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Flow cytometric and microscopic evaluation of post-thawed ram semen cryopreserved in chemically defined home-made or commercial extenders

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Abstract. The present study was designed to determine the effect of three different extenders on ram sperm quality during a freeze–thawing procedure using flow cytometric and microscopic evaluations. Several *in vitro* qualitative analyses of post-thawed sperm parameters including motility and velocity parameters, plasma membrane functionality, total abnormality, capacitation status, acrosome integrity, mitochondrial activity and apoptosis features were considered. In the breeding season, seven ejaculates from each Zandi ram were collected routinely twice a week. Following semen collection, samples were pooled and equally divided into three aliquots. Each aliquot was diluted and frozen with one of the following extenders: (1) Tris-based extender containing 1.5% (w/v) soybean lecithin (TSL), as a chemically defined extender, (2) Bioxcell, a commercial soybean lecithin-based extender, and (3) Tris-based extender containing 20% (v/v) egg yolk (TEY). The results of the present study indicated no differences in total [TSL (55.8 ± 2.02%) vs TEY (50.2 ± 2.02%; $P < 0.05$)] and progressive motility of spermatozoa [TSL (26.2 ± 1.36%) vs Bioxcell (22.4 ± 1.36%; $P < 0.05$)]. Semen freezing by means of TSL resulted in a higher percentage of live spermatozoa (39.42 ± 1.81%) compared with TEY (29.17 ± 1.81%; $P < 0.05$), and a higher percentage of functional plasma membrane (50.8 ± 192%) compared with TEY (44 ± 1.92%) and Bioxcell (38.8 ± 1.92%; $P < 0.05$). The effect of extenders on sperm capacitation status showed that the percentage of post-thawed capacitated spermatozoa was higher in TEY (61.9 ± 1.48%) compared with that in TSL (56.6 ± 1.48%; $P < 0.05$). The evaluation of post-thawed spermatozoa indicated that the percentage of live spermatozoa with active mitochondria was higher in TSL (53.05 ± 2.31%) compared with Bioxcell (45.92 ± 2.31; $P < 0.05$) and the percentage of intact acrosome spermatozoa was higher in TSL (84.55 ± 2.51%) compared with TEY (74.91 ± 2.51%; $P < 0.05$). The use of TSL and Bioxcell extenders reduced the percentage of apoptotic spermatozoa (40.82 ± 2.07% and 42.22 ± 2.07%, respectively), compared with TEY (51.34 ± 2.07%; $P < 0.05$). Post-thawing dead spermatozoa were increased when semen was frozen by Bioxcell (25.69 ± 1.28%). The results of this study showed that TSL extender may provide stabile milieu and conditions for ram sperm cryopreservation compared with Bioxcell and TEY extenders. Whether TSL extender can improve the artificial insemination results remains, however, an open question.

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Introduction

Sperm cryopreservation is an increasingly important area in assisted reproductive techniques (Holt 2000). The type of extender and its composition play a key role in maintaining or enhancing the quality and fertility of cryopreserved semen (Watson 1981; Aires *et al*. 2003). Extenders that are used for semen cooling and freezing contain several components and additives, which may provide a suitable milieu for sperm storage (Curry 2000; Salamon and Maxwell 2000). Components of the extenders including lipoproteins and phospholipids with a high molecular weight or non-permeating cryoprotectant agents (egg yolk, milk, and soybean lecithin) serve to prevent cold shock and provide membrane stabilisation during the freeze–thawing process. The stabilisation of sperm membranes has an important role in improvement of post-thawed sperm quality (Maxwell and Watson 1996; Barbas and Mascarenhas 2009). Recently, researchers have shown that lethal and sub-lethal damages to sperm structure during freezing have led to poor fertility of post-thawed sperm (Watson 2000). Over the past 40 years, various extenders have been used to evaluate their protective potential in the cooled or frozen state. Egg yolk-based semen extenders have been at the heart of understanding of semen cryoprotectant for almost all farm animals especially sheep (Watson 1981). Lecithin is the lipid portion of low density lipoprotein, which is one of the most important and effective components of egg yolk. It is a potent cryoprotectant, which preserves the membrane phospholipid integrity during cryopreservation (Moussa *et al*. 2002). However, the existing

accounts fail to resolve the contradiction between the variable composition of the egg yolk and evaluation of its beneficial component (Aboagla and Terada 2004; Forouzanfar *et al*. 2010). Another major drawback of using egg yolk in extenders is that the egg yolk microbial contamination and subsequent production of endotoxin may lead to a dramatic reduction in sperm fertility (Bousseau *et al*. 1998). Egg yolk has also some micro-elements that result in an increase in extender's viscosity and reduced fertility (van Wagtendonk-de Leeuw *et al*. 2000). However, several commercial lecithin-based extenders [Andromed (Minitub, Tiefenbach, Germany) and Bioxcell (IMV, L'Aigle, France)] with unknown formulation have been used for ram semen cryopreservation (Gil *et al*. 2003; Khalifa *et al*. 2013). In recent years, the home-made soybean lecithin-based semen extender has been used for sperm cryopreservation in some species such as ram (Forouzanfar *et al*. 2010; Del Valle *et al*. 2012), goat (Vidal *et al*. 2013), stallion (Papa *et al*. 2011), and cat (Vick *et al*. 2012). The use of this extender decreases the extender variability and diseases transmission potential compared with egg yolk-based extenders. Therefore, the goal of the present study was to compare the quality of post-thawed ram semen, diluted with Tris-soybean lecithin, commercial Bioxcell and Tris-egg yolk extenders by means of some important *in vitro* evaluations including motility and velocity parameters, plasma membrane functionality, total abnormality, capacitation status, acrosome integrity, mitochondrial activity and apoptosis features.

Material and methods

The chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA) and Merck (Darmstadt, Germany).

