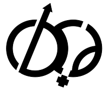

Anim. Reprod., v.7, n.2, p.70-74, Apr./Jun. 2010

Water-induced hypo-osmotic test for the evaluation of canine sperm membrane integrity

A.T. Quintela[1], I.R.S. Oliveira[2], A.O. Souza[1], A.L. Gusmão[3], A.R. Silva[2,4]

[1]Centro-Vet Clínica Veterinária, Salvador, BA, Brasil.
[2]Laboratório de Conservação de Germoplasma Animal, UFERSA, Mossoró, RN, Brasil.
[3]Laboratório de Reprodução Animal, EMV-UFBA, Salvador, BA.

Abstract

The purpose of this study was to investigate the efficiency of the water test to evaluate the functional membrane integrity of ejaculated canine sperm after collecting, cooling, and freezing-thawing. Twenty-five ejaculates were obtained from 12 stud dogs by digital manipulation. Sperm-rich fractions were evaluated, diluted, cooled at 5ºC and frozen-thawed. A conventional hypo-osmotic swelling test (HOST) using a fructose solution (60 mOsm/l) was compared with a HOST using distilled water (0 mOsm/l), for evaluation of the functional integrity of the plasma membrane in fresh, cooled, and frozen-thawed semen. Distilled water detected a higher percentage of reacted sperm in fresh semen (91.2 ± 0.1%) than the conventional HOST (90.6 ± 0.1%; $P < 0.05$). For cooled and frozen-thawed semen, the water test was as efficient as the conventional HOST (81.2 ± 0.3% and 80.3 ± 0.3%, respectively), for detecting functional membrane integrity ($P > 0.05$). Regardless of the test used, there was a decrease in the mean percentage of reacted sperm after equilibrium and freezing-thawing ($P < 0.05$). In conclusion, this study demonstrated that water can efficiently replace conventional fructose-based hypo-osmotic media for evaluation of the functional integrity of the plasma membrane of ejaculated fresh, cooled, and frozen–thawed canine sperm.

Keywords: canine, hypo-osmotic swelling test, sperm, water test.

Introduction

The plasma membrane is involved in several essential processes that occur in the cell, such as physiological mechanisms involved in the maintenance and survival of the spermatozoa in the female genital tract, sperm capacitation, and fertilization (Mocé and Graham, 2008). In order to evaluate the plasma membrane functionality of the human sperm, Jeyendran *et al*. (1984) proposed the use of a test in which sperm were subjected to adverse conditions of osmolarity. Sperm were evaluated for their response (curling or coiling of the sperm flagellum) when exposed to the hypo-osmotic solution. This test was named as the hypo-osmotic swelling test (HOST), and the sperm presenting a coiled or curled tail was deemed to having a functional plasma membrane.

In spite of the HOST being a simple test, it is considered an indicator of fertility in some species, as the viability of the sperm membrane is a basic requirement for fertilization (Mocé and Graham, 2008). Furthermore, it is used as a complementary test to *in vitro* evaluation of frozen semen, due to its high accuracy. This is possible because the sperm suffer damages that lead to alterations in the plasma membrane and loss in viability during the cooling and freezing - thawing procedures (Watson, 2000).

Studies in humans (Jeyendran *et al.*, 1984), horses (Nie and Wenzel, 2001), goats (Fonseca *et al.*, 2005), and rabbits (Amorim *et al*., 2009) reported that HOST should be conducted by using solutions with osmolarity varying from 50 to 150 mOsm/l. Conversely, water-based HOST that uses distilled water as a hypo-osmotic solution was developed as a simple and quick method for human spermatozoa (Lomeo and Giambersio, 1991). Currently, the water test has also been reported as an efficient test for the evaluation of the stallion sperm (Dell'Aqua *et al*., 2002; Vasconcelos *et al*., 2010).

In dogs, hypo-osmotic solutions at 60 (Michael *et al*., 2009) to 150 mOsm/l (Silva *et al*., 2006) have been currently used in the HOST. Furthermore, a HOST using ultrapure water has been used previously only for the evaluation of fresh epididymal canine sperm (Hishinuma and Sekine, 2003). The incorporation of water-based HOST in the routine evaluation of canine semen would reduce costs and facilitate the process. Accordingly, we have investigated the efficiency of the water test to evaluate the functional membrane integrity of canine sperm immediately after ejaculation, cooling, and freezing-thawing procedures.

