IN VITRO EVALUATION OF GOAT CAUDA EPIDIDYMAL SPERM, COOLED IN DIFFERENT EXTENDERS AT 4 ºC

AVALIAÇÃO *IN VITRO* DE ESPERMATOZOIDES DA CAUDA DO EPIDÍDIMO DE CAPRINOS, RESFRIADO A 4 ºC EM DIFERENTES DILUIDORES

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ADDITIONAL KEYWORDS

Coconut water. Diluent.

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Água de coco. Diluidor.

SUMMARY

Collection of spermatozoa obtained from the epididymis cauda (CES) is a viable option to preserve of genetic material from threatened species and for use in assisted reproduction. The aim of the present study was to assess the *in vitro* effect of four different extenders on spermatozoa from goat epididymis cauda, cooled at 4 ºC. Epididymal sperm were recovered from the cauda by flushing six pairs of epididymis. The samples of each pair were mixed, subdivided into four aliquots and diluted in coconut water-egg yolk (CW-EY), physiologic solution with 0.5 % glucose and egg yolk (PSG-EY), UHT skim milk (SM), and UHT skim milk with egg yolk (SM-EY). The samples were cooled at 4 ºC for 2, 12, 24 and 48 hours. After storage, in each period, the semen was evaluated by the heat resistance test for sperm motility, vigor and total morphological alterations. The motility degradation rate was calculated at the end of each time period. Sperm viability decreased with time in the four extenders. Epididymal sperm diluted in CW-EY showed higher values for vigor and motility, although these parameters did not differ from the sperm kept in the PSG-EY up to 48 hours at 4 °C, except for spermatic vigor. However, the PSG-EY and SM extenders caused greater morphologic damage to epididymal sperm after 12 and 24 hours, respectively. Extenders based on skim milk provided less spermatic cell stability during 48 hours. In conclusion, CW-EY extender was the most efficient extender to maintain CES viability at 4 ºC.

RESUMO

A colheita de espermatozóides da cauda do epidídimo (CES) é uma opção viável de preservação de material genético de espécies ameaçadas de extinção, bem como para uso em reprodução assistida. Este trabalho teve como objetivo verificar a eficiência de quatro diferentes diluidores sobre os espermatozóides da cauda do epidídimo de caprinos, resfriado a 4 ºC. Os espermatozoides epididimários foram recuperados da cauda de seis pares de epidídimo. As amostras de cada par foram misturadas e subdivididas em quatro alíquotas que foram diluídas na água de coco-gema (CW-EY), solução fisiológica glicosada 0,5 %-gema (PSG), leite desnatado UHT (ME) e leite desnatado UHT-gema (ME-EY). As amostras foram resfriadas a 4 °C por 2, 12, 24 e 48 horas. Após o armazenamento, em cada período, o CES foi avaliado através do teste de termoresistência quanto à motilidade, vigor espermático e alterações morfológicas total. A taxa de degradação da motilidade foi calculada no final de cada período. A viabilidade espermática diminuiu com o tempo de refrigeração nos quatro diluidores. Os CES diluídos na CW-EY apresentaram maiores valores para vigor e motilidade, embora estes parâmetros não deferissem daqueles mantidos no PSG por até 48 horas, exceto para vigor espermático. Entretanto, os diluidores PSG e ME ocasionaram maiores danos morfológicos aos CES a partir de 12 e 48 horas, respectivamente. Os diluidores ME e ME-EY proporcionaram à célula espermática menor estabilidade durante 48 h de conservação.

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Concluiu-se que o CW-EY foi o mais eficiente em manter a viabilidade dos CES a 4 ºC.

INTRODUCTION

Obtaining caudal epididymal sperm is an important technique in the propagation and conservation of animal specimens with high genetic values after serious injury or from dead animals (Dong *et al*., 2008), endangered species (Santiago-Moreno *et al.,* 2006) and pets (Leibo and Songsasen, 2002). In addition, if the epididymal sperm are obtained immediately after death, the gamete remains alive for 24 to 48 hours and is viable for fertilization (Dong *et al*., 2008). In this context, assisted reproductive techniques, such as artificial insemination (Santiago-Moreno *et al*., 2006), *in vitro* fertilization (Blash *et al*., 2000) and intracytoplasmic sperm injection (Jimenez-Macedo *et al*., 2005) have been reported in the literature as biotechnology that has given satisfactory results.

