DOI: 10.5216/cab.v12i1.4692

EFFECT OF MINIMUM CONTAMINATION (MC) AND MODIFIED EGG YOLK LACTOSE (MLEY) EXTENDERS ON CANINE SEMEN VIABILITY AFTER COOLING AT 4ºC FOR 24 HOURS IN EQUITAINER®

BIANCA PEREZ BARCELLOS[1], MARCO ANTÔNIO ROCHA[2], LUCIANO JAMAS[3], FELIPE ZANDONADI BRANDÃO[4], LUIS GARCIA NOGUEIRA[5], TANIA GOES DE PINHO[6]

[1] Post-graduated by Universidade Federal Fluminense (UFF)
[2] Military Police of Rio de Janeiro state
[3]Adjunct Professors of the Departament of Pathology and Veterinary Clinic, Reproductive Physiology (UFF)

ABSTRACT

The aim of this study was to compare the viability of canine semen after dilution in Minimum Contamination (MC) and Modified Lactose-Egg Yolk (MLEY) extenders and incubation at 4ºC for 12 and 24 hours. Thirteen ejaculates were collected from five adult dogs by digital manipulation. Macroscopic and microscopic characteristics were assessed right after collection. Semen was divided to be diluted (1:3) in MC and MLEY and subsequently chilled in Equitainer®. Seminal parameters of motility and spermatic morphology, membrane integrity (hyposmotic test), spermatic viability and spontaneous acrosome reaction (Trypan-blue Giemsa stain) were evaluated in fresh and chilled semen after 12 and 24 hours of incubation at 4ºC. No difference between extenders was identified in semen conservation after 12 hours. After 24 hours just spermatic motility was different ($p>0.05$). But, after both periods of conservation, semen diluted in LGM maintained most of the characteristics verified in the fresh semen. The mean of true and false acrosome reaction did not exceed 2% in both semen extenders, which demonstrates no influence of media component and incubation period on these phenomena at 4ºC. In conclusion, these results indicate that both extenders can be used in canine semen conservation at 4ºC in container during 12 hours without significant changes in semen characteristics.

KEYWORDS: dogs; sperm; cooling.

EFEITO DOS MEIOS DILUÍDORES MMC (MÍNIMA CONTAMINAÇÃO) E LGM (LACTOSE-GEMA MODIFICADO) NA VIABILIDADE DO SÊMEN DE CÃES CONSERVADO POR 24 HORAS EM EQUITAINER®

RESUMO

Objetivou-se com este estudo comparar a viabilidade do sêmen de cães diluído nos meios Mínima Contaminação (MMC) e Lactose-gema Modificado (LGM), conservado a 4ºC por 12 e 24 horas. Foram utilizados treze ejaculados de cinco cães adultos, obtidos através de manipulação digital. Avaliaram-se volume do ejaculado, concentração espermática, motilidade progressiva e vigor espermático no sêmen fresco imediatamente após a coleta. A seguir o sêmen foi diluído (1:3) em MMC e LGM e posteriormente refrigerado em Equitainer®. Motilidade progressiva e vigor espermáticos, patologia espermática, integridade da membrana plasmática, viabilidade espermática e integridade acrossômica foram avaliados no sêmen fresco e após 12 e 24 horas de refrigeração a 4ºC. Não houve

diferença entre os meios na conservação do sêmen após doze horas de refrigeração. Decorridas 24 horas, apenas a motilidade progressiva foi diferente entre os tratamentos (p>0,05). Porém, observou-se que o meio LGM manteve, após esses períodos, a maioria das características do sêmen fresco (p>0,05). As taxas médias de reação acrossômica verdadeira e falsa não ultrapassaram 2% em ambos os meios, o que demonstra que a 4ºC os componentes dos meios diluidores e a conservação não afetaram a integridade acrossômica. Concluiu-se que ambos os meios podem ser utilizados na conservação sob refrigeração (4ºC em contêiner de transporte) do sêmen de cães por um período de até doze horas, sem que ocorram mudanças significativas nas características verificadas no sêmen fresco.

PALAVRAS-CHAVE: Refrigeração; espermatozóides; caninos.

