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Effect of seminal plasma and egg yolk concentration on freezability of goat semen

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ABSTRACT - The objective of this study was to evaluate the effects of egg yolk and seminal plasma on the viability of cryopreserved goat semen. To this end, four fertile Saanen bucks, aged between 10 months and 1 year, and weighing 18 to 25 kg, were used. Semen was collected from each buck by the artificial vagina method at the end of breeding season (June-July). The extender used was the yolk citrate, which was split into two equal aliquots: 5% egg yolk (2.5 mL egg yolk: 47.5 mL citrate solution) were added to one of the samples and 10% egg yolk (5.0 mL egg yolk: 45.0 mL citrate solution) were added to another. The sperm motility and vigor after thawing and post thermal resistance test (TRT) were evaluated and the data were subjected to analysis of variance and means were compared by the F test at 5.0% probability. The observed values for motility and vigor after thawing and post thermal resistance test (TRT), fast and slow, according to the presence of seminal plasma and egg yolk percentage were: 5% egg yolk with plasma (25.0% and 3.3; 1.60% and 0.7; 12.36% and 1.6, respectively); 5% egg yolk without plasma (23.61% and 3.1; 1.25% and 0.2; 9.93% and 1.3, respectively); 10% egg yolk with plasma (30.8% and 3.3; 4.4% and 1.9; 19.5% and 2.7, respectively); and 10% egg yolk without plasma (13.4% and 2.5; 4.1% and 0.5; 17.0% and 1.0, respectively). There were significant differences between the analyzed data in relation to semen with or without plasma at different percentages of egg yolk, and the group that presented the best results was 10% egg yolk citrate in extender with plasma. The presence of seminal plasma and higher concentration of egg yolk in extender provide a higher viability of cryopreserved goat semen.

Key Words: buck, centrifugation, extender

Introduction

Goat farming acts as an important tool in social development activities, mainly because of the easy adaptability and great importance of goats as a source of food to needy populations, especially in tropical countries. In this context, the intensification of reproductive management and genetic improvement are essential to the expansion of the activity in a competitive way, in which assisted-reproduction programs and the use of reproductive biotechnologies are tools to optimize this process (Bicudo et al., 2003).

Semen cryopreservation, when associated with artificial insemination, represents an efficient mechanism to the promotion and diffusion of genetic material. Because of this, many extenders have been used to cryopreserve goat semen, such as citrate-yolk and tris-yolk, and a larger amount of the substance promotes cell membrane stability against heat shock during the cooling and freezing processes (Watson, 1995; Bispo, 2009).

Goat semen has a particularity to be considered for its cryopreservation, and the interaction of seminal plasma with egg yolk can be detrimental to sperm of this species. This occurs because the seminal fluid has an enzyme secreted by the bulbourethral glands, which in the presence of the yolk forms lisolecitins through hydrolysis, becoming toxic to the sperm (Roy, 1957).

Thus, more research is needed to evaluate the interaction of the seminal plasma with the egg yolk in extender, because many studies determined that the plasma removal is beneficial regarding the use of the extender containing egg yolk (Ustuner et al., 2009). On the other hand, Cabrera et al. (2005) observed that the use of non-centrifuged goat semen in extender containing yolk promoted better results when compared with centrifuged semen. Therefore, the objective of this study was to evaluate the effect of seminal plasma and different percentages of egg yolk on *in vitro* viability of cryopreserved goat semen.

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Material and Methods

This study complies with the basic principles of research involving use of animals and is in agreement with the ethical principles and the animal welfare, in accordance with resolution 715 of 06/20/2002 from CFMV.

This experiment was carried out at the Department of Animal Evaluation and Reproduction, Institute of Animal Science at Universidade Federal do Rio de Janeiro, located in Seropedica, state of Rio de Janeiro, Brazil, from February to July 2012. The city is located at 22º45'53"S, 43º41'56"W and 33 m of altitude above sea level.

