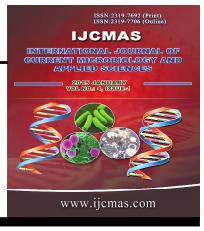
Int.J.Curr.Microbiol.App.Sci (2015) 4(3): 752-761

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 3 (2015) pp. 752-761**
http://www.ijcmas.com



Original Research Article

Effect of TFC-based extenders with soybean lecithin and/or low concentration of glycerol on the quality of goat chilled-stored semen

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Keywords

Goat, Semen Extender, Soybean Lecithin, Glycerol

A B S T R A C T

The aim of this study was to evaluate the effect of semen extender with soybean lecithin and/or low concentration of glycerol on the quality of goat chilled-stored semen. After collection the ejaculates were divided in three equal parts, diluted with Tris-fructose-citric acid (TFC) extender containing 1.5% soybean lecithin (SL), 1.5% soybean lecithin and 1.5% glycerol (SL-GL) or 1.5% glycerol (GL) and stored at temperature 0-4^{0}C for 48 hours. The semen parameters were recorded at different intervals during chilling and after incubation in a water bath at 35^{0}C. The spermatozoa fertility was tested *in vivo*. The present study revealed similar sperm motility and viability of the semen until 6 hours of storage, followed by dramatically decrease of the values ($P<0.05$) for TFC-GL extender. Between 24 and 48 hour, the motility and the viability of the semen diluted with TFC-SL-GL extender differed significantly ($P<0.05$) than the registered for the other extenders. Additionally, the motility ≥50% for the most extended incubation period and preserved sperm fertility were determined. In conclusion, the TFC-based extender containing soybean lecithin and low glycerol provided the best motility and viability of the chilled-stored spermatozoa and preserved their fertilization capacity.

Introduction

The artificial insemination (AI) in goats is biotechnological method providing augmentation of the genetic merit in caprine flocks (Leboeuf *et al.*, 2000). In previously synchronized goats, the double artificial insemination with liquid semen at 8 - or 12 - hour intervals or timed AI with chilled or frozen semen has been commonly performed (Lopez-Sebastian *et al.*, 2007; Menchaca and Rubianes, 2007; Martemucci and D'Alessandro, 2011). In many of the cases, AI with frozen semen is too expensive and conception rate is poor that limits its application. Therefore, artificial insemination with liquid semen stored in low temperature (0 to 5^{0}C) has been showed as alternative option for achievement of good conception and pregnancy rate (Roca *et al.*, 1997; Leethongdee, 2010; Phutikanit *et al.*, 2011).

One of the most important elements in handling and storage of the semen was the

preparation of eligible semen extender, ensuring high survivability and fertility of the spermatozoa for a long period (Purdy, 2006; Mara *et al.,* 2007). In most cases the diluents used for chilling or freezing of semen included egg yolk, skimmed milk, glycerol or their combination (Maxwell and Watson, 1996; Sharafi *et al.,* 2009; Kulaksiz *et al.,* 2013). Regardless of the good fertility rate after adding of egg yolk, milk or glycerol, some limitations for use of these protectors have been detected in goats (Leboeuf *et al.,* 2000). A specific problem with egg yolk and skimmed milk based extenders was the production of egg yolk coagulating enzyme and protein fraction (SBUIII) from the bulbourethral gland in bucks that depressed the spermatozoa during the time of their storage. Animal ingredients have been noted as source of microorganisms, releasing endotoxins that decrease fertilization ability of sperm (Bousseau *et al.,* 1998; Bittencourt *et al.,* 2008). High concentration of glycerol had a negative effect on the sperm membrane integrity of spermatozoa (Deka and Rao, 1986; Abdelhakeam *et al.,* 1991; Garcia *et al.,* 2012).

However, Tris-fructose- cirtric acid-based extender (TFC) with low concentration of glycerol protected the buck spermatozoa for up to 192 h after collection (Qureshi *et al.,* 2013).