INTRODUCTION

The use of cooled semen in artificial insemination (AI) in canine species has increased exponentially. Cooling process aims at conserving semen for a longer period, making its transportation viable. During the cooling process, sperm cells metabolism decreases in order to reduce both energy losses and the formation of toxic metabolites in storing and transportation (AMANN & PICKETT, 1987; HOLT, 2000). Semen cooling is a complex process that encompasses structural changes in sperm cell, which can cause irreversible damages to the spermatozoon. Changes in plasmatic membrane fluidity and in calcium influx in the cell occur, leading to the break of plasmatic and acrosomal membranes. Such changes are known as heat shock (TARTAGLIONE & RITTA, 2004).

The success of semen conservation under cooling depends on several factors, such as the use of an appropriate extender, whose components protect the spermatozoon against the heat shock and do not allow the occurrence of early sperm capacitation, slow cooling and temperature maintenance rates, minimizing damages to the membrane and to the sperm cell metabolism (AMANN & GRAHAN,1993). The most commonly used extenders in semen conservation process are those that present macromolecules in milk and/or egg yolk, which protect cellular membrane (HOLT, 2000).

Egg yolk contains lectin, a biological-origin component more often used in the composition of semen extenders (ENGLAND, 1993). It seems that its protective activity against damaging effects of cell cooling comes from the stabilizing activity of the sperm membrane by its phospholipid components, which are responsible for restoring phospholipid cell loss and preventing membrane breakings (ROTA *et al*., 1995; HOLT, 2000). PHILLIPS & LARDY (1993) observed that the lipoproteins present in egg yolk promote spermatozoon protection against the heat shock caused by the cooling process (MAXWEEL & SALOMON, 1993).

A low density lipoprotein (LDL) was previously isolated and identified as an effective component to preserve and protect the spermatozoon. According to several authors, there is better motility when a medium with higher amounts of LDL is used (MOUSSA *et al*., 2002; AMIRAT *et al*., 2004).

Milk is also often used due to its buffering capacity and to the protective activity against heat shock by lactose and its proteins. Skim milk-glucose-based extender used in equine semen cooling has been being used in canine semen cooling as well (ENGLAND & PONZIO, 1996).

Minimum Contamination (MC), Tris-yolk and Modified Lactose-Egg Yolk (MLEY) extenders have been tested and used in dog´s semen cooling (IVANOVA-KICHEVA *et al*., 1997; CUNHA & LOPES, 2000; SILVA *et al*., 2001). IVANOVA-KICHEVA *et al*. (1997) reported a slight superiority of MLEY for conserving the studied semen characteristics.

Different tests have been used in order to assess the quality of conserved semen; however, it is agreeded that no *in vitro* test can predict the exact fertilization potential of fresh or conserved semen (ARRUDA *et al*., 2003). Seminal parameters, such as progressive motility, sperm vigor, sperm morphology, plasmatic membrane integrity and acrosomal membrane integrity have been indicated for the evaluation of domestic animals´semen (CUNHA *et al*., 2005). Progressive motility and sperm vigor reflect indirectly the fertilizing capacity of sperm (VANNUCCHI *et al*., 1998). Plasmatic and

acrosomal membrane integrity, verified by the hypoosmotic test and by staining for the evaluation of sperm capacitation by acrosome reaction, is also used as a parameter to measure the oocyte-penetrating capacity of the spermatozoa (JEYENDRAN *et al*., 1984). Vital staining methods are used to assess the percentage of live spermatozoa in semen (DIDION *et al*., 1989).

Therefore, the purpose of this study was to verify sperm viability after dilution in extenders (MC and MLEY) commonly used for dogs´semen conservation, after 12 and 24 hours of cooling (4 ºC). Progressive motility, vigor, percentage of live cells and plasmatic and acrosomal membrane integrity were compared.

MATERIAL AND METHODS

Thirteen ejaculates (three collections in three dogs and two collections in two dogs) from five adult, healthy German Shepard and Beagel dogs were used. The animals weighed between 15 and 40 kg and came from the military police kennel. They were fed commercial ration (Champ®) twice a day and water at will. After andrological examination and drain on sperm reserves, semen was collected from each animal every seven days, during spring, by manual manipulation. Only the second sperm fraction and part of the third sperm fraction (from one to two first sperm gushes) were used.

The ejaculate was immediately evaluated regarding volume, sperm concentration, progressive motility, sperm vigor, sperm pathology, plasmatic membrane integrity, sperm viability and acrosomal membrane integrity, being kept in bain-marie at 37ºC until the end of the dilution procedure. Fresh semen minimal parameters for posterior dilution were 70% progressive motility and vigor 3.

Ejaculates were divided into three equal parts and slowly diluted (1:3 dilution rate) in Minimal Contamination (KENNEY *et al*., 1975) and Lactose-Egg Yolk (SILVA FILHO *et al*., 1997) extenders. After dilution, samples were stored in 1.5-mL plastic tubes.

Container for commercial transportation (Equitainer II®), whose temperature curve decreases from 37ºC to 5°C in ten hours (average decrease of 0.3°C) was used for semen cooling and maintenance at 4ºC for 24 hours.

Progressive motility, sperm vigor, sperm pathology, plasmatic membrane integrity, sperm viability and acrosomal membrane integrity were evaluated again in cooled semen after 12 and 24 hours. After semen re-evaluation, semen aliquots were pre-heated

Performed tests

Sperm concentration was determined by using a Neubauer hemocytometer chamber in optical microscope with 400x augmentation; sperm samples were used in 1:20 dilution.

Progressive motility and sperm vigor were subjectively evaluated in a common optical microscopy, being motility determined as the percentage of spermatozoa in rectlineal progressive movement, and vigor was classified in a scale ranging from zero to five, according to the intensity of the observed movement (HENRY & NEVES,1998). Sperm pathology was evaluated by means of wet preparation usind phase contrast microscope at 1000x augmentation, in immersion, in 200 cells. Percentage of major, minor and total defects were determined (BLOM, 1973).

Plamatic membrane integrity was determined by the hyposmotic test (KUMI-DIAKA, 1993). A 50 µL semen aliquot was diluted in 450 µL of hyposmotic solution 150 mosmol/L (1:10 dilution rate), previously heated at 37ºC, being then incubated in bain-marie for 30 minutes at 37ºC. After incubation, one drop of the sample was placed between the slide and the cover slip, heated at 37°C, and it was evaluated by optical microscopy with 400x augmentation for 100 cells counting. The result was expressed in percentage of intact cells (which presented tail folding due to osmolarity changes).

The evaluation of sperm viability and acrosome integrity was carried out by means of Trypan-blue-Giemsa double staining cytochemistry (DIDION *et al*., 1989). A 100 µL semen aliquot was diluted in 100 µL Trypan blue 0.2%, previously heated at 37ºC.

Sample was incubated for 30 minutes in bain-marie at 37ºC. After incubation, one drop of semen was placed on each slide previously heated at 37ºC on a heater board, to make the air-dried thin smears. These slides were incubated in Giemsa 10%

with distilled water pH 6.8 – 6.9, for 24 hours. The smears were washed in running water and air-dried. The reading of the smears was carried out by 100 cells counting in optical microscope with 400x augmentation. This technique enabled the differentiation of four classes of spermatozoa: dead spermatozoon with intact acrosome – blue head and dark pink acrosome; dead acrosome-reacted spermatozoon (false acrosome reaction) – blue or purple head and discolored acrosome (absent); live acrosome-intact spermatozoon (viable) – pinkish or white head and pinkish acrosome; live acrosome-reacted spermatozoon (real acrosome reaction) – pinkish or white head and discolored acrosome.

In the statistical analysis, all semen characteristics were evaluated regarding the kind of extender and time of conservation. Data were submitted to analysis of variance (ANOVA) and means were compared by SNK (Student-Newman-Keuls) test, using the Software GraphPad Instat 3.05 32 bit for Win 95/NT. The same statistical system was used to find means and standard deviations. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Mean sperm volume and concentration verified in dogs´ejaculates were 3.08 (± 0.08) mL and 496 and 92 (± 304,92) x 10^6sptz/mL, respectively. There were no changes in the volume of the dogs´s ejaculates, since the methodology used determines the collection of the sperm fraction and of part of the third fraction, aiming at standardizing the volume for subsequent dilution. Changes ($p < 0.05$) in sperm concentration were observed among the animals. Individual changes were reported by ENGLAND & PONZIO (1996), who observed concentrations ranging between 400 x 106 and 900 x 10^6 sptz/mL.