At ejaculation, ruminant seminal plasma contains factors that positively or negatively influence the fertilizing capacity of the spermatozoa (Yamashiro *et al*., 2006). An example of this negative interaction was observed in goat semen dilution, when the cryosurvival in extenders contained egg yolk (Roy, 1957) or milk (Nunes, 1982). The phospholipases A2 (PLA2) are enzymes present in seminal plasma that hydrolyze egg yolk lecithin in fatty acids and lysolecithin (Roy, 1957; Iritani *et al.,* 1964), and react with the phosphocaseinate fraction of skimmed milk-based extenders (Pellicer-Rubio *et al*., 1997), producing compounds toxic to the spermatozoa.

Therefore, optimizing cryopreservation extenders is a fundamental issue for properly performing germplasm banking of wild species. However, there are few comparative studies about the effect of extenders routinely used in ejaculated spermatozoa cryopreservation on caudal epididymal sperm viability, and there are no domain protocols for cryopreservation in the goat species, mainly because the use of coconut water extender, which is poor in phospholipids, has simplified the technology of storing liquid goat semen at 5 °C (Nunes, 1998). Therefore, the objective of the present study was to test the effect of different extenders on goat cauda epididymal sperm when cooled at 4 ºC for 48 h.

MATERIAL AND METHODS

Epididymal samples were collected from six crossbred male goats, average 22.4 ± 5.9 months of age, weighing 36.2 ± 6.9 kg and with 24.7 ± 1.3 cm scrotal circumference. These animals are characterized by their small size and high adaptability in tropical environments (Catunda *et al*., 2011). All the animals were adults and kept in individual stalls in a ventilated building and were fed uniformly according to the NRC (1981).

The pair of epididymis (n=6) were obtained by surgical castration. In brief, the animals were sedated with 2 % xylazine hydrochloride (Rompum®, Bayer), then 2 % lidocaine hydrochloride was applied (Anesthetic L Pearson, Eurofarma) in the spermatic cord and the scrotum skin to induce local anesthesia. Subsequently, the testicles and epididymis were removed, refrigerated to 5 ºC and taken to the laboratory. The epididymis were dissected and separated from the testes. The sperm were recovered from the cauda by flushing, as recommended by Martinez-Pastor *et al*. (2006) with retrograde washing of the *vas deferens* and epididymis cauda. A syringe was used loaded with 4 mL of the saline solution cannulated to the *vas deferens* using a blunted 21G needle. The cauda was perfused with the saline solution and air afterwards, until all the contents were flushed out of the epididymis cauda. An average of 0.2 mL cauda epididymal sperm (CES) content was recovered. After the retrograde washing of the pair of epididymis, a mix (*pool*) was made from the recovered solution of each animal.

The sample was then centrifuged in a Sigma refrigerated centrifuge (model 4K15) at 34170 g/20 min/+4 °C to remove the saline solution and epididymal fluid. The supernatant was carefully removed and the *pellet* re-suspended in four different extenders to reach 200 $\times 10^6$ spermatozoa/mL. The sperm concentration of the *pool* was determined in a hemacytometer using diluted semen samples (1: 400; Evans and Maxwell, 1987).

Different extenders were used: (1) Coconut water egg yolk extender or CW-EY (50mL coconut water -green variety and average age of six months, 50 mL 2.5 % sodium citrate solution and 2.5 % egg yolk (v/v) in 100 mL distilled water q.s.p.), prepared according to Nunes (1998); (2) Physiologic solution with 0.5 % glucose and egg yolk (v/v) or PSEY; (3) Ultra-heat-treated long-life, UHT ME (PARMALAT S/A) and (4) Milk-based extender or ME-EY consisting of long-life ultra-heat-treated milk with 2.5 % egg yolk (v/v). After preparation, the extenders were incubated at 5 °C until use, and were heated to 38 °C for use at the time of the experiment.

