



Assessment of different solutions and incubation times in the hypoosmotic swelling test of frozen/thawed semen in Piau boars

R.O. Pinho^{1,5}, H.H. Shiomi², D.M.A. Lima³, J.B. Siqueira³, M.H.C. Barros⁴, P.S. Lopes¹, S.E.F. Guimarães¹, J.D. Guimarães²

¹Animal Science Department, Federal University of Viçosa, MG, Brazil.

²Veterinary Medicine Department, Federal University of Viçosa, MG, Brazil.

³Veterinary Medicine Department, Federal University of Espírito Santo, Alegre, ES, Brazil.

⁴Minitube of Brazil, Porto Alegre, RS, Brazil.

Abstract

The objective of this study was to assess the effect of several solutions and incubation times in the hypoosmotic swelling test in predicting the *in vitro* quality of the frozen/thawed semen of Piau boars. Four samples of frozen semen from five different Piau boars were used. For the assessment of the hypoosmotic test, three incubation times were used (5, 30 and 60 min) in three different hypoosmotic solutions (BTS at 75 mOsm/Kg, sucrose at 100 mOsm/Kg and fructose and sodium citrate at 100 mOsm/Kg), thus, samples were submitted to each hypoosmotic solution in three incubation times. The means of sperm motility, spermatic vigor and live sperm by supravital staining for the assessed samples were, respectively, $37.5 \pm 7.2\%$, 2.8 ± 0.3 and $37.4 \pm 7.8\%$ in thawing and $15.5 \pm 7.6\%$, 1.7 ± 0.5 and $22.6 \pm 6.6\%$ after 120 min of thermo-resistance test ($P < 0.05$). There was no difference between the mean values obtained for reactive sperm in the different incubation times and hypoosmotic solutions, and also no interaction between time and solutions. The results obtained in the present study demonstrated that the hypoosmotic test can be used in assessing the quality of frozen/thawed semen, besides offering alternatives for hypoosmotic solutions and different incubation times in performing the hypoosmotic test to predict the sperm plasmatic membrane quality in boars.

Keywords: complementary tests, sperm, swine.

Introduction

In Brazil, Piau is considered the most important naturalized national breed, being the one with the most number of studies and published papers (Garcia and Barbosa, 1994). Therefore, the maintenance of naturalized boar breeds becomes fundamental because they have their own adaptation characteristics to the Brazilian ecosystems, besides building potential sources of new genetic variants for the national swine culture (Sollero *et al.*, 2009).

The association of motility and sperm morphology is among the main criterion used in breeding assessment (Parkinson, 2004), but the use of complementary tests is necessary to identify the cases of sub-fertility (Kastelic and Thundathil, 2008), as shown by Larsson and Rodriguez-Martinez (2000) who used complementary tests such as oocyte penetration tests in the triage of young animals and potential semen donors.

The complementary hypoosmotic test evaluates the functionality of the sperm plasmatic membranes. However, it is not used routinely in breeding soundness evaluation since there are no established parameters for the classification of breeders. Studies must still be done for a better understanding of the results, especially due to the lack of technique standardization or protocols applied in several researches (Martins *et al.*, 2011).

The capacitation process, acrosome reaction and fertilization need a biochemically active membrane (Fraser *et al.*, 2001), and the supravital and hypoosmotic tests can be used to assess spermatic membrane integrity. Different aspects in sperm plasmatic membrane are assessed in each test. The supravital staining determines if the membrane is physically damaged and the hypoosmotic test assesses the membrane's biochemical activity (Correa and Zavos, 1994).

The protocol for performing the hypoosmotic test was first proposed by Jeyendran *et al.* (1984) in humans, and has been adapted by several researchers for bovine (Correa and Zavos, 1994; Revel and Mrode, 1994; Bacinoglu *et al.*, 2008; Mocé and Graham, 2008; Martins *et al.*, 2011), equine (Melo and Henry, 1999; Melo *et al.*, 2003; Alves *et al.*, 2005) and swine (Vazquez *et al.*, 1997; Fraser *et al.*, 2001; Perez-Llano *et al.*, 2001, 2003; Samardzija *et al.*, 2008). Such studies had the objective of assessing different hypoosmotic solutions (osmolarity), in the technology imposed on the semen and incubation time at which the semen is subjected, seeking the determination of the best hypoosmotic test protocol for the several species of domestic animals.