Materials and Methods

Animals

The procedures conducted in this research were in agreement with the international animal care regulations. Twenty-five ejaculates were obtained from 12 proven stud dogs (four Labrador retrievers, four Brazilian mastiffs, and four American pit bull terriers). The dogs were maintained in individual pens in private

[4]Corresponding author: legio2000@yahoo.com
Phone/Fax: +55(84)3315-1760
Received: March 8, 2010
Accepted: August 27, 2010

kennels in Salvador, BA, Brazil (12º 58'S-38º 30'W). They were 2 to 7 years old and were fed dry commercial food twice daily, with free access to water.

Semen collection and initial evaluation

Two ejaculates were collected from each dog, except from one dog that provided three ejaculates. Ejaculates were obtained by digital manipulation at one week intervals. The sperm rich fractions were kept at 37ºC and analyzed using routine semen analysis procedures (Seager and Platz, 1977). The semen volume was recorded and sperm concentration was determined using a Neubauer counting chamber (hemocytometer). The percentage of the total and progressive motile sperm and the sperm vigor (0-5; 0 characterized by no movement and five characterized by progressive forward movement) were subjectively assessed using bright field microscopy under 100X and 400X magnification. For morphological evaluation, a semen aliquot was diluted in 10% buffered formalin and wet mount semen smears were made. The sperm morphology was evaluated under phase-contrast microscopy under 400X magnifications by counting 200 cells.

Evaluation of the functional integrity of plasma membrane

For evaluation of sperm membrane functional integrity, the semen samples were divided in two aliquots and subjected to conventional and water-based HOST. For the conventional HOST, the first semen aliquot (0.1 ml semen) was diluted into 1 ml fructose solution presenting 60 mOsm/l (Michael *et al*., 2009). For the water test, a second aliquot of 0.1 ml of semen was diluted in 1.0 ml distilled water with 0 mOsm/l (Lomeo and Giambersio, 1991). The osmolarity of the solutions was determined by using a micro-osmometer (Type '13/13DR - Autocal, Roebling, Berlin, Germany). Both aliquots were incubated in a water bath at 37ºC for 45 min. Evaluations were conducted under phase-contrast microscopy (200X) by operators unaware of the experimental design. The cells were classified as reacted and non-reacted according to the presence or absence of coiled tails, respectively (Kumi-Diaka and Badtram, 1994). The percentage of reacted sperm was subtracted from the percentage of tail defects verified by the sperm morphology evaluation.

Semen processing

The reagents used in this study were obtained from Sigma-Audrich (St. Louis, MO, USA). An extender consisting of 3.028 g Tris-hydroximethyl-aminomethane (Tris), 1.78 g monohydrated citric acid, and 1.25 g D-fructose dissolved in 100 ml ultrapure water was used (Silva *et al*., 2002). The osmolarity of this solution was 295 mOsm/l and the pH was 6.6.

Immediately after initial evaluation, each sperm-rich fraction of the semen was diluted 1:1 with skimmed milk extender (Kenney *et al*., 1975), and centrifuged at 800 *g* for 5 min. The supernatant was discarded and the pellet resuspended in Tris plus egg yolk (20%) and glycerol (5%) to a final concentration of 80 x 10^6 spermatozoa/ml. The samples were packed into 0.5 ml plastic straws and equilibrated in a refrigerator at 5°C for 60 min. After cooling, one straw was rewarmed and evaluated for functional membrane integrity using both hypo-osmotic solutions. The other straws were placed horizontally in a thermal box for 20 min, positioned 6 cm above the liquid nitrogen (N_2) level, and reached a temperature close to -70ºC in the vapor. Finally, the straws were immersed into liquid N_2 for storage. After one week, the frozen semen straws were thawed in a water bath at 37ºC for 30 sec and thereafter evaluated for sperm motility, vigor, and morphology, and for the functional integrity of the membrane, by conventional and water-based HOST, as reported for fresh semen.

