Fertility and Flow Cytometry Study of Frozen-thawed Sperm in Cryopreservation Medium Supplemented with Soybean lecithin

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Abstract

Semen cryopreservation can provide genetic resources for a large number of females from a small number of superior males. Optimization of cryopreservation media to achieve the highest quality of post-thaw semen is crucial. Soybean lecithin has evaluated as a plant-based cryoprotectant for substitution of egg yolk in ram semen extender. Flow cytometric and fertility assessments were applied following cryopreservation procedure in two experimental groups (SL group: extender containing 1% w/v soybean lecithin and EY group: extender containing 20% v/v egg yolk). The higher percentage of live sperm and the lower percentage of dead sperm were obtained in SL (47.66 ± 1.38 , 52.33 ± 1.69 , respectively) extender compared to EY (41.16 ± 1.38 , 58.83 ± 1.69). For motion characteristics, plasma membrane integrity, acrosome integrity and mitochondria activity, no significant difference was observed between SL and EY extenders. In artificial insemination experiment, there was no significant difference in pregnancy rate, lambing rate and twining rate between SL and EY extenders. It can be concluded that SL extender can be an efficient alternative extender to preserve ram sperm during cryopreservation procedure without adverse effects.

Keywords: Cryopreservation; Soybean lecithin; Flow cytometry; Artificial insemination.

Introduction

In recent years, efforts to optimize ram semen cryopreservation have increased to improve reproductive performance [4,32]. Although frozen-thawed ram semen is a valuable tool to distribute genetic material, low pregnancy rate after artificial insemination has limited this technique [25]. Semen cryopreservation leads to structural and biomedical stress on sperm, resulted to reduce quality of post-thawing sperm [10,14,30]. Cryoprotectants are the most

effective part of freezing process which can influence on the sperm performance [11]. Soybean lecithin (SL) has recently been successfully applied as an extracellular cryoprotectant for cryopreservation of semen in ram [11,30], goat [27], bull [1], buffalo [2] and human [23]. In recent years, SL has been a replacement for egg yolk (EY) due to increment the risk of microbial contamination of egg yolk base extenders [31]. In addition, EY increases the agglutination and pre-capacitation of sperm that has crucially effects on sperm quality [9,16]. Furthermore, wide varieties of EY compositions make a difficulty to standardization of extenders [9]. Our previous study indicated the ability of SL to improve the quality and fertility potential of post-thawed semen [11]. SL also improved the mitochondria potential and acrosome integrity of ram sperm [9] and reduced the lipid peroxidation of goat semen [27]. However, cellular mechanism of SL on sperm structure and *in vivo* fertility potential have not been completely clarified. Therefore, the purpose of this study was to consider intracellular events after freeze-thawing using flow cytometry procedure as well as fertility potential using artificial insemination. For this purpose, motion characteristics, membrane integrity, viability and apoptosis status, acrosomal status and mitochondria activity were assessed after freeze-thawing using SL. Artificial insemination was also applied to consider the fertility potential of post-thawed ram semen.

Materials and methods

Extender preparation

The basic hand-made cryopreservation medium was composed of 2.71 g Tris (Merck, Darmstadt, Germany), 1.0 g fructose (Merck), 1.4 g citric acid (Merck), 7 % glycerol (v/v), 100 IU penicillin and 1 mg streptomycin. The osmolality and PH were set at 320 mosm/kg water and 7.2, respectively. The plant origin extender (SL) consisted of basic medium supplemented with 1%

soybean originated phosphatidyl choline. The animal origin extender (EY) as the control extender was composed of the basic medium supplemented with 20% egg yolk.

Semen collection and processing

Semen was collected using artificial vagina twice a week from ten mature Zandi rams (3 and 4 years of age) in six replicates. Semen was evaluated after ejaculation and accepted for experiment if the following parameters were observed: volume ranging between 1 - 2 ml; sperm concentration of 3×10^9 sperm/ml and the percentage of total motility of sperm was higher than seventy percent. In order to eliminate individual differences, semen samples were pooled and then aliquoted into two equal parts and diluted with 2 extenders (SL and EY). The diluted semen was gradually cooled to 4°C for 90 min and subsequently loaded in 0.25 ml French straws (Biovet, L'Agile France) to obtain 50×100^6 sperm per each straw. Then the straws were exposed to static nitrogen vapor (-70 °C) for 10 min, plunged into liquid nitrogen (LN₂) and stored in LN₂ until thawed and used for evaluation of sperm parameters and *in vivo* fertilization.

Evaluation of sperm parameters after freeze-thawing

Motion characteristics were analyzed using sperm class analyzer (Animal Version 12.3 CEROS, Hamilton-Thorne Biosciences, Beverly, MA, USA). Total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, m/s), straight-line velocity (VSL, m/s), curvilinear velocity (VCL, m/s), amplitude of lateral head displacement (ALH, m), beat/cross frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %) were surveyed after freeze-thawing [22].