After dilution, a 250 µL sample from each suspension (CES + extenders) was incubated at 38 °C in a water bath to evaluate the heat resistance test for vigor (scale from 0 to 5), sperm motility (0-100 %) and total morphological alterations at 5, 60 and 120 minutes, using optical microscopy (Cortell, 1981; CBRA, 1998), which corresponded to T0 fresh semen. The remainder of the diluted semen was collected in a closed glass tube and incubated in a beaker at ambient temperature. It was cooled gradually to 4 ºC and stored in a refrigerator for up to 48 h to evaluate the caudal epididymal sperm characteristics at 2, 12, 24 and 48 hours after storage. At the end of each time the motility degradation rate (MDR) was calculated by the following formula (Campos *et al.*, 2004):

$$MDR = \frac{\text{Vigor (5 min)} - \text{Vigor (120 min)}}{\text{Vigor (5 min)}} \times 100$$

The percentage of abnormal sperm (PAS) was determined after staining cells with bromophenol blue (Medeiros *et al.,* 2006), counting 200 cells/treatment (Colas, 1980) in one sample from each treatment and storage time (T0, T2, T12, T24 and T48) after 120 min incubation.

The statistical program SAS @ was used to analyze the data. Percentage data underwent angular transformation (arc sine square root percentage transformation) before analysis. Analysis of variance was performed using randomized block designs to evaluate the effect of different extenders on sperm characteristics (sperm motility, vigor, MDR and PAS). The means of these variables across treatments and storage times were compared using the t Student statistical test, with 5 % probability of error.

RESULTS

Tables I and **II** show patterns of cauda epididymal sperm characteristics in the four different extenders at 4 ºC for 48 hours conservation. No significant difference ($p>0.05$) was observed regarding spermatic vigor during 48 hours storage using semen diluted in CW-EY and ME-EY. However, a significantly decreased ($p<0.05$) vigor was observed in the PSG-EY and ME extenders during/after 24 h storage **(table I)**. The sperm motility decreased significantly ($p<0.05$) 24 h after storage in the PSG-EY, ME and ME-EY extenders and after 48 h storage in CW-EY extender **(table I)**.

Significant increase ($p<0.05$) in the sperm cell morphology from cauda epididymis was observed after 12 hours cooling in the PSG-EY extender and 48 hours in the ME extender. However, significant alterations were not found ($p>0.05$) in the sperm cell morphology from cauda epididymis when preserved in CW-EY and ME-EY over time **(table II)**. The motility degradation rate increased significantly ($p<0.05$) in the PSG-EY and ME extenders after 12 and 48 hours of cooling, respectively.

Table I. *Effect of extender type and storage time (St) on the spermatic vigor and motility of goat cauda epididymal sperm.* (Efeito do tipo de diluidor e do tempo de armazenamento sobre o vigor e motilidade espermática de espermatozoides epididimário da cauda do epidídimo de caprinos).

St	Vigor				Motility			
(h)	CW-EY	PSG-EY	ME	ME-EY	CW-EY	PSG-EY	ME	ME-EY
0	2.67 ± 0.38^{Aab}	2.86 ± 0.29^{Aa}	2.22 ± 0.40^{Ac}	2.31 ± 0.49^{Abc}	66.11 ± 6.80^{Aab}	70.00 ± 7.30^{Aa}	58.89 ± 6.55^{Ab}	62.50 ± 6.30^{Aab}
2	2.75 ± 0.47^{Aa}	2.56 ± 0.51A^{Bab}	2.03 ± 0.29^{Abc}	2.25 ± 0.48^{Abc}	66,11 ± 6.80^{Aa}	62.22 ± 11.29^{ABab}	57.78 ± 6.56^{ABb}	61.67 ± 6.91^{Aab}
12	2.75 ± 0.31^{Aa}	2.50 ± 0.21A^{Bab}	1.92 ± 0.14^{ABc}	2.14 ± 0.19^{Abc}	65.00 ± 6.23^{Aa}	63.34 ± 7.60^{ABCab}	53.06 ± 6.69^{ABCc}	57.78 ± 8.07^{ABabc}
24	2.64 ± 0.27^{Aa}	2.36 ± 0.16B^{Cab}	1.78 ± 0.17^{Bc}	2.00 ± 0.38^{Abc}	60.28 ± 6.19A^{Ba}	55.00 ± 7.53^{BDab}	50.56 ± 4.91^{BCb}	52.22 ± 6.21^{Bab}
48	2.53 ± 0.36^{Aa}	2.08 ± 0.29^{Cb}	1.72 ± 0.42^{Bb}	1.95 ± 0.25^{Ab}	54.72 ± 3.71^{Ba}	48.34 ± 6.91^{Da}	48.33 ± 10.90^{Ca}	51.67 ± 3.49^{Ba}

