Anim. Reprod., v.10, n.4, p.684-688, Oct./Dec. 2013.

Hypoosmotic swelling test in young Nelore bulls classified as sound and unsound for breeding

L.F. Martins[1], R.O. Pinho[2,7], J.B. Siqueira[3], D.S. Costa[4], S.E.F. Guimarães[2], T. Miranda Neto[5], J.D. Guimarães[6]

[1]Veterinary Medicine Department, Paranaense University, Umuarama, PR, Brazil.
[2]Animal Science Department, Federal University of Viçosa, MG, Brazil.
[3]Veterinary Medicine Departament, Federal University of Espírito Santo, Alegre, ES, Brazil.
[4]Veterinary Medicine Departament, Federal University of Mato Grosso do Sul, Campo Grande, MS, Brazil.
[5]Agro-Livestock CFM Ltda., São José do Rio Preto, SP, Brazil.
[6]Veterinary Medicine Department, Federal University of Viçosa, MG, Brazil.

Abstract

This work aimed to study the correlations between the physical characteristics of semen, sperm morphology and the biochemical integrity of the plasmatic membrane of raw sperm in young Nelore bulls (*Bos taurus indicus*). A total of 420 bulls between 18 and 22 months of age were examined for breeding soundness according to the criteria established by the Brazilian College of Animal Reproduction. After physical and morphological evaluation, semen was tested with a hypoosmotic test. To accomplish this, 10 µl of fresh semen was incubated in 1 ml of hypoosmotic solution (150 mOsm/kg) for 60 min at 37°C. After the breeding soundness evaluation, 83% (350/420) of the bulls were classified as sound for breeding. There were no significant differences between the mean testicular biometry, scrotal circumference and the percentage of spermatozoa reactive to the hypoosmotic test (43.9 ± 21.1% *vs*. 43.6 ± 19.3%) in bulls classified as sound and unsound for breeding ($P > 0.05$), although there were significant differences between the averages for all physical and morphological aspects of the sperm between the two groups ($P < 0.05$). The hypoosmotic test did not correlate with the main characteristics of semen quality ($P > 0.05$). In this study, the hypoosmotic test was not an efficient predictor of the reproductive potential of young Nelore bulls.

Keywords: *Bos taurus indicus*, breeding soundness examination, complementary tests.

Introduction

After the pubertal phase, marked quantitative and qualitative changes in sperm production occur, and sperm production stabilizes when the bull reaches sexual maturity (Ellis *et al.*, 2005). An increase in semen volume, progressive sperm motility, spermatic vigor, sperm concentration and a decrease in the total number of sperm abnormalities are observed in this period (Evans *et al.*, 1995). Kennedy *et al.* (2002) used 3,648 young taurine and zebu bulls to demonstrate that low sperm quality is the most important cause of reproductive failure. Chacón *et al.* (1999) in Costa Rica found that 23.9% were deemed unsound for breeding due to poor semen quality (n = 898 taurine and zebu breeds and crossbred reared in field conditions).

The association between sperm morphology and motility in bulls with good testicular development and normal libido is among the main criteria used in the evaluation of breeding soundness (Parkinson *et al.*, 2004). However, other tests are necessary in order to identify subfertility cases (Kastelic and Thundathil, 2008).

Several methods have been developed to predict fertility. However, this prediction is difficult when the viability of spermatozoa varies widely between ejaculates. One ejaculate is not representative of a bull's reproductive life (Rodriguez-Martinez, 2006). Rodriguez-Martinez (2003) recommends several additional tests which can be used for a first screening in young animals to detect future male semen donors. The hypoosmotic swelling test is used to evaluate plasma membrane in many species: human (Jeyendran *et al*., 1984), horses (Melo and Henry, 1999; Alves *et al*., 2005), dogs (Kumi-Diaka, 1993), ovine (Oberst *et al*., 2003) and goat (Martins *et al*., 2006, 2010). The hypoosmotic swelling test is inexpensive, easy to perform and evaluates a fundamental characteristic of the plasma membrane, its biochemical integrity. The hypoosmotic swelling test involves an influx of water into the interior of the cells and consequent alteration of the cell volume and plasmatic membrane, resulting in a folding of the sperm flagellum (Jeyendran *et al*., 1984). The processes of the acrossome reaction and fertilization require a biochemically active membrane (Fraser *et al*., 2005), and relationships between high fertility and the hypoosmotic swelling test samples of frozen bull semen were found in artificial insemination programs (Revell and Mrode, 1994; Bacinoglu *et al*., 2008).

This study aimed to evaluate the relationship

[7]Corresponding author: rogerio_op@yahoo.com.br
Phone: +55(31)3891-0572
Received: June 27, 2012
Accepted: September 6, 2013

between physical semen characteristics and morphological features of sperm with the hypoosmotic swelling test in raw semen of young Nelore bulls classified as sound and unsound for breeding.

Materials and Methods

A total of 420 young Nelore bulls were examined for breeding soundness according to the criteria established by the Brazilian College of Animal Reproduction - CBRA (Manual…, 1998). The bulls were between 18 and 22 months old, with good body condition scores and were raised extensively on predominant *Brachiaria decumbens* pasture up to 14 months of age, and then confined and fed corn silage, mineral salt and water *ad libitum* until the time of the breeding soundness evaluation. The bulls were raised in confinement and were fed corn silage and sorghum and housed on a farm located in São Paulo State in Brazil (located at latitude 20-21° South and longitude 50-51° West), with an average temperature of 24ºC and an annual rainfall of 1,189 mm^3.

The vesicular glands were evaluated for size, symmetry, lobulation and consistency to detect possible changes. Testicular size was determined through the measurement of the scrotal circumference and the testicular length and width. Semen was collected by electroejaculation. Ejaculates were evaluated for physical characteristics and sperm morphology. Hypoosmotic swelling test was performed on an aliquot of semen. The physical evaluation of the semen was performed by analyzing the mass motility (0-5), progressive sperm motility (0-100%) and sperm vigor (0-5), visualized by optical microscopy.

The sperm morphology analysis was performed by phase contrast microscopy, an aliquot of the ejaculate was added to a tube containing 1 ml of saline formaldehyde-buffered solution at a volume sufficient to muddy the solution and visualized at a magnification of 1000X under a drop of immersion oil. Approximately 400 cells were counted per ejaculate sample, and sperm defects were measured as a percentage according to the classification criteria adopted by Blom (1973) and recommended by the CBRA (Manual…, 1998).

The Brazilian College of Animal Reproduction (Manual…, 1998) recommends that values above 70% for progressive sperm motility, major sperm defects below 15%, and total sperm abnormalities below 30% to rank bulls as sound for breeding. Major and minor sperm defects were classified by Blom (1973).

The hypoosmotic test was performed according to the protocol adopted by Revel and Mrode (1994), where 10 µl of semen was incubated for 1 h at 37ºC in 1 ml of hypoosmotic solution with 150 mOsm/kg (7.35 g sodium citrate and 13.51 g fructose in 1 liter of distilled water). After the incubation period, 0.5 ml of saline formaldehyde-buffered solution was added to fix the sperm, which were then examined by phase contrast microscopy. A total of 100 sperm were counted at a magnification of 1000X, and the percentage of sperm that had their tail curving along the expanded membrane was measured. The percentage of reactive spermatozoa was then calculated by subtracting the percentage of tail defects recorded in the raw semen (Melo and Henry, 1999).

For the statistical analysis, SAEG version 9.1 (2007) software was used. Descriptive analysis regarding the averages and standard deviations were performed for all parameters. The Lilliefors test was used to verify the normality of the variables. The homogeneity of variances was studied using the Cochran-Bartlett test. Analysis of variance was used to detect the differences between the animals classified as sound and unsound breeders in terms of physical and morphological features and the hypoosmotic test. The differences were detected when there was no effect with the F test (5%). The Wilcoxon test (5%) was performed to analyze the effect of breeding groups for all physical aspects studied. Pearson simple correlations (5% of significance) were performed between all variables.

Results

After the breeding soundness examination, 83% (350/420) of the young bulls were classified as sound, and 17% (70/420) were classified as unsound for breeding. There were no significant differences between the average scrotal circumference of sound and unsound animals ($P > 0.05$).

Significant differences were found between sound and unsound animals, respectively, in terms of the percentage of proximal cytoplasmic droplets (1.3 *vs*. 5.1%), acrosomal abnormalities (2.8 *vs*. 8.1%) and bent tails (3.2 *vs*. 8.4%). The percentage of tail defects observed in the morphological analysis of semen was different among the breeding soundness groups ($P < 0.05$; Table 1), with a high coefficient of variation for the trait (112.8).

No differences were found between the mean percentage of spermatozoa reactive to the hypoosmotic test in young bulls classified as sound and unsound for breeding ($P > 0.05$; Table 1). When analyzing the group of unsound bulls with the worst physical and morphologic sperm features, no differences were found between the mean percentages of spermatozoa reactive to the hypoosmotic test ($P > 0.05$; Table 1). No Pearson correlation was observed between the hypoosmotic test and variables.

Table 1. Scrotal circumference, percentage of reactive spermatozoa according to the hypoosmotic test and physical characteristics and sperm morphology aspects of raw semen of adult Nelore bulls classified as sound and unsound for breeding.

Parameters	Sound bulls	Unsound bulls	Unsound bulls (poor semen quality)	General
Number of animals	350	70	39	420
Scrotal circumference (cm)	33.5 ± 2.1^{a}	33.5 ± 2.6^{a}	33.9 ± 2.2^{a}	33.5 ± 2.2
Hypoosmotic swelling test (%)	43.9 ± 21.1^{a}	43.6 ± 19.3^{a}	44.1 ± 17.9^{a}	43.9 ± 20.8
Sperm progressive motility (%)	72.0 ± 8.6^{A}	55.4 ± 21^{B}	54.8 ± 19.5^{B}	69.0 ± 13.9
Sperm vigor (0-5)	3.0 ± 0.34^{A}	2.4 ± 0.6^{B}	2.5 ± 0.6^{B}	2.9 ± 0.46
Mass motility (0-5)	1.3 ± 1.1^{A}	0.5 ± 0.8^{B}	0.5 ± 0.7^{B}	1.2 ± 1.1
Tail defects (%)	3.0 ± 2.2^{B}	8.4 ± 8.3^{A}	10.3 ± 9.2^{A}	3.9 ± 4.4
Major defects (%)	11.6 ± 4.6^{B}	33.8 ± 18.5^{A}	42.2 ± 19.1^{A}	15.3 ± 11.9
Minor defects (%)	3.7 ± 1.8^{B}	6.2 ± 4.9^{A}	11.6 ± 10.8^{A}	4.1 ± 2.8
Total defects (%)	15.3 ± 5.4^{B}	40.1 ± 20.4^{A}	53.9 ± 17.8^{A}	19.4 ± 13.3

^{a,b,c}different lower case letters in the same line indicate a significant difference ($P < 0.05$) by the F test at 5%; ^{A,B,C}different capital letters in the same column indicate a significant difference ($P < 0.05$) by Wilcoxon 5%.

Discussion

The average scrotal circumference was considered very good for the age of the animals used in this study, according to the criteria established by the Brazilian College of Animal Reproduction (Manual…, 1998) and was higher than the averages reported by other authors for young Nelore bulls of the same age (Valentim *et al.*, 2002; Brito *et al.*, 2004; Dias *et al.*, 2006; Salvador *et al.*, 2008; Silveira *et al.*, 2010). The percentage of animals deemed sound for breeding at 18 to 22 months of age (83%) was higher than those reported by other authors. Dias *et al.* (2006) evaluated young Nelore bulls at 2 years of age and found that 26.3% were sexually mature, which is fewer than in the present experiment. Silveira *et al.* (2010) used 5,903 Nelore bulls with an average age of 21 months and found that 78.3% of the animals were sexually mature. It is noteworthy that in this experiment the animals were classified as unsound only by semen analysis.

