higher compared to SL0.5, SL2 and EYT extenders. Also, SL1, SL1.5 and SL2.5 extenders contain low percentages of early-apoptotic spermatozoa compared to EYT extender. Our result is agreement with finding of Del Valle et al. (2012) who showed that lecithin is able to effectively protect sperm against freezing-induced cryoinjury, because its addition resulted in increased proportions of viable and non-apoptotic spermatozoa. In the present study, we did not evaluate the lipid peroxidation in spermatozoa, but it seems that SL-based extenders can prevent apoptosis in spermatozoa via decreasing lipid peroxidation in plasma membrane (Alvarez and Storey 1992; Hammerstedt 1993), which may cause apoptosis in these cells. The results of this study show that the percentage of A^+/PI^- spermatozoa is a few higher than those in modified protocols. Also, in the present study, the higher percentage of A^+/PI^- spermatozoa is associated with the higher percentage of live spermatozoa with active mitochondria. These results may be due to incubation of sperm suspension on ice for the A/PI, but not for the R123/PI assessment.

Conclusion

The results of this study indicate that SL-based extender can be an efficient alternative extender to preserve spermatozoa during freeze-thawing process. Between SL-based extenders tested in this study, SL1.5 extender has been provided an optimal environment and condition for improving the quality of post-thawed ram spermatozoa by means of improved motility, functional membrane integrity, viability. Of course, further studies are needed to improve and modify SL1.5 extender.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

Experiment was designed by M. Zhandi and A. Zare-Shahneh. Semen freezing and post-thawed sperm evaluations were carried out by M. Emamverdi, M. Sharafi and A. Akbari-Sharif. Manuscript was written by M. Emamverdi and M. Zhandi.

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