Four young Saanen bucks, aged between 10 months and 1 year, weighing on average 20 kg, were kept in individual pens, fed forage and concentrate twice daily and water *ad libitum*. The control of endoparasites was accomplished with Ripercol Solution® (Fort Dodge) at every fifteen days until achieving total parasite control.

The experimental design used in this experiment was completely randomized with a 4 × 2 × 2 (four animals × two egg yolk concentrations × presence or absence of seminal plasma) factorial arrangement.

The semen collections were carried out during a month, with two weekly collections for each of the animals by the artificial vagina method during handling. The collected samples were used to evaluate the seminal characteristics (volume, turbulence, motility and vigor) and subsequently discarded. Semen was collected between June and July, and two collections were made per week over three weeks until freezing. Before the collection, the prepuce area was sanitized externally with mild soap and water and internally with 0.9% warmed physiological solution to reduce possible semen contaminations. Goat semen collections occurred on the same days to avoid variations between ejaculates due to weather, feeding and management.

Six semen collections from each animal were performed. On each experimental day, collection was made from three of four goats used in this study, and on each day, one animal was not used (rotation). Right after collection, with an average interval of ten minutes between each ejaculate, the semen of each bull was taken to the laboratory and kept in a water bath at 37 °C, and volume was determined in the same collection graduated tube.

Evaluations of motility, vigor and sperm concentration were conducted with an optical microscope. The gross motility was evaluated by depositing a drop of pure semen (20 μL) in a glass slide warmed at 37 °C under an optical microscope with 4x magnification, where the samples received a score that ranged from 0 to 5. Motility (0 to 100%) and vigor (0 to 5) were evaluated by depositing a drop of diluted semen on the citrate-yolk extender warmed at 37 °C between slide and coverslip, also warmed at 37 °C, and the sample was evaluated under an optical microscope with 40x magnification.

After each semen analysis, a "pool" of the ejaculates was made, in which the motility and vigor were re-evaluated, and sperm concentration was re-calculated. To determine the concentration, a drop of semen diluted in water at the ratio of 1:9 (10 μL of semen in 90 μL of water) was placed in a Neubauer chamber, in which the count of sperm cells was performed. After that, the number of doses was calculated, considering an insemination dose with 40 million spermatozoa.

After seminal analysis, the pool composed of the semen of each animal was divided into four samples containing 0.250 mL each. Two pure-semen samples were centrifuged for 15 min at 800 g rotation speed to separate the seminal plasma, in which the supernatant was discarded and the pellet was resuspended in citrate yolk extender (1.47 g sodium citrate, 0.03 g penicillin and 50 mL distilled water). Resuspended samples were split into two equal aliquots; 5% of yolk (2.5 mL yolk:47.5 mL citrate solution with penicillin) were added to one of the samples and 10% of yolk (5.0 mL yolk:45.0 mL citrate solution with penicillin) were added to another. Each semen sample (with and without seminal plasma) was split into two equal volumes, to which the extender with the different percentages of yolk was added.

After dilution, the semen was cryopreserved by the conventional method (refrigerator). This method consists of using two fractions (fraction A and fraction B). Fraction A is composed of extender and semen, containing different concentrations of yolk, prepared at room temperature and kept in a refrigerator at 5 °C. Fraction B is composed of the same extender used in the fraction A, with 12% of glycerol, also kept in the refrigerator.

After 01h30min in the refrigerator at 5 °C, fraction B was added to fraction A in three equal parts at intervals of five minutes each to avoid the osmotic shock of semen with extender containing glycerol. After four hours in the refrigerator, straws were loaded with the part containing two fractions (fraction A + fraction B). This loading was performed in 0.5 mL identified, closed straws with polyvinyl alcohol in the refrigerator, with the aid of a syringe used to add the extender in the straws, in which they were exposed to liquid nitrogen vapor at approximately −120 °C for 20 min. After this period, straws were immersed in liquid nitrogen (−196 °C) and stored in cryopreservation canister. Five straws of pool (with and without seminal plasma) were frozen for each match and for each yolk percentage, amounting to 360 samples at the end of experiment.

