USE OF WATER TEST TO ASSESS THE SPERM MEMBRANE FUNCTIONAL INTEGRITY IN CRYOPRESERVED BULL SEMEN

Uso de la prueba de agua para medir la integridad funcional de la membrana espermática en semen criopreservado de toros

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ABSTRACT

The hypoosmotic swelling test (HOST) has been used to asses the sperm membrane functional integrity in bulls. However, for human, goat and dog semen, the water test (WT) has been proposed as a replacement to HOST, because WT uses distilled water and only five minutes of incubation. The aim of the present study was to compare the HOST and the WT to assess the sperm membrane functional integrity of cryopreserved bull semen. The percentage of sperm with swelling tail was higher in WT (54.85 ± 13.63%, P<0.0001) than HOST (42.90 ± 12.89%), but both were correlated positively (r = 0.77, P<0.0001). In comparison with viability of thawed semen (52.52 ± 9.79%), only WT diminished significantly this parameter to 45.95 ± 8.69%, P<0.0001. Acrosome integrity of thawed semen (82.35 ± 8.21%) decreased significantly after HOST (57.87 ± 12.67%) and WT (39.00 ± 16.88%). Acrosome integrity in thawed semen was correlated positively with results after HOST (r = 0.52; P = 0.0126) and WT (r = 0.52; P = 0.0115). In conclusion, the WT can assess the sperm membrane functional integrity of cryopreserved bull semen and is able to replace the HOST.

Key words: Sperm membrane, functional integrity, hipoosmotic test, water test.

RESUMEN

La prueba de hinchamiento hipoosmótica (HOST) ha sido usada para medir la integridad funcional de la membrana espermática en toros. Sin embargo, en humanos, caprinos y perros, la prueba de agua (WT) ha sido propuesta como un reemplazo para el HOST, debido a que usa agua destilada y solo cinco minutos de incubación. El propósito de este estudio fue comparar el HOST y el WT para medir la integridad funcional de la membrana espermática en semen criopreservado de toros. El porcentaje de colas enrolladas fue mayor en el WT (54,85 ± 13,63%, P<0,0001) que en el HOST (42,90 ± 12,89%), pero ambas pruebas se correlacionaron positivamente (r = 0,77, P<0,0001). En comparación con la viabilidad del semen descongelado (52,52 ± 9,79%) solo el WT disminuyó significativamente este parámetro a 45,95 ± 8,69%, P<0,0001. La integridad acrosómica en el semen descongelado (82,35 ± 8,21%) disminuyó significativamente luego del HOST (57,87 ± 12,67%) y el WT (39,00 ± 16,88%), sin embargo, la integridad acrosomica del semen descongelado se correlacionó positivamente con los resultados luego del HOST (r = 0,52; P = 0,0126) y el WT (r = 0,52; P = 0,0115). En conclusión, el WT puede medir la integridad funcional de la membrana espermática en semen criopreservado de toros y puede ser alternativa al HOST.

Palabras clave: Membrana espermática, integridad funcional, prueba hipoosmótica, prueba de agua, espermatozoide.

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INTRODUCTION

Sperm membrane is involved in several process needed for fertilization. Evaluation of this structure is of great interest in cryopreserved semen, because sperm membrane is the primary site of damage by cryopreservation process [3, 5]. These damage effects include a reduction in viability and motility [3], cryocapacitation [5], chromatin fragmentation [15] and a reduc-

tion in the capacity of sperm to attach to the oviductal ephitelial cells [11]. This situation could to explain why there is need to use eight times more cryopreserved sperm to reach the same level of fertility than with fresh semen [23].

Different supravital stain, fluorometric or not, have been employed to asses the sperm membrane integrity [2], whereas with the aim to measure the functional integrity of sperm membrane, Jeyendran et al. [13] developed the Hypoosmotic Swelling Test (HOST), this test is based in the capacity of water in a hypoosmotic solution to flow freely into the cell with a functional plasma membrane, with sperm responding to this challenge with the tail swelling. This test has been used to assess the functional integrity of sperm membrane in bull semen [2, 6, 20, 22], and seems to be a more precise parameter than supravital stains [24].