Therefore, the present study had the objective of assessing the effect of several solutions and

⁵Corresponding author: rogerio_op@yahoo.com.br
Phone: +55(31)3899-2325

Received: February 2, 2013

Accepted: November 8, 2013



incubation times in the hypoosmotic test in predicting the *in vitro* quality of frozen/thawed semen in Piau boars.

Materials and Methods

The experiment took place at the Genetic Improvement Pig Farm of the Animal Science Department at the Federal University of Viçosa, MG (DZO/UFV). Four frozen semen ejaculates from five adult boars of the Piau breed, suitable for reproduction by breeding soundness evaluation were used, totalizing 20 samples (four ejaculates per boar).

The semen collections were made using the gloved-hand technique (Hancock and Hovell, 1959) and the aid of a mobile dummy or a female in estrous. The ejaculates were collected in disposable 700 ml plastic cups, properly sterilized, protected in thermal packages previously heated at 38°C and assembled with a filter, attached to the cup with a string in order to retain the gelatinous fraction of the semen.

Immediately after semen collections the sperm motility (expressed in percentage) and the spermatic vigor (scale from 0 to 5) were evaluated placing an aliquot of 10 µl of fresh semen between two glass slides pre-heated at 38°C. Semen was evaluated by a single person in an optic microscope.

For cryopreservation of the semen, the methodology described by Bianchi *et al.* (2008) was used. The rich fraction was diluted in 1:1 BTS (Beltsville Thawing Solution) and kept at room temperature for 60 min. Later, it was cooled at 15°C for 60 min and, at the end of this period, centrifuged at 800 G for 10 min. After centrifugation, the supernatant was discarded and the pellets were re-suspended with the freezing extender (80% of β-lactose solution, 20% of egg yolk and 100 µg/ml of canamycin sulfate; Sigma-Aldrich). The re-suspended pellet was kept at 5°C for 90 min and after this period the freezing extenders were added (72.5% lactose at 11%, 20% egg yolk, 6% glycerol and 1.5% of Orvus-es-paste; Sigma-Aldrich), until reaching a final concentration of 200×10^6 spermatozoa/straw and 1.3% of glycerol and packaged in 0.5 ml straws. After packaging, the straws were kept 5 cm above liquid nitrogen (at nitrogen vapor; -120°C) for 20 min and later submerged in liquid nitrogen (-196°C).

The semen samples were thawed at 38°C, for 20 sec and re-suspended with 1.25 ml of BTS extender (Maxwell and Johnson, 1997). The thermo-resistance test (TRT) was done by incubating the post-thawed semen at 38°C for 120 min, assessing sperm motility, spermatic vigor and percentage of live sperm by supravital staining as described below, immediately after thawing and with 120 min of testing.

For the assessment of the hypoosmotic test an

aliquot of 100 µl of the thawed semen in BTS extender was incubated at 38°C for 5, 30 and 60 min with 1 ml of three hypoosmotic solutions, BTS at 75 mOsm/Kg (Pérez-Llano *et al.*, 2001), sucrose at 100 mOsm/Kg (Barros *et al.*, 2012) and fructose and sodium citrate at 100 mOsm/Kg (Samardzija *et al.*, 2008). At the end of the incubation period the samples were fixed with 0.5 ml of buffered formal-saline solution.

Later an aliquot of 20 µl of the solution was placed between two glass slides for counting 100 sperm cells in contrast microscopy at 1000X magnification. The cells were classified by the presence or not of swollen or coiled tails and the result was determined in percentage, using the formula: Hypoosmotic (%) = (% of alterations in the caudal region after the hypoosmotic test) - (% of alterations in the caudal region before the hypoosmotic test) by sperm morphology assessment (Melo and Henry, 1999).

The assessment of live and dead cells was made by the supravital staining test using eosin (1%) and negrosin (5%) solution, according to Bamba (1988). For that, a sample of 10 µl of frozen semen was mixed with 10 µl of stain, over a pre-heated glass slide at 38°C. Next, a smear was made in the slide, and after 20 to 30 sec, a total of 100 cells were counted under optic microscopy at a 200X magnification and the ones that were not stained were classified as living cells, and those that became pink-red were classified as dead.

The statistical analysis was done using the statistical program SAEG 9.1 (SAEG, 2007). A descriptive statistical design was done (mean, standard-deviation) for all of the studied characteristics. The quantitative data were evaluated by analysis of variance (ANOVA), and when there was significance by the “F” test, the means were compared by the SNK test, with a 5% error probability. The Pearson simple correlation analysis was applied to verify the relation of the seminal characteristic and the complementary tests.