In term of membrane integrity, hypo osmotic swelling test (HOST) was used according to Revell and Mrode with a slight modification [24]. Twenty micro liters of semen were added to 200 µl of

hypo osmotic solution (9.0 g fructose, 4.9 g trisodium citrate, in 1000ml of H₂O with 100 m osm/kg water). The samples were evaluated under the invert light microscope after 45 min incubation at room temperature. Two hundred sperm were recorded in 4 different microscopic fields. Then, the number of sperm with swollen and non-swollen tails were recorded as intact and damaged membrane, respectively. After calculation, the percentage of sperm with integrated membrane was reported in Fig. 1.

Annexin V and PI kit was used for detection of phosphatidyl serine externalization as an index of apoptosis changes in sperm cells. For this purpose, sperm was prepared according to the study of Emamverdi et al (2013). Following flow cytometric procedure, sperm subpopulations were classified into four groups: i) viable non-apoptotic cells: negative for both Annexin-V and PI (A⁻/PI⁻); ii) early apoptotic cells: positive for Annexin-V but negative for PI (A⁺/PI⁻); iii) late apoptotic cells: positive for both Annexin-V and PI (A⁺/PI⁺); and IV) necrotic cells: negative for Annexin-V but positive for PI (A⁻/PI⁺). Finally, the viable sperm and early apoptotic sperm was counted as live sperm and late apoptotic sperm and necrotic sperm was counted as dead sperm (Fig. 1).

A procedure described by Thys et al. (2009) with some modifications was applied to evaluate acrosome integrity [33]. Five hundred µl of sperm samples (1×10^6 sperm/ml) were placed into a 1.5 ml microtube. The contents were centrifuged ($600 \times g$, 10 min) and the resultant sperm pellet was dissolved in 100 µl of 96% ethanol. After 15 min, the sperm was fixed and 10 µl of mixed sperm were placed in a thin line on a glass slide to evaporate the remaining ethanol. Then, 30 µl of isothiocyanate-conjugated Pisum sativum agglutinin (PSA-FITC; 50 µl/ml) was added to the sperm which was placed on the top of the sperm. Then the glass slide was incubated for 20 min and subsequently dripped 10 times in distilled water, allowed to dry and then was mounted with

glycerol. We assessed 200 sperm per slide by a fluorescence microscope (BX51; Olympus) at 400x magnification. Green head sperm were considered as intact, those with no fluorescence in the head area or with a green fluorescent band at the equatorial level were recorded as damaged or disrupted acrosome, respectively.

The percentage of sperm with functional mitochondria was evaluated by Rhodamine-123 (R123; Invitrogen TM, Eugene, OR, USA) and PI according to shahverdi et al (2014) with a slight modification [29]. Ten microliters of Rhodamin-123 solution (0.01 mg/ml) was added to 500 µl of diluted semen samples by Tris buffer (50 x 10⁶ spermatozoa/ml) and incubated for 20 min in the dark. Then, Samples were centrifuged at 500 x g (3 min), and sperm pellet was resuspended in 500 µl Tris buffer. Next, 10 µl of PI (1 mg/ml) was added to sperm suspension. The sperm were analysed with a FacsCalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer. For each sample, 10000 events were recorded. The percentage of sperm with functional mitochondria was identified by positive signal for R123 and negative signal for PI.

Artificial insemination

Two hundred Iranian fat tailed Zandi ewes with 55±5 kg weight and 3-4 years old were used to determine the effects of extenders on fertilizing ability of sperm. The ewes received CIDR (Easy-Breed™, CIDR®, New Zealand) for 12 days and 500 IU eCG (Sanofi Animal Health, Libourne Cedex, France) intramuscularly at the time of CIDR removal and 54 h after CIDR removal assigned to two equal groups (100 ewes per group) for artificial insemination. Laparoscopic artificial insemination was used for ewe insemination at the fixed time. Two straws were thawed to obtain the concentration of 100×10^6 sperm for each insemination. Pregnancy

diagnosis performed by an ultrasound unit that equipped with a 3.5 MHz sector probe in day 50 after insemination. After parturition, lambing rate and twinning rate were calculated.

Statistical analysis

Sperm data was analyzed by GLM procedure of SAS software (version 9.1; SAS Institute, 2002, Cary, NC, USA). The T-test was used to compare the LSM for sperm parameters. Reproductive parameters were analyzed via GENMOD procedure using Chi-Square.

Result

Results of motion characteristics of frozen-thawed semen using two extenders containing SL and EY have been shown in Table 1. No significant differences were observed for total motility and progressive motility between the SL extender (44.83 ± 1.56 , 28.83 ± 1.4 , respectively) and EY extender (43.33 ± 1.56 , 28.5 ± 1.4 , respectively). Also, for VAP, VSL, VCL, ALH, BCF, STR and LIN, no significance difference was observed between SL and EY.

The significant ($P \leq 0.05$) higher percentage of live sperm was observed in SL extender compared to EY extender (47.66 vs. 41.16, SEM 1.38, Fig. 1). Moreover, the higher significant ($P \leq 0.05$) percentage of dead sperm was obtained in EY compared to SL based extender (58.83 vs. 52.33, SEM 1.69, Fig. 1).

