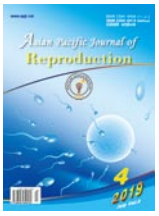

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Soybean lecithin-based extender improves Damascus goat sperm cryopreservation and fertilizing potential following artificial insemination

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ABSTRACT

Objective: To study the effect of adding different concentrations of soybean lecithin in Tris based extender and to compare the results of Tris soybean lecithin extender with two commercial diluents (egg yolk-based: BullXcell and plant-based: OptiXcell) for Damascus goat sperm cryopreservation.

Methods: The ejaculates from 4 mature male Damascus goats were obtained by using an artificial vagina. Semen samples were pooled and diluted in Tris extender supplemented with soybean lecithin at different concentrations of 1.5%, 3.0%, 6.0% and 10.0% to get better concentration to be used for further experiments, with a final concentration of 240×10^6 spermatozoa/mL. Semen samples were packed in straws (0.25 mL), frozen by using an automated system and stored in liquid nitrogen at -196 ℃ for 48 h. After thawing (37 ℃/30 s), the samples were evaluated for sperm quality parameters, including sperm motility, membrane integrity and acrosome integrity. Malondialdehyde concentration was estimated as a marker for lipid peroxidation. Based on the previous investigations, only Tris extender supplemented with 3.0% soybean lecithin (based on its positive results) was used *versus* BullXcell and OptiXcell for sperm ultrastructure evaluation and artificial insemination by using electron microscope and artificial insemination of the synchronized does.

Results: There was no significant difference between Tris-soybean lecithin at 3.0% and BullXcell/OptiXcell diluents in post-thaw sperm parameters and fertility following artificial insemination; meanwhile, the other concentrations of soybean lecithin (1.5%, 6.0% and 10%) showed lower sperm parameters following cryopreservation.

Conclusions: Using of Tris-soybean lecithin based extender at the level of 3.0% can be an appropriate alternative to either BullXcell or OptiXcell for Damascus goat sperm cryopreservation.

1. Introduction

Goat semen cryopreservation and artificial insemination tend to enhance the genetic potential of the offspring by manipulating sperm of superior bucks[1]. Like other animals, semen cryopreservation in goat has some drawbacks as it leads to cellular and functional destruction in sperm cells, lipid peroxidation and cryoprotectants toxicity due to the temperature and osmotic variation[2]. Addition

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of 10 or 15 mg/mL bovine serum albumin had a beneficial effect on post-thaw parameters of Mahabadi goat sperm[3].

The role of semen extender is to protect sperm against cold shock and promote the stabilization of the plasma membrane, the reduction of the deleterious properties of pH and osmolarity changes during the freeze-thawing process[4]. Supplementation of 10 mM cysteine to soya lecithin based semen extender of ram resulted in improvement of post-thaw parameters of ram sperm[5]. Also, it was reported that using 1 mM butylated hydroxytoluene had a crucial role in goat sperm cryopreservation[6]. No significant difference was reported in terms of viability, motility and rate of blastocyst production following diluting ram semen with either 1% lecithin-7% glycerol and Bioxcell[7].

Egg yolk is one of the most extensive cryoprotectants used during semen cryopreservation, as it protects the plasma membrane and acrosome in association with the other components[4]. No significant difference was observed by using egg yolk and soy lecithin extenders in either sperm parameters or pregnancy and lambing rates in rams[8]. In addition, it is supposed that lecithin existing in the egg yolk protects the plasma membrane by restoring the missing phospholipids during heat shock[9]. But, egg yolk is considered a predisposing factor for microbial contamination, and it also interacts with egg yolk coagulating enzyme existing in seminal plasma[10]. Egg yolk coagulating enzyme has been categorized as phospholipase A that hydrolyzes egg yolk lecithin into fatty acids and lysolecithin which is toxic to goat spermatozoa. This toxic effect can be partially eliminated by removal of the seminal plasma, resulting in an increase in the percentage of live and motile spermatozoa[9]. The risk of endotoxins released by microorganisms associated with the use of either egg yolk or milk during semen processing can reduce the fertilizing potential of spermatozoa[11,12]. As the success of artificial insemination mainly depends on appropriate semen collection and processing, many authors suggested using soybean lecithin as an alternative to animal origin based-egg yolk during semen processing in goats[13], stallion[14], boar[15], bull[16] and cat[17].

