

Effects of Centrifugation and Different Levels of Soybean Lecithin-Based Extender on Post-Thaw Sperm Quality of Ghezel Ram Semen

Research Article

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ABSTRACT

The objective of this study was to specify the effects of centrifugation and different surface of soybean lecithin-based extender on post-thaw Sperm quality of Ghezel ram semen. Semen samples were gathered from 5 mature Ghezel ram two/week for 3 weeks. After initial evaluation, the approved semen samples were pooled together and split into equal parts in Falcon tubes. Three samples were combined with Tris buffer at 30 °C and then centrifuged to remove the seminal plasma. After centrifugation and removal of the supernatant, samples were diluted with Tris-citrate-fructose extender + different concentrations of soybean lecithin (1%, 1.5% and 2% weight/volume) with 7% glycerol; and residual one samples, which were not centrifuged, were diluted with the (1.5% weight/volume) soybean lecithin. Samples then cooled to 5 °C and frozen in 0.25 mL French straws and straws were thawed in a 37 °C water bath. The results of different concentrations of soybean lecithin showed that total motility (TM) and motion parameters of average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL) and lateral head displacement (ALH) and the percentage of spermatozoa with intact acrosomes were significantly higher in soybean lecithin (SL) 1.5% compared to other semen extenders ($P<0.05$). Total motility and sperm-motion parameters (VAP, VSL, VCL and ALH), in SL1.5% without centrifugation were higher than the groups with 1%, 1.5% or 2% and with centrifugation ($P<0.05$). Generally, the results suggest that SL1.5% extender was better (with or without centrifugation) than other extenders in most *in vitro* evaluated sperm parameters.

KEY WORDS centrifugation, freeze-thawing, soybean lecithin, sperm.

INTRODUCTION

Semen cryopreservation is influenced by many factors such as the efficiency of the antibiotics used in the extenders, the quality control of reagents, and the hygiene during semen processing, and the quality of the extenders (Ruigh *et al.* 2006). There is an increased universal concern regarding microbiological safety. Therefore, recent studies are in progress aiming to develop chemically defined extenders free of compounds of animal origins. Therefore new research has focused on the use of plant-based extenders. Semen extender solutions are generally completed with avian egg-

yolk. The main difficulty of using an animal-derived material in the structure of the cryopreservation media is the sanitary risk, transmission of diseases (Chelucci *et al.* 2015) and microbial contamination which allows subsequently production of endotoxins (Vidal *et al.* 2013). Moreover, egg yolk has been found to be an antigenic matter and incites antibodies in systemic circulation and the reproductive tract (Üstüner *et al.* 2014). Soybean lecithin may be used as an alternative to egg yolk in some species. But the precise mechanism of its effect on the sperm plasma membrane is still not completely clear. It has been suggested that the lecithin protection mechanism is because of the replace-

ment of sperm membrane phospholipids, with the reduction in the freezing point. On the other hand, it may surround the sperm with a layer around the plasma membrane and preventing the formation of intracellular ice crystals and avoiding the mechanical damage on the sperm membranes (Zhang *et al.* 2009). The cell membrane of ram sperm has a high proportion of unsaturated/saturated fatty acids, and low cholesterol / phospholipids molar ratio, which can overstate the oxidative damage due to reactive oxygen species (ROS) production (Bucak *et al.* 2007). In additions during sperm storage, sperm membranes cholesterol content altered non-physiologically due to cool and storing (Purdy and Graham, 2004). During capacitation *in vitro*, part of the cholesterol effluxes and the plasma membrane becomes disordered and resulted in increased bilayer penetrance (Cross, 2003) and fluidity (Gadella and Harrison, 2002).

Researchers have shown that seminal plasma proteins induce cholesterol efflux of sperm membranes in the ram (Roostaei *et al.* 2015). Although centrifugation causes potential sperm damage, but use of centrifugation could eliminate ejaculated contaminants, such as abnormal and dead sperm. Dead spermatozoa produce ROS which exert harmful effects on spermatozoa during cryopreservation (Upreti *et al.* 1999). The purpose of present study was to evaluate the effects of 2 freezing protocols and different level of soybean lecithin-based extender in rams on total motility, progressive motility, motility patterns, malondialdehyde (MDA), and membrane integrity.