Results

The means of sperm motility, spermatic vigor and live sperm by supravital staining for the samples assessed at thawing were $37.5 \pm 7.2\%$; 2.8 ± 0.3 and $37.4 \pm 7.8\%$; respectively and $15.5 \pm 7.6\%$; 1.7 ± 0.5 and $22.6 \pm 6.6\%$ after 120 min of TRT ($P < 0.05$), with a mean reduction in motility of $22 \pm 9.2\%$, in spermatic vigor of 1.1 ± 0.6 and in live sperm of $14.8 \pm 9.8\%$ from 0 to 120 min after thawing.

There was no difference between the mean values obtained for the reactive sperm cells in the different incubation times and the studied hypoosmotic solutions (Table 1), as well as no interaction between the times and solutions ($P > 0.05$).



Table 1. Hypoosmotic swelling tests in frozen/thawed semen of Piau boars, according to different incubation times and hypoosmotic solutions*.

Incubation times (minutes)	Solutions			
	BTS (Beltsville Thawing Solution)	Sucrose	Fructose + Sodium citrate	General
5	22.7 ± 12.4	20.1 ± 12.2	23.3 ± 9.5	22.0 ± 11.3
30	22.7 ± 11.5	24.4 ± 11.3	24.4 ± 12.6	23.8 ± 11.7
60	21.8 ± 9.5	26.0 ± 11.1	25.3 ± 11.2	24.4 ± 10.6
General	22.4 ± 11.0	23.5 ± 11.6	24.4 ± 11.0	23.4 ± 11.2

*ANOVA ($P > 0.05$); Percentages of mean ± sd = Mean and standard deviation.

There was a high correlation of sperm motility and live sperm ($r = 0.52$) and medium to high correlation with the results of the hypoosmotic test performed with BTS ($r = 0.48$) and sucrose solution ($r = 0.53$), while no correlation was observed when fructose or citrate solution were used. Regarding the mean values obtained in the supravital staining, there was only correlation with the results from the BTS solution ($r = 0.40$).

Discussion

In Piau breed, Barros *et al.* (2012) obtained higher mean values of motility ($49.5 \pm 12.1\%$) and vigor (3.4 ± 0.4) in relation to the post-thawing semen, although with similar results on live sperm ($39.9 \pm 10.4\%$) and lower hypoosmotic results ($18.8 \pm 5.7\%$) when compared to those of the present study (Table 1). In swine, some authors have used the TRT to assess the fertility of frozen/thawed semen in several freezing protocols, incubating the semen at 37°C for a period from 2 to 4 h (Larsson and Einarsson, 1976; Bwanga *et al.*, 1991; Ohata *et al.*, 2001; 2005; Peláez *et al.*, 2006; Roca *et al.*, 2006).

Similarly to the present study, Ohata *et al.* (2001, 2005) and Bianchi *et al.* (2008), working with commercial lineage swine, observed reduction in motility and vigor, but with higher values after thawing and after 120 min of incubation. Decrease in motility of the sperm during TRT was also seen by Bwanga *et al.* (1991) for frozen semen in 0.25 and 0.5 ml straws and by Peláez *et al.* (2006) in incubation periods from 2 to 4 h at 37°C , respectively. The decrease in sperm motility seen at the end of TRT demonstrates great sensibility of the swine sperm to the cryopreservation process, which can be due to the loss of intracellular components (water, ATP, potassium, and/or enzymes), or structural lesions in the sperm tails.

Pérez-Llano *et al.* (2001, 2003) did not observe differences regarding the incubation times used. In the same manner, Vazquez *et al.* (1997) and Alves *et al.* (2005) did not see differences in the results of hypoosmotic test in relation to the incubation times and solutions used in working with swine and equine semen, respectively. Martins *et al.* (2011) did not see differences in relation to the incubation times in bovine.

In an assessment study of equine semen conducted by Melo and Henry (1999), correlations of 0.70

were recorded between the two tests mentioned above (supravital and hypoosmotic), besides a correlation between each of the tests and physical parameters of the semen (Supravital x physical parameters = 0.75; Hypoosmotic x physical parameters = 0.68).