The current study aimed to obtain an optimum concentration of soybean lecithin to be added during semen processing of Damascus goat in comparison with the well-established protocols of using either plant-based diluent (OptiXcell) or egg yolk based diluent (BullXcell) during cryopreservation and emphasis our results by making artificial insemination as a field trial.

2. Materials and methods

2.1. Chemicals and reagents

The used chemicals OptiXcell, BullXcell and Tris buffer components were obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA).

2.2. Animals

Four mature Damascus bucks aged from 2 to 3 years and weighing up to 55-60 kg were used. The goats were maintained under appropriate conditions of feeding and management at the farm of Animal Reproduction Research Institute, Egyptian Ministry of Agriculture. The bucks were thoroughly examined for general and reproductive health conditions, and water was provided *ad libitum*.

Animal care processes were monitored and the ethical protocol was licensed by the Ethical Committee for Animal Use at Reproduction Research Institute (protocol number: 769-3-4-2018).

2.3. Semen collection and initial evaluation

Two ejaculates were weekly collected from each buck *via* a standard artificial vagina in the presence of a well-restrained doe as a stimulus for three months. Immediately after collection, semen samples were initially evaluated. Each semen sample was evaluated for volume, individual sperm motility, concentration and morphology. The accepted semen samples were pooled and cryopreserved, with volume varying between 0.5-2.0 mL, motility percentage higher than 70%, concentration of (2-3)×10^9 sperm/mL and less than 10% abnormal sperm in total as described by Salmani *et al*[18].

2.4. Semen extenders and processing

Semen samples from all goats were pooled to get rid of any individual differences. The pooled sample was centrifuged twice (1 750 × *g* for 10 min) in Tris buffer (3.605 g Tris; 2.024 g citric acid; 1.488 g fructose, 100 mL double-distilled water; pH 6.8) at a volume ratio of 1 : 9 (v : v; semen : Tris) in order to remove seminal plasma. Following centrifugation and removal of the supernatant, the sediment was extended (1 : 5) with Tris extender and divided into six aliquots. The first aliquot was diluted with BullXcell (IMV Technologies, France), the second aliquot was diluted with OptiXcell (IMV Technologies, France) as described by Murphy *et al*[19], and the other four aliquots were diluted with Tris-soybean lecithin based extender at levels of 1.5%, 3.0%, 6.0% and 10.0% soybean. Tris-soybean extender consisted of 3.63 g Tris, 1.80 g citric acid, 0.50 g glucose, 1.5%, 3.0%, 6.0% or 10.0% soybean, 5.00 mL glycerol, 0.05 g streptomycin, 0.25 g lincospectin and completed with bi-distilled water up to 100 mL. The extended semen was cooled gradually to 5 ℃, 1 ℃ per 3 min in a cold handling cabinet with the help of crushed ice cubes. The extended semen was maintained at 5 ℃ in the cold handling cabinet for 2 h for equilibration. After equilibration, 0.25 mL French straws and polyvinyl powder of different colors were used for filling and sealing the extended semen. The straws were placed horizontally 5 cm above the surface of nitrogen vapor at -120 ℃ in a thermo box for 15 min. Immediately after freezing, the straws were collected in a goblet containing liquid

nitrogen and then plunged into liquid nitrogen container for storage at -196 ℃[19]. The frozen straws were carried out after 48 h of freezing at 37 ℃ for 30 s in a water bath for thawing.

2.5. Experimental design

2.5.1. Evaluation of post-thawing sperm motility, acrosome integrity and plasma membrane integrity

Progressive sperm motility was assessed in five different fields of the microscopical slide by using a hot staged microscope (40× magnification) for each semen sample[20]. Percentages of acrosomal integrity were determined by silver nitrate staining technique as described by El-Amrawi *et al*[21]. The percentage of damaged acrosomes was counted in at least 100 sperm cells per slide. Membrane integrity was evaluated by using the hypo-osmotic swelling test as described by Zubair *et al*[22]. Briefly, a hypo-osmotic solution that was composed of 9.0 g fructose plus 4.9 g sodium citrate mixed with 1 000 mL of distilled water was prepared prior to use. Subsequently, 10 μL of semen was incubated in 100 μL hypo-osmotic solution at 37 ℃ for 30 min. Thereafter, 0.1 mL of the mixture was located on a warmed slide and overlapped with a coverslip. Two hundred spermatozoa were observed under the Celestron PentaView LCD digital microscope (400× magnification). Swelled spermatozoa with a distinctive coiled tail were recorded as intact plasma membrane.