TABLE 1: Means ± SD of fresh semen characteristics of dogs diluted in Minimum Contamination (MC) and Modified Lactose-Egg yolk (MLEY) extenders after 12 and 24 hours of cooling (4ºC) in Equitainer®

Seminal characteristics	Fresh semen (n=13)	Semen diluted in MC extender (n=13)		Semen diluted in MLEY extender (n=13)	
		12 hours of cooling	24 hours of cooling	12 hours of cooling	24 hours of cooling
PM (%)	89.61(± 4.77)[a]	51.15(± 26.00)[b]	28.08(±26.34)[b]	70.00(±12.74)[ab]	56.54(±27.80[c])
V (1-5)	4.80(± 0.38)[a]	2.85(± 0.85)[b]	1.85 (± 1.39)[b]	3.46(± 0.70)[b]	2.65(± 1.31)[b]
TD (%)	31.84 (± 5.64)[a]	40.66(±12.01)[a]	52.15(±16.04)[b]	36.77(±8.70)[a]	44.54(±12.53)[ab]
MI (%)	86.54 (± 3.60)[a]	70.46 (±5.29)[b]	62.77 (±9.00)[b]	77.53(±5.70)[ab]	69.85 (±17.25)[b]
SV (%)	92.23 (± 6.37)[a]	81.46(±11.91)[b]	75.62(±12.50)[b]	90.30(±7.01)[ab]	83.39 (± 8.48)[ab]
TAR (%)	0.38 (± 1.12)[a]	0.31 (±1.11)[a]	0.31 (±0.85)[a]	0.15 (±0.55)[a]	0.31 (±0.75)[a]
FAR (%)	0.23 (± 0.60)[a]	0.23 (±0.60)[a]	0.38 (±0.77)[a]	0.38 (±0.96)[a]	0.46 (±1.13) [a]

Different letters in the same line, for each characteristic, indicate Letras diferentes na mesma linha, para cada $p < 0.05$ by SNK test.
PM: Progressive motility; V: Sperm vigor; TD: Total defects; MI: plasmatic membrane integrity; SV: Sperm viability (live acrosome-intact spermatozoon); TAR: True acrosome reaction (live acrosome-reacted spermatozoon); FAR: False acrosome reaction (dead acrosome-reacted spermatozoon).

In Table 1, fresh semen parameters are appropriate to be used in artificial insemination (AI) (VERSTEGEN *et al*., 2005), except sperm pathology, with means above the limit (31.84 (± 5.64), because it was not considered a criterion for selecting the ejaculates. The mean percentage of major and minor defects was 22.77 (± 2.98)% and 9.08 (± 4.37)%, respectively. Total sperm pathology values found in this study were higher than the ones verified by VERSTEGEN *et al*. (2005) and RIJSSELAERE *et al*. (2002). These authors verified less than 15% of total defects, which might be due to the animals´ safety-training-exercise routine, leading to different level of stress (HATAMOTO *et al*., 2006; BAPTISTA SOBRINHO *et al*., 2009).

Nevertheless, means of progressive motility (PM) and vigor (PV) in fresh semen were high, possibly due to the ejaculates previous selection (progressive motility higher than 70% and minimum vigor 3). Progressive motility as well as sperm pathology are used as selection parameters for ejaculates that will be stored (cooled and/or frozen) because they have high correlation with heat shock resistance (VERSTEGEN *et al*., 2005). On the other hand, TARTAGLIONE & RITTA (2004) reported that plasmatic membrane integrity and functionality as well as acrosomal membrane integrity were superior parameters than morphology and sperm motility to predict fertility of bulls´semen. Percentages of viable spermatozoa with intact plasmatic and acrosomal membranes were also high in fresh semen.

After 12 hours of conservation of the ejaculates, better preservation of the characteristics was observed in semen diluted in MLEY than in fresh semen. However, semen diluted in MC did not show the same behavior, possible because of heat shock protection promoted by egg yolk components in MLEY extender (ENGLAND, 1993), whose phospholipids promote the stabilization of the plasmatic membranes, restoring it after the phospholipids loss, which occur during the cooling process, preventing breakings (ROTA *et al*., 1995; HOLT, 2000).