Statistical analyses

Twenty-five replicates were performed for each treatment (conventional and water-based HOST). Statistical analyses were conducted with the *Statistical Analysis System* (SAS 6.10, SAS Institute Inc., Cary, NC, USA). The results were expressed as mean ± SD. Data were checked for normality by the Kolmogorov-Smirnov test with Lilliefor's correction and for homocedasticity with the Levene's test. An experimental design at random blocks was used and the treatments (fresh x frozen-thawed semen; conventional x water-based HOST) were compared using the Tukey test. The results were considered significant when $P < 0.05$.

Results

The mean (± SD) volumes for the sperm-rich semen fractions (n = 25) were 1.5 ± 0.5 ml, whereas, mean (± SD) sperm concentrations were 768 ± 182.2 x 10^6 sperm/ml. Other sperm characteristics obtained following semen analysis are shown in Table 1. A significant reduction in sperm quality was verified after freezing–thawing ($P < 0.05$).

Table 2 shows the results for the functional integrity of plasma membrane in fresh, cooled, and frozen-thawed sperm. Significant differences between hypo-osmotic solutions were detected only for fresh semen ($P < 0.05$). A decrease in functional sperm membrane integrity was verified in the use of both tests after freezing–thawing ($P < 0.05$).

Table 1. Mean (± SD) values for sperm characteristics in fresh and frozen-thawed canine semen (n = 25 ejaculates).

	Fresh semen	Frozen-thawed semen
Total motility (%)	91 ± 2.1^{a}	36 ± 9.6^{b}
Progressive motility (%)	89.2 ± 2.8^{a}	25.2 ± 9.2^{b}
Vigor (0-5)	5 ± 0^{a}	2.1 ± 0.5^{b}
Normal morphology (%)	93.6 ± 1.6^{a}	67.1 ± 6.1^{b}

^{a,b}Within a row, means without a common superscript differ (P < 0.05).

Table 2. Mean (± SD) values for functional integrity of plasma membrane of fresh, cooled and frozen-thawed canine sperm evaluated by conventional hypoosmotic swelling test (HOST) or water test(n = 25 ejaculates).

Evaluation	HOST	Water test
Fresh semen (%)	90.6 ± 0.1^{aA}	91.2 ± 0.1^{bA}
Cooled semen (%)	80.3 ± 0.3^{aB}	81.2 ± 0.3^{aB}
Frozen-thawed semen (%)	53.4 ± 0.7^{aC}	53.8 ± 0.7^{aC}

^{a,b}Within a row, means without a common superscript differ (P < 0.05).
^{A,B}Within a column, means without a common superscript differ (P < 0.05).

Discussion

The response of spermatozoa to a hypo-osmotic solution is reported to vary according to the species. In dogs, correlations between osmotic response and sperm motility have been reported (Rota *et al*., 2005; Silva *et al*., 2006). However, a relationship between conventional HOST response and fertility of canine sperm has not yet been established (Peña-Martinez, 2004), as reported for humans (Jeyendran *et al*., 1992).

A HOST that used distilled water as a hypo-osmotic solution was developed as a simple and quick method for human spermatozoa (Lomeo and Giambersio, 1991). For dogs, water-based HOST was previously reported as an efficient method to analyze the functional integrity of plasma membrane in fresh canine epididymal sperm (Hishinuma and Sekine, 2003). However, it is known that the physiological properties of canine epididymal spermatozoa differ from those of ejaculated spermatozoa. These differences include the presence or absence of sensitization with prostatic fluid (PF). The prostate is the sole accessory reproductive organ in dogs, and PF is mixed with spermatozoa at ejaculation, but the effect of PF on sperm has not yet been clarified (Hori *et al*., 2005).

We demonstrate that water-based HOST is also efficient for evaluating plasma membrane integrity of fresh and frozen-thawed canine sperm. In addition, distilled water presents the advantage to be relatively inexpensive and easier to manipulate than sugar-based solutions currently used for the conventional HOST. Further studies must also be conducted in order to reduce the time of incubation of sperm in distilled water, as Pinto and Kozink (2008) recommended a decrease in incubation time of the HOST for canine sperm to as short as 1 min.