2.5.2. Biochemical assay of malondialdehyde (MDA) concentration as a marker for lipid peroxidation

MDA concentrations were assayed by using thiobarbituric acid reaction as previously established by Placer *et al*[23]. Briefly, an aliquot (500 μL) of each semen sample was centrifuged at 800×*g* for 10 min; sperm pellets were obtained and resuspended in phosphate buffer saline and recentrifuged twice, following last centrifugation, and 1 mL of deionized water was added to spermatozoa followed by snap-freezing and storage at -70 ℃ until be used. MDA concentrations were measured by using thiobarbituric acid reaction, and the quantification of thiobarbituric acid reactive substances was assayed by comparing the absorption with the standard curve of MDA equivalents generated by the acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane.

2.5.3. Sperm cells ultrastructure evaluation

Based on the previous results, only post-thawing sperm extended with BullXcell, OptiXcell and Tris soybean lecithin 3.0% were subjected to ultrastructure evaluation. The ultrastructure of spermatozoon was done by transmission-electron microscope. Post-thawed straws were centrifuged several times (600×*g* for 5 min) with 0.1 M sodium cacodylate (pH 7.2) and then fixed for 12 h in a solution comprising 2.5% glutaraldehyde, sodium cacodylate buffer and 2.5% paraformaldehyde. Washing was done in a similar buffer and fixed in a solution containing 1.0% osmium tetroxide, 0.8% potassium ferricyanide and 2 mM $CaCl_2$ in 0.1 M cacodylate buffer. Dehdration of the samples was done by using an increased acetone series and inserted in SPIN-PON resin (Embed 812 was from Sigma-Aldrich). Polymerization was done at 60 ℃ for 72 h. Ultrathin cuts were gained through an ultra-microtome, located on 300-mesh nickel grids, contrasted with lead citrate and 5.0% uranyl acetate[24]. A descriptive evaluation of the spermatozoon structures was carried out.

2.5.4. Artificial insemination of synchronized goats as a fertility test

Depending on the obtained results, only semen diluted with OptiXcell extender, BullXcell extender and Tris extender supplemented with 3.0% soybean lecithin were used for artificial insemination. Damascus female goats (n=115) were synchronized with intra vaginal sponges containing 30 mg of fluorogestone acetate for 12 days. At the day of sponges' removal, all goats were treated intramuscularly with 500 IU of pregnant mare serum gonadotropin. The animals with uniform age, body weight, feeding plan were separated into three groups. Group I (n=39) was inseminated with semen frozen in OptiXcell extender, group II (n=40) inseminated with semen frozen in BullXcell extender and group III (n=36) inseminated with semen frozen in Tris extender supplemented with 3.0% soybean lecithin. Timed artificial insemination was done twice (36 and 48 h after sponge removal) by the cervical technique according to Ritar *et al*[25]. The pregnancy was detected by an ultrasound machine equipped with a 5-12 MHz linear probe. Two transrectal examinations (day 20 and day 30 after the second artificial insemination) were carried out for exclusion of the embryonic death.

2.6. Statistical analysis

Ten replicates were done for each trial. Descriptive statistics were performed to calculate the mean and standard deviation. A simple one-way analysis of variance test was performed. Data were presented as the mean ± standard deviation (mean ± SD) and analyzed by one-way analysis of variance followed by Tukey's multiple comparisons test.

3. Results

3.1. Effect of using different types of semen extender on post-thawing sperm motility, acrosomal integrity and plasma membrane integrity

The percentage of post-thawing sperm motility was significantly (P<0.05) higher in case of using BullXcell and OptiXcell as extenders than using Tris 1.5% and Tris 6.0% and also more significantly (P<0.01) higher than using Tris 10.0%. But, there was no obvious significant difference while comparing both BullXcell and OptiXcell with Tris 3.0%. The percentage of defected acrosome

was significantly (P<0.05) lower while using BullXcell and OptiXcell than using Tris 1.5% and Tris 6.0% and more significantly (P<0.01) lower than using Tris 10.0%. Interestingly, no significant difference was noted while comparing both BullXcell and OptiXcell with Tris 3.0%. The percentage of intact plasma membrane was significantly (P<0.05) higher when using BullXcell and OptiXcell than Tris 1.5% and Tris 6.0%, while no significant difference was recorded between using either BullXcell or OptiXcell and Tris 3.0% (Table 1).

3.2. Effect of using different types of semen extender on MDA concentration

There was no significant difference in MDA concentrations while using OptiXcell, BullXcell, Tris 1.5% and Tris 3.0% semen extenders. MDA concentration was significantly (P<0.05) higher when using Tris 6.0% as extender and more significantly (P<0.01) higher while using Tris 10.0% extender (Table 1).

3.3. Ultrastructure evaluation

Electron microscopy images of sagittal sections through cryopreserved goat sperm cells head cryopreserved with Bullxcell, Optixcell and Tris 3.0% extender illustrated a well-defined head and preserved acrosome covering partially the nucleus and intact plasma membrane. The nucleus content was homogenous in the electron density. Also, outer and inner acrosomal membranes were intact and the subacrosomal space was evident (Figure 1).

Electron micrograph of a cross-section through the mid-piece region from frozen-thawed goat sperm cells cryopreserved with BullXell, OptiXcell and Tris 3.0% extender illustrated normal mitochondrial organization and good mitochondrial dense electron spaces (Figure 2).

3.4. Effect of type of semen extender on fertility following post-thawing artificial insemination

There was no significant difference in either conception rates or the number of does lambing single or the number of does lambing twins when the female goats were artificially inseminated with frozen-thawed semen extended with either OptiXcell or BullXcell or Tris 3.0% (Table 2).

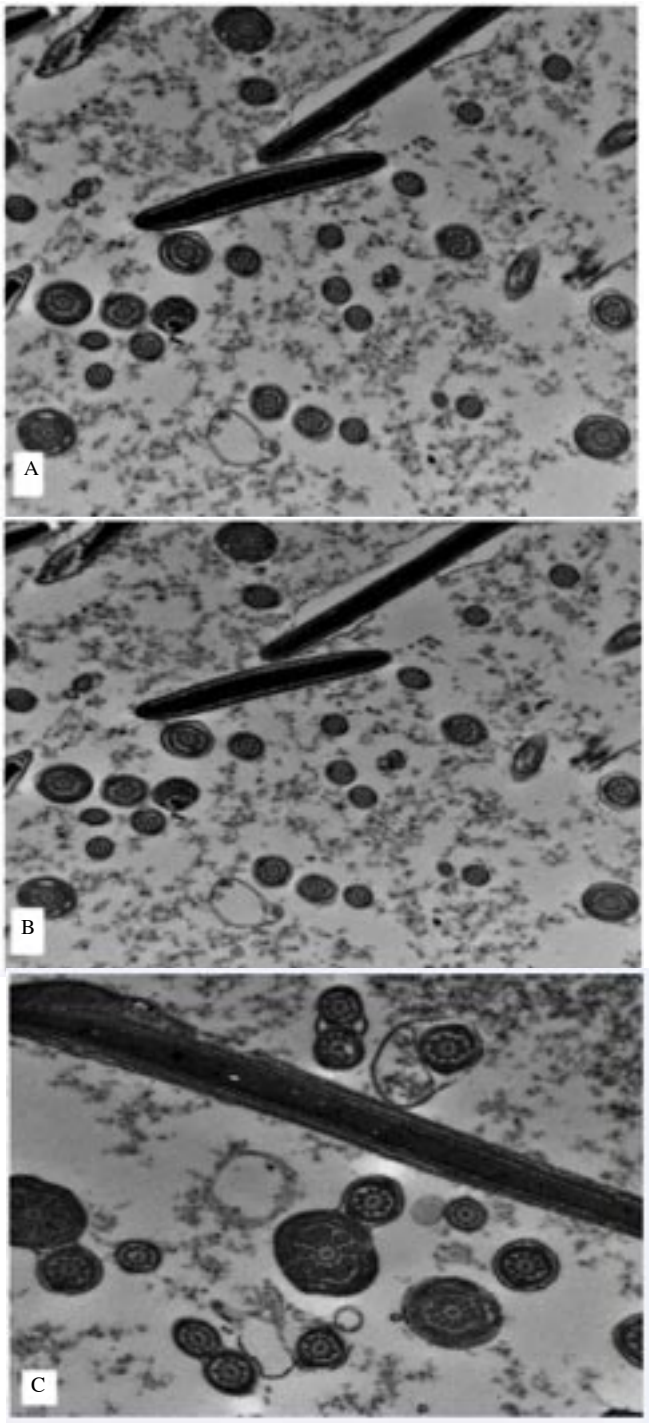

Figure 1. Electron microscopic images of sagittal sections through cryopreserved goat sperm cells head cryopreserved with Bullxcell (A), Optixcell (B) and Tris 3.0% extender (C) illustrate a well-defined head and preserved acrosome covering partially the nucleus and intact plasma membrane.