Brito *et al.* (2004) studied sexual maturation in Nelore bulls between 18 and 22 months of age and in comparison with this study, observed a lower average of progressive sperm motility (55%) in sexually mature young bulls, although the percentages of major sperm defects (16.8%), minor defects (8.8%) and total defects (25.6%) were very similar to this study. The results obtained by Silveira *et al.* (2010) for progressive sperm motility (69.5%), major sperm defects (15.8%) and total defects (22.1%) were similar to the results of this study for the same age, breed and production system.

The average percentage of spermatozoa reactive in the hypoosmotic test was low compared to those observed by other authors. Martins *et al.* (2011) studied six adult Nelore bulls and found that 60.3% of the spermatozoa were reactive in the hypoosmotic test for raw semen and 30.8% for frozen semen, showing damage to the plasma membrane caused by the cryopreservation process. Vera-Munoz *et al.* (2009) used adult crossbred bulls and found a mean of 68.1% of spermatozoa reactive to the hypoosmotic test in raw semen and 48.8% in frozen/thawed semen. The higher average of reactive spermatozoa in this experiment can be explained by the higher functioning of the testes and epididymis in the formation and maturation of the sperm plasma membrane of adult bulls and the absence of sexual rest. The tail of the epididymis serves to keep the sperm viable and if defective, the dead sperm may release enzymes that interfere with the viability of the spermatozoa remaining in the epididymis (Jones, 2004). In addition, these enzymes may cause damage to the plasma membrane of the spermatozoa (Marengo, 2008), an event that could explain the means and low reactivity in the hypoosmotic swelling test in this experiment.

Another issue is the maturation of the spermatic plasma membrane. Lunstra and Echterncamp (1982) used a physical integrity test of the plasma membrane and observed differences between the percentage of abnormal sperm morphology in the semen before and after puberty but found no difference in the percentages of viable sperm in supravital staining. These results could be explained by the fact that the young bulls studied were in the final stages of puberty and had not yet reached sexual maturity. To support this, the most frequent types of sperm defects found in the morphological analysis were proximal cytoplasmic droplets, bent tails and acrosomal defects, sperm defects often found in young bulls at the end of puberty (Parkinson, 2004).

The results of the hypoosmotic test of raw semen were not correlated with any physical characteristics or sperm morphological features, in agreement with Martins *et al.* (2011). However, these authors observed positive correlations between the results of the hypoosmotic test of frozen semen and the progressive sperm motility, as well as between the results of the hypoosmotic test and the sperm vigor after thawing ($r = 0.38$ and $r = 0.34$, respectively). Siqueira *et al.* (2007) found a positive correlation ($r = 0.21$) when evaluating the frozen semen of adult Nelore bulls,

although this correlation was lower than the high positive correlation (r = 0.96) found by Vera-Munoz *et al.* (2009).

Most studies perform the hypoosmotic test with frozen/thawed semen to measure the biochemical integrity of the plasma membrane and obtain different results regarding the fertility of the samples in artificial insemination programs. Correa *et al.* (1997) found differences between the mean percentage of reactive spermatozoa in the hypoosmotic test of bulls classified as low and high fertility, with results of 31.7 and 41.9%, respectively. However, Bacinoglu *et al.* (2008) found no differences in hypoosmotic test results between bulls with high and low fertility in an artificial insemination program. In this experiment no differences were found between young bulls classified as sound and unsound for breeding, because they were in sexual rest and final sexual maturation stage, and these factors probably influenced the integrity of sperm plasma membrane.

The functional integrity of the sperm plasma membrane should be considered routinely in the evaluation of frozen/thawed semen and has demonstrated relationships with the high fertility of frozen bovine semen samples in artificial insemination programs (Revell and Mrode, 1994, Correa *et al.* 1997; Bacinoglu *et al.*, 2008). Spermiograms provide additional information regarding another aspect of the plasma membrane quality when compared to tests of physical integrity. The true fertilization potential of frozen samples to be marketed cannot be measured, but low quality samples can be identified using additional tests (Rodriguez-Martinez, 2006; Moce and Graham, 2008).