Forty-eight hours after semen cryopreservation, three straws from each repetition and each animal were thawed in a water bath at 37 °C for 30 s to evaluate motility and vigor post-thawing. After thawing, the samples were subjected to fast and slow thermal resistance tests (TRTF and TRTS). To this end, the content of the straw, after thawing, was kept for 30 min and 2 h in a water bath at 46 ºC and 37 °C in TRTF and TRTS, respectively.

Data obtained from experiments were subjected to analyses of variance and the means were compared by the F test, with P<0.05. Analyses were performed using the SAS (version 9.0) software.

Results and Discussion

The volume, motility and sperm vigor of the fresh semen from each animal were evaluated (Tables 1 and 2), and these evaluations were repeated for the obtained pool, with sperm concentration evaluated only in the ejaculate pool. Average volume, motility, and sperm vigor were 0.57, 79.8%±5.1 and 3.6±0.62, respectively. These values for motility and vigor were similar to those found by Bispo (2005), which were 82.33%±9.79 and 3.93±0.41, respectively. The observed values are in accordance with the recommended standards for goat semen (CBRA, 1998).

The mean values obtained for motility and vigor are above the standard required for fresh goat semen preconized by CBRA (1998), which is 70% and 3.0, respectively. There were significant differences (P<0.05) for motility and vigor among animals. This difference can be explained by individual particularities of each animal, due to age difference among goats, and thus more developed reproductive systems, with the handling of all animals in the experiment being exactly the same.

Table 1 - Means and standard deviation for volume, motility, and vigor of fresh semen from each Saanen buck used in the experiment

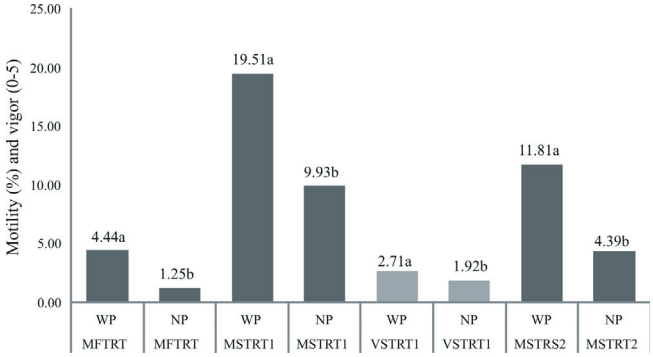
Goat	Volume (mL)	Motility (%)	Vigor (0-5)
1	0.7±0.08a	83.7±4.8a	4.1±0.6a
2	0.6±0.0a	80.0±0.0b	3.5±0.7b
3	0.7±0.2a	78.3±7.5b	3.4±0.7b
4	0.3±0.2b	77.5±8.2b	3.4±0.5b

Means followed by different letters in the same column differ by the F test (P<0.05).

The average sperm concentration obtained in Saanen bucks is about 3.0 × 10^6 sperm/mL, ranging between 1.0 and 5.0 × 10^6 sperm/mL (Castelo et al., 2008). Therefore, the results are in agreement with the observed standards for this species.

There was significant a difference (P<0.05) for motility after fast TRT (MTRTF), motility after slow TRT 1 h (MTRTS1), vigor after slow TRT 1 h (VTRTS1) and motility after slow TRT 2 h (MTRTS2) regarding semen with and without seminal plasma (Figure 1). These results showed that the sperm with seminal plasma provided a better goat semen quality, regardless of the amount of egg yolk in the yolk citrate extender, which is in agreement with Azerêdo et al. (2001), Gil et al. (2002) and Viana et al. (2006), who reported deleterious effects to sperm after centrifugation for plasma removal and subsequent addition of the extender containing egg yolk. On the other hand, results from this study are not in agreement with those observed by Memon et al. (1985), Ritar and Salamon (1982) and Sariozkan et al. (2010), who reported beneficial effects of centrifugation and seminal plasma removal on the quality of goat semen.