Table 1. Effect of type of semen extender on post-thawing progressive sperm motility, defected acrosome, plasma membrane integrity and malondialdehyde concentrations.

Parameters	BullXcell	OptiXcell	Tris			
			1.5%	3.0%	6.0%	10.0%
Sperm quality						
Post-thawing motility (%)	61.67 ± 6.02^{a}	60.00 ± 7.65^{a}	38.33 ± 4.02^{b}	50.00 ± 5.78^{ab}	36.67 ± 4.20^{bc}	33.33 ± 9.29^{c}
Defective acrosome (%)	15.33 ± 1.46^{c}	14.00 ± 1.73^{c}	24.67 ± 2.34^{ab}	17.67 ± 2.61^{bc}	25.33 ± 2.96^{ab}	29.00 ± 3.46^{a}
Plasma membrane integrity (%)	51.66 ± 4.42^{a}	50.00 ± 8.67^{a}	26.67 ± 8.82^{bc}	43.33 ± 6.02^{ab}	23.33 ± 7.28^{bc}	20.00 ± 5.78^{c}
Malondialdehyde concentration (nmol/10^{9})	12.18 ± 1.36^{bc}	9.64 ±1.55^{c}	13.89 ± 1.45^{bc}	12.07 ± 1.05^{bc}	15.62 ± 2.16^{ab}	19.75 ± 2.38^{a}

Values of different superscripts (a, b, c) in each line are significantly different at P<0.05.

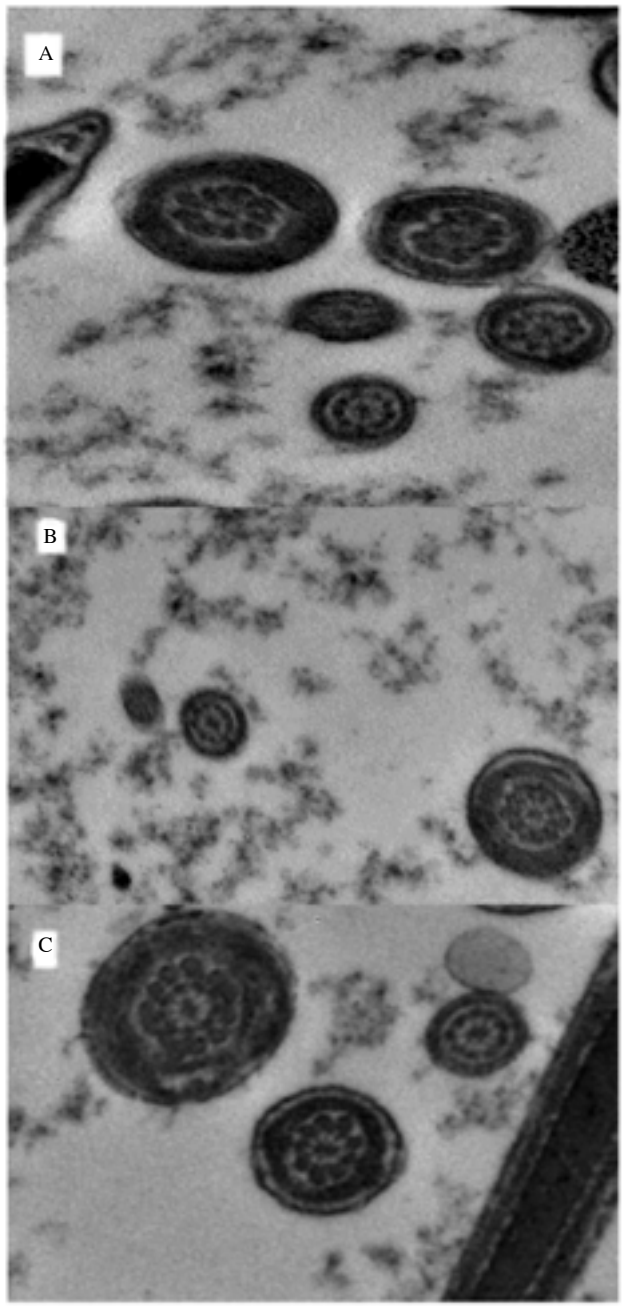

Figure 2. Electron micrograph of a cross-section through the mid-piece region from frozen-thawed goat sperm cells cryopreserved with BullXell (A), OptiXcell (B) and Tris 3.0% extender (C) illustrates normal mitochondrial organization and good mitochondrial dense electron spaces.