Lomeo and Giambresio [18] proposed the Water Test (WT) to asses the sperm membrane functional integrity, substituting the hypoosmotic solution in the HOST by distilled water. Many studies with human semen comparing HOST and WT reported a high correlation coefficient among these tests [1, 4, 9, 10, 17]. However, contrasting to human semen, scarce studies have been published with semen from animals; a high correlation coefficient was observed among HOST and WT (r=0.86, P=0.0004) when evaluating canine (*canis familiaris*) epididymal semen [12]; whereas in goat (*Capra hircus*) fresh semen different percentages of tail coiling sperms was observed among HOST and WT, but a positive correlation was observed (r=0.65, P<0.001) [19].

Given that in several studies a high correlation has been observed WT and other sperm parameters of sperm quality like concentration, motility and viability [1, 10, 12, 18], WT has been proposed as a suitable test to assess the functional integrity of sperm membrane and to substitute to the HOST [4, 17]. Moreover, taking in mind that WT is carried out using distilled water and during five minutes at 37°C, substitution of HOST by WT could be a real, practical and inexpensive possibility [18] and could be included in the routine sperm evaluation to estimate the sperm quality. Therefore, the aim of this study was to compare the HOST and the WT to determine the functional integrity of sperm membrane of cryopreserved semen from bulls.

MATERIALS AND METHODS

Animals used and semen processing

Two ejaculates from 6 fertile sound Brahman bulls (*Bos indicus-taurus*) were obtained during the same week, from the Artificial Insemination Center VIATECA at Machiques County, Zulia State, Venezuela. Ejaculates were collected with artificial vagina. Sperm concentration was determined with a photometer (SpermaCue®, Minitube, Germany) and motility were assessed subjectively. Semen extender was prepared with skim milk, egg yolk medium (20%) and 7% of glycerol. Semen dilution was carried out in two stages to reach a final concentration of ~30×10^6 motile sperm/mL. After dilution and equilibration at 5°C, semen was loaded into 0.5 mL straws, which were frozen in liquid nitrogen vapours, 4 cm above its surface for 10 min, and then plunged into liquid nitrogen.

Evaluation of thawed semen

Viability and acrosome integrity. Immediately after straw thawing in a water bath (Gemmy Industrial Corp., Taiwan, model YCW-03S) at 37°C by 30 seconds, sperm viability was measured with eosin-nigrosin stain [25], mixing 15 µL of semen and 10 µL of stain by 30 seconds. Spermatozoa were smeared onto a pre-warmed glass slide and air dried. The percentage of viable (unstained) and dead (stained) sperm were determined by smear observation under a light microscope (Globe 1600, Germany) at a 1000x magnification. Acrosome integrity was assessed using the Coomassie blue stain [16].

Evaluation of sperm membrane. Sperm membrane functional integrity was assessed with HOST and WT. For HOST, 30 µL of cryopreserved semen was mixed with 300 µL of hypoosmotic solution of NaCl (154 mOsm) and incubated in water bath at 37°C for 30 min. For WT, 30 µL of cryopreserved semen was mixed with 300 µL of distilled water and incubated in water bath at 37°C for 5 min. After incubation, 15 µL of semen were mixed with 10 µL of eosin-nigrosin stain and slides were prepared from each sample. In both, HOST and WT the swelling phenomena of sperm tail irrespective of the types of tail coiling were determined according to Jeyendran et al. [13]. Additionally, percentages of viable sperm and intact acrosome after WT and HOST were determined.

Statistical analysis

At least 200 cells were counted in duplicate for each smear and percentages of viability, acrosome intact and tail reactive sperm were determined immediately after thaw (thawed semen) and after HOST and WT. Statistical data analysis was carried out using Statistical Analysis System for Windows, software 8.2 (*SAS Inst. Inc.*; Carry, NC. USA) and percentages of viability, acrosome intact and tail reactive sperm were transformed through square arcsine method to obtain a normal distribution. Data were analysed through general lineal model analysis of variance (GLM procedure) and results are shown as corrected means ± SD. A Spearman's correlation test was used to establish correlation among measured parameters.