Animal and semen collection

Four (3–5 years old) sexually mature and healthy rams that were kept at the Breeding Station of Zandi Sheep (Varamin, Iran) under similar conditions, were selected for semen collection. A total of 28 ejaculates (seven ejaculates for each ram) were collected twice a week (two ejaculates from all rams during one week and on the same days) during the breeding season using an artificial vagina. Semen volume and concentration (using a calibrated semen collection tube and a Neubauer hemocytometer, respectively), sperm motility and morphology (using objective observation and Hancock solution, respectively), were assessed immediately after collection (Emamverdi *et al.* 2013). Ejaculates which met the following criteria were selected: volume of 0.5–2 mL; semen concentration: $>3 \times 10^9$ spermatozoa/mL; total motility: >80%, and abnormal spermatozoa: <10%. Ejaculates obtained from all rams on the same days were pooled ($n = 7$) together in each replicate to avoid individual variability of rams and were used for the experiment.

Extenders

Three different extenders were used and prepared as follows: (1) Tris-soybean lecithin-based (TSL) extender was prepared via addition of 1.5% (w/v) soybean lecithin (Sigma Chemical Co, Cat. #P3544) to a Tris buffer [Tris (27.1 g/L), fructose (10 g/L), citric acid (14 g/L) with glycerol (7%, v/v) at the pH of 6.8 and an osmolarity of 320 mOsm/kg; Emamverdi *et al*. 2013], (2) Tris-egg yolk-based (TEY) extender was prepared via addition of 20% (v/v) egg yolk to the abovementioned Tris buffer, and (3) Bioxcell was prepared by combination of one part of the commercial extender with four parts of pre-warmed Milli-Q water at 37°C.

Semen extending, freezing, and thawing

The pooled semen was divided into three equal aliquots and diluted (one-step method) with the three extenders to a final concentration of 280×10^6 spermatozoa/mL. The diluted semen was gradually cooled at 5°C for 2 h. The cooled semen was aspirated into 0.25-mL straws (IMV) and sealed with pulp hematocrit (Memmert, Cat. #6388). Before plunging the straws into the liquid nitrogen for freezing, the straws were placed at 5 cm above the liquid nitrogen vapour for 12 min. The thawing process was conducted using a water bath at 37°C for 30 s.

Post-thawed semen evaluation

Sperm motility and velocity parameters

Frozen semen was thawed and diluted in Tris buffer (in one step) to 10×10^6 spermatozoa/mL and loaded on the counting chamber (Leja, Nieuw-Vennep, The Netherlands) at 37°C. The sperm motility and velocity parameters were evaluated by means of a computer-assisted sperm analysis (CASA; CEROS version 12.3; Hamilton-Thorne Biosciences, Beverly, MA, USA) system. Table 1 shows the setting of CASA software for ram semen analysis. The following nine motility parameters were measured for each sample: 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, %), and linearity (LIN, %).

Plasma membrane functionality

Plasma membrane functionality was assessed by means of the hypo-osmotic swelling test as described by Revell and Mrode (1994) with some modifications. Briefly, 10 μL of semen was mixed with 100 μL of a hypo-osmotic solution [fructose (9 g/L), sodium citrate (4.9 g/L), osmolarity: 100 mOsm/Kg] and

Table 1. Settings for the Hamilton Thorne CEROS animal software (version 12.3) used to assess ram sperm kinematics

Parameter	Setting
Frame rate (Hz)	60
Frame acquired (no.)	30
Minimum contrast	70
Cell size (pixels)	5
Cell intensity	55
Path velocity (μ/s)	75
STR (%)	80
VAP cat off (μ/s)	21.09
VSL cat off (μ/s)	6
Slow cells	Statics
Temperature (°C)	37

incubated at 37°C for 30 min. After incubation, a smear was prepared and 300 spermatozoa were counted by phase-contrast microscope (CKX41; Olympus, Tokyo, Japan) at 400× magnification and sperm with swollen and coiled tails were recorded.

Total abnormality

Total morphological abnormalities were assessed by a phase-contrast microscopic examination (CKX41; Olympus) at 400× magnification according to the procedure used by Schafer and Holzmann (2000). Three drops of semen (~60 µL) were added to test tubes containing 1 mL of Hancock solution [formalin 37% (62.5 mL), saline solution (150 mL), buffer solution (150 mL) and double-distilled water (500 mL)]. To detect acrosome, head, tail and other abnormalities, 10 µL of the prepared sample was placed on a slide and covered with a coverslip. Three-hundred spermatozoa on each slide were counted.

Capacitation status

The fluorescent chlortetracycline (CTC) staining was performed to characterise the capacitation status (Perez *et al*. 1996). A CTC working solution (750 mM) was freshly prepared in a buffer containing Tris (20 mM), NaCl (130 mM), and D,L-cysteine (5 mM) at a pH of 7.8. Five µL of semen was mixed with 20 µL of CTC working solution. After 20 s, the reaction was stopped by adding 5 µL 1% (v/v) glutaraldehyde in TRIS-HCl (1 M; pH 7.8). Smears were prepared on a clean microscope slide and were covered with a 24 × 48-mm coverslip, sealed with colourless enamel, and stored at 4°C in the dark. At least 200 spermatozoa per slide were evaluated using a microscope (BX51; Olympus) equipped with fluorescence illumination and an ultraviolet filter (excitation at 400–440 nm and emission at 455 nm) at 400× magnification.