The positive results for the semen with the seminal plasma can be explained by the fact that this experiment occurred at the end of the breeding season, when levels of phospholipase A are in lower amounts in seminal plasma when compared with semen collected off the breeding season. According to Nunes (1982) and Brito (2008), during



WP - with plasma; NP - no plasma; MFTRT - motility after fast thermal resistance test; MSTRT - motility after slow thermal resistance test; VSTRT - vigor after slow thermal resistance test.

Figure 1 - Effect of seminal plasma on pool of cryopreserved semen from four Saanen bucks on the MTRTF, MTRTS1, VTRTS1 and MTRTS2 traits.

Table 2 - Means for motility (%) and sperm concentration of the pool of young ejaculates from Saanen bucks

	Collection days					
	1	2	3	4	5	6
Motility (%)	70	70	70	85	80	80
Concentration (sperm/mL)	3.620 × 10^6	2.740 × 10^6	3.060 × 10^6	3.250 × 10^6	4.910 × 10^6	4.070 × 10^6

the non-breeding season, the bulbourethral glands increase their activity by high plasma concentrations of prolactin and produce more phospholipase A.

The age of breeding may also have influenced the results in this study because young bucks (10 months to 1 year) were used. Young animals are still in the development of the reproductive system with lower production of phospholipase A when compared with adult animals, which already have the well-developed bulbourethral glands.

There was significant a difference ($P<0.05$) for motility after fast TRT (MTRTF), motility after slow TRT 1 h (MTTRS1) and motility after slow TRT 2 h (MTRTS2) regarding semen with citrate extender containing 5% and 10% egg yolk (Figure 2). These results show that the extender containing 10% egg yolk provided better results, regardless of the presence of seminal plasma. There was no significant difference ($P>0.05$) between the samples with different percentages of yolk for vigor after slow TRT 1 h (VTRTS1).

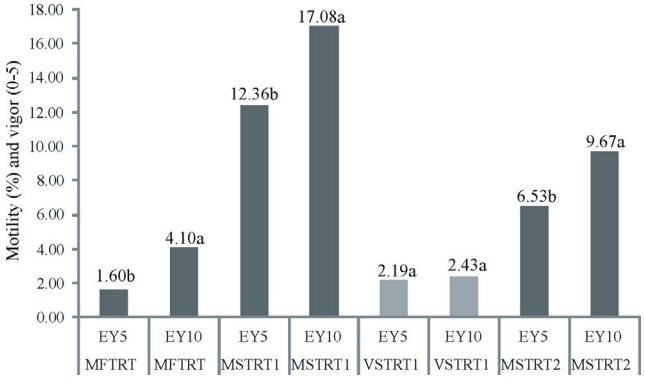
According to Ferrari and Barnabe (1999), the semen samples frozen in tris yolk extender with 2.6% egg yolk showed higher results for progressive motility (31.6%) after the thermal resistance test when compared with the skim-milk extender, which was not confirmed in this experiment because the extender that has a higher percentage of yolk (10%) provided better results in fast and slow TRT (1 h and 2 h). This difference may be because the extender used in the study of these authors (tris extender) was different from that used in the present study (citrate extender), and citrate extender with 10% egg yolk can provide higher protection for sperm cryopreservation to increase the osmotic pressure, resulting in cell dehydration with consequent reduction of intracellular ice formation. Memon et al. (1985) reported decreased survival of cryopreserved semen in extender containing 11% egg yolk.

Daskin and Tekin (1996) observed that thawed goat semen showed higher motility in an extender with 20% egg yolk in relation to extender without egg yolk during the breeding season. Compared with the extender with different percentages of yolk, it is observed that a higher amount of yolk in citrate extender may have provided a better protection in cryopreservation by the presence of phospholipids, which act on the cell surface by repairing the loss of phospholipids that occurs during heat shock in order to avoid disruption of the cell membrane (Bispo, 2005).

Regarding motility after thawing (Figure 3), there was significant difference ($P<0.05$) when comparing the semen with and without plasma in citrate yolk extender containing 5% and 10% egg yolk. The extender containing 10% egg yolk provides better results in the presence of seminal plasma on the centrifuged samples. However, extender with 5% egg yolk provided better results for motility when compared with semen with 10% egg yolk in extender without seminal plasma.