CW-EY= coconut water-egg yolk; PSG-EY= physiologic solution with 0.5 % glucose-egg yolk; ME= milk extender; ME-EY= milk extender-egg yolk. Uppercase letters: comparison between storage time (p<0.05); Lowercase letters: comparison between extenders (p<0.05).

Table II. *Effect of extender type and storage time (St) on the motility degradation rate and cell morphology of goat cauda epididymal sperm.* (Efeito do tipo de diluidor e do tempo de armazenamento sobre a taxa de degradação da motilidade e percentagem de espermatozoides anormais de espermatozoides epididimário da cauda do epidídimo de caprinos).

St	Motility degradation rate				Percentage of abnormal sperm			
(h)	CW-EY	PSG-EY	ME	ME-EY	CW-EY	PSG-EY	ME	ME-EY
0	22.15 ± 10.12^{Aa}	30.16 ± 2.46^{Aa}	35.28 ± 19.45^{Ba}	40.32 ± 13.64^{Aa}	35.60 ± 20.22^{Aa}	18.33 ± 10.48^{Ba}	21.17 ± 9.47^{Ba}	22.70 ± 11.99^{Aa}
2	25.47 ± 17.66^{Aa}	33.30 ± 5.2A^{Ba}	42.50 ± 14.44^{ABa}	43.65 ± 11.79^{Aa}	35.83 ± 10.52^{Aa}	33.25 ± 16.98A^{Bba}	26.13 ± 20.54^{ABa}	22.33 ± 13.49^{Aa}
12	16.03 ± 9.31^{Aa}	28.33 ± 7.81^{Ba}	35.83 ± 21.08^{Ba}	51.11 ± 10.68^{Aa}	34.83 ± 18.22^{Aa}	36.80 ± 20.18^{Aa}	22.88 ± 14.75^{Ba}	25.33 ± 14.44^{Aa}
24	15.00 ± 7.53^{Aa}	22.20 ± 5.4^{Ba}	40.00 ± 13.78^{ABa}	38.06 ± 15.14^{Aa}	38.08 ± 12.69^{Aa}	38.92 ± 12.31^{Aa}	34.90 ± 11.34^{ABa}	28.33 ± 10.55^{Aa}
48	6.27 ± 9.81^{Aa}	22.5 ± 16.05^{Ba}	44.17 ± 29.23^{Aa}	41.67 ± 13.29^{Aa}	43.92 ± 18,28^{Aa}	48.83 ± 12.04^{Aa}	42.25 ± 15.47^{Aa}	35.75 ± 11.28^{Aa}

CW-EY= coconut water-egg yolk; PSG-EY= physiologic solution with 0.5 % glucose-egg yolk; ME= milk extender; ME-EY= milk extender-egg yolk. Uppercase letters: comparison between storage time (p<0.05); Lowercase letters: comparison between extenders (p<0.05).

There were differences between extenders for spermatic vigor and motility **(table I)**, but no significant difference (p>0.05) was detected between extenders in terms of percentage of abnormal sperm and motility degradation rate **(table II)**. Spermatic vigor was significantly higher (p<0.05) in semen diluted with CW-EY over the conservation time, after 2 and 48 hours of conservation when compared to the ME, ME-EY and PSG-EY extenders, respectively **(table I)**. Differences were also detected between PSG-EY and ME up tol 24 hours of conservation, while higher values were observed in the PSG-EY extender. Regarding sperm motility, the PSG-EY and CW-EY extenders provided the best results compared to semen diluted with ME up to 12 and 24 hours of storage **(table I)**.

DISCUSSION

Spermatic cell processing using adequate extenders is one of the key points for successful assisted reproduction (Azawi *et al*., 1993; Mara *et al*., 2007). In the present study, the cauda epididymal sperm characteristics were compared in four such extenders during storage for 48 hours at 4 °C.

The effect of cooling at 4 °C on storage time was verified on all the characteristics from cauda epididymal sperm (sperm motility, vigor, morphology and motility degradation rate). The reduction in characteristics observed in the present experiment during storage at 4 °C may have been due to a sum of factors, such as the partially irreversible damage to the epididymal sperm membranes that results in the loss of vital cell constituents, and consequently reduced sperm metabolic rate, motility and spermatozoa survival time in the female reproductive tract (Blackshaw and Salisbury, 1957; Holt, 2000; De Pauw *et al*., 2003); and reduced extracellular pH, which can cause a gradual decline in cell metabolic activity (Corteel, 1974; Vishwanath and Shannon, 2000) because there is a build-up of lactic acid produced by bacterial growth and their own aerobic metabolic activity (Miki *et al*., 2004), and heat shock due to temperature changes to which the sperm are subjected (Azawi *et al*., 1993; Varisli *et al*., 2009).

As expected, there were differences among extenders regarding conservation time for spermatic vigor and motility. In ejaculated sperm from different species, the effect of different extenders at 4 ºC on sperm characteristics is well known (Azawi *et al*., 1993; Mara *et al*., 2007). In these studies, the differences have been related to the extender composition. The present results clearly demonstrate that coconut water based extender affords a more adequate medium to sustain the viability of epididymal sperm storage at 4 °C, because of it could maintain the sperm characteristics for a period of up to 48 hours better than other extenders (**table I** and **II**). These best results can be explained by coconut water composition. Coconut water and egg yolk are usual components of semen extenders for goats (Campos *et al*., 2003; 2004). Coconut water is poor in phospholipids and rich in complex organic molecules, such as proline, glycine, glutamic acid and indole-acetic acid (IAA) which protect and extend the spermatozoa life span, based on cell membrane protection that reinforces its molecular structure (Nunes and Combarnous, 1995). Egg yolk is well known for its protective action on sperm membranes against heat shock during storage at 5 ºC (Watson and Martin, 1975; Leeuw *et al*., 1993), which is due to the low-density lipoprotein fraction (Moussa *et al*., 2002).

In addition, it was observed that IAA present in coconut water had a beneficial effect on boar sperm acrosomal integrity , but it is assumed that other molecules present in coconut water also play a protective role (Toniolli *et al.,* 1996). Azevêdo and Toniolli (1999) also reported that sperm motility and vigor increased with the addition of indole-3-acetic acid to skim milk extender. Several studies have

demonstrated that coconut water after correcting osmolality and pH is effective in the maintenance of in vitro and *in vivo* spermatic cell characteristics (Nunes, 1998). Therefore, we believe that the best results observed with the CW-EY extender can be attributed the characteristics mentioned above, as well as the presence of sodium citrate that exerts a buffering power preventing rapid changes in the medium pH (Holt, 2000). Recently, Barros and Toniolli (2011) reviewed the potential effects of coconut water on semen technology and reported that its utilization as an extender is important because it is an easily prepared and cheap alternative for semen transport over small distances and for use in artificial insemination programs in several species.

Initially, it was reported that isotonic sodium chloride was the first extender used to dilute semen from domestic animals to evaluate sperm metabolic activity (Salisbury and Nakabayashi, 1957) and as a constituent to evaluate sperm concentration (Evans and Maxwell, 1987). The present study shows that PSG-EY extender can be used to refrigerate cauda epididymal spermatozoa, since similar results in terms of MDR and PAS tests were observed compared to CW-EY. However, more studies should be conducted to improve the viability of the PSG-EY extender over conservation time, because of a significant decrease ($p<0.05$) in the sperm motility and vigor, sperm cell morphology and motility degradation rate during storage (**tables I** and **II**). Some properties should be taken into consideration when elaborating an extender, such as membrane protective capacity against possible damage by heat shock and mechanical injuries during sperm transportation. The extender should also provide nutrients and stabilize the medium pH and osmotic pressure (Leboeuf *et al*., 2000; Verstegen *et al*., 2005).