Acrosome integrity

Pisum sativum agglutinin (PSA) is a lectin obtained from pea plant that binds to α-mannose and α-galactose moieties of the acrosomal matrix (Gillan *et al*. 2005). The PSA fluorescent dye is conjugated to fluorescein isothiocyanate (FITC-PSA) and used for acrosome integrity assessment (Thys *et al*. 2009). Five-hundred µL of sperm suspension (1×10^6 spermatozoa/mL) were added to a micro-tube and centrifuged at 600*g* for 10 min (25°C). The supernatant was removed and the sperm pellet was dissolved in 50 µL 96% ethanol. The spermatozoa were fixed for 30 min (4°C) and mixed completely by pipetting. A narrow line of sperm suspension with an approximate volume of 20 µL was prepared on a glass slide. The ethanol then was left to be evaporated within a period of 10–15 min. Thereafter, 20 µL of PSA (50 µL/mL) was set on the top of sperm on the glass slide. The glass slides were incubated (10–15 min at 4°C) and sank 15 times in distilled water. Then, glass slides were allowed to dry and mounted with anti-fad (10 µL) and covered with a 24 × 24-mm coverslip. Two-hundred spermatozoa per slide were evaluated by a using a microscope (BX51; Olympus) equipped with fluorescence illumination and a FITC filter (excitation at 455–500 nm and emission at 560–570 nm) at 400× magnification. Sperm with green fluorescence in their whole head region were considered to have intact acrosome, while spermatozoa without any detectable green fluorescence or with a narrow line of fluorescence irradiance in their head segment were considered as sperm with disrupted or damaged acrosome.

Mitochondrial activity

The proportion of thawed sperm with functional mitochondria was evaluated by Rhodamine-123 (R123; Invitrogen, Eugene, OR, USA) and Propidium Iodide (PI; Sigma-Aldrich, Cat. #287075). Briefly, 10 µL Rhodamine-123 solution (0.01 mg/mL distillated water) was added to 500 µL of semen samples (50×10^6 spermatozoa/mL) and incubated for 20 min in the dark. Samples were then centrifuged at 500*g* (3 min, 25°C) and the sperm pellet was re-suspended in 500 µL Tris buffer. Then, 5 µL of PI (1 mg/mL) was added to the sperm suspension (Partyka *et al*. 2011). The sperm were analysed with a FACS-Calibur (Becton Dickinson, San Khosoz, CA, USA) flow cytometer. For each sample, 10 000 events were collected and sperm subpopulations were classified as: (1) live spermatozoa with active mitochondria ($R123^+PI^-$), and (2) dead spermatozoa (PI^+) (Fig. 1).

Phosphatidylserine (PS) translocation assay

Annexin-V is a calcium dependent probe that has recently been used for tracking externalisation 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 a calcium buffer solution and readjusted at the concentration of 1×10^6 spermatozoa/mL. 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 the sperm suspension and incubated on ice for at least 10 min. Following flow cytometery,

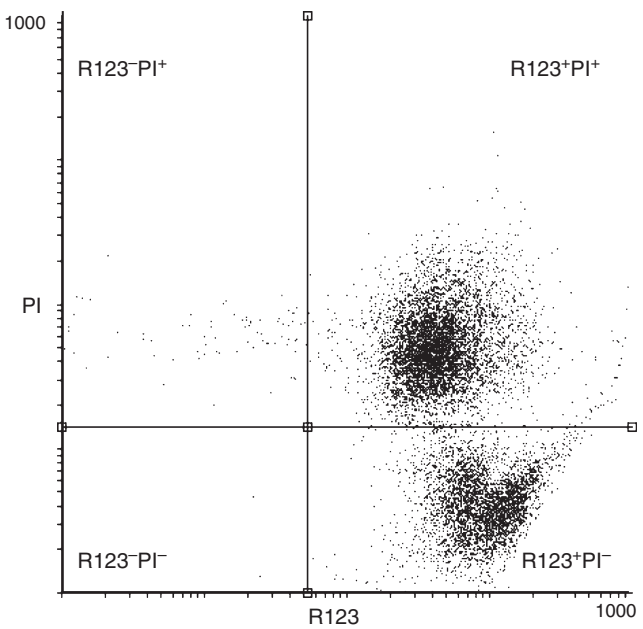

Fig. 1. Flow cytometric detection of ram spermatozoa stained with Rhodamine123 and PI. $R123^-PI^-$ quadrant contains events without fluorescence; $R123^+PI^-$ quadrant contains spermatozoa with functional mitochondria; $R123^-PI^+$ and $R123^+PI^+$ quadrants contain dead spermatozoa.

sperm subpopulations were classified as: (1) live spermatozoa (Annexin$^-$PI$^-$), (2) apoptotic spermatozoa (Annexin$^+$PI$^-$), and (3) dead spermatozoa (PI$^+$) (Fig. 2). The sperm were analysed with a FACS-Calibur (Becton Dickinson) flow cytometer. For each sample, 10 000 events were collected.

Statistical analyses

Proc GLM of SAS software (SAS Institute 2002) was used for analyses of all data. The results were represented as LSM ± s.e.m. The Tukey's test was used to compare LSM. Differences with values of $P < 0.05$ were considered to be statistically significant. The statistical model of the trial was as follows:

$$Y_{ij} = \mu + T_i + e_{ij},$$

where Y_{ij} is the observed dependent variables, μ is mean of population, T_i stands for the effect of ith extender (i = 1, 2 and 3), and e_{ij} represents the random residual error.

Results

Sperm motility and velocity

The results of the total motility in post-thawed spermatozoa showed no variation between TEY (47.6 ± 2.02) and either TSL (55.8 ± 2.02) or Bioxcell (47.6 ± 2.02) extenders. However, the total motility was significantly reduced in Bioxcell when compared with that of TSL extenders ($P < 0.05$). Moreover, ram spermatozoa cryopreserved in TSL extender showed a significantly higher proportion of post-thawed progressive motility (26.2 ± 1.36) compared with those diluted in TEY (21.6 ± 1.36; $P < 0.05$). Regarding the progressive motility parameter no variation was noted between Bioxcell (22.4 ± 1.36) and both TEY (21.6 ± 1.36) and TSL (26.2 ± 1.36) extenders. Interestingly, no significant differences were found between extenders for VAP, VSL, ALH, and STR (Table 2).

Plasma membrane functionality

Evaluation of spermatozoa to identify sperm with functional plasma membrane (Fig. 3) showed that using TSL extender led to significantly higher percentage of sperm with functional plasma membrane (50.8 ± 1.92%) compared with those noticed for TEY (44 ± 1.92%) and Bioxcell (38.8 ± 1.92%; $P < 0.05$).

Total abnormality

The percentage of total abnormality in post-thawed spermatozoa showed no difference among the TSL (20.80 ± 1.02), TEY (22.38 ± 1.02) and Bioxcell (21.34 ± 1.02) extenders (Fig. 3).

Capacitation status

Determination of post-thawing sperm capacitation status revealed that using TSL, TEY, and Bioxcell extenders had no effect on the percentage of non-capacitated (F) and acrosome-reacted (AR) spermatozoa (Table 3), whereas the percentage of

Table 2. The effect of different extenders on post-thawing motility and velocity parameters of ram spermatozoa (LSM ± s.e.m.)

Within rows, values followed by different letters are significantly different ($P < 0.05$). TSL: Tris-soybean lecithin-based extender; TEY: Tris-egg yolk-based extender

Parameter (unit)	Extenders			s.e.m.
	TSL	TEY	Bioxcell	
TM (%)	55.8a	50.2ab	47.6b	2.02
PM (%)	26.2a	21.6b	22.4ab	1.36
VAP (μm/s)	92.32	87.44	89.58	2.46
VSL (μm/s)	64.62	61.30	58.66	3.31
VCL (μm/s)	172.84a	141.16c	149.46b	2.51
ALH (μm)	8.46	7.44	7.22	0.45
BCF (Hz)	29.92a	25.6b	24.3b	1.22
STR (%)	69.6	69.8	65.4	3.12
LIN (%)	37.2b	43.2a	39ab	1.89

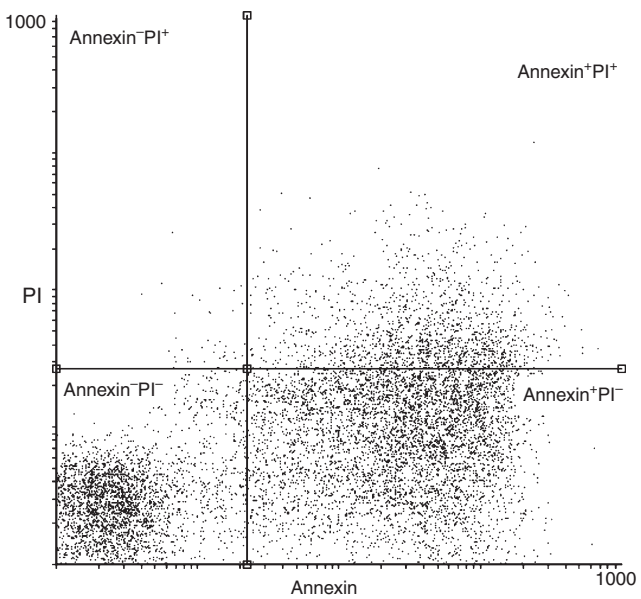

Fig. 2. Flow cytometric detection of ram spermatozoa stained with Annexine-V and PI. Annexin$^-$PI$^-$ quadrant contains events without fluorescence; Annexin$^+$PI$^-$ quadrant contains apoptotic spermatozoa; Annexin$^-$PI$^+$ and Annexin$^+$PI$^+$ quadrants contains dead spermatozoa.

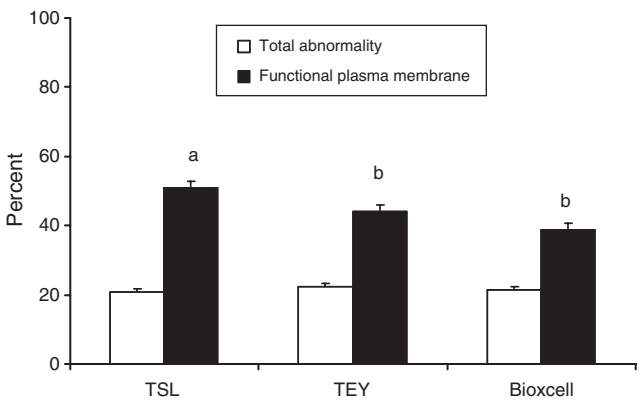

Fig. 3. The effect of different semen extenders on sperm abnormality and functional membrane integrity (LSM ± s.e.m.). Bars with different letters are significantly different ($P < 0.05$). TSL: Tris-soybean lecithin-based extender; TEY: Tris-egg yolk-based extender.

post-thawing capacitated spermatozoa (B) in TEY extender (61.9 ± 1.48%) was significantly increased compared with TSL extender (56.6 ± 1.48%; $P < 0.05$). Also, in post-thawing capacitated spermatozoa no variation was observed between Bioxcell (58.2 ± 1.48) and either TSL (56.6 ± 1.48) or TEY (61.9 ± 1.48) extenders.

Acrosome integrity

The results of acrosome integrity showed that TSL extender (84.55 ± 2.51) resulted in a significantly higher percentage of sperm with intact acrosome compared with TEY (74.91 ± 2.51) ($P < 0.05$), but no difference was observed between Bioxcell and both TSL and TEY extenders (Fig. 4).

Spermatozoa with active mitochondria

As shown in Fig. 4, TSL (53.05 ± 2.31) resulted in a significantly higher percentage of live spermatozoa with active mitochondria compared with Bioxcell (45.92 ± 2.31%) ($P < 0.05$). No difference, however, was found between TEY and both TSL and Bioxcell extenders.