The mammalian sperm were rather susceptible of oxidative stress during the chilling or freezing procedures that decreased membrane integrity and sperm motility (Aitken, 1998; Maxwell and Watson, 1996; Ortega *et al.,* 2003). Recently, many researches (Phutikanit *et al.,* 2011; Vidal *et al.,* 2013; Salmani *et al.,* 2013) have been reported of the replacement of the animal origin components with a soybean lecithin (SL). This antioxidant represents a mixture of phosphatidylcholine and different fatty acids and protects the sperm cells membrane by restoring the phospholipids lost during heat shock (Farstad, 1996; Oke *et al.,* 2010). Data connected with effect of TFC-based extenders with soybean lecithin or low concentration of glycerol on semen quality of buck from Bulgarian White milk breed are not available.

The aim of this study was to evaluate the effect of semen extender with soybean lecithin and/or low concentration of glycerol on the quality (motility and viability) of goat semen after storage at temperature regimen 0-4^{0}C and incubation at temperature 35^{0}C. In addition, to test the fertility of chilled-stored spermatozoa with the best parameters.

Material and Methods

Male animals and semen collection

The study was carried out with three bucks from Bulgarian White milk breed, between 2-3 years of age, weighing 55-60 kg, reared in individual boxes under uniform conditions and an immunoprophylaxis regimen. All males were clinical healthy determined by preliminary andrological examination.

Prior to the experiment, an abstinence period of 14 days was provided. The animals were located at latitude of 42° 25' N, and the experiment was done during the breeding season (September).

A total of 9 ejaculates were collected (three from each buck early morning, on three consecutive days) by the artificial vagina method in the presence of a goat in estrus as a teaser. The average ambient temperature for the period of semen collection was 22±1.4^{0}C.

Immediately after collection, color, vigour on a scale from 0 to 5 (Evans and Maxwell, 1987), pH and sperm volume in graduated collection tube were evaluated, and the semen was placed in a water bath at 37 ^{0} C. Each ejaculate was assessed individually and used when the following parameters were determined: lack of changes in normal color; vigour >3; pH 6.4-6.8, motility ≥70% and sperm concentration ≥1.5×10^{9} /ml.

Semen dilution and storage

After the initial assessment of sperm motility, concentration and sperm pathology each fresh ejaculate was divided in three aliquots, and diluted with three different Tris - fructose - citric acid based extenders. The TFC consisted of Tris -hydroxymethyl aminomethane 2.4g, fructose 1g, citric acid 1.4g and aqua bidestillata up to 100 ml. Extenders (Ext.) 1, 2 and 3 included soybean lecithin 1.5% (w/v) (SL), soybean lecithin 1.5 % (w/v) + glycerol 1.5% (v/v) (SL-GL) and glycerol 1.5% (v/v) (GL), respectively. The pH was adjusted to 6.8 and all extenders were stored in a refrigerator.

Before the dilution, the fresh semen and the extenders were kept together for 5 min in a water bath at 37 ^{0} C for equilibration. After that each part of the ejaculate was diluted to a final concentration of 400 × 10^{6} sperm/ml. Equal volume (0.4 ml) extended semen from different ejaculates of each donor was put into pre-warmed at 37^{0} C eppendorf tubes and they were stored at temperature 0-4^{0}C. The sperm motility was recorded at 0, 0.5, 1, 2, 2.5, 3, 6, 12, 24, 36 and 48 hours after storage, while sperm viability was estimated at 0, 3, 6, 12 and 24 h (progressive motility ≥50%). For each interval, one eppendorf tube with diluted semen from different extenders was used for evaluation.

Semen evaluation

The sperm concentration was determined by a calibrated for small ruminant semen Photometer SpermaCue (Minitüb GmbH, Germany). Motility of semen was assessed by microscopic examination using of "Motic Image Plus" digital software system (Motic China Group Ltd, 2001 - 2004), including a microscope with a heating stage and oil immersion objective, digital camera and relevant software. The motility rate was calculated as a percentage of motile spermatozoa into a sperm drop of 5 µL, placed on a warmed slide (37^{0}C), covered with 20 mm × 20 mm coverslip and observed at a magnification of 200-400×. For each sample, at least 5 microscopic viewfields were observed by two qualified operators. The average value of the three consecutive observations was calculated as the final motility (Ax *et al.,* 2000).

The sperm viability was evaluated after the eosin-nigrosin staining and presented as a percentage by the counting of 200 spermatozoa under microscope at a 400×magnification. The non stained sperm cells were accepted as alive, whereas stained were considered to be dead (Evans and Maxwell, 1987). Additionally, the motility of chilled for 12 hours semen and incubated in a water bath at 35 ^{0} C was assessed.

Fertility test

Pregnancy was considered as a reliable indicator for presence of semen fertility. The insemination protocol was based on the results obtained after motility and viability rate determination. It was designed to test the fertility of diluted semen, stored for 12 h at temperature 0-4^{0}C with motility and viability ≥50%. Only semen diluted with extender 1 and 2 was used for AI, because the motility and viability in extender 3 were

unsatisfactory (<50%). Twelve goats from Bulgarian White milk breed were synchronized with intravaginal sponges containing 30 mg of fluorogestone acetate (Syncro-part, Ceva Animal Health, France) for 12 days. After that the sponges were withdrawn and all goats were treated intramusculary with 500 IU of pregnant mare serum gonadotropine (PMSG) (Folligon, MSD Animal Health, Bulgaria).

The animals with uniform age, body weigh, feeding plan were separated into two groups. Group I (n=6) was inseminated with 0.5 ml (400×10^6 sperm/ml) chilled-stored semen diluted with Ext. 1 and group II (n=6) received the same dose, but diluted with Ext.2. Twice timed AI (36 and 48 h after sponge removal) was made by the cervical technique of Ritar et al. (1990). The pregnancy was detected by an ultrasound scanner (A5 Vet SonoScape Co. LTD, Shenzhen, China), equipped with a 5-12 MHz linear probe. Two transrectal examinations (Day 20 and Day 30 after the second AI) were carried out for exclusion of embryonic death.

Statistical analysis

The results were processed by statistical program Statistica version7.0 (Stat-Soft 1984-2000 Inc., Tulsa, OK, USA). The data was expressed as mean±Standard deviation (Mean±SD). Analysis of variance (ANOVA) was used to evaluate the extender type and the time for storage effects on semen motility and viability during the chilling, and after reanimation. Tukey`s post hoc test was used to compare the differences between values. Statistical significance was considered at $P < 0.05$.

Results and Discussion

The mean volume, concentration, motility and pH of the fresh semen were 1.87±0.18 ml, $3.57\pm0.32\text{x}10^9$/ml, 85±5% and 6.77±0.06%, respectively. Significant differences between semen parameters of the first, the second and the third ejaculates for each buck were not determined (P=0.8).

The sperm motility during the storage of semen at temperature 0-4^{0}C are presented on Figure 1. The type of extender and the time of storage did not influence significant on the sperm motility between 0 and 3 h (P=0.6). The mean values were acceptable and varied between 83.3±5.8 % and 71.7±2.9% for Ext. 1, 81.7±2.9% and 68.3±7.6% for Ext. 2 and 81.7±2.9% and 63.3±2.9% for Ext. 3. After this period, the sperm motility of the semen diluted with Ext. 1 and 2 decreased slowly for 24 hours and reached 50.0±10.0% and 51.0±12.6 %, respectively. On the contrary, the motility rate in Ext. 3, dropped dramatically (P<0.05) between 6 to 48 h from 48.3±2.9% to 10.0±1.2%. The values after use of the first two diluents differed significantly (P<0.05) than the percentage for the third extender, from 6 h onwards. The motility for Ext. 2 at 36 h and 48 h (50.0±10.0% and 45.7±6.4%) was greater (P<0.05) than those (40±10% and 36.4±10.4%) for Ext 1.

The time of storage had no influence on the sperm viability from 0 to 6 h (Table 1). Considerable drop (P<0.05) was registered 12 hour after chilling for Ext. 3. At the same time, the sperm viability (70.7±1.2% and 64.3±4.0%) diluted with the first and the second extender was higher than that (46.0±5.3%) of the third extender. After 24 h, the percentage of live sperm cells was the highest for extender 2 (67.7±2.5%), followed by 59.3±2.1% for Ext. 1 and 36.7±4.2% for Ext. 3 (P<0.05).

The motility of chilled for 12 h semen did not change during the first 30 min after incubation of a water bath at 35^{0}C (Figure

2). The spermatozoa diluted with extender 3 had the lowest motility that decreased from 33.3±7.6% to 0 % until the 3rd hour after incubation. Similar tendency was determined for extender with SL 1.5 % for extender with SL 1.5 % from 1.5 h onward. The best results (≥50% motile sperm cells during the first 2 hours) were registered after dilution with SL-GL extender. Significant differences (P<0.05) were established between the motility for Ext. 1 and 2 versus Ext. 3 up to 2.5 h, and Ext. 1 versus Ext. 2 from 1.5 to 3 hour.

The results attributed to *in vivo* fertility test showed presence of a preserved fertilization capacity of spermatozoa for both extenders. On day 20 after artificial insemination, the pregnancy rate in the first and the second groups was 83.3% and 50%, respectively (Table 2). One case (16.7 %) of embryonic death was registered in the second group during the last ultrasonography. On Day 30 the percentage of pregnant goats in the first group (83.3 %) was significantly (P<0.05) greater than those in second group (33.3%).

Various experiments for storage of the goat semen at low temperature regimens have been reported until now (Maxwell and Salamon, 1993; Leboeuf *et al.,* 2000; Purdy, 2006). In most studies the effects of different diluents on sperm fertility have been evaluated *in vitro*, while the *in vivo* tests of their fertilization capacity are limited. This study demonstrates the positive influence of the TFC-based extender with SL and low concentration of glycerol on the motility, the viability and the fertility of chilled-stored goat semen.

The slow reduction of the sperm motility until the 3rd h after storage at temperature 0 - 4 ^{0} C and the insignificant differences between the percentages of live spermatozoa during the first 6 hours showed a good protective ability of all diluents. Similar motility (63.1+14.4%) and viability (75.2+12.9%) have been registered after dilution of buck semen with TFC- 3% SL extender and equilibration at 4^{0}C for 4 hours (Phutikanit *et al.,* 2011). Significantly (P<0.05) greater sperm motility and viability for extenders consisting of SL than those for extender with low glycerol only from the 6th h onward were evidence for positive effect of the used concentration of soybean lecithin. The lowest motility (33.3±7.6 %) obtained after semen dilution with Ext. 1 at 12 h, additionally supports the above mentioned assertion. This result was in agreement with previous reports for the protective properties of the soybean lecithin on chilled (Phutikanit *et al.,* 2011) or frozen (Vidal *et al.,* 2013) buck semen. Its anti-oxidative effect resulted in a decrease of the lipid peroxidation, removal of the free radicals and elimination of their adverse effects on the spermatozoa (Bucak *et al.,* 2008; Oke *et al.,* 2010).

The current data showed that TRIS-based extenders with SL or SL-GL could be provide acceptable sperm motility and enough live spermatozoa (≥50%) for 24 hours after storage of the semen at temperature 0-4^{0}C, where as extender including low glycerol only conducted to significant (P<0.05) decrease of the sperm motility and the viability. In contrast to the obtained result, Qureshi *et al.* (2013) reported that TFC extender with glycerol 1.4% protected spermatozoa motility for up to 192 h after storage in refrigerator. Regardless of that, a drastic reduction was observed during the first 24 h, as between 6 and 192 h the sperm motility decreased significantly from 56.43% to 10.71%. Probable reason for this discrepancy with the presented information could be individual peculiarity of the ejaculates collected from different donors. Different

freezability and fertility of the semen collected from different males have been recorded by Leboeuf *et al.* (2000) and they classified the bucks as "good" or "bad" freezers. Dorado *et al.* (2010) recommended identification of ejaculates as suitable or not suitable to be based on evaluation of fresh and post-thaw semen parameters.

The of sperm motility from 24 to 48 h and the percentage live spermatozoa at 24 h after storage in low temperature for Ext. 2 ($P<0.05$) were indictor that SL improves quality of the TFC-based extender with low concentration of glycerol. This hypothesis was supported by the significantly ($P<0.05$) better sperm motility of the semen diluted with Ext. 1 and 2 than with Ext. 3 during the time of storage in a water bath at 35^0 C.

The protective effect of the antioxidants on sperm plasma membrane has been detected in different studies (Oke *et al.*, 2010; Vidal *et al.*, 2013). According to Del Valle *et al.* (2012), however, the SL induced serious damage of the inner mitochondrial membrane in ram spermatozoa that became more evident along with thawing time. Therefore, they may negatively affect sperm motility and fertilizing capacity, but can not be detected by classic sperm quality analyses. The glycerol is a penetrating cryoprotectant used to freeze of spermatozoa, but in higher concentration it had chemical and osmotic toxicities on the spermatozoa membrane (Purdy, 2006; Garcia *et al.*, 2012). Zeng *et al.* (2014) concluded that lower concentrations of glycerol (2-3%) have the best anti-apoptotic effects in boar semen, but the exact cryoprotective or anti-freezing mechanism of glycerol remains unknown.

In our study, the low glycerol or soybean lecithin separately had no clear positive effect, but together they increased significantly ($P<0.05$) the motility and the viability of the buck spermatozoa. Besides, their combination ensured higher motility for a longer period ($P<0.05$) after incubation at temperature 35^0C, compared to those for extender with SL only. This data indicated synergistic effect of both protectors - SL1.5% and GL1.5%. Future investigations could clarify intimate mechanism for improvement of the motility, viability and fertility of buck sperm cells by simultaneously inclusion of these components in the TFC-based extender.

Numerous studies have demonstrated that the stored semen has good motility, but not always a high fertility (Windsor, 1997; Dorado *et al.*, 2010). If spermatozoa membranes undergo different damages during storage, they lose fertilization ability due to a problem during the capacitation and the acrosomal reaction (Maxwell and Watson, 1996). The fertility test showed that buck spermatozoa handled with TFC-based extender containing SL or SL-GL remained fertile at lest 12 h after chilling-storing procedures. The use of semen diluted with Ext. 1 and Ext. 2 until 1 and 2 hours after incubation, respectively, could be considered as important prerequisite for successful conception. After that the motility decline rapidly that could influence negatively on semen quality. This suggestion coincided with data reported by Phutikanit *et al.* (2011). Thus, the goat semen diluted with Ext. 1 and especially Ext. 2 could be transported or chilled and used in twice artificial insemination 8 or 12 h apart.

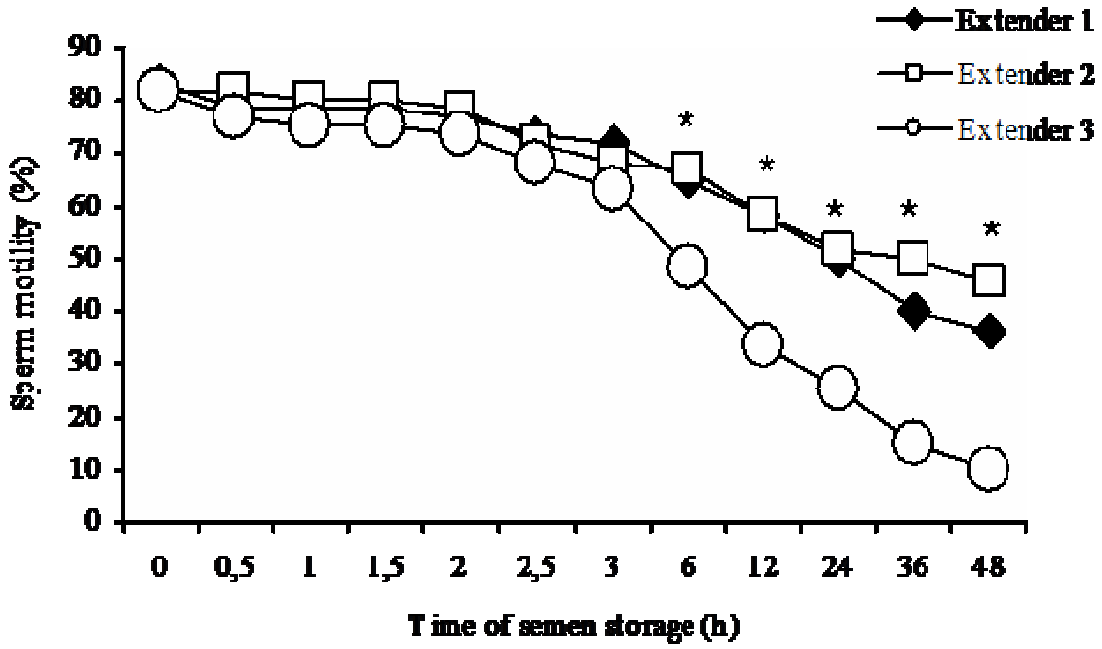
In conclusion, dilution with TFC-based extender containing 1.5% soybean lecithin and 1.5% glycerol provided the best motility, viability of chilled-stored spermatozoa and preserved their fertilization capacity.