It can be verified in Table 1 that there was no significant difference between the characteristics of fresh semen and of semen diluted for 12 hours, except sperm vigor ($p<0.05$). Although it occurred, mean vigor was higher than 3. The means of all the characteristics of semen diluted in MLEY after 12 hours of preservation are within the parameters recommended for AI (CONCANNON & BATTISTA, 1989; VERSTEGEN *et al*., 2005), except sperm pathology (36.77%), probably because this characteristic was 31.84% in fresh semen. However, neither values differed significantly. The most frequent defects observed after conservation were tail folding, maybe due to the heat shock (PARKS & GRAHN, 1992). The characteristics of semen diluted in MC were inferior ($p<0.05$) than the ones of fresh semen after 12 hours of conservation, except acrosome reaction and sperm pathology. Despite that, most characteristics are within the patterns for AI (VERSTEGEN *et al*., 2005).

The means of the characteristics of semen diluted in MLEY after 24 hours of conservation were not different from those found in semen conserved for 12 hours. There was a significant decrease ($p<0.05$) of progressive motility only, which remained above 50%. Some characteristics, however, differed from those of fresh semen. After 24 hours, neither extenders differed statistically from each other, and only progressive motility was lower in semen conserved in MC extender (56.54 (± 7.00). Besides, after 24 hours, semen conserved in both extenders presented satisfactory values regarding sperm viability, plasmatic membrane integrity and percentage of sperm showing acrosome reaction.

The amount of spermatozoa showing true or false acrosome reaction (AR) observed in fresh semen was low. In fact, finding significant number of capacitated acrosome-reacted spermatozoa in fresh semen is not expected (CORMIER *et al*., 1996) It is known that time, temperature and

extender components can induce spontaneous acrosome reaction (SIRIVAIDYAPONG *et al*., 2000). However, the mean rates of true and false acrosome reaction did not exceed 1% after storage under refrigeration in both extenders, which shows that, after 24 hours at 4°C, the extender components and the conservation did not induce these phenomena and prevented damage to the acrosome. IGUER-OUADA & VERSTEGEN (2001) also verified less than 5% of acrosome reaction after dog´s semen dilution in TRIS-Egg-Yolk extender and conservation for 72 hours at 4ºC.

Both sperm viability (SV) and plasmatic membrane integrity (PMI) remained relatively high after 24 hours of conservation in the samples diluted in both extenders. RIJSSELAERE *et al*. (2002) observed around 93% of live spermatozoa presenting intact acrosome after dog´s semen dilution in TRIS-Egg-yolk extender and conservation for 72 hours at 4ºC.

Although there were some changes in semen quality after conservation, many characteristics remained satisfactory in semen diluted in MLEY after 24 hours of refrigeration. CUNHA and LOPES (2000) verified 50% progressive motility and vigor 5 after 24 hours of conservation of dog´s semen diluted in Glicine-Egg Yolk extender, showing no differences when compared to fresh semen characteristics. VERSTEGEN *et al*. (2005) did not find significant decrease of these parameters after 24 hours of conservation in TRIS-Egg yolk-Glucose at 4ºC. In dogs, 60% spermatozoa with progressive motility and 10% or less presenting acrosome reaction can be considered good parameters for semen quality after refrigeration (VERSTEGEN *et al*., 2005). CONCANNON & BATTISTA (1989) suggested that at least 40 to 50% PM is necessary to achieve success in AI. It is known that extenders are very important to minimize changes that might occur during heat shock in semen conservation process; however, it is also important to begin with high quality semen samples to assure longer preservation of the necessary characteristics to fecundation.

We emphasize the importance of including sperm morphological evaluation in ejaculates selection because progressive motility does not always reflect some semen pathological conditions nor sperm viability (CASTRO *et al*., 2007). In case the animals selection included sperm pathology, it is likewise that the results of semen conservation after 24 hours would be closer to the fresh semen results.

CONCLUSION

It can be concluded that under the conditions of this experiment both extenders can be used of dogs´semen conservation for at least 12 hours at 4ºC in transportation container, without major changes of the characteristics verified in fresh semen.