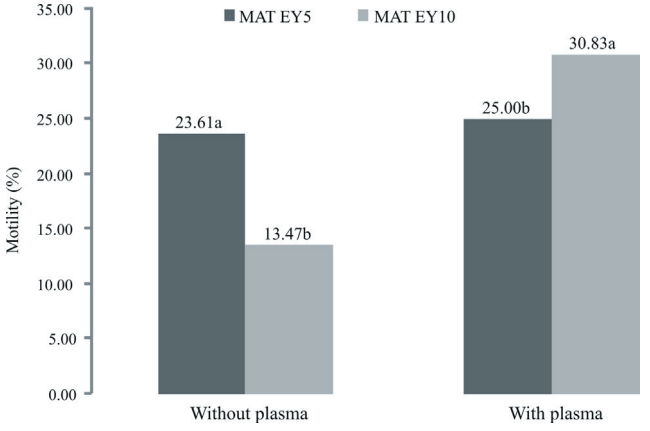
The best result for motility after thawing may have occurred because the seminal plasma with higher concentration of egg yolk provides the protection to sperm on the cryopreservation process by stabilizing the membrane and avoiding its disruption during freezing.

The results obtained in this study for post-thaw motility were lower than those obtained by Siqueira et al. (2009). These authors used extender with low concentration of egg yolk in semen cooled for 24 h and observed 62.50% for motility. However, it was observed that the motility assessed



EY - egg yolk; MFTRT - motility after fast thermal resistance test; MSTRT - motility after slow thermal resistance test; VSTRT - vigor after slow thermal resistance test.

Figure 2 - Effect of egg yolk percentage in pool of semen from four Saanen bucks in citrate yolk extender with 5% and 10% egg yolk on the MFTRT, MSTRT1, VSTRT1 and MSTRT2 traits.



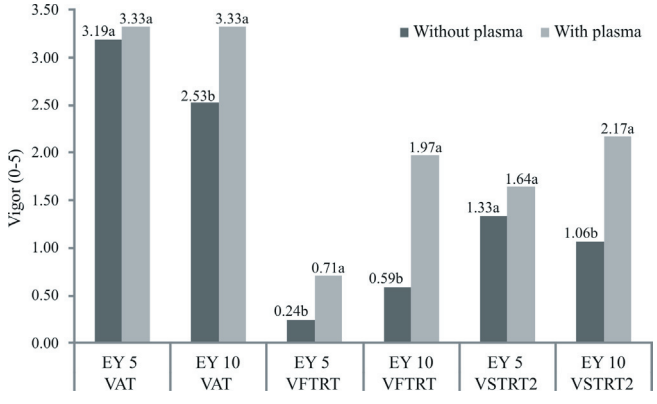
EY - egg yolk; MAT - motility after thawing.

Figure 3 - Motility after thawing by semen from four Saanen bucks with and without plasma with different percentages of egg yolk (5% to 10%).

in the study of these authors is related to the cooled semen, using a method different from that of the present study, which causes less damage to the sperm compared with the frozen semen. The same was reported by Siqueira (2006), who observed 62.5% for motility in cooled semen when using unwashed semen and tris extender with 2.5% egg yolk. In the cryopreservation process, sperm cells undergo crioinjuries, which can provide decreased sperm motility after thawing.

Regarding vigor after fast TRT (VTRTF), vigor after slow TRT (VTRTS) and vigor after thawing (VAT) after two hours (Figure 4), there was no significant difference (P>0.05) between semen with or without plasma in extender containing 5% egg yolk. However, these differences occurred in relation to semen with and without plasma in extender with 10% egg yolk, such that the extender with 10% egg yolk provided better results when compared with the extender containing 5% yolk. The proposed hypothesis is that the higher concentration of yolk with the seminal plasma provided higher protection to the sperm cell in cryopreservation process, which is not in agreement with some authors (Ustuner et al., 2009), who obserbed that semen in tris extender containing 18% egg yolk with plasma provided lower motility (16.7%) when compared with tris extender containing 6% (35.7%) and 12% (47.7%) egg yolk.

Dias (2010) evaluated different extenders for cryopreservation of goat semen without centrifugation and observed that tris extender containing lower concentrations of yolk in relation to the current study (2.5%) provided better results for vigor after slow TRT (1.0±0.2) when compared with other extenders used, such as ringer lactate (0.2±0.1)



EY - egg yolk; VAT - vigor after thawing; VFTRT - vigor after fast thermal resistance test; VSTRT - vigor after slow thermal resistance test.

Figure 4 - Vigor after thawing (VAT), vigor after fast TRT (VTRTF) and vigor after slow TRT 2 h (VTRTS2) by semen with and without plasma in two percentages of egg yolk (5% and 10%) from Saanen bucks.

and sodium citrate 2.92% (0.1±0.1). However, these results differ from the present study because the averages for vigor after slow TRT were higher than those obtained by Dias (2010) (1.64 with 5% egg yolk and 2.17 with 10% egg yolk). Nunes et al. (1982) observed positive results for the presence of seminal plasma on sperm survival subjected to freezing, supported by Azerêdo et al. (2001), who observed that the removal of seminal plasma decreased motility and vigor of frozen semen goat. According to Corteel et al. (1980), there are differences between collections of semen during season, even with or without the favorable effect of ejaculate centrifugation, since the cells withstand the cryopreservation process better during breeding season, as in this study, in which the samples were collected at the end of the reproductive period (between June and July).

The removal of the seminal plasma may be deleterious to sperm by decreasing the presence of antioxidants in plasma. The lack of these substances may promote the action of reactive oxygen species, which decrease sperm motility, viability and sperm DNA integrity.

Despite the difference observed between the different treatments, the quality of non-centrifuged semen in citrate extender with 10% egg yolk remained in the recommended standards for use in artificial insemination programs. However, fertility tests can be made to evaluate the efficiency of semen subjected to the cryopreservation process.

Conclusions

Higher concentrations of yolk extender (10%) provide better viability to goat semen cryopreserved at −196 °C. Removal of seminal plasma does not contribute to the freezing of goat semen in citrate extender with 10% egg yolk. Cryopreservation of semen from young Saanen bucks (10 months to 1 year) provides better results in the breeding season, in which the release of phospholipase A by bulbourethral glands is decreased.

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References

Azerêdo, G. A.; Esper, C. R. and Resende, K. T. 2001. Evaluation of plasma membrane integrity of frozen thawed goat spermatozoa with or without seminal plasma. Small Ruminant Research 41:257-263.

Bicudo, S. D.; Souza, D. B. and Takada, L. 2003. Possibilidades e limitações da inseminação com sêmen ovino refrigerado e biotécnicas associadas como estratégias de intensificação do manejo reprodutivo. In: Anais do 15º Congresso Brasileiro de Reprodução Animal, Porto Seguro. Colégio Brasileiro de Reprodução Animal, Belo Horizonte.

Bispo, C. A. S. 2005. Avaliação "in vitro" do sêmen caprino resfriado a 5°C em função de curvas de resfriamento e diluidores. Dissertação (M.Sc.). Universidade Federal de Viçosa, Viçosa, MG.

Bispo, C. A. S. 2009. Fertilidade do sêmen caprino resfriado ou congelado em diferentes concentrações de gema de ovo no diluente. Tese (D.Sc.). Universidade Federal de Viçosa, Viçosa, MG.

Brito, B. G. M. 2008. Viabilidade do sêmen caprino conservado em três diferentes diluidores: Efeito da concentração inicial de frutose no plasma seminal. Dissertação (M.Sc.). Universidade Federal do Ceará, Fortaleza.

Cabrera, F.; González, F.; Batista, M.; Calero, P.; Medrano, A. and Gracia, A. 2005. The effect of removal seminal plasma, egg yolk level and season on sperm freezability of Canary buck (*Capra hircus*). Reproduction in Domestic Animals 40:191-195.