In bovine, Zúccari *et al.* (2009) observed a correlation of 0.59 between eosin/nigrosin and hypoosmotic tests, besides correlations of sperm motility with the hypoosmotic test of 0.78 and with eosin/nigrosin of 0.52. Samardzija *et al.* (2008) on the other hand, using different osmolarities of the hypoosmotic solution in the assessment of Yorkshire and Pietrain breeds semen, observed higher correlations between sperm motility and live sperm by supravital staining ($r = 0.98$) and with the hypoosmotic test ($r = 0.99$), the hypoosmotic test, and the live sperm ($r = 0.98$), observing better results for the fructose-citrate solution with osmolarity of 100 mOsm/kg. However, Barros *et al.* (2012) did not find any correlation between supravital and hypoosmotic tests. These medium and high correlations were expected, being that the sperm motility depends partially on the functional integrity of the sperm membrane, and partially on other biochemical activities, as well as the spermatid metabolism (Jeyendran *et al.*, 1984).

The variation on correlations between the hypoosmotic test and other parameters assessed in the several studies done by other authors is probably due to the property differences in the applied tests and evaluations (Barros *et al.*, 2012). According to Vazquez *et al.* (1997), the hypoosmotic test assesses the functional integrity of the plasmatic membrane (based on the osmotic properties of the plasmatic membrane), while the supravital staining shows the structural integrity of the spermatid membrane (based on the permeability of the great molecules of the stains).

Still according to Vazquez *et al.* (1997), the hypoosmotic test used with other membrane integrity tests is an important complementary analysis in the detection of alterations in the plasmatic membrane in swine sperm given that some are considered with intact membrane in the supravital staining and do not maintain biochemical activity of the plasmatic membrane when incubated in a hypoosmotic solution.

Since there was no difference between the incubation times and solutions, it is possible to use all the solutions and incubation times in performing the



test, without alterations in the assessment of swine sperm membranes, also indicating the reactive sperm coil of the tail at the beginning of incubation, needing no longer than 5 minutes for the hypoosmotic test. Therefore, the use of BTS solution for the hypoosmotic test would be more practical and less costly, since it is an extender used routinely in pig farms that manage artificial insemination.

The results obtained in the present study show that the hypoosmotic test can be used in the assessment of the quality of frozen/thawed semen, besides offering alternatives for hypoosmotic solutions and different incubation times in performing the hypoosmotic test to predict the in vitro quality of the plasmatic membrane in the sperm of Piau boars.

Acknowledgments

To Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), for their financial support.