Phosphatidylserine translocation

The percentage of live spermatozoa was significantly higher in TSL (39.42 ± 1.81) compared with that in TEY (29.17 ± 1.81) extenders ($P < 0.05$). Using TSL and Bioxcell extenders was associated with a significantly decreased proportion of apoptotic spermatozoa (40.82 ± 2.07 and 42.22 ± 2.07, respectively), compared with TEY (51.34 ± 2.07) ($P < 0.05$). Thawed dead spermatozoa in TSL and TEY were significantly lower than Bioxcell (Table 4; $P < 0.05$).

Table 3. The effect of different extenders on post-thawing uncapacitated (F pattern), capacitated (B pattern) and acrosome-reacted (AR pattern) ram spermatozoa (LSM ± s.e.m.)

Within rows, values followed by different letters are significantly different ($P < 0.05$). TSL: Tris-soybean lecithin-based extender; TEY: Tris-egg yolk-based extender

Parameter	Extender			s.e.m.
	TSL	TEY	Bioxcell	
F pattern	18	15	15.8	1.29
B pattern	56.6b	61.9a	58.2ab	1.48
AR pattern	25.4	23.1	26	1.77

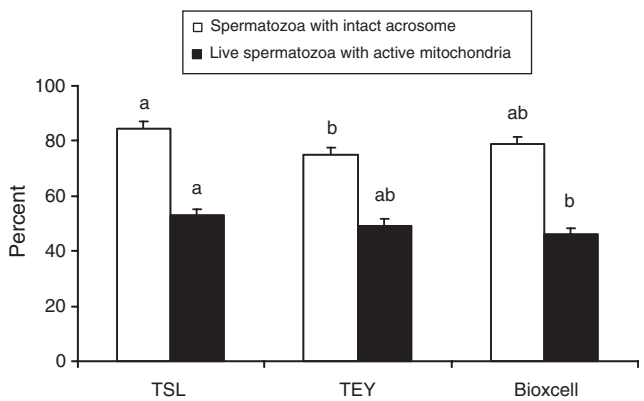

Fig. 4. The effect of different semen extenders on acrosome intactness and active mitochondria in post-thawed ram spermatozoa (LSM ± s.e.m.). Bars with different letters are significantly different ($P < 0.05$). TSL: Tris-soybean lecithin-based extender; TEY: Tris-egg yolk-based extender.

Discussion

In most of recent studies, improvement of ram semen cryopreservation using chemically defined and home-made soybean lecithin-based extenders has been the subject of many past studies (Forouzanfar *et al*. 2010; Del Valle *et al*. 2012; Emamverdi *et al*. 2013; Salmani *et al*. 2013). Using these extenders may lead to a reduction in extender variability and less potential for disease transmission compared with the type of extenders containing components of animal origin. Chemical and physical properties of the extender affect quality of post-thawed spermatozoa (Holt 2000). It has been reported that using soybean lecithin-based extender has had a similar or great positive impact on sperm quality of bull (van Wagtendonk-de Leeuw *et al*. 2000; Aires *et al*. 2003), man (Reed *et al*. 2009), buck (Vidal *et al*. 2013), stallion (Papa *et al*. 2011) and ram (Gil *et al*. 2003; Fukui *et al*. 2008; Forouzanfar *et al*. 2010), especially following the thawing procedure.

In this study, the highest percentages of total motility were found in TSL-based extender compared with the Bioxcell, but it was not significantly different from that of TEY-based extender. Moreover, ram spermatozoa cryopreserved in TSL extenders resulted in a significantly higher percentage of progressive motility compared with TEY. Also, there were significant differences among extenders for VCL, BCF and LIN. According to some reports (Aires *et al*. 2003; Salmani *et al*. 2013), using soybean lecithin significantly increased the post-thawed sperm motility compared with egg yolk extender. The micro-particles of the egg yolk, may lead to an increased viscosity of extender, which may consequently reduce sperm motility (Forouzanfar *et al*. 2010). Also, some substances in egg yolk such as yolk granules of yolk inhibit sperm respiration and reduced motility (Moussa *et al*. 2002).

A biochemically active plasmalemma is required for the processes of capacitation, acrosome reaction and the oocyte penetration (Amirat *et al*. 2004). In this study, the ratio of spermatozoa with functional plasma membrane in TSL-based extender was significantly higher than TEY and Bioxcell, but there were no significant differences between TEY and Bioxcell. The plasma membrane is often considered to be the primary site

Table 4. The effect of different extenders on subpopulations of post-thawing sperm detected with the Annexin-V (A)/propidium iodide (PI) assay after flow cytometry analysis (LSM ± s.e.m.)

Within rows, values followed by different letters are significantly different ($P < 0.05$). TSL: Tris-soybean lecithin-based extender; TEY: Tris-egg yolk-based extender

Sperm subpopulations	Extenders			s.e.m.
	TSL	TEY	Bioxcell	
Live spermatozoa (A^-/PI^-)	39.42a	29.17b	32.08ab	1.81
Apoptotic spermatozoa (A^+/PI^-)	40.82b	51.34a	42.22b	2.07
Dead spermatozoa (PI^+)	19.76b	19.49b	25.69a	1.28

at which injury in the sperm is initiated (Watson 1981). It has been suggested that soybean and egg yolk lecithin protect phospholipids ingredients in sperm membrane by occupying the surface of sperm plasma membrane, thereby increasing the freezing tolerance (Quinn *et al*. 1980; Watson 1981; Graham and Foote 1987; Moussa *et al*. 2002). Graham and Foote (1987) reported that exogenous phospholipids can replace some of the sperm membrane phospholipids during cryoinjury and showed that phosphatidylcholine and phosphatidylserine are the most effective components of phospholipids to protect the spermatozoa. Therefore, it seems that TSL extender provided optimum exogenous source of phospholipids for the maintenance of ram sperm membrane phospholipids. Furthermore, it has been indicated that plasma membrane functionality may be improved in goat spermatozoa by soybean lecithin-based extender (Vidal *et al*. 2013). In agreement to our findings, Akhter *et al*. (2010) suggested that functional plasma membrane in post-thawed buffalo spermatozoa was not significantly different between Bioxcell and egg yolk-based extenders.