Castelo, T. S.; Frota, T. R. and Silva, A. R. 2008. Considerações sobre a criopreservação do sêmen de caprinos. Acta Veterinária Brasílica 2:67-75.

CBRA - Colégio Brasileiro de Reprodução Animal. 1998. Manual para exame andrológico e avaliação de sêmen animal. 2.ed. CBRA, Belo Horizonte.

Corteel, J. M.; Baril, G. and Leboeuf, B. 1980. Residual seasonal variations in fertility in selected deep-frozen ejaculates of European dairy male goats. p.422-425. In: Proceedings of the International Congress on Animal Reproduction and Artificial Insemination. v.5. ICAR, Madrid.

Daskin, A. and Tekin, N. 1996. The effect of egg-yolk on the quality of frozen Angora buck semen. Turkey Journal Veterinary Animal Science 20:395-398.

Dias, J. C. O. 2010. Adição de Ringer Lactato, citrato de sódio 2,92% e solução Tris em sêmen caprino. Dissertação (M.Sc.). Universidade Federal de Viçosa, Viçosa, MG.

Ferrari, S. and Barnabe, V. H. 1999. Efeito de dois tipos de diluidores e dois métodos de congelação na qualidade do sêmen criopreservado de caprinos. Brazilian Journal Veterinary Research and Animal Science 36:199-204.

Gil, J.; Rodriguez-Irazoqui, M.; Soderquist, L. and Martinez, H. R. 2002. Influence of centrifugation or low extension rates pre freezing on the fertility of sêmen after cervical insemination. Theriogenology 57:1781-1792.

Memon, M. A.; Bretzlaff, K. N. and Ott, R. S. 1985. Effect of washing on motility and acrosome morphology of frozen-thawed goat spermatozoa. American Journal of Veterinary Research 46:473-475.

Nunes, J. F.; Corteel, J. M.; Combarnous, Y. and Baril, G. 1982. Role du plasma seminal dans la survie in vitro des spermatozoids de bouc. Reproduction, Nutrition, Development 22:611-620.

Ritar, A. J. and Salamon, S. 1982. Effects of seminal plasma and of its removal and of egg yolk in the dilluent on the survival of fresh and frozen-thawed spermatozoa of the Angora goat. Australian Journal of Biological Science 35:305-312.

Roy, A. 1957. Egg yolk coagulating enzyme in the semen and Cowper's gland of the goat. Nature 179:318-319.

Sariözkan, S.; Bucak, M. N.; Tuncer, P. B.; Taşdemir, U.; Kinet, H. and Ulutaş, P. A. 2010. Effects of different extenders and centrifugation/washing on post thaw microscopic-oxidative stress parameters and fertilizing ability of Angora buck sperm. Theriogenology 73:316-323.

Siqueira, A. P. 2006. Inseminação artificial em caprinos com sêmen resfriado. Dissertação (M.Sc.). Escola de Veterinária/Universidade Federal de Minas Gerais, Belo Horizonte.

Siqueira, A. P.; Fonseca, J. F.; Silva Filho, J. M.; Bruschi, J. H.; Viana, J. H. M.; Palhares, M. S.; Bruschi, M. C. M. and Peixoto, M. P. 2009. Parâmetros reprodutivos de cabras Toggenburg inseminadas com sêmen resfriado, após diluição em meio à base de gema de ovo. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 61:299-305.

Ustuner, B.; Gunay, U. and Nur, A. 2009. Effect of seminal plasma, egg yolk, and season on the freezability of Saanen buck semen. Bull Veterinary Institute Pulawy 53:369-374.

Viana, A. K. S.; Chalhoub, M.; Ribeiro Filho, A. L.; Almeida, A. K.; Portela, A. P. M.; Bittencourt, R. F.; Alves, S. G. G.; Bittencourt, T. C. C. and Quintela, A. T. 2006. Avaliação *in vitro* do sêmen caprino resfriado, com ou sem centrifugação do plasma seminal e diluído em leite desnatado-glicose e tris-gema de ovo. Ciência Animal Brasileira 7:67-76.

Watson, P. F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reproduction, Fertility and Development 7:871-891.