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from Sigma Co (St. Louis, MO, USA) and Merck (Darmstadt, Germany), unless otherwise indicated.

Animal and semen collection

Semen samples were collected from 5 sexually mature Ghezel rams (3-4 years of age). The rams were kept on the farm at a livestock research station at University of Tabriz (Iran, Tabriz). Ejaculates were collected twice a week using an artificial vagina during the breeding season. Semen evaluation was performed within approximately 60 min.

To remove individual effects, ejaculates containing sperm with > 75% progressive motility, a volume of 0.75-2 mL, sperm concentrations greater than 3×10^9 sperm/mL, and sperm abnormalities of less than 10%, were pooled.

Semen processing

The basic extender used in this study was composed of 223.71 mM Tris, 55.50 mM fructose, and 72.87 mM citric acid (320 mOsm/kg, pH 7.2). Different levels of soybean lecithin (P3644 Sigma L-phosphatidylcholine from soy-

bean) were added to the basic extender at (w/v). Each mixed ejaculates from Ghezel rams was pooled and split into four equal parts. Three samples were diluted with Tris buffer and then centrifuged ($300 \times g/10$ min) at 30 °C to remove seminal plasma. After centrifugation, samples were diluted with a Tris-based extender containing 1%, 1.5% and 2% soybean lecithin and (7%) glycerol. One of the samples was not centrifuged and was diluted with the (1/5% w/v) soybean lecithin. The diluted semen samples were aspirated into 0.25 mL straws for semen (IMV, L'Aigle, France), and sealed with haematocrite sealing clay and slowly cooled to 4 °C for 2 h. After cooling, the straws were horizontally placed on an aluminium rack and frozen in liquid nitrogen vapour, about 4 cm above the surface of the liquid nitrogen, for 7 min, and then immersed into the liquid nitrogen for storage. The cryopreserved samples remained in liquid nitrogen for 4 weeks. For post-thawing quality assessment, the frozen straws were thawed individually (37 °C for 30 s) in a water bath.

Semen evaluation

Sperm motility

Sperm motility characteristics were assessed by computer assisted sperm analysis (CASA, VideoTesT-Sperm 3.1, S t. Petersburg, Russia). The sample was examined at magnification $\times 20$ using a phase contrast microscope with a warmed stage (37 °C), (Labomed LX400; Labomed Inc., Culver City, CA, USA).

Sperm viability

Sperm viability was determined by means of the nigrosin-eosin staining procedure. The stain contained eosin-Y 1.67 g, nigrosin 10 g, and sodium citrate 2.9 g, which were dissolved in 100 mL of twice-distilled water. For this objective, sperm suspension smears were prepared by mixing 10 μ L of sperm sample with 20 μ L of stain on a warm slide and spreading the stain with a second slide; the viable and nonviable sperm were calculated by counting 200 total sperm using phase-contrast microscopy $\times 400$.

Sperm morphology

Sperm abnormalities were evaluated using Hancock solution. Semen (10 μ L) was homogenized with 1 mL of Hancock's solution (Hancock solution (HS) preparing by mixing formalin (62.5 mL), sodium saline solution (150 mL), buffer solution (150 mL) and double-distilled water (500 mL) which were prepared as follows: Sodium saline solution: 9.01 g NaCl in 500 mL of double-distilled water, buffer solution: (1) 21.7 g $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ in 500 mL of double-distilled water, (2) 22.254 g KH_2PO_4 in 500 mL of double-distilled water, 100 mL of (1) and 50 mL of (2) were mixed to obtain 150 mL of buffer solution and examined using phase-contrast microscopy $\times 400$.

Sperm (200/slide) were assessed and the percentage of total sperm abnormalities (head abnormalities, detached heads, abnormal mid-pieces, and tail defects) was determined.