RESULTS AND DISCUSSION

Percentages of viability and acrosome integrity in thawed semen were 52.52 ± 9.79% y 82.35 ± 8.21%, respectively. Percentages of sperm responding to hypoosmotic challenge differed significantly between the tests (P<0.0001). Percentage of sperm with swelling tail was significantly lower after HOST (42.90 ± 12.89%) than after WT (54.85 ± 13.63%, P<0.0001),

but both tests were correlated positively (r = 0.77, P<0.0001). Viability after HOST and WT differed significantly (53.19 ± 8.24% vs. 45.95 ± 8.69%, respectively, P<0.0001), but only WT diminished the viability in comparison with thawed semen (P=0.05). Percentage of sperm with intact acrosome was higher after HOST than after WT (57.87 ± 12.67% vs. 39.00 ± 16.88%, P<0.0001) and both test diminished acrosome integrity in comparison with thawed semen. A positive and significant correlation was observed between percentage of intact acrosome in thawed semen and intact acrosome after HOST (r = 0.52; P = 0.0126) and WT (r = 0.52; P = 0.0115).

The hypoosmotic swelling test [13] has been commonly used to asses the functional integrity of sperm membrane form several species including bull [2, 6, 19, 22], while in human, goat and dog semen the WT has been proposed as an alternative method to asses the functional integrity of sperm membrane [1, 4, 9, 10, 12, 17, 19]. However, to the current knowledge this is the first study in Venezuela using the WT to asses the functional membrane integrity in bull semen.

In the present study, the percentage of sperm with swelled tails was higher in WT than HOST, and this is in agreement with Fazano et al. [9] using human semen, but is opposite to the findings of Nur et al. [19] whom using buck fresh semen observed a higher percentage of sperm with swollen tails in HOST. Others studies did not observe a significant difference among HOST and WT [1, 4, 10]. However, when the type of swelling was considered, percentage of swelling in the WT was significantly higher than HOST [10, 12]. The differences among WT and HOST observed in the present study could be explained by the fact that the sperm acts as an osmometer and that the sperm response is proportional to the decrease in external osmolarity [7, 8]. Taken together, these observations and the results of present study it is possible to suggest than HOST at 154 mOsm could be an insufficient hypoosomotic challenge to some bull sperm, probably because the damage produced to the membrane by cryopreservation process is not similar to all sperm; when sperm viability is measured using a combination of eosin-nigrosin stain plus HOST, four sperm populations are observed (unstained-swelling; unstained-non-swelling; stained-swelling; and stained-non-swelling), indicating that membrane resistance is different between the sperm tail and head, and with unstained-swelling sperm correlating with bull field fertility (Quintero-Moreno A., personal communication).

In human, goat and dog semen positive and significant correlation coefficients have been observed between HOST and WT [1, 4, 9, 10, 12, 17, 19] and a similar situation has been observed in this trial, nevertheless, no significant correlations were observed among sperm with swelling tail assessed with HOST or WT and viability, contrasting with others studies [1, 2, 10, 12, 18, 20].

In the present study WT affected negatively the viability, (–12.50%), while the HOST did not affected this parameter. In buffalo (*Bubalus bubalis*) semen, the challenge to 50 mOsm diminished the viability in 59 and 73% in fresh and frozen-thawed semen, respectively [14]. In addition, acrosome integrity was diminished in both test HOST and WT (29.72% and 52.64%, respectively). These results could indicate that sperm plasmalemma and acrosome have different osmotic resistance, being the acrosome membrane less resistant to the reduced osmolarity, which could be a consequence of cryopreservation process [21]. However, a positive correlation between intact acrosome in thawed semen and acrosome integrity after HOST and WT was observed, therefore, acrosome resistance to hypoosmotic conditions could be used as criteria to asses the sperm quality and the reproductive potential of bulls.

CONCLUSIONS

Results of the present study suggest that sperm response to hypoosmotic challenge depends on osmolarity, with WT producing a higher percentage of sperm with tail swelling than HOST, and this suggest than HOST at 154 mOsm could be sub-estimating the membrane functional integrity in some sperm. Additionally, because both tests were positively correlated, the WT represent an inexpensive and practical alternative to replace the HOST. Moreover, further research is needed to establish the correlation between the results of WT and other parameters of sperm quality as well as with the reproductive potential of bulls.

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