References

- Alves SGG, Ribeiro Filho AL, Snoeck PPN, Chalhoub M, Bittencourt RF, Portela AP, Almeida AK, Melo MIV, Henry M. 2005. Efeito da solução, da fixação em formol-salina e do tempo de incubação sobre os resultados do teste hiposmótico para sêmen equino congelado. *Ciênc Anim Bras*, 6:219-225.
- Bacinoglu S, Tas M, Cirit U, Ozdas O, 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.
- Bamba K. 1988. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. *Theriogenology*, 29:1245-1251.
- Barros MHC, Shiomi HH, Amorim LS, Guimarães SEF, Lopes PS, Siqueira JB, Guimarães JD. 2012. Criopreservação de sêmen de suínos da raça Piau submetido a três protocolos de congelamento. *Rev Bras Zootec*, 41:914-922.
- Bianchi I, Calderam K, Maschio EF, Madeira EM, Ulguim RR, Rambo G, Corrêa EK, Júnior TL, Deschamps JC, Corrêa MN. 2008. Inseminação artificial intra-uterina em leitoas com sêmen criopreservado com dimetilacetamida e glicerol. *Ciênc Rural*, 38:1978-1983.
- Bwanga CO, Einarsson S, Rodríguez-Martínez H. 1991. Cryopreservation of boar semen: II. Effect of cooling rate and duration of freezing point plateau on boar semen frozen in mini- and maxi-straws and plastic bags. *Acta Vet Scand*, 32:455-461.
- Correa JR, Zavos PM. 1994. The hypoosmotic swelling test: its employment as assay to frozen-thawed bovine sperm membrane. *Theriogenology*, 42:351-360.
- Fraser L, Gorszcaruk K, Strzezek J. 2001. Relationship between motility and membrane integrity of boar spermatozoa in media varying in osmolality. *Reprod Domest Anim*, 36:325-329.
- Garcia SK, Barbosa AS. 1994. Características etológicas, biométricas e seminais de varrões da raça Piau. *Arq Bras Vet Zootec*, 46:279-289.
- Hancock JL, Hovell GJR. 1959. The collection of boar semen. *Vet Rec*, 71:664-665.
- Jeyendran RS, Van Der Ven HH, Perez-Pelaez M, Crabo 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*, 70:219-228.
- Kastelic JP, Thundathil JC. 2008. Breeding soundness evaluation and semen analysis for predicting bull fertility. *Reprod Domest Anim*, 43:368-373.
- Larsson K, Einarsson S. 1976. Influence of boars on the relationship between fertility and post thawing sperm quality of deep frozen boar spermatozoa. *Acta Vet Scand*, 17:74-82.
- Larsson B, Rodríguez-Martínez H. 2000. Can we use in vitro fertilization tests to predict semen fertility? *Anim Reprod Sci*, 61:327-336.
- Martins LF, Pinho RO, Paraizo RM, 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*, 40:1519-1525.
- Maxwell WMC, Johnson LA. 1997. Membrane status of boar spermatozoa after cooling or cryopreservation. *Theriogenology*, 48:209-219.
- Melo MIV, Henry M. 1999. Teste hiposmótico na avaliação de sêmen equino. *Arq Bras Vet Zootec*, 51:71-78.
- Melo MIV, Snoeck PPN, Bispo C, Henry M. 2003. Efeito da solução e do tempo de incubação sobre os resultados do teste hiposmótico para o sêmen equino congelado. *Rev Bras Reprod Anim*, 27:379-380.
- Mocé E, Graham JK. 2008. In vitro evaluation of sperm quality. *Anim Reprod Sci*, 105:104-118.
- Ohata PM, Wentz I, Bernardi ML, Castagna C, Bortolozzo FP. 2001. Viability of frozen swine semen submitted to a pre-freezing equilibrium time in the presence or absence of seminal plasma. *Arq Fac Vet*, 29:123-129.
- Ohata PM, Bernardi ML, Reis GR, Bortolozzo FP, Wentz I. 2005. Congelabilidade do sêmen suíno de acordo com o período de equilíbrio pré-congelamento e da sensibilidade ao resfriamento. *Arch Vet Sci*, 10:69-74.
- Parkinson TJ. 2004. Evaluation of fertility and infertility in natural service bulls. *Vet J*, 168:215-229.
- Peláez J, Breininger E, Alegre B, Peña FJ, Domínguez JC. 2006. In vitro evaluation of the quality and fertilizing capacity of boar semen frozen in 0,25 ml straws. *Reprod Domest Anim*, 41:153-161.
- Pérez-Llano B, Lorenzo JL, Yenes P, Trejo A, García-Casado P. 2001. A short hypoosmotic swelling test for the prediction of boar sperm fertility.



Theriogenology, 56:387-398.

Pérez-Llano B, Yenes P, García-Casado P. 2003. Four subpopulations of boar spermatozoa defined according to their response to the short hypoosmotic swelling test and acrosome status during incubation at 37°C. *Theriogenology*, 60:1401-1407.

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

Roca J, Hernández M, Carvajal G, Vázquez JM, Martínez EA. 2006. Factors influencing boar sperm cryosurvival. *J Anim Sci*, 84:2692-2699.

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

Samardžija M, Dobranic T, Kruslin S, Cergolj M, Karadžole M, Prvanović N, Grizelj J. 2008. The use of the hypoosmotic swelling test and supravital staining

in evaluation of sperm quality in boars. *Vet Arh*, 78:279-287.

Sollero BP, Paiva SR, Faria DA, Guimarães SEF, Castro STR, Egito AA, Albuquerque MSM, Piovezan U, Bertani GR, Mariante AS. 2009. Genetic diversity of Brazilian pig breeds evidenced by microsatellite markers. *Livest Sci*, 123:8-15.

Vázquez JM, Martínez EA, Martínez P, García-Artiga C, Roca J. 1997. Hypoosmotic swelling of boar spermatozoa compared to other methods for analysing the sperm membrane. *Theriogenology*, 47:913-922.

Zúccari CESN, Leite PA, Passos TS, Carrijo PR, Kiefer C. 2009. Correlação entre métodos de avaliação da integridade da membrana plasmática do espermatozóide bovino criopreservado. *Rev Bras Saúde Prod Anim*, 10:678-684.