The mean percentage of sperm that reacted to the water test was greater than that verified by the conventional HOST for fresh semen (P < 0.05). However, we emphasize that a difference lower than 2% between the treatments may not have biological relevance for this parameter. Distilled water has been previously reported to be a superior medium for detection of the functional integrity of plasma membrane in equine sperm (Dell'Aqua *et al*., 2002), when compared with other sugar-based solutions. It is suggested that the plasma membrane of equine spermatozoa present high permeability to water, due to the presence of a porous membrane and protein water channels (Neild *et al*., 1999). We speculate that these characteristics are also possibly present in the canine sperm, which facilitates its permeability to water.

For cooled and frozen-thawed canine sperm, the water test was as efficient as the conventional HOST in evaluating the functional integrity of the plasma membrane. Similar results were reported for human frozen-thawed semen (Lin *et al*., 1998), in which distilled water was shown to be as efficient as sugar- and electrolyte-based hypo-osmotic solutions, for detection of sperm membrane integrity. In the present study, a significant decrease in the functional integrity of the plasma membrane was verified in both hypo-osmotic solutions during the processes of cooling (pre-freezing equilibration) and freezing-thawing of the canine semen. This decrease was also observed by other authors during the cooling (Lopes *et al*., 2009) and freezing (Silva *et al*., 2006; Pinto and Kozink, 2008) of canine semen. During these procedures, the sperm cells pass through a serious of drastic changes in their physical and chemical environment (Watson, 2000). First, the temperature falls close to the freezing point of water, which leads to structural changes in the lipid bi-layer, altering the plasma membrane. Due to this, a reorganization of the membrane components occurs and some lipids, which are linked to membrane proteins, aggregate among themselves and allow the freed membrane proteins to aggregate other substances. All these changes can provoke alterations in the permeability of the plasma membrane, and

consequently, cause its rupture (Hermansson and Linde-Forsberg, 2006). Besides, glycerol can also promote physical-chemical alterations that can provoke the rupture of the plasma membrane or even the loss of important membrane proteins (Holt, 2000).

Differences in temperature and osmolarity among freezing media and the sperm provide great variation in the water volume in the cell, leading to a stress mechanism on the cell membrane. The transition phase that occurs during the freezing alters the ultra-structure of the membrane and creates regions that are free of proteins, which reduces the cryopreservative efficiency. Reorganization of the lipid membrane of the sperm seems to disturb the lipid-lipid or lipid-protein interactions that are necessary for the perfect functioning of the membrane (Watson, 2000). In addition, the freezing media can lead to the rupture of the membrane, as it can cause osmotic stress, membrane alterations, and changes in the microtubule structure of the sperm tail (Correa and Zavos, 1994). Furthermore, the sperm are also exposed to osmotic stress when the time for thawing is insufficient for the efflux of excess cryoprotectants. Therefore, the sperm cells swell and rupture as the media become abruptly diluted due to the melting of the extracellular ice (Pegg, 2002).

In conclusion, it has been demonstrated that the water test can be efficiently used for the evaluation of the functional integrity of the plasma membrane for ejaculated canine sperm after collecting, cooling, and freezing-thawing. The use of this test provides an inexpensive and simple alternative to other HOST protocols that utilize sugar-based solutions. The potential relationship between the water test and the fertility of canine sperm needs to be further investigated.

References

Amorim EAM, Torres CAA, Graham JK, Amorim LS, Santos LVL. 2009. The hypoosmotic swelling test in fresh rabbit spermatozoa. *Anim Reprod Sci*, 111:338-343.

Correa JR, Zavos PM. 1994. The hypoosmotic swelling test: its employment as assay to frozen-thawed bovine sperm membrane. *Theriogenology*, 42:351-360.

Dell'Aqua Jr JA, Papa FO, Zahn FS, Alvarenga MA, Leonardo H. 2002. Novo teste osmótico de avaliação da integridade da membrana plasmática de sêmen congelado eqüino. *Rev Bras Reprod Anim,* 26:189-191.

Fonseca JF, Torres CAA, Maffili VV, Borges AM, Santos ADF, Rodrigues MT, Oliveira RFM. 2005. The hypoosmotic swelling test in fresh goat spermatozoa. *Anim Reprod*, 2:139-144.

Hermansson U, Linde-Forsberg C. 2006. Freezing of stored, chilled dog spermatozoa. *Theriogenology*, 65:584-593.