4. Discussion

The present study revealed that the post-thawing sperm motility was superior while using BullXcell, OptiXcell and Tris soybean lecithin at 3% concentration as extenders during semen processing to any other extenders. The protective role of soybean lecithin during the freeze-thawing process was previously explained by Waterhouse *et al*[26] as the phospholipids play a crucial role in decreasing the ice crystal formation throughout reducing the freezing point; moreover, the phospholipids can protect sperm plasma membrane by minimizing plasmalogen replacement. Maintenance of the structure and function of the sperm plasma membrane can be achieved through using an exogenous source of phospholipid that can replace the natural phospholipids present in the sperm plasma membrane, and it is suggested that the exogenous phospholipids could coat the outer sperm plasma membrane during the freeze-thawing process[14].

The percentage of soybean lecithin added to the extender is a critical point as higher concentrations may be toxic while the lower concentration may not be sufficient for protection[3]. This can illustrate why soybean lecithin at 3.0% concentration was better than other concentrations. In the same way, Baliarti *et al*[27] concluded that using 3.0% soy lecithin as extender tended to increase the post-thawing motility and acrosomal integrity of ram sperm.

The current study revealed that using either Tris soy lecithin 3.0%, BullXcell or OptiXcell resulted in significantly lower MDA concentrations than other extenders. This may be attributed to the protective effect of the phospholipid and the optimum concentration of the added soy lecithin. Makker *et al*[28] mentioned that MDA is considered as a lipid peroxidative byproduct that can illustrate the peroxidative destruction of the sperm cells. Soy lecithin was proved to contain antioxidant composites as glutathione helps in reduction of lipid peroxidation and inhibition of MDA creation during semen processing[29].

Sperm plasma membrane impairment during freeze-thawing process can explain the reduced ratio of post-thawing sperm motility, the increased ratio of damaged acrosomes and the higher MDA concentrations of using Tris soy lecithin 1.5%, 6.0% and 10.0%, despite the fact that the methods used for determination of MDA concentration are not well standardized especially in goat sperm, but

Table 2. Effect of type of extender on fertility following post-thawing artificial insemination.

Parameters	OptiXcell	BullXcell	Tris 3.0%
Number of inseminated does	39	40	36
Number of conceived does [*n*(%)]	24(61.54)	23(57.50)	20(55.56)
Number of does lambing single [*n*(%)]	15(62.50)	13(56.52)	12(60.00)
Number of does lambing twins [*n*(%)]	9(37.50)	10(43.48)	8(40.00)

our investigations were emphasized by previous results conducted on sperm of human beings[30].

Our results showed that the effect of the freeze-thawing process on sperm ultrastructure was similar when semen was extended with BullXcell, OptiXcell and Tris 3.0% diluents; this may be attributed to the role of the added soy lecithin at 3.0% concentration. Hashida *et al*[31] concluded that the process of cryopreservation negatively affects goat sperm ultrastructure, resulting in impaired fertilizing capacity. Baliarti *et al*[27] concluded that using 3.0% soy lecithin can protect plasma membrane and acrosomal integrity of ram sperm.

In our study, there is no significant difference in the conception rates, number of does lambing single and number of does lambing twins while using BullXcell, OptiXcell and Tris soy lecithin 3.0%; this may be attributed to the parallel post-thawing results obtained by using those extenders and the protective effect of soy lecithin at 3.0% level that could maintain the post-thawing motility and membrane integrity. On the one hand, Fukui *et al*[32] found that the lambing rate was 64.5% for ram sperm cryopreserved using egg yolk-based diluent and 56.7% for sperm cryopreserved by soybean lecithin-based diluent. On the other hand, Ricker *et al*[33] concluded that using diluents containing egg yolk resulted in higher fertility rates than using the commercial extender of soy lecithin (55% and 36%, respectively). Several authors reported that using the commercial extender of soy lecithin can provide parallel fertility rates if compared with either egg yolk based extender or milk diluent in sheep[34]; these findings were confirmed by Khalifa *et al*[35] who reported that using soy lecithin resulted in a reasonable fertility outcome that can reach to 71% due to its cryoprotective effect on ram sperm.

Using of Tris-soybean lecithin based extender at a level of 3.0% can maintain post-thawing sperm characteristics, plasma membrane integrity, safeguard the mitochondrial sheath and preserve the fertilizing capacity of Damascus goat spermatozoa following cryopreservation.

Conflict of interest statement

All the authors declare that there is no conflict of interest.

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