ACKNOWLEDGEMTS

We acknowledge the 12th Squad of the Military Police of Rio de Janeiro State for the essential collaboration with this project, as well as CAPES for the financial support.

REFERENCES

AMANN, R.P.; GRAHAN, J.K. Spermatozoa function. In: Mckinnon, A.O.Voss, J.L**. Equine Reproduction**, p.715-745, 1993.

AMANN, R.P.; PICKET, B.W. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. **Equine Veterinary Science**., v. 7, n. 3, p. 145-173, 1987.

AMIRAT, L.; TAINTURIER, D.; JEANNEAU, L.; THORIN, C.; GERARD, O. COURTENS, L.J.; ANTON, M. Bull semen en vitro fertility after cryopreservation using egg yolk LDL: a comparison with Optidyl, a commercial egg yolk extender. **Theriogenology**, v. 61, p.895-907, 2004

ARRUDA, R.P.; BALL, B.A.; GRAVANCE, C.G.; LIU, I.K.M. Avaliação dos efeitos de diluidores e crioprotetores para espermatozóides de garanhões utilizando análise computadorizada da motilidade (CASA) e citometria de

fluxo. **Acta Scientiae Veterinatiae**, 31 Supl., p.228, 2003.

BLOM, E. The ultraestructural of some characteristic sperm defects and proposal a new classification of the Bull spermiogram. **Nordisk Veterinaer Medicin**., Copenhagen, v.25, n.7, p.383-391, 1973.

CASTRO, A. C. N. **Comparação entre os crioprotetores glicerol e etilenoglicol na congelaçãodo sêmen canino**. 2003, 69p. Dissertação (Mestrado em Medicina Veterinária)- Faculdade de Veterinária, Universidade Federal Fluminense, Niteroi. 2003.

HATAMOTO, L. K.. **Efeito do estresse e da suplementação com vitamina E sobre os parâmetros seminais, peroxidação lipídica de componentes seminais e atividade das enzimas antioxidante presentes no plasma seminal de cães**. 2004. Tese (Doutorado em Reprodução Animal) Faculdade de Veterinária e Zootecnia da Universidae de São Paulo, São Paulo, 2004.

CONCANNON, P.W.; BATTISTA, M. **Canine semen freezing and artificial insemination**. In: KIRK: Current veterinary therapy – small animal practice. 10 ed. Philadelphia: W.B.Saunders, 1989, p.1247-59.

CORMIER, N.; SIRARD, M.A.; BAILEY, J.L. Premature capacitation of bovine spermatozoa is initiated by cryopreservation. **Journal Andrology** v.18, p.461-468, 1996.

CUNHA, I. C. N. **Estudo da viabilidade do processo de refrigeração do sêmen canino à base de leite e glicina gema.** Dissertação (Mestrado em Reprodução Animal) Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, São Paulo, 1997.

CUNHA, I.C.N. **Criopreservação do sêmen de cães**. 2002, 149p. Tese (Doutorado em Reprodução Animal) Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, São Paulo, 2002.

DIDION, B.A., DOBRINSKY, J.R., GILES, J.R., GRAVES, C.N. Staining procedure to detect viability and the true acrosome reaction in spermatozoa of various species. **Gamete Research.,** 22: 51-57, 1989.

ENGLAND, G.C.W. Cryopreservation of dog semen: a review. **Journal Reproduction Fertility,** v.47, p.243-255, 1993.

ENGLAND, G.C.W.; PONZIO, P. Comparison of the quality of frozen-thawed and cooled-re-warmed dog's semen. **Theriogenology**, v.46, p.165-171, 1996.

HOLT, W.V. Basics aspects of frozen storage of semen. **Animimal Reproduction Science,** v.62, p.3-22, 2000.

HENRY, M., NEVES, J. P. **Manual para exame andrológico e avaliação de sêmen animal**. CBRA, 2 ed., Belo Horizonte, 1998, 49p.

IGUER-OUADA, M.; VERSTEGEN, J.P. Long-term conservation of chilled canine semen: effect of commercial and laboratory-prepared extenders. **Theriogenology**, v.55, p.671-684, 2001.

IVANOVA KICHEVA, M. G.;BOBADOV, N.; SOMLEV, B. Cryopreservation of canine semen in pellets and in 5-ml aluminum tubes using three estenders. **Theriogenology**, v.48, p. 1343-1349, 1997.

JEYENDRAN, R. S., VAN DER VEM, H. H., PEREZ-PELAEZ, M. et al. Development of an assay to asses the functional integrity of the human sperm membrane and its relationship to the other semen characteristics. **Journal Reproduction Fertility**. v. 70, p. 219 - 228, 1984.

KENNEY, R. M., BERGMAN, R. V., COOPER, W. L. et al. Minimal contamination techniques for breeding mares: technique and preliminary findings. **Procedures. American Association Equine Practice** v. 21, p. 327 – 336, 1975.

KUMI - DIAKA, J. Subjecting canine semen to the hypo-osmotic test. **Theriogenology**, v.39, p.1279-1289, 1993.

MAGNAGO, L.G.P. **Avaliação física e morfológica do sêmen de cães da raça pastor alemão,resfriado a 5ºC.** Belo Horizonte, 2000, 79p. Dissertação (Mestrado em medicina Veterinária)- Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte. 2000.

MAXWELL, W.M.C., SALAMON, S. Liquid storage of ram semen: a review. **Reproduction, Fertility and Development** v.5, p.613-638, 1993.

MOUSSA, M.; MARTINET.; TRIMECHE, A; et al. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen thawed Bull semen. **Theriogenology**, v.57, p.1695-1706, 2002.

PARKS, J.E.; GRAHAM, J.K. Effects of cryopreservation procedures on sperm membranes. **Theriogenology**, v.38, n. 2, p.209-222, 1992.

PHILLIPS, P.H., LARDY, H.A.A. Yolk buffer pabulum for the preservation of bull semen. **Journal Dairy Science,** v.23, p.399-404, 1993.

RIJSSELAERE, A.; VAN SOOM, D.; MAES, A.; DE KRUIF, A. Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. **Theriogenology**, v.57, p.1669-1681, 2002.

ROTA, A.; STROM, B.; LINDE-FORSBERG, C. Effects of seminal plasma and three extenders on canine semen stored at 4ºC. **Theriogenology**, v.44, p.885-900, 1995.

SILVA, A.R.; CARDOSO, R.C.S.; UCHOA, D.C.; SILVA, L.D.M. Efeitos das etapas de cripreservação sobre a qualidade do sêmen canino diluído em Tris. **Revista. Brasileira de Reprodução Animal**., v.25, n.3, p.474-475, 2001.

SILVA FILHO; PALHARES, M. S.; BERGMAN,J. A. G. Inseminação artificial em eqüinos incluindo transporte de

sêmen. In: Cong. Bras. Rep. Anim., 7, 1987, Belo Horizonte. **Anais**. Belo Horizonte, CBRA, p. 78, 1987.

SIRIVAIDYAPONG, S.; CHENG, F. P.; MARCS, A.; VOORHOUT, W. F.; BEVERS, M. M.;COLENBRANDER, B. Effect of sperm diluents on the acrosome reactions in canine sperm. **Theriogenology**, v.53, p.789-802, 2000.

TARTAGLIONE, C.M.; RITTA, M.N. Prognostic value of spermatic parameters as predictors of in vitro fertility of frozen-thawed bull semen. **Theriogenology**, v.62, p.1245-1252, 2004.

VANNUCCHI, C.I.; SATZINGER, S.; SANTOS, S.E.C. *Avaliação seminal em cães – aspectos práticos.* Ver. Clin. Vet., n.15, p.22-26, 1998.

VERSTEGEN, J.P.; ONCLIN, K.; IGUER-OUADA, M. Long-term motility and fertility conservation of chilled canine semen using egg yolk added Tris-glucose extender: In vitro and in vivo studies. **Theriogenology**, v.64, p.720-733, 2005.

VISHWANATH, R.; SHANNON, P. Storage of bovine semen in liquid and frozen state. **Animal Reprodction Science,** v.62, p.23-53, 2000.

Submitted on: September, 9, 2008. Accepted on: January, 18, 2011