Sperm membrane integrity

The hypo-osmotic swelling test was used to evaluate functional sperm plasma membrane integrity after freeze-thawing. The assay was performed by mixing 15 μ L of semen with 150 μ L of a 100 mOsm hypo-osmotic solution (9 g/L fructose+4.9 g/L sodium citrate in 1 L of twice-distilled water) at 37 °C, for 30 min. A total of 0.2 μ L of the mixture was placed on a microscope slide and mounted with a coverslip, and sperm were immediately evaluated under a phase-contrast microscope \times 400. Sperm with (200/slide) swollen and non-swollen tails were subsequently recorded.

Malondialdehyde concentrations

The amount of malondialdehyde (MDA) in the semen samples, as an index of lipid peroxidation, was measured with the thiobarbituric acid reaction; one ml of the diluted semen (250×10^6 spermatozoa/mL; three straws per each replicate) was mixed with one (1, same as others) mL of cold 20% (w/v) trichloroacetic acid (TCA) to precipitate protein. To inhibit lipid oxidation, one mL of butylated hydroxytoluene (2% BHT solution in ethanol) and one mL of EDTA (1 mM final concentration) were added to the sample before TCA precipitation. The precipitate was pelleted by centrifuging (1200 g for 15 min), and one mL of the supernatant was incubated with one ml of 0.67% (w/v) thiobarbituric acid in a boiling water bath at 100 °C for 10 min. After cooling, the absorbance was evaluated with a spectrophotometer (T80 UV/VIS PG Instruments Ltd, UK) at 532 nm, and the results were reported as nmol/mL.

Acrosome integrity

The method described by [Thys *et al.* \(2009\)](#) with some rectification was used for evaluation of acrosomal integrity. Five hundred μ L of sperm suspension was added to the 1.5-mL microtube and centrifuged (600 g, 10 min). The supernatant was removed, and the sperm pellet was dissolved in 100 μ L 96% ethanol (in the same microtube). The sperm were fixed for 30 min (4 °C) and thoroughly mixed by pipetting. Ten μ L of sperm suspension was put on a glass slide (in a thin line *vs.* a droplet), and the ethanol allowed to evaporate. Then, 20 μ L of PSA (50 μ L/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, they were allowed to dry and were mounted with glycerol and covered with a 24 \times 24 mm coverslip. Two hundred sperm per slide were assessed by a fluorescence microscope (Olympus IX81 fluorescence microscope, Olympus Optical Co., Ltd., Tokyo,

Japan) at 400 \times magnification. Sperm heads with green fluorescence, no fluorescence, or a green fluorescent band at the equatorial level were recorded as intact or damaged/disrupted acrosome, respectively.

Statistical analysis

All data were analyzed by the GLM procedure of SAS 9.2 ([SAS, 2002](#)). The experiment was conducted as a completely randomized 1-way ANOVA (1/5% soybean lecithin without centrifugation and 1%, 1/5% and 2% soybean lecithin with centrifugation). Results are reported as means \pm SE. Statistical differences between/among the various treatment group means were determined by Tukey's test. Differences were considered to be statistically significant at ($P < 0.05$).

RESULTS AND DISCUSSION

The evaluations showed that total motility and some motion parameters (VAP, VSL, VCL and ALH) in post-thawed spermatozoa were affected by different extenders (Table 1); and SL1.5% (without centrifugation) extender resulted in higher TM, VAP, VSL, VCL and ALH compared to groups containing 1%, 1.5% or 2% lecithin with centrifugation, ($P < 0.05$).

Other motion parameters (PM, BCF and LIN) were not significantly different among groups. The results of the current study revealed that a significant effect was found between different freezing protocols and different levels of soybean lecithin with respect to sperm abnormality (Table 2).

Sperm abnormality in the SL1.5% (without centrifugation) extender was significantly lower than the groups containing 1%, 1.5% or 2% lecithin with centrifugation ($P < 0.05$). Viability, plasma membrane integrity, and lipid peroxidation were not significantly affected by different treatments and the freezing protocol.

The sperm with intact acrosomes in the 1.5% groups (without centrifugation) were significantly greater in number than in the groups containing 1%, 1.5% or 2% lecithin with centrifugation ($P < 0.05$) (Figure 1).

Sperm heads with green fluorescence, no fluorescence, or a green fluorescent band at the equatorial level were recorded as intact and damaged acrosomes, respectively (Figure 2). The percentage of total motility and other motion parameters (VAP, VSL, VCL and ALH) in the 1.5% group (without centrifugation) was significantly higher than that in groups containing 1%, 1.5% or 2% lecithin with centrifugation.