With these results, it was concluded that the hypoosmotic test cannot be used alone to classify young Nelore bulls as sound or unsound for breeding. Although an important complementary test in the evaluation of frozen semen, it cannot be used routinely in the examination of breeding soundness because there are no established standards for the percentage of reactive spermatozoa for the classification of the reproductive status of young Nelore bulls.

Acknowledgments

The authors are grateful to CFM Agropecuária Ltda. for their collaboration, donation of semen samples and contribution with results. The authors also thank Capes (Coordenação de Aperfeiçoamento de Pessoal de nível Superior) and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) for their financial support.

References

Alves SGG, Ribeiro Filho AL, Snoeck PPN, Chalhoub M, Bittencourt RF, Portela APM, Almeida AK, Melo MIV, Henry ME. 2005. Efeito da solução, da fixação em formol-salina e do tempo de incubação sobre os resultados do teste hiposmótico para sêmen eqüino congelado. *Ciênc Anim Bras*, 6:219-225.

Bacinoglu S, Tas M, Cirit U, Ozdas BO, Ak K. 2008. The potential fertility estimation capacity of the hypoosmotic swelling test, the thermal stress test and a modified cervical mucus penetration test in the bovine. *Anim Reprod Sci*, 104:38-46.

Blom E. 1973. The ultrastructure of some characteristic sperm defects and a proposal for a new classification of the bull spermiogram. *Nord Vet Med*, 25:383-390.

Brito LF, Silva AE, Unanian MM, Dode MAN, Barbosa RT, Kastelic JP. 2004. Sexual development in early and late maturing *Bos indicus* and *Bos indicus* x *Bos taurus* crossbred bulls in Brazil. *Theriogenology*, 62:1198-1217.

Chacón J, Pérez E, Muller E, Soderquist L, Rodriguez-Martinez H. 1999. Breeding soundness evaluation of extensively managed bulls in Costa Rica. *Theriogenology*, 52:221-231.

Correa JR, Pace MM, Zavos PM. 1997. Relationship among frozen-thawed sperm characteristics assessed via routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. *Theriogenology*, 48:721-731.

Dias, JC, Andrade VJ, Fridrich AB, Salvador DF, Vale Filho VR, Corrêa AB, Silva MA. 2006. Estimativas de parâmetros genéticos de características reprodutivas de touros Nelores, de dois e três anos de idade. *Arq Bras Med Vet Zootec*, 58:388-393.

Ellis RW, Rupp GP, Chenoweth PJ, Cundiff LV, Lunstra DD. 2005. Fertility of yearling beef bulls during mating. *Theriogenology*, 64:657-678.

Evans ACO, Davies FJ, Nasser LF, Bowman P, Rawlings NC. 1995. Differences in early patterns of gonadotrophin secretion between early and late maturing bulls and changes in semen characteristics at puberty. *Theriogenology*, 43:569-578.

Fraser, LR, Adeoya-Osiguwa S, Baxendale RW, Samra Mededovic S, Osiguwa OO. 2005. First messenger regulation of mamalian sperm function via Adenylyl cyclase/cAMP. *J Reprod Dev*, 51:37-46.

Jeyendran RS, Van Der Ven HH, Perez-Pelaez M, Grabo BG, Zaneveld 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*, 70:219-225.

Jones R. 2004. Sperm survival vesus degradation in the mammalian epididymis. a hypothesis. *Biol Reprod*, 71:1405-1411.

Kastelic JP, Thundathil JC. 2008. Breeding soundess evaluation and semen analysis for predicting bull fertility. *Reprod Domest Anim*, 43:368-373.

Kennedy SP, Spitzer JC, Hopkins FM, Higdon HL, Bridges WC. 2002. Breeding soundness evaluation of 3648 yearling beef bulls using the 1993 Society for Theriogenology guidelines. *Theriogenology*, 58:947-961.

Kumi-Diaka J. 1993. Subjeting canine semen to the hypo-osmotic test. *Theriogenology*, 39:1279-1289.

Lunstra DD, Echternkamp SE. 1982. Puberty in beef bulls. acrosome morphology and semen quality in bulls of different breeds. *J Anim Sci*, 55:638-648.