In this study, the percentage of total abnormality in post-thawed spermatozoa was not affected by the three different extenders. Primary sperm abnormalities occur during spermatogenesis (Chenoweth 2005). However, cold shock during semen cryopreservation may bring about some abnormalities in sperm. It has been reported that there was no significant difference in sperm morphology after freeze–thawing process in extenders containing soy extract (Perez-Garnelo *et al*. 2006) and Bioxcell (Akhter *et al*. 2010) compared with egg yolk.

An unanticipated finding previously reported by researchers was that damage caused by the freeze–thawing process may induce acrosome reaction and capacitation. Therefore, evaluation of capacitation and acrosome statuses in post-thawed spermatozoa are crucial (Moce and Graham 2008). Several studies indicated that freezing and thawing led to a dramatic increase in the number of spermatozoa showing the capacitated B pattern but had a little effect on the numbers of acrosome-reacted spermatozoa (Perez *et al*. 1996; Gillan *et al*. 1997). The results of the present study confirm the previous reports on the effect of freezing on capacitation status in spermatozoa. The proportion of spermatozoa with B pattern (capacitated) in TEY extender was significantly higher than that of TSL extender. A possible explanation for this finding might be that egg yolk normally contains progesterone, which causes early sperm capacitation (Bencharif *et al*. 2008). The differences between the percentage of post-thawed spermatozoa with F and AR patterns were not significant in three different extenders. Also, in the current study, the percentage of uncapacitated spermatozoa was lower compared with the mean value reported by Gil *et al*. (2000), but it was closer to the values reported in our previous studies (Najafi *et al*. 2013; Zanganeh *et al*. 2013). This discrepancy is due to the assessments of capacitation status in live spermatozoa by Gil *et al*. (2000).

Acrosome plays a key role in fertilisation. Therefore, sperm with damaged acrosome is considered unfertile (Amirat *et al*. 2004). The stained spermatozoa by FITC-PSA fluorescent dye showed that the percentage of spermatozoa with intact acrosome in TSL extender was significantly higher than TEY extender, but the difference was not significant compared with that of Bioxcell. Previous studies showed that egg yolk decreased the percentage of intact acrosome in the ram (Watson 1981), bull (Amirat *et al*. 2005) and goat spermatozoa (Aboagla and Terada 2004). It has also been reported that using Biociphos Plus (a soybean lecithin-based extender) was associated with a higher percentage of spermatozoa with intact acrosome compared with Triladyl (an egg yolk-based extender) (Amirat *et al*. 2005). The latter authors suggested that the presence of higher calcium ions in the egg yolk might be responsible for the acrosomal damage. However, it has been reported that different levels of lecithin in the semen freezing medium had no effect on acrosome integrity in goat spermatozoa (Vidal *et al*. 2013).

In this study, flow cytometric evaluation used for investigating sperm mitochondrial activity and apoptosis status. Evaluation of post-thawed spermatozoa with R123 and PI staining showed that the percentage of live spermatozoa with active mitochondria was significantly higher in TSL extender compared with Bioxcell; however, there was no significant difference between TSL and TEY extenders. In accordance with Kasai *et al*. (2002), the current results showed an association between mitochondrial activity and sperm total motility. However, Vidal *et al*. (2013) reported that mitochondrial activity was not altered by either soybean lecithin or skim milk-based extenders in post-thawed goat spermatozoa.

In normal cells, the PS is located on the inner surface of the plasma membrane. Induction of apoptosis results in the translocation of PS from the inner to the outer surface of the plasma membrane (Anzar *et al*. 2002; Marti *et al*. 2008). Freezing of ram semen with different extenders showed that the percentage of live spermatozoa was significantly higher in TSL compared with TEY extender. Several researchers reported that soybean lecithin based-extenders are more capable of maintaining a higher percentage of live spermatozoa compared with those containing egg yolk (Forouzanfar *et al*. 2010; Del Valle *et al*. 2012). Extended and frozen semen with TSL and Bioxcell resulted in a significant reduction in the percentage of apoptotic spermatozoa compared with TEY. Marti *et al*. (2008) reported that caspase enzymes were activated in the initial phase of apoptosis, whereas the translocation of PS is restricted to the later stage of apoptosis. This change may detrimentally affect the plasma membrane. It seems that extenders, containing soybean lecithin (TSL and Bioxcell), may constitute a protective film around the cell to protect sperm against cold shock and mechanical damages during the freeze–thawing process (Quinn *et al*. 1980). Also, dead spermatozoa significantly increased in Bioxcell compared with TSL and TEY extenders after the freeze–thawing process. The results obtained from the PS translocation test regarding spermatozoa viability indicated that using different extenders have led to recovered spermatozoa with a different percentage of viability following the thawing process. The soy extender provided a significant improvement in percentages of normal, non-capacitated sperm after thawing, possibly indicating a superior sperm membrane protection compared with well known egg yolk-based formulations (Vick *et al*. 2012). In line with the current findings, it has been shown that lecithin in ram semen extender may increase the proportion of viable and non-apoptotic spermatozoa in fresh and frozen–thawed sperm

(Del Valle *et al*. 2012). The results of the present study revealed that Bioxcell increased the ratio of post-thawed dead spermatozoa compared with those in TSL and TEY extenders. The reason for the higher percentage of post-thawed dead spermatozoa in Bioxcell may be attributed to an inadequate lecithin concentration, regarding that the Bioxcell was set up for the freezing of bull semen.

Conclusion

The most critical finding from the present study is that TSL extender may provide a stabile milieu and conditions to better preserve ram semen during the freezing and thawing process. The results also indicated that post-thawed sperm progressive motility and several velocity parameters, plasma membrane functionality, acrosome integrity, viable and non-apoptotic spermatozoa were higher in home-made soybean lecithin-based extender. Further works are needed to unravel the efficiency of TSL extender to improve artificial insemination results when using frozen semen.