Table.1 Sperm viability (%) of stored semen at 0-40 C for 24 hours

Time of storage (h)	Extender 1 n=9 Mean±SD	Extender 2 n=9 Mean±SD	Extender 3 n=9 Mean±SD
0	83.3 ± 3.9^{a}	85.2 ± 2.8^{a}	80.3 ± 6.7^{a}
3	78.7 ± 5.0^{a}	81.7 ± 2.1^{a}	77.7 ± 4.0^{a}
6	75.7 ± 2.5^{a}	78.7 ± 5.0^{a}	62.2 ± 2.0^{a}
12	64.3 ± 4.0^{Ab}	70.7 ± 1.2^{Aa}	46.0 ± 5.3^{Bb}
24	59.3 ± 2.1^{Ac}	67.7 ± 2.5^{Bb}	36.7 ± 4.2^{Cc}

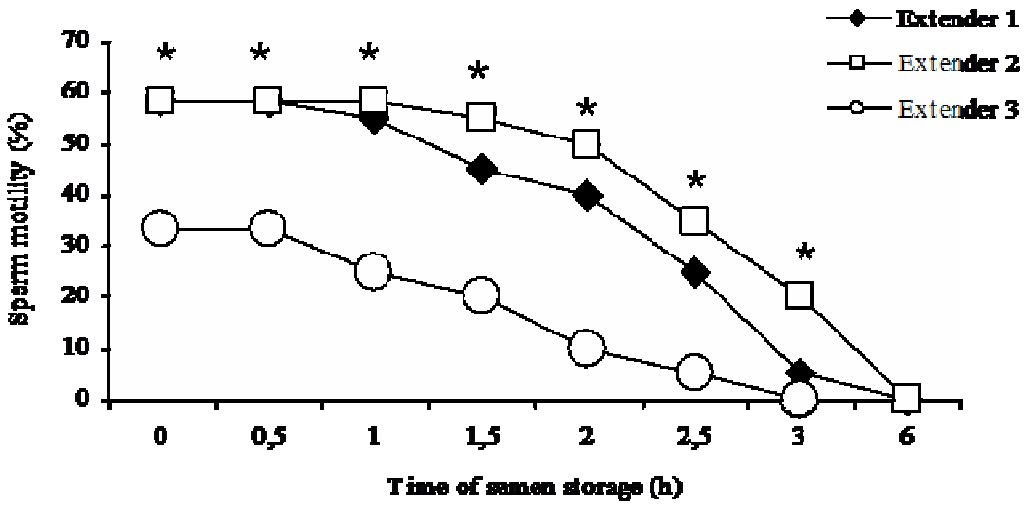
Different capital letters in the same row indicate significant differences P<0.05
Different lowercase letters in the same column indicate significant differences P<0.05

Figure.1 Sperm motility of diluted semen during storage at temperature 0-40C for 48 hours



Marked hours (*) indicate significant (P<0.05) differences among the extenders:
6 - 48 h Ext.1, 2 vs. Ext.3; 36 - 48 h Ext. 1 vs. Ext. 2

Figure.2 Sperm motility of chilled semen during incubation in a water bath at 35°C



Marked hours (*) indicate significant (P<0.05) differences among the extenders:
0 - 2.5 h Ext. 1, 2 vs. Ext. 3; 1.5 - 3 h Ext. 1 vs. Ext. 2

Acknowledgements

I would like to give special thanks to all people from Department of Obstetrics, Reproduction and Reproductive Disorders, FVM, Trakia University for their help and comments on the preparation of this manuscript.

References

Abdelhakeam, A.A., Graham, E.F., Vazquez, I.A. 1991. Studies on the presence and absence of glycerol in unfrozen and frozen ram semen: fertility trials and the effect of dilution methods on freezing ram semen in the absence of glycerol. *Cryobiology,* 28: 36-42.

Aitken, R.J., Gordon, E., Harkiss, D., Twigg, J.P., Milne, P., Jennings, Z., Irvine, D.S. 1998. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol. Reprod.,* 59: 1037-1046.

Ax, R.L., Dally, M. A., Lenz, R.W., Love, C.C., Varner, D.D., Hafez, B., Bellin, M.E. 2000. Semen evaluation. In: Hafez, B., Hafez, E.S.E. (Eds.), Reproduction in Farm Animals, 7th ed. Lippincott Williams and Wilkins, Philadelphia, pp. 365-375

Bittencourt, R.F., Ribeiro Filho, A.L., Chalhoub, M., Alves, S.G.G., Vasconcelos, M.F., Biscarde, C.E., Leal, L.S., Oba, E. 2008. Efeito de um quelantede cálcio, um detergente e da lecitina de soja sobre a qualidade do sêmen caprino congelado-descongelado. *Braz. J. Vet. Res. Anim. Sci.,* 45: 305-312.

Bousseau, S., Brillard, J.P., Guienne, B.M.L., Guérin, 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.

Bucak, M.N., Atessahin, A., Yüce, A. 2008. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Rum. Res.*, 75: 128-134.

Deka, B.B., Rao, A.R. 1986. Effect of extenders and thawing methods on post-thawing preservation of goat semen. *Ind. Vet. J.*, 6: 591-594.

Del Valle, I., Gomez-DurAn, A., Holt, W.V., Muino-Blanco, T., Cebrian-Perez. 2012. Soy lecithin interferes with mitochondrial function in frozen-thawed ram spermatozoa. *J. Androl.*, 33: 717-725.

Dorado, J., Munoz-Serrano A., Hidalgo, M. 2010. The effect of cryopreservation on goat semen characteristics related to sperm freezability. *Anim. Reprod. Sci.*, 121: 115-123.

Evans, G., Maxwell, W.M.C. 1987. Salamon's Artificial Insemination of Sheep and Goats. Butterworths, Sidney, pp. 107-141.

Farstad, W. 1996. Semen cryopreservation in dogs and foxes. *Anim. Reprod., Sci.*, 42: 251-260.

Futino, D., Mendes, M., Matos, W., Mondadori, R., Lucci, C. 2010. Glycerol, methylformamide and dimethylformamide in canine semen cryopreservation. *Reprod. Domest. Anim.*, 45, 214-220.

Garcia, B.M., Ortega F.S., Aparicio, I.M., Miro-Moran, A., Morillo Rodriguez, A., Gallardo Bolanos, J.M., Gonzalez Fernandez, L., Balao da Silva, C.M., Rodriguez Martinez, K., Tapia, J.A., Pena, F.J. 2012. Toxicity of glycerol for the stallion spermatozoa: effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential, *Theriogenology*, 77: 1280-1289.

Kulaksiz, R., Ari, U.Ç., Daşkin, A., Üner, A.G. 2013. The effect of different glycerol concentrations on freezability of semen from angora, kilis and saanen goats. *Slovak J. Anim. Sci.*, 46: 39-44.

Leboeuf, B., Restall, B., Salamon, S. 2000. Production and storage of goat semen for artificial insemination. *Anim. Reprod. Sci.*, 62: 113-141.

Leethongdee, S. 2010. Development of trans-cervical artificial insemination in sheep with special reference to anatomy of cervix. *Suranaree J. Sci. Technol.*, 17: 57-69.

Lopez-Sebastian, A., Gonzalez-Bulnes, A., Carrizosa, J.A., Urrutia, B., Dıaz-Delfa, C., Santiago-Moreno, J., Gomez-Brunet, A. 2007. New estrus synchronization and artificial insemination protocol for goats based on male exposure, progesterone and cloprostenol during the non-breeding season. *Theriogenology*, 68: 1081-1087.

Mara, L., Dattena, M., Pilichi, S., Sanna, D., Branca, A., Cappai, P. 2007. Effect of different diluents on sperm motility: Collection and goat semen fertility. Evaluation of semen. *Anim. Reprod. Sci.*, 102: 152-157.

Martemucci, G., D'Alessandro, A.G. 2011. Induction/synchronization of oestrus and ovulation in dairy goats with different short term treatments and fixed time intrauterine or exocervical insemination system. *Anim. Reprod. Sci.*, 126: 187-194.

Maxwell, W.M.C., Salamon, S. 1993. Liquid storage of ram semen: A review. *Reprod. Fertil. Dev.* 14, 83-89.

Maxwell, W.M.C., Watson, P.F., 1996. Recent progress in the preservation of ram semen. *Anim. Reprod. Sci.*, 42: 55-65.

Menchaca, A., Rubianes, E. 2007. Pregnancy rate obtained with short-term protocol for timed artificial insemination in goats. *Reprod. Domest. Anim.*, 42: 590-593.

Oke, M., Jacob, J.K., Paliyath, G. 2010. Effect of soybean lecithin in enhancing fruit juice/sauce quality. *Food Res. Int.*, 43: 232-240.

Ortega, A.M., Izquierdo, A.C., Gómez, J.J.H., Olivares-Corichi, I.M., Torres, V.M.M., Méndez, J.J.V. 2003. Peroxidación lipídica y antioxidantes enla preservación de semen. *Interciencia*, 28: 699-704.

Phutikanit, N., Sangkrachang, E., Suwimonteerabutr, J., Singlor, J. 2011. Effect of sources and concentrations of soybean phosphatidylcholine on diluted goat semen equilibrated at 4 ºC. *J. Agric. Sci. Tech.,* A 1: 1170-1173.

Purdy, P.H. 2006. A review on goat sperm cryopreservation. *Small Rum. Res.*, 63: 215-225.

Qureshi, M.S., Khan, D., Mushtaq, A., Afridi, S.S. 2013. Effect of extenders, postdilution intervals, and seasons on semen quality in dairy goats. Turk J Vet Anim Sci., 37, 147-152.

Ritar, A.J., Ball, P.D., O'May, P.J. 1990. Artificial insemination of Cashmere goats: effects on fertility and fecundity of intravaginal treatment, method and time of insemination, semen freezing process, number of motile spermatozoa and age of female. Reprod. Fertil. Dev., 2: 377-384.

Roca, J., Carrizosa, J.A., Campos, I., Lafuente, A., Vazquez, J.M., Martinez, E. 1997. Viability and fertility of unwashed Murciano-Granadina goat spermatozoa diluted in Tris-egg yolk extender and stored at 5 °C. *Small Rum. Res.*, 25: 2147-2153.

Salmani, H., Nabi, M.M., Vaseghi-Dodaran, H., Rahman, M.B., Mohammadi-Sangcheshmeh, A., Shakeri, M., Towhidi, A., Shahneh, A.Z., Zhandi, M. 2013. Effect of glutathione in soybean lecithin-based semen extender on goat semen quality after freeze-thawing. *Small Rum. Res*., 112: 123-127.

Sharafi, M., Eghbalsaied, S., Nili, N., Nasr-Esfahani, M.H. 2009. Ram semen in vitro fertility after cryopreservation using soybean lecithin and egg yolk based extenders. *Reprod. Domest. Anim.*, 44: 90-95.

Vidal, A.H., Batista, A.M., Bento da Silva, E.C., Gomes, W.A., Pelinca, M.A, Silva, S.V., Guerra, M.M.P. 2013. Soybean lecithin-based extender as an alternative for goat sperm cryopreservation. *Small Rum. Res.*, 109: 47-51.

Windsor, D.P. 1997. Mitochondrial function and ram sperm fertility. *Reprod. Fertil. Dev.*, 9: 279-284.

Zeng, C., Tang K., He, L., Peng, W., Ding, L., Fang, D., Zhang, Y. 2014. Effects of glycerol on apoptotic signaling pathways during boar spermatozoa cryopreservation. *Cryobiology*, 68: 395-404.