Hishinuma M, Sekine J. 2003. Evaluation of membrane integrity of canine epididymal spermatozoa by short hypoosmotic swelling test with ultrapure water. *J Vet Med Sci*, 65:817-820.

Holt WV. 2000. Basic aspects of frozen storage of semen. *Anim Reprod Sci,* 62:3-22.

Hori T, Hagiuda K, Kawakami E, Tsutsui T. 2005. Unilateral intrauterine insemination with prostatic fluid-sensitized frozen caudal epididymal sperm in beagle dogs. *Theriogenology*, 63:1573-1583.

Jeyendran RS, Van der Ver HH, Perez-Pelaez M, Crabo BG, Zaeveld LJD. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil,* 70:219-228.

Jeyendran RS, Van der Ven HH, Zaneveld LJD. 1992. The hypoosmotic swelling test: an update. *Arch Androl*, 29:105-116.

Kenney RM, Bergman RV, Cooper WL, Morse GW. 1975. Minimal contamination techniques for breeding mares: technique and preliminary findings. *Proc Am Assoc Equine Pract*, 21:327-336.

Kumi-Diaka J, Badtram G. 1994. Effect of storage on sperm membrane integrity and other functional characteristics of canine spermatozoa: *in vitro* bioassay for canine semen. *Theriogenology*, 41:1355-1366.

Lin MH, Morshedi M, Srisombut C, Nassar A, Oehningher S. 1998. Plasma membrane integrity of cryopreserved human sperm: an investigation of the results of the hypoosmotic swelling test, the water test and eosin-Y staining. *Fertil Steril*, 70:1148-1155.

Lomeo AM, Giambérsio AM. 1991. Water-test: a simple method to assess sperm-membrane integrity. *Int J Androl,* 14:278-282.

Lopes G, Simões A, Ferreira P, Martins-Bessa A, Rocha A. 2009. Differences in preservation of canine chilled semen using different transport containers. *Anim Reprod Sci*, 112:158-163.

Michael AJ, Alexopoulos C, Pontiki EA, Hadjipavlou-Litina DJ, Saratsis P, Ververidis HN, Boscos CM. 2009. Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. *Anim Reprod Sci*, 112:119-135.

Mocé E, Graham JK. 2008. *In vitro* evaluation of sperm quality. *Anim Reprod Sci*, 105:104-118.

Neild D, Chaves G, Flores M, Mora N, Beconi M, Aguero A. 1999. The hypoosmotic swelling test in equine spermatozoa. *Theriogenology,* 51:721-727.

Nie GJ, Wenzel JGW. 2001. Adaptation of the hypoosmotic swelling test to assess functional integrity of stallion spermatozoa plasma membranes. *Theriogenology*, 55:1005-1018.

Pegg DE. 2002. The history and principles of cryopreservation. *Semin Reprod Med,* 20:5-13.

Peña-Martinez AI. 2004. Canine fresh and cryopreserved semen evaluation. *Anim Reprod Sci*, 82/83:209-224.

Pinto CRF, Kozink DM. 2008. Simplified hypoosmotic swelling test (HOST) of fresh and frozen-thawed canine

spermatozoa. *Anim Reprod Sci*, 104:450-455.
Rota A, Rota A, Martine M, Milani C, Romagnoli S. 2005. Evaluation of dog semen quality after slow (biological freezer) or rapid (nitrogen vapours) freezing. *Reprod Nutr Dev*, 45:29-37.
Seager SWJ, Platz CC. 1977. Artificial insemination and frozen semen in the dog. *Vet Clin North Am*, 7:757-764.
Silva AR, Cardoso RCS, Uchoa DC, Silva LDM. 2002. Effect of trisbuffer, egg yolk and glycerol on canine semen freezing. *Vet J*, 164:244-246.
Silva AR, Cardoso RCS, Silva LDM. 2006. Influence of temperature during glycerol addition and post-thaw dilution on the quality of canine frozen semen. *Reprod Domest Anim*, 41:74-78.
Vasconcelos AB, Santana MA, Santos AM, Santoro MM, Lagares MA. 2010. Metabolic evaluation of cooled equine spermatozoa. *Andrologia*, 42:106-111.
Watson PF. 2000. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci*, 60/61:481-492.