The extenders based on skim milk did not satisfactorily preserve cauda epididymal spermatozoa quality. Although the skim milk and egg yolk extender has similar patterns in terms of spermatic vigor, abnormal sperm percentage and motility degradation rate during 48 hours of storage in CW-EY extender, the spermatic motility of the spermatozoa in this extender decreased significantly ($p<0.05$) 24 h after storage. In addition, the values of the characteristics analyzed were significantly lower ($p<0.05$) compared to sperm cryopreserved in coconut water and PSG-EY. Previous studies with sperm cells from the ejaculate have warned about the short life span of goat sperm stored with skim milk (Corteel, 1974; Pellicer-Rubio *et al*., 1997). Viana *et al*. (2006) worked with different extenders and found a significant decrease ($p<0.05$) in sperm motility and vigor, as well as increase in the acrosomal damage and number of abnormal sperm after 3 and 24 hours conservation, respectively, in semen stored in skim milk-glucose. However, our results indicated that epididymal sperm viability can be maintained for up to 24 hours of storage (**tables I** and **II**).

Campos *et al*. (2003 and 2004) found that storage at 4 ºC reduced sperm motility and vigor after 24 hours of conservation when diluted in coconut and milk-egg yolk. In this study, we verified that only the sperm motility was altered from 48 hours of storage when preserved in coconut water-egg yolk extender (**table I**). In fact, the ejaculated sperm in ruminants is particularly susceptible to heat shock because of their large amount of unsaturated phospholipids, low cholesterol:phospholipid ratios (Darin-Bennett and White, 1977) and higher membrane permeability (Scott *et al*., 1967).

We did not observe significant difference ($p>0.05$) in the percentage of abnormal sperm and motility degradation rate between extenders in any conservation time. These results differed from those reported by Pereira-Rocha (2012) who observed interaction between extender and conservation time for this characteristic. The motility degradation rate increased significantly ($p<0.05$) only in the ME extender after 48

hours of cooling from 35.3 ± 19.4 to 44.2 ± 29.2, this result was associated with a greater decrease in the spermatozoa vigor in this extender. These results were similar to those obtained by Azevêdo and Toniolli (1999) who showed that after 48 hours of storage there was an increase in MDR in goat semen when diluted in skim milk. Thus, these findings indicated that extenders based on skim milk are not indicated for conservation of goat cauda epididymal sperm.

CONCLUSION

The extenders based on coconut water - egg yolk with sodium citrate 2.5 % were efficient for maintaining sperm cells traits from cauda epididymis at 4 ºC during 48 h.

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REFERENCES

Azawi, O.I.; Al-Dahash, S.Y.A. and Juma, F.T. 1993. Effect of different diluents on Shami goat semen. *Small Ruminant Res*, 9: 347-352.

Azevêdo, D.M.M.R. e Toniolli, R. 1999. Água de coco estabilizada suplementada com antibióticos e ácido 3-indol acético na conservação de sêmen de caprinos Marota. *Cienc Anim*, 9: 37-42.

Barros, T.B. e Toniolli, R. 2011. Uso potencial da água de coco na tecnologia de sêmen. *Rev Bras Reprod Anim*, 35: 400-407.

Blackshaw, A.W. and Salisbury, G.W. 1957. Factors influencing metabolic activity of bull spermatozoa. II. Cold-shock and its prevention. *J Dairy Sci,* 40: 1099-1106.

Blash, S.; Melican, D. and Gavin, W. 2000. Cryopreservation of epididymal sperm obtained at necropsy from goats. *Theriogenology*, 54: 899-905.

Campos, A.C.N.; Nunes, J.F.; Monteiro, A.W.U.; Pinheiro, J.H.T.; Ferreira, M.A.L.; Araújo, A.A. e Cruz, J.F. 2003. Conservação do sêmen caprino a 4º C durante o período seco e chuvoso no Nordeste do Brasil. *Rev Bras Reprod Anim*, 27: 620-624.

Campos, A.C.N.; Nunes, J.F.; Monteiro, A.W.U.; Figueirêdo, E.L.; Pinheiro, J.H.T.; Ferreira, M.A.L. e Araújo, A.A. 2004. Viabilidade do sêmen caprino lavado e não lavado diluído em água de coco e armazenado a 4 ºC. *Rev Bras Ciênc Vet,* 11: 178-182.