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References

Aboagla EME, Terada T (2004) Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. *Theriogenology* **62**, 1160–1172. doi:10.1016/j.theriogenology.2004.01.013

Aires VA, Hinsch KD, Mueller-Schloesser F, Bogner K, Mueller-Schloesser S, Hinsch E (2003) In vitro and in vivo comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. *Theriogenology* **60**, 269–279. doi:10.1016/S0093-691X(02)01369-9

Akhter S, Ansari MS, Rakha BA, Andrabi SM, Iqbal S, Ullah N (2010) Cryopreservation of buffalo (*Bubalus bubalis*) semen in Bioxcell extender. *Theriogenology* **74**, 951–955. doi:10.1016/j.theriogenology.2010.04.024

Amirat L, Tainturier D, Jeanneau L, Thorin C, Gérard O, Courtens JL, Anton M (2004) Bull semen in vitro fertility after cryopreservation using egg yolk LDL: a comparison with Optidyl, a commercial egg yolk extender. *Theriogenology* **61**, 895–907. doi:10.1016/S0093-691X(03)00259-0

Amirat L, Anton M, Tainturier D, Chatagnon G, Battut I, Courtens JL (2005) Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. *Reproduction (Cambridge, England)* **129**, 535–543. doi:10.1530/rep.1.00011

Anzar M, He L, Buhr MM, Kroetsch TG, Pauls KP (2002) Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biology of Reproduction* **66**, 354–360. doi:10.1095/biolreprod66.2.354

Barbas JP, Mascarenhas RD (2009) Cryopreservation of domestic animal sperm cells. *Cell and Tissue Banking* **10**, 49–62. doi:10.1007/s10561-008-9081-4

Bencharif D, Amirat L, Anton M, Schmitt E, Desherces S, Delhomme G, Langlois ML, Barrière P, Larrat M, Tainturier D (2008) The advantages of LDL (low density lipoproteins) in the cryopreservation of canine semen. *Theriogenology* **70**, 1478–1488. doi:10.1016/j.theriogenology.2008.06.095

Bousseau S, Brillard JP, Marguant-Le Guienne B, Guerin B, Camus A, Lechat M (1998) Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology* **50**, 699–706. doi:10.1016/S0093-691X(98)00175-7

Chenoweth PJ (2005) Genetic sperm defects. *Theriogenology* **64**, 457–468. doi:10.1016/j.theriogenology.2005.05.005

Curry MR (2000) Cryopreservation of semen from domestic livestock. *Reviews of Reproduction* **5**, 46–52. doi:10.1530/ror.0.0050046

Del Valle I, Gomez-Duran A, Holt WV, Muino-Blanco T, Cebrian-Perez JA (2012) Soy lecithin interferes with mitochondrial function in frozen-thawed ram spermatozoa. *Journal of Andrology* **33**, 717–725. doi:10.2164/jandrol.111.014944

Emamverdi M, Zhandi M, Zare Shahneh A, Sharafi M, Akbari-Sharif A (2013) Optimization of ram semen cryopreservation using a chemically defined soybean lecithin-based extender. *Reproduction in Domestic Animals* **48**, 899–904. doi:10.1111/rda.12183

Forouzanfar M, Sharafi M, Hosseini SM, Ostadhosseini S, Hajian M, Hosseini L, Abedi P, Nili N, Rahmani HR, Nasr-Esfahani MH (2010) In vitro comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. *Theriogenology* **73**, 480–487. doi:10.1016/j.theriogenology.2009.10.005

Fukui Y, Kohno H, Togari T, Hiwasa M, Okabe K (2008) Fertility after artificial insemination using a soybean-based semen extender in sheep. *The Journal of Reproduction and Development* **54**, 286–289. doi:10.1262/jrd.20004

Gil J, Soderquist L, Rodriguez-Martinez H (2000) Influence of centrifugation and different extenders on post-thaw sperm quality of ram semen. *Theriogenology* **54**, 93–108. doi:10.1016/S0093-691X(00)00328-9

Gil J, Rodriguez-Irazoqui M, Lundeheim N, Soderquist L, Rodriguez-Martinez H (2003) Fertility of ram semen frozen in Bioexcell and used for cervical artificial insemination. *Theriogenology* **59**, 1157–1170. doi:10.1016/S0093-691X(02)01178-0

Gillan L, Evans G, Maxwell WMC (1997) Capacitation status and fertility of fresh and frozen-thawed ram spermatozoa. *Reproduction, Fertility and Development* **9**, 481–487. doi:10.1071/R96046

Gillan L, Evans G, Maxwell WMC (2005) Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology* **63**, 445–457. doi:10.1016/j.theriogenology.2004.09.024

Graham J, Foote R (1987) Effect of several lipids, fatty acyl chain length, and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. *Cryobiology* **24**, 42–52. doi:10.1016/0011-2240(87)90005-8

Holt WV (2000) Basic aspects of frozen storage of semen. *Animal Reproduction Science* **62**, 3–22. doi:10.1016/S0378-4320(00)00152-4

Kasai T, Ogawa K, Mizuno K, Nagai S, Uchida Y, Ohta S, Fujie M, Suzuki K, Hirata S, Hoshi K (2002) Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. *Asian Journal of Andrology* **4**, 97–103.

Khalifa T, Lymberopoulos A, Theodosiadou E (2013) Association of soybean-based extenders with field fertility of stored ram (*Ovis aries*) semen: a randomized double-blind parallel group design. *Theriogenology* **79**, 517–527. doi:10.1016/j.theriogenology.2012.11.009

Marti E, Perez-Pe R, Colas C, Muino-Blanco T, Cebrian-Perez J (2008) Study of apoptosis-related markers in ram spermatozoa. *Animal Reproduction Science* **106**, 113–132. doi:10.1016/j.anireprosci.2007.04.009

Maxwell WMC, Watson PF (1996) Recent progress in the preservation of ram semen. *Animal Reproduction Science* **42**, 55–65. doi:10.1016/0378-4320(96)01544-8

Moce E, Graham JK (2008) In vitro evaluation of sperm quality. *Animal Reproduction Science* **105**, 104–118. doi:10.1016/j.anireprosci.2007.11.016

Moussa M, Marinet V, Trimeche A, Tainturier D, Anton M (2002) Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. *Theriogenology* **57**, 1695–1706. doi:10.1016/S0093-691X(02)00682-9

Najafi A, Zhandi M, Towhidi A, Sharafi M, Akbari Sharif A, Khodaei Motlagh M, Martinez-Pastor F (2013) Trehalose and glycerol have a dose-dependent synergistic effect on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology* **66**, 275–282. doi:10.1016/j.cryobiol.2013.03.002

Papa FO, Felicio GB, Melo-Ona CM, Alvarenga MA, De Vita B, Trinque C, Puoli-Filho NJ, Dell'Aqua JA, Jr (2011) Replacing egg yolk with soybean lecithin in the cryopreservation of stallion semen. *Animal Reproduction Science* **129**, 73–77. doi:10.1016/j.anireprosci.2011.10.006

Partyka A, Łukaszewicz E, Niżański W (2011) Flow cytometric assessment of fresh and frozen-thawed Canada goose (*Branta canadensis*) semen. *Theriogenology* **76**, 843–850. doi:10.1016/j.theriogenology.2011.04.016

Perez LJ, Valcarcel A, de Las Heras MA, Moses DF, Baldassarre H (1996) In vitro capacitation and induction of acrosomal exocytosis in ram spermatozoa as assessed by the chlortetracycline assay. *Theriogenology* **45**, 1037–1046. doi:10.1016/0093-691X(96)00031-3

Perez-Garnelo SS, Oter M, Borque C, Talavera C, Delclaux M, Martinez-Nevado E, Palasz AT, De la Fuente J (2006) Post-thaw viability of European bison (*Bison bonasus*) semen frozen with extenders containing egg yolk or lipids of plant origin and examined with a heterologous in vitro fertilization assay. *Journal of Zoo and Wildlife Medicine* **37**, 116–125. doi:10.1638/05-039.1

Quinn P, Chow P, White I (1980) Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. *Journal of Reproduction and Fertility* **60**, 403–407. doi:10.1530/jrf.0.0600403

Reed ML, Ezeh PC, Hamic A, Thompson DJ, Caperton CL (2009) Soy lecithin replaces egg yolk for cryopreservation of human sperm without adversely affecting postthaw motility, morphology, sperm DNA integrity, or sperm binding to hyaluronate. *Fertility and Sterility* **92**, 1787–1790. doi:10.1016/j.fertnstert.2009.05.026

Revell SG, Mrode RA (1994) An osmotic resistance test for bovine semen. *Animal Reproduction Science* **36**, 77–86. doi:10.1016/0378-4320(94)90055-8

Salamon S, Maxwell WM (2000) Storage of ram semen. *Animal Reproduction Science* **62**, 77–111. doi:10.1016/S0378-4320(00)00155-X

Salmani H, Nabi MM, Vaseghi-Dodaran H, Rahman MB, Mohammadi-Sangcheshmeh A, Shakeri M, Towhidi A, Shahneh AZ, Zhandi M (2013) Effect of glutathione in soybean lecithin-based semen extender on goat semen quality after freeze-thawing. *Small Ruminant Research* **112**, 123–127. doi:10.1016/j.smallrumres.2012.12.015

SAS Institute (2002) 'SAS proprietary software release 9.0.' (SAS Inst. Inc.: Cary, NC)

Schafer S, Holzmann A (2000) The use of transmigration and Spermac stain to evaluate epididymal cat spermatozoa. *Animal Reproduction Science* **59**, 201–211. doi:10.1016/S0378-4320(00)00073-7

Thys M, Nauwynck H, Maes D, Hoogewijs M, Vercauteren D, Rijsselaere T, Favoreel H, Van Soom A (2009) Expression and putative function of fibronectin and its receptor (integrin $\alpha5\beta1$) in male and female gametes during bovine fertilization in vitro. *Reproduction (Cambridge, England)* **138**, 471–482. doi:10.1530/REP-09-0094

van Wagtendonk-de Leeuw AM, Haring RM, Kaal-Lansbergen LM, den Daas JH (2000) Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. *Theriogenology* **54**, 57–67. doi:10.1016/S0093-691X(00)00324-1

Vick MM, Bateman HL, Lambo CA, Swanson WF (2012) Improved cryopreservation of domestic cat sperm in a chemically defined medium. *Theriogenology* **78**, 2120–2128. doi:10.1016/j.theriogenology.2012.08.009

Vidal AH, Batista AM, da Silva ECB, Gomes WA, Pelinca MA, Silva SV, Guerra MMP (2013) Soybean lecithin-based extender as an alternative for goat sperm cryopreservation. *Small Ruminant Research* **109**, 47–51. doi:10.1016/j.smallrumres.2012.07.022

Watson PF (1981) The roles of lipid and protein in the protection of ram spermatozoa at 5 C by egg-yolk lipoprotein. *Journal of Reproduction and Fertility* **62**, 483–492. doi:10.1530/jrf.0.0620483

Watson PF (2000) The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science* **60–61**, 481–492. doi:10.1016/S0378-4320(00)00099-3

Zanganeh Z, Zhandi M, Zare-Shahneh A, Najafi A, Nabi MM, Mohammadi-Sangcheshmeh A (2013) Dose rosemary aqueous extract improve buck semen cryopreservation? *Small Ruminant Research* **114**, 120–125. doi:10.1016/j.smallrumres.2013.05.015