The semen centrifugation treatment had no significant effect on sperm progressive motility, lipid peroxidation, or viability of ram sperm after freeze-thawing.

Table 1 The effect of different levels of soybean lecithin and freezing and thawing protocol on sperm motility parameters

Items	%	TM (%)	PM (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	LIN (%)	STR (%)	ALH (µm)	BCF (HZ)
Un-centrifuged	1.5	62.6 ^a	26.8	78.72 ^a	63.81 ^a	134.47 ^a	47.44	81	8.05 ^a	24.73
	1	55.4 ^{bc}	22.4	74.30 ^b	58.4 ^{ab}	117.11 ^b	49.88	7.70	7.18 ^{ab}	24.78
Centrifuged	1.5	58.2 ^{ab}	25	75.81 ^{ab}	61.27 ^a	133.25 ^a	46	80.86	7.54 ^{ab}	25.92
	2	52 ^c	19.6	69.44 ^c	52.75 ^b	112.50 ^c	46.91	76.03	6.28 ^b	24.72
SEM		1.230	2.028	1.082	1.778	0.941	1.492	2.448	0.355	1.258

TM: total motility; PM: progressive motility; VAP: average path velocity; VSL: straight-line velocity; VCL: curvilinear velocity; LIN: linearity; STR: Straightness; ALH: amplitude of lateral head and BCF: beat cross frequency.

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Table 2 The effect of different levels soybean lecithin and freezing and thawing protocol on sperm assessment parameters

Items	%	Viability (%)	Membrane integrity (%)	Abnormal forms (%)	MDA (nmol/mL)
Un-centrifuged	1.5	66.4	53.2	16.74 ^b	2.4
	1	61.60	51.6	19.2 ^a	2.38
Centrifuged	1.5	64.20	52.8	17.70 ^{ab}	2.32
	2	59.2	49	19.24 ^a	2.4
SEM		3.331	2.033	0.552	0.164

MDA: malondialdehyde.

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

SEM: standard error of the means.

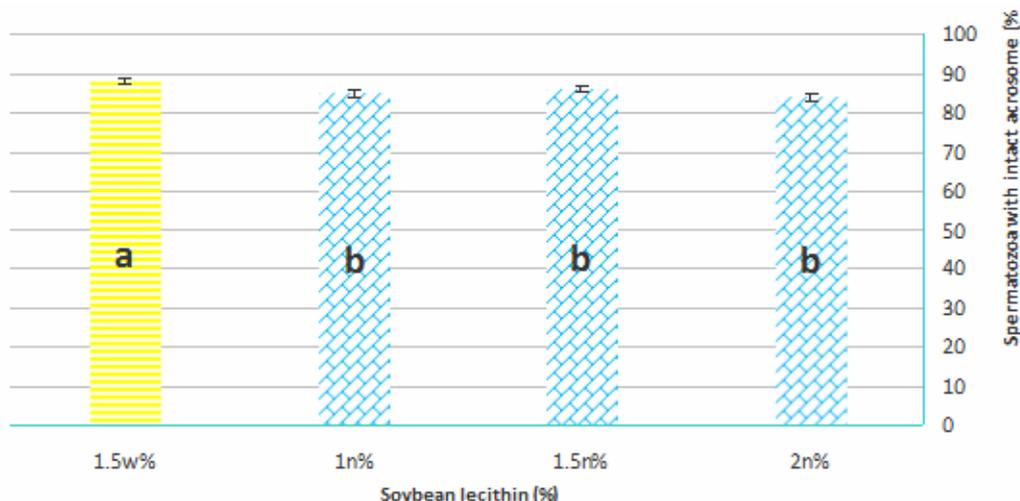


Figure 1 The Effect of different levels of soybean lecithin and tow freezing protocol on sperm intact acrosome after freezing-thawing process

Bars with different letters denote significant differences (P<0.05)

This phenomenon indicated that it was not necessary to centrifuge ram semen diluted with soybean lecithin in order to remove the seminal plasma. The percentage of abnormal sperm observed in the non-centrifuged sample was lower than in the centrifuged samples. Studies have shown that centrifugal force can affect motility and the membrane integrity of sheep (Gil *et al.* 1999) and goat (Ritar, 1993) spermatozoa. These reports indicate that methods involving mechanical stress, (such as centrifugation), are harmful to the viability of goat sperm. The plasma membrane integrity of spermatozoa is necessary to maintain sperm functionality during storage in the female’s reproductive tract (Holt and North, 1994), and the disruption of plasma membrane integrity caused by disarrangement of lipids within the mem-

brane during cryopreservation may induce further cellular damage and consequently lead to sperm death (Holt and North, 1994). In the present study, SL 1.5 resulted in a non-significant higher percentage of sperm with functional membrane integrity compared to other SL-based extenders. It has been reported that the functional membrane integrity of spermatozoa bears a direct relationship with sperm motility (Salmani *et al.* 2013). Therefore, it appears that a part of the beneficial effect of SL1.5 on total motility and some motion parameters of sperm may be related to plasma membrane integrity. The number of sperm with intact acrosomes in the 1.5% group without centrifugation was significantly higher than in groups containing 1%, 1.5% or 2% lecithin with centrifugation.

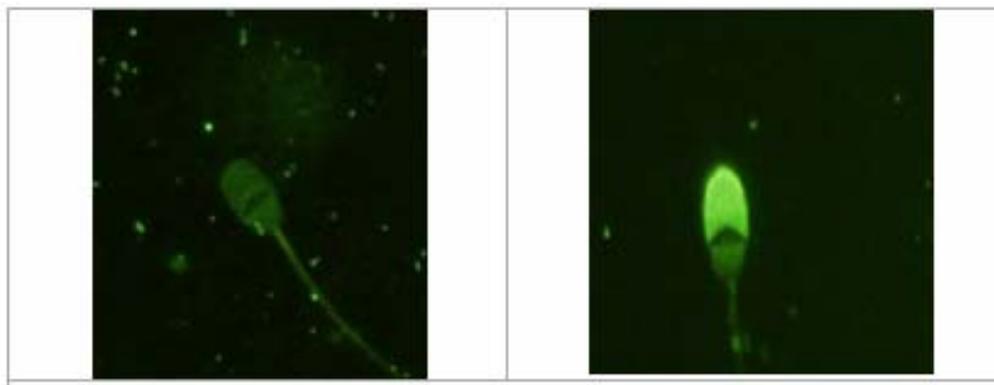


Figure 2 Sperm acrosome integrated (right) and non-integrated (left)

Acrosomal injuries are generally caused by the formation of large ice crystals within the cell, damaging the internal structures and plasma membrane (Futino *et al.* 2010). Though the mechanism by which soybean lecithin protects sperm during freeze-thawing remains unclear, two hypotheses to explain the phenomenon have been offered. Phospholipids, being the main component of membranes, play important physiologic functions in reducing the freezing point of water, thus avoiding the formation of large ice crystals, and minimizing the replacement of plasmalogens to lessen the possible mechanical detriment to the sperm membrane (Waterhouse *et al.* 2006).

Therefore, phospholipids of foreign origin in the extenders can replace the membrane phospholipids and maintain the structure and function of the plasma membrane (Zhang *et al.* 2009). Another hypothesis accepted by a group of researchers is that soybean lecithin does not enter the plasma membrane to elicit changes in its composition, but may rather surround the membrane and prevent the formation of intracellular ice crystals and protect the cell membrane against mechanical damage during freeze-thawing (Zhang *et al.* 2009).

Our results suggest that centrifugation and higher SL concentrations were harmful to acrosomal membrane integrity, whereas the lower concentration was insufficient in providing appropriate protection.

CONCLUSION

This study indicated that centrifugation of ram semen diluted with soybean lecithin was not necessary to remove the seminal plasma, and that a 1.5% soybean lecithin level in the extender was an optimal level to decrease the percentage of abnormal ram sperm and increase the total motility, other motion parameters (VAP, VSL, VCL and ALH), and the number of sperm with intact acrosomes after freeze-thawing.

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