Catunda, A.G.V.; Aguiar, G.V.; Lima, I.C.S.; Pereira, J.F.; Pitombeira, R.S.S.; Andrade, I.R.A.; Martins, G.A.; Souza, C.E.A.; Nunes, J.F.; Moura, A.A.N. and Campos, A.C.N. 2011. Characterization of ejaculates from crossbreed male goats raised in tropical environment. *Rev Port Ciên Vet*, 110: 59-67.

CBRA. 1998. Colégio Brasileiro de Reprodução Animal. Manual para exame andrológico e avaliação de sêmen animal. 2ª ed. CBRA. Belo Horizonte. 49 pp.

Colas, G. 1980. Variations saisonnieres de la qualité du sperme chez le belier Ile-de-France I. Etude de la morphologie cellulaire et de la motilité massale. *Reprod Nutr Dev*, 20: 1789-1799.

Corteel, J.M. 1974. Viabilité des spermatozoides de bouc conservés el congelés avec ou sans leur plasma sèminal. Effect du glucose. *Ann Biol Anim Biochem Biophys*, 14: 741-745.

Corteel, J.M. 1981. Collection, processing and artificial insemination of goat semen. In: C. Gall (Ed.). Goat Production. Academic Press. London. pp. 171-191.

Darin-Bennett, A. and White, I. 1977. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold shock. *Cryobiology*, 14: 466-470.

De Pauw, I.M.C.; Van Soom, A.; Mintiens, K.; Verberckmoes, S. and Kruif, A. 2003. *In vitro* survival of bovine spermatozoa stored at room temperature under epididymal conditions. *Theriogenology*, 59: 1093-1107.

Dong, Q.; Rodenburg, S.E.; Huang, C. and Vandevoort, C.A. 2008. Cryopreservation of rhesus monkey (*Macaca mulatta*) epididymal spermatozoa before and after refrigerated storage. *J Androl,* 29: 283-292.

Evans, G. and Maxwell, W.M.C. 1987. Salamon's artificial insemination of sheep and goats.