Manual para Exame Andrológico e Avaliação de Sêmen Animal. 1998. 2nd ed. Belo Horizonte, MG: CBRA. 48 pp.

Marengo SR. 2008. Maturing the sperm. Unique mechanisms for modifying integral proteins in the sperm plasma membrane. *Anim Reprod Sci*, 105:52-63.

Martins LF, Baracat-Pereira MC, Guimarães JD, Costa EP, Silveira TS, Torres CAA, Rodrigues, MT, Braz VB. 2006. Avaliação espermática e da concentração de proteínas solúveis no plasma seminal de bodes da raça Alpina em regime de monta controlada. *Rev Bras Zootec*, 35(supl):1653-1659.

Martins LF, Baracat-Pereira MC, Guimarães JD, Guimarães SEF, Pinho RO. 2010. Semen quality and concentration of soluble proteins in the seminal plasma of Alpine bucks. *Rev Bras Saúde Prod Anim*, 11:547-554.

Martins LF, Pinho RO, Paraizo RM, de Oliveira RR, Castilho EF, Guimarães JD. 2011. Avaliação de diferentes osmolaridades de soluções hiposmóticas e tempos de incubação no teste hiposmótico do sêmen de touros Nelore. *Rev Bras Zootec*, 40:2019-2025.

Melo MIV, Henry M. 1999. Teste hiposmótico na avaliação de sêmen eqüino. *Arq Bras Vet Zootec*, 51:71-78.

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

Oberst ER, Jobim MIM, Mattos RC, Kroth E, Lara G, Smiderie W, Bronzatto M. 2003. Teste hiposmótico e sua relação com outros métodos da avaliação da integridade da membrana espermática do carneiro. *Rev Bras Reprod Anim*, 27:375-376.

Parkinson TJ. 2004. Evaluation of fertility and infertility in natural service bulls. *Vet J*, 168:215-229.

Revell SG, Mrode RA. 1994. An osmotic resistance test for bovine semen. *Anim Reprod Sci*, 36:77-86.

Rodriguez-Martinez H. 2003. Laboratory semen assessment and prediction of fertility. Still utopia? *Reprod Domest Anim*, 38:312-318.

Rodriguez-Martinez H. 2006. Can we increase the estimative value of semen assessment? *Reprod Domest Anim*, 41:2-10.

SAEG: sistema para análises estatísticas. 2007. versão 9.1. Viçosa: UFV. 68 pp.

Salvador DF, Andrade VJ, Vale Filho VR, Dias JC, Nogueira LAG. 2008. Associação entre o perfil andrológico e a congelação de sêmen de touros da raça Nelore aos dois anos de idade, pré-selecionados pela classificação andrológica por pontos (CAP). *Arq Bras Med Vet Zootec*, 60:587-593.

Silveira TS, Siqueira JB, Guimarães SEF, De Paula TAR, Miranda Neto, T, Guimarães JD. 2010. Maturação sexual e parâmetros reprodutivos em touros da raça Nelore criados em sistema extensive. *Rev Bras Zootec*, 39:503-511.

Siqueira JB, Guimarães JD, Costa EP, Henry M, Torres CAA, Silva MVGB, Silveira TS. 2007. Relação da taxa de gestação com sêmen bovino congelado e testes de avaliação espermática in vitro. *Rev Bras Zootec*, 36:387-395.

Valentim R, Arruda RP, Barnabé RC, Alencar MM. 2002. Biometria testicular de touros Nelore (*Bos taurus indicus*) e touros cruzados Nelore-europeu (*Bos taurus indicus* x *Bos taurus taurus*) aos 20 e 24 meses. *Braz J Vet Res Anim Sci*, 39:113-120.

Vera-Munoz O, Amirat-Briand L, Diaz T, Vásquez L, Shcmidt E, Desherces S, Anton M, Bencharif D, Tainturier D. 2009. Effect of semen dilution to low-sperm number per dose on motility and functionality of cryopreserved bovine spermatozoa using low-density lipoproteins (LDL) extender. Comparison to Triladyl® and Bioxcell®. *Theriogenology*, 71:895-900.