Percentage of mitochondria activity and acrosome integrity of frozen-thawed sperm using SL and EY extenders have been shown in Fig 2. No significant difference was observed for mitochondria activity and acrosome integrity of frozen-thawed sperm between two groups.

Results of fertility potential of frozen-thawed semen after artificial insemination are presented in Table 2. There were no significant differences in terms of pregnancy rate (45 vs. 40 %),

parturition rate (40 vs. 40 %), lambing rate (40 vs. 40 %) and twinning rate (0 vs. 0 %) for SL and EY groups, respectively.

Discussion

In recent years, there have been many discussions for elimination of animal origin materials from semen cryopreservation media [8,19,21]. Egg yolk is an animal origin cryoprotectant for protection of sperm in freezing process [20]. However, possible problems of egg yolk such as bacterial contamination and quality variation have been reported [8,17]. Therefore, it seems that replacement of egg yolk by an alternative cryoprotectant has several advantages, such as improvement of consistency in the semen extenders components, hygienic risks elimination and improvement of fertility after freeze-thawing [31].

Our study showed that the 1% SL and 20% EY extenders have the similar effects on the post-thawed quality of sperm, so it can be concluded that egg yolk and soybean lecithin have similar effective fractions for protection of sperm in freezing process. In this regard, Benchierif et al reported that phosphatidyl choline (lecithin) is the common fraction of egg yolk and soybean that prevents cold shock [5]. This result is in agreement with Masoudi et al who reported the optimum level of SL for ram is 1% (v/v) [18]. However, for goat semen cryopreservation, optimized level of SL has reported in 1.5% (v/v) [28]. This discrepancy may be related to the difference between species in case of seminal plasma composition which may effect on the need of soybean lecithin in the extender [18].

Viability assessment using flow cytometer showed that SL produced higher percentage of live sperm and lower percentage of dead sperm compared to EY. It seems that SL had more protective effects on plasma membrane of sperm resulted in lower destruction of intercellular sperm organs [15]. Moreover, in the present study, SL obtained lower apoptotic sperm after

freezing that is in agreement with Emamverdi et al who reported that soybean lecithin can prevent apoptotic events in the ram spermatozoa [9]. Furthermore, for goat sperm, soybean lecithin showed an increment in the number of viable sperm after cryopreservation [28]. Despite the various *in vitro* assessments of sperm after freezing, we completed our evaluation in farm using artificial insemination because there is a high correlation between farm results and fertility potential [12].

In this study, overall results of artificial insemination was desirable when it compared to similar reports [25,26] and also overall of outputs of AI followed the *in vitro* pattern of sperm motility and viability suggesting the similar effects of SL and EY.

Although pregnancy rate was numerically higher in SL extender, this difference was not significant. Obtaining the same results for fertility in SL compared to EY are indices of optimum protection of sperm against cryopreservation by soybean lecithin. The ability of lecithin as a cryoprotectant is described by Bergeron and Manjunath who suggested an interaction between seminal plasma proteins and phosphatidyl choline in extender. The seminal plasma proteins which are known as RSP have negative effects on the sperm during cryopreservation, and, attachment of phosphatidyl choline and RSP can lead to a better protection of sperm [6,7]. Moreover, lecithin can create a protective layer at the surface of sperm membrane against ice crystal [20]. Moreover, Graham and Foote stated that phosphatidyl choline could substitute some phospholipids of spermatozoa membrane during cryopreservation [13].

In conclusion, soybean lecithin can successfully be used instead of egg yolk as a Supplement for ram cryopreservation medium, without adverse effects on post-thawed sperm quality and fertility.

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Groups	Total	Progressive	VAP	VSL	VCL	ALH	BCF	STR	LIN
	motility (%)	motility (%)	($\mu\text{m/s}$)	($\mu\text{m/s}$)	($\mu\text{m/s}$)	(μm)	(Hz)	(%)	(%)
SL	44.83	28.83	98.66	71.65	171.63	9.11	28.0	72.6	41.7
EY	43.33	28.50	97.86	70.80	168.66	8.96	28.5	72.6	41.9
SEM	1.56	1.40	2.34	0.93	1.84	0.33	0.68	1.73	2.71

Table1: Mean (%) motion parameters of frozen-thawed ram sperm in two extenders containing soybean lecithin and egg yolk.

Motion characteristics were assessed by Sperm Class analyzer (SCA)

Table 2. Fertility potential of frozen-thawed semen in two extenders after artificial insemination.

Extender	Pregnancy rate (%)	Parturition rate (%)	Lambing Rate (%)	Twining Rate (%)
SL	45 (45/100)	40 (40/100)	40 (40/100)	0 (0/100)
EY	40 (40/100)	40 (40/100)	40 (40/100)	0 (0/100)

The number in parentheses indicates the number of ewes/total in each group.

Fig 1. Means of membrane integrity, viability and apoptotic percentage of frozen-thawed ram sperm in two extenders containing soybean lecithin and egg yolk.

Fig 2. Means of mitochondrial activity and acrosome integrity of frozen-thawed ram sperm in two extenders containing soybean lecithin and egg yolk.