Butterworth Publishers. Sydney. 194 pp.
Holt, W.V. 2000. Basic aspects of frozen storage of semen. *Anim Reprod Sci*, 62: 3-22.
Iritani, A.; Nishikawa, Y. and Nagasawa, S. 1964. Studies on the egg-yolk coagulating enzyme in goat semen. VII. Variations in the enzyme activity of the semen between breeding season and non-breeding season, and in each ejaculate collected three times successively. *Jpn J Anim Reprod*, 10: 52-56.
Jimenez-Macedo, A.R.; Izquierdo, D.; Anguita, B. and Paramio, M.T. 2005. Comparison between intracytoplasmic sperm injection and *in vitro* fertilization employing oocytes derived from prepubertal goats. *Theriogenology*, 64: 1249-1262.
Leboeuf, B.; Restall, B. and Salamon, S. 2000. Production and storage of goat semen for artificial insemination. *Anim Reprod Sci*, 62: 113-141.
Leeuw, F.E.; Leeuw, A.M.; Den Daas, J.H.G.; Colenbrander, B. and Verkleij, A.J. 1993. Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. *Cryobiology*, 30: 32-44.
Leibo, S.P. and Songsasen, N. 2002. Cryopreservation of gametes and embryos of non-domestic species. *Theriogenology*, 57: 303-326.
Mara, L.; Dattena, M.; Pilichi, S.; Sanna, D.; Branca, A. and Cappa, P. 2007. Effect of different diluents on goat semen fertility. *Anim Reprod Sci,* 102: 152-157.
Martinez-Pastor, F.; Garcia-Macias, V.; Alvarez, M.; Chamorro, C.; Herraez, P.; Paz, P. and Anel, L. 2006. Comparison of two methods for obtaining spermatozoa from the cauda epididymis of Iberian red deer. *Theriogenology*, 65: 471-485.
Medeiros, A.A.; Araújo, A.A.; Moura, A.A.A.; Cavalcante, J.M.M.; Figueiredo, E.L. e Rodrigues, L.F.S. 2006. Utilização do azul de bromofenol conservado a 4 ºC e 29 ºC, como método coloração vital para avaliação do espermatozóide ovino. *Rev Ciênc Agrár*, 46: 287-297.
Miki, K.; Qu, W.; Goulding, E.H.; Willis, W.D.; Bunch, D.O.; Strader, L.F.; Perreault, S.D.; Eddy, E.M. and O'brien, D.A. 2004. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm specific glycolytic enzyme is required for sperm motility and male fertility. *Proc Natl Acad Sci*, 101: 16501-16506.
Moussa, M.; Martinet, V.; Tainturier, D. and Anton, M. 2002. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective affect on frozen-thawed bull semen. *Theriogenology,* 57: 1695-1706.
NRC. 1981. National Requirement Council. Nutrient requirements of goats: Angora, dairy, and meat goats in temperate and tropical countries. In: D.C. Washington (Ed.). National Academy Press. 87 pp.
Nunes, J.F. 1982. Étude des effets du plasma seminal sur la survie *in vitro* des spermatozoïdes de bouc. Tese (Doutorado). Université Paris VI. França.
Nunes, J.F. 1998. Utilização da água de coco como diluidor do sêmen de animais domésticos e do homem. *Rev Bras Reprod Anim*, 22: 109-112.
Nunes, J.F. e Combarnous, Y. 1995. Utilização da água de coco e de suas frações ativas como diluidor de sêmen de mamíferos domésticos. *Cienc Anim*, 2: 15-21.
Pellicer-Rubio, M.T.; Magallon, T. and Combarnous, Y. 1997. Deterioration of goat sperm viability in milk extenders is due to a bulbourethral 60-kilodalton glicoprotein with triglyceride lipase activity. *Biol Reprod*, 27: 1023-1031.
Pereira-Rocha, J.F. 2012. Estudo da composição bioquímico do plasma seminal de caprinos e do seu efeito sobre os espermatozoides epididimarios e ejaculados. Dissertação (Master Thesis). Universidade Federal do Ceará. Fortaleza. Brasil.
Roy, A. 1957. Egg yolk coagulating enzyme in the semen and Cowper's gland of the goat. *Nature,* 179: 318-319.
Salisbury, G.W. and Nakabayashi, N.T. 1957. Effect of phosphate and chloride ions on aerobic metabolism of bovine spermatozoa. *J Exp Biol*, 34: 52-59.
Santiago-Moreno, J.; Toledano-Díaz, A.; Pulido-Pastor, A.; Dorado, J.; Gómez-Brunet, A. and López-Sebastián, A. 2006. Effect of egg yolk concentration on cryopreserving Spanish ibex (*Capra pyrenaica*) epididymal spermatozoa. *Theriogenology*, 66: 1219-1226.
Scott, T.W.; Voglmayr, J.K. and Setchell, B.P. 1967. Lipid composition and metabolism in testicular and ejaculated ram spermatozoa.

Biochem J, 102: 456-461.

Toniolli, R.; Bussière, J.; Courot, M.; Magistrini, M. and Combarnous, Y. 1996. Effect of indole-3-acetic acid (plant auxin) on the preservation at 15 ºC of boar semen for artificial insemination. *Reprod Nutr Dev*, 36: 503-511.

Varisli, O.; Uguz, C.; Agca, C. and Agca, Y. 2009. Motility and acrosomal integrity comparisons between electro-ejaculated and epididymal ram sperm after exposure to a range of anisosmotic solutions, cryoprotective agents and low temperatures. *Anim Reprod Sci*, 110: 256-268.

Verstegen, J.P.; Onclina, K. and Iguer-Ouadab, M. 2005. Long-term motility and fertility conservation of chilled canine semen using egg yolk added tris-glucose extender: *in vitro* and *in vivo* studies. *Theriogenology*, 64: 720-733.

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. e 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. *Cienc Anim Bras*, 7: 67-76.

Vishwanath, R. and Shannon, P. 2000. Storage of bovine semen in liquid and frozen state. *Anim Reprod Sci*, 62: 23-53.

Watson, P.F. and Martin, I.C. 1975. Influence of some fractions of egg yolk on the survival of ram spermatozoa at 5 ºC. *Aust J Biol Sci*, 28: 145-152.

Yamashiro, H.; Kumamoto, K.; Wang, H.; Yamashita, Y. and Terada, T. 2006. Effect of semen collection in extender solution on the characteristics of goat spermatozoa. *J Reprod Dev*, 52: 397-406.