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The use of the hypoosmotic swelling test and supravital staining in evaluation of sperm quality in boars

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ABSTRACT

Semen samples from ten boars, Yorkshire and Pietrain breed, aged between two and six years were used. The sperm quality was evaluated using the following parameters: the motility (subjective), the structural supravital staining (eosine/nigrosine) and the functional integrity of sperm membrane (HOS test). The majority of HOS reactive or swelled up spermatozoa were gained in a hypoosmolar solution of 100 mOsm/kg by incubation of 30 minutes. Between middle values for HOS reactive (swollen up) spermatozoa in hypoosmolar solutions with different osmolarity, significant differences ($P<0.05$) were determined. Using a hypoosmotic solution with different osmolarity 100 mOsm/kg, significantly strong, positive correlations between HOS and motility ($r = 0.98$; $P<0.05$) were determined, between HOS test and supravital staining eosine/nigrosine ($r = 0.99$; $P<0.05$) and between motility and supravital staining eosine/nigrosine ($r = 0.98$; $P<0.05$). The results of our research have shown that the HOS test and supravital staining eosine/nigrosine could be used for routine evaluation of the functional and structural integrity of diluted boar sperm.

Key words: boar, semen, hypoosmotic test, eosine/nigrosine staining

Introduction

Standard methods of semen analysis provide basic information on spermatogenesis, sperm cell longevity and fertility of the analysed individuals (MADEJA et al., 2003). In any case, the standard parameters used in evaluation of fertility in males have limited

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capacity for prediction of their fertilizing potential (AMANN, 1989). For a long time evaluation of sperm motility was considered to be a reliable sperm quality indicator (KJAESTAD et al., 1993). However, the best evaluation of fertility can be achieved with a combination of results from evaluation of plasma membrane integrity and motility (HENKEL et al., 1993). Because of the great importance of sperm membrane in fertilization, considerable attention is given to the membrane integrity in spermiogram evaluation. To evaluate the plasma membrane integrity, two simple basic tests are used: supravital staining (eosine/nigrosine) and the hypoosmotic swelling test (HOS) (CURRY and WATSON, 1994). The commonly used staining test (eosine/nigrosine) for assessing sperm membrane measures only its structural integrity. Some physiological processes during fertilization (capacitation, acrosomal reaction, fusion of sperm and ovum) demand active membrane, and it is impossible to have fertilization with physically inactive membrane (JEYENDRAN et al., 1984). JEYENDRAN et al. (1984) started to use HOS in evaluation of plasma membrane functional integrity in humans. After that, the HOS test was used in sperm quality evaluation of dogs (KUMI-DIAKA, 1993; DOBRANIĆ et al., 2005), bulls (SAMARDŽIJA et al., 2005; SAMARDŽIJA et al., 2006), rams (WATSON and DUNCAN, 1988), boars (RODRIGUEZ-GIL and RIGAU, 1996) and stallions (VIDAMENT et al., 1998). The HOS test is based on the swelling ability of functioning sperms after being exposed to the hypoosmotic solution (CABRITA et al., 1999). In the hypoosmolar solution, fluid is transfered into the cell through plasma membrane of spermatozoa. Trying to achieve a balance between intracellular and extracellular spaces, functionally intact membranes begin to swell starting at the tail of the spermatozoa. Such spermatozoa are denoted as swelled or HOS reactive (HOS+) signifying functionally intact membranes. Spermatozoa with functionally defected membrane do not swell and their tails do not invaginate (JEYENDRAN et al., 1984). The osmolarity of the solution should be sufficient to provoke the best effect without the lysis of the sperm membrane (ROTA et al., 1999). Fertilization of oocyte will not happen if the sperm membrane is biochemically inactive even if it stays structurally intact, so the HOS test is a better indicator of fertilization potential than supravital staining (TAMULI and WATSON, 1992). Supravital staining is based on the fact that the membranes of dead spermatozoa allow the passage of the red stain into the cytoplasm, but membranes of live spermatozoa do not allow this. This means that all dead spermatozoa in ejaculate will be coloured while live spermatozoa will stay colorless (HERAK, 1991). The objective of this study was to verify the effectiveness of the HOS test in boars and compared the HOS test and supravital staining (eosine/nigrosine) in evaluation of sperm quality in boars.

Material and methods

In our research we used the semen from ten sexually mature boars of Yorkshire and Pietren breeds between the ages of 2 and 6 years. The boars were owned by private

pig farm "Dubravica, Zaprešić, Croatia". All boars were used in reproduction. After collection of the semen, in eight boars a dramatically low percentage of sperm motility was noticed. Because of that they sent us ejaculates of all eight problematic boars and also ejaculates from the two boars with normal, physiologic percentage of motility, as a control. Semen was collected using manual fixation of penis. The second and the third fraction of ejaculates were collected for the evaluation of the spermiogram. After semen collection ejaculates were diluted by helatonic dilution and sent to us for analyses.

Sperm evaluation. The parameters that were evaluated immediately after collection included: motility, and evaluation of plasma membrane structural and fuctional integrity.

The motility was determined by taking a drop of the semen on a warmed glass slide (37 °C) using a light microscope.

The sperm morphology and live/dead status was determined according to Bloom's method (eosine/nigrisine) (HERAK, 1991): 5% solution of eosine and 10% solution of nigrosinewere used. On clean, warmed glass (to avoid temperature shock) one drop of sperm (5 μL), two drops of eosine (10 μL) and four drops of nigrosine (20 μL) were put together, the solutions were mixed and then the smears were made for microscopic examination, using a phase contrast microscope at 10×, 40× and oil immersion magnifications.

Evaluation of the functional integrity of sperm membrane was determined by HOS test. Before the test the efficacy of the HOS test was checked using a fructose solution and Na-citrate solution with different osmolarity (100, 150, 200, 250 and 300 mOsm/kg). It enabled us to obtain a hypoosmolar solution with optimal osmolarity which would give us the most HOS reactive (HOS+) spermatozoa. After that mixtures of sperm and hypoosmolar solution were incubated at 35 °C and 37 °C at 15 minute intervals, for a total of 120 minutes. This was done to determine the time and temperature needed for the most HOS reactive spermatozoa. The experiment was repeated three times in order to obtain a consistent result. Based on the preliminary results, a hypoosmolar solution was chosen of 100 mOsm/kg and an incubation temperature of 37 °C for 30 minutes to be used in our studies. The HOS was performed, according to the protocol of JEYENDRAN et al. (1984). Briefly, 50 μL of semen was added to 1 mL of 100 mOsmol solution, mixed and incubated at 37 °C for 60 minutes. A drop of incubated suspension was put onto a glass slide, covered with coverslip and examined at 30×, 40× microscope magnification. At least 200 sperm cells were counted on each slide.

Statistical analysis of data. The results of the research were processed by the ANOVA statistical method, Tukey HSD tests of post-hoc analysis and also correlations between the sperm parameters were determined.

Results

The results of this study are summarized in Tables 1 and 2 and Figs 1 and 2. Evaluating the sperm parameters, at first, we were focused on evaluation of the motility and then we determined the structural membrane integrity by supravital staining by Bloom (eosine/nigrosine), and the functional integrity of membrane using the HOS test. The value of the motility was between 20-90%, averaging 44%. The eosine/nigrosine supravital staining method gave an average of 34.78% yielded spermatozoa with structurally intact plasma membrane. Using the hypoosmolar solution with osmolarity of 100 mOsm/kg in the HOS test we obtained values from 6.18% to 42.35% with functionally intact plasma membrane and the mean value was 16.96%. Correlations between the HOS test, eosine/nigrosine supravital staining and the sperm motility are shown in Figs 1 and 2.

Table 1. Mean percentage of HOS reactive sperms in response to hypoosmotic solutions with varying osmolarities

	Osmolarity (mOsm/kg)				
Hipoosmotic	100	150	200	250	300
Solution	11.89%*	10.45%	10.89%	10.08%	10.34%

P<0.05*

Table 2. Sperm quality parameters

Boar	motility %	eosine/nigrosine % live	HOS % active
1	30	23.75	11.64
2	30	24.51	9.15
3	20	18.87	6.18
4	20	16.89	7.79
5	40	19.83	7.97
6	30	24.76	10.29
7	40	29.35	18.42
8	40	26.98	14.87
9	90	79.67	40.89
10	90	82.15	42.35
Mean	43	34.68	16.96
SEM	8.17	7.8	4.27

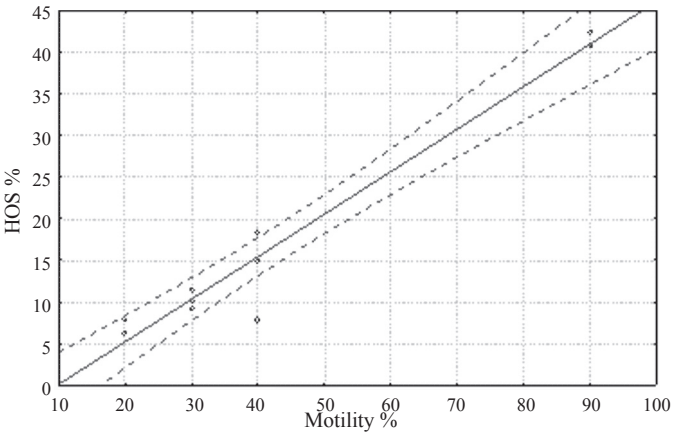

Fig. 1. The correlation between the motility and HOS test

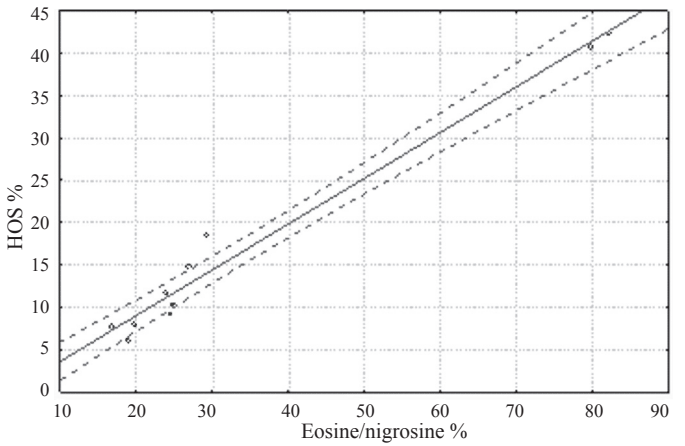

Fig. 2. The correlation between eosine/nigrosine staining and HOS test

Discussion

The development of simple tests to assess functional activity of spermatozoa should be useful in the diagnosis of sperm quality. Correlation between the sperm fertility and motility is pretty controversal, most likely due to different test conditions (GADEA et al., 1998). One of the most important aims in spermiology is the research of new methods in sperm evaluation, to discover their characteristic changes, that would indicate infertility or subfertility (HAMMERSTEDT, 1996). The functional and structural integrity of sperm

membrane are crucial for the viability of spermatozoa (LECHNIAK et al., 2002). Many authors consider that the best evaluation of fertility can be achieved with evaluation of sperm membrane integrity (HENKEL et al., 1993). During spermatogenesis spermatozoa acquire hard capsules which also give them a defined shape, and during maturation in the epididimis, that capsule is covered by a thin lipoprotein membrane. The quality of the capsule determines the viability and vitality of spermatozoa. We usually use supravital staining to evaluate fresh semen, but it is also possible to use it for diluted semen, to determine the percentage of live spermatozoa. Similarly, we can determine different abnormal shapes of the spermatozoa (HERAK, 1991). The HOS test has been used to evaluate functional integrity of plasma membrane of spermatozoa. The HOS test has much better efficacy than supravital (eosine/nigrosine) staining. The evaluation of functional status of the sperm membrane by the HOS test is a better indicator of their fertilization capacity (ZAVOS, 1990). The HOS test can indicate whether the sperm membrane is biochemically active or not (VAZQUEZ et al., 1997). In a hypoosmolar solution, liquid is transferred into the cell through the plasma membrane of the spermatozoa. Trying to achieve a balance between intra and extracellular space, the functionally intact membrane swells, beginning with the fibres in the sperm tail. As the membranes swell, the spermatozoa curl and invaginate (JEYENDRAN et al., 1984). Osmolarity of the solution should be sufficient to induce the best effect without lysing the sperms. Different solutions and osmolarites are detected for samples of fresh and frozen-thawed sperm of bulls (ROTA et al., 1999), stallions, bucks (NEILD et al., 1999) and dogs (KUMI-DIAKA, 1993; DOBRANIĆ et al., 2005). In the present study the largest number of swelled spermatozoa (HOS reactive) was observed in the hypoosmolar solution of fructose and Na-citrate with osmolarity of 100 mOsm/kg. This is in agreement with CABRITA et al. (1999) who obtained a result of 100 mOsm/kg in a maximum number of clearly identifiable swollen spermatozoa and maintained sperm membrane integrity during the assay in the apparatus. Between the middle values of swelled spermatozoa in hypoosmolar solutions of different osmolarity significant differences ($P<0.05$) were determined; which agrees with the results given by (KUMI-DIAKA, 1993; DOBRANIĆ et al., 2005) for dog sperm, and also for the results given by NEILD et al. (1999) for stallion sperm. In our research very high positive correlations between the sperm quality parameters was established. High correlations between the HOS reactive, morphologically normal and progressively motile spermatozoa were expected because sperm motility partially depends on the functional integrity of the membrane and partially on other biochemical activities such as sperm metabolism (JEYENDRAN et al., 1984). CABRITA et al. (1999) analysed the results of the HOS test and the percentage of live sperms after stain dye Hoechst 33258. They found a good correlation between them ($r = 0.65$), what is in agreement with our results ($r = 0.99$). The results of average motility of 43.0%, obtained in our research, are significantly lower than the results of ZOU and YANG (2000) who gained an average of 92% motile spermatozoa, and BAMBA

(1988) with average motility of 93.1 %. Those results were expected, since the 80% of the ejaculate used in our research were from the problematic boars. The mean HOS score (16.96%) observed in this experiment was lower than previously reported for boar ZOU and YANG (2000) (59.5%) and VAZQUEZ et al. (1997) (42.2 till 68.3%). Also, results higher than ours (34.68%), but lower than the mentioned authors were obtained by LECHNIAK et al. (2002). These authors explained this as due to the fact that during the HOS procedure spermatozoa are subjected to stress conditions and the test selects cells with the most resistant membranes. Our lower HOS values in our research are due to ejaculates of problematic boars, 8 out of 10 (80%). Results congruent with the results of our research r = 0.98 were given by KUMI-DIAKA (1993) and DOBRANIĆ et al. (2005) who determined efficacy of the HOS test and supravital staining in dogs and found a high correlation between the percentage of motile spermatozoa and HOS reactive spermatozoa r = 0.94 and r = 0.82, respectively. In determining the efficacy of the HOS test for bull sperm together with supravital staining, CORREA and ZAVOS (1994) found a high correlation between the percentage of spermatozoa with structurally intact membrane and HOS reactive spermatozoa (r = 0.81). The results of our studies are in agreement with these observations (r = 0.99; $P<0.05$). The mean results of live sperm after eosine/ nigrosine supravital staining were lower than the results obtained by GADEA and MATAS (2000). However, by comparing their values of live spermatozoa after eosine/nigrosine staining (between 81.07 till 83.57%) with the results of 2 normal ejaculates used in our research as a control, we established values similar to ours (79.87 and 82.15%). The results of our research show that the HOS test and supravital staining eosine/nigrosine could be used for routine evaluation of the functional and structural integrity of boars' diluted sperm.

References

AMANN, R. P. (1989): Can the fertility potential of a semen sample be predicted accurately? J. Androl. 10, 89-98.

BAMBA, K. (1988): Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. Theriogenol. 29, 1245-1251.

CABRITA, E., R. ALVAREZ, E. ANEL, M. P. HERRAEZ (1999): The hypoosmotic sweelling test performed with coulter counter: a method to assay functional integrity of sperm membrane in rainbow trout. Anim. Reprod. Sci. 55, 279-287.

CORREA, J. R., P. M. ZAVOS (1994): The hypoosmotic swelling test: Its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. Theriogenol. 42, 351-360.

CURRY, M. R., P. F. WATSON (1994): Osmotic effects on ram and human sperm membranes in relation to thawing injury. Cryobiol. 31, 39-46.

DOBRANIĆ, T., M. SAMARDŽIJA, M. CERGOLJ, N. PRVANOVIĆ (2005): Determination of membrane integrity on dogs sperm. Vet. arhiv 75, 23-30.

GADEA, J., C. MATAS, X. LUCAS (1998): Prediction of porcine semen fertility by homologous *in vitro* penetration (hIVP) assay. Anim. Reprod. Sci. 56, 95-108.

GADEA, J., C. MATAS (2000): Sperm factors related to *in vitro* penetration of porcine oocytes. Theriogenol. 54, 1343-1357.

HAMMERSTEDT, R. H. (1996): Evaluation of sperm quality: identification of the subfertile male and courses of action. Anim. Reprod. Sci. 42, 77-87.

HENKEL, R., C. MÜLLER, W. MISKA, H. GIPS, W.-B. SCHILL (1993): Determination of the acrosome reaction in human spermatozoa is predictive of fertilization *in vitro*. Hum. Reprod. 8, 2128-2132.

HERAK, M. (1991): Umjetno osjemenjivanje domaćih životinja; Reprodukcija domaćih životinja. In: Veterinarski priručnik, pp. 181-209.

JEYENDRAN, R. S., H. H. VAN DER VEN, M. PEREZ-PELAEZ, B. G. CRABO, L. J. D. ZANEVELD (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.

KJAESTAD, H., E. ROPSTAD, K. ANDERSEN BERG (1993): Evaluation of spermatological parameters used to predict the fertility of frozen bull semen. Acta. Vet. Scand. 34, 299-303.

KUMI-DIAKA, J. (1993): Subjecting canine semen to the hypo-osmotic test. Theriogenol. 39, 1279-1289.

LECHNIAK, D., A. KEDZIERSKI, D. STANISLAWSKI (2002): The use of HOS test to evaluate membrane functionality of boar sperm capacitated *in vitro*. Reprod. Dom. Anim. 37, 379-380.

MADEJA, Z., M. WAROCZYK, T. STRABEL, D. LECHNIAK (2003): Use of the hypo-osmotic swelling test for evaluating bull and boar semen quality. Medycyna Weterynaryjna 59, 1115-1118.

NEILD, D., G. CHAVES, M. FLORES, N. MORA, M. BECONI, A. AGUERO (1999): Hypoosmotic test in equine spermatozoa. Theriogenol. 51, 721-727.

RODRIGUEZ-GIL, J. E., T. RIGAU (1996): Effects of ouabain on the response to osmotic changes in dog and boar spermatozoa. Theriogenol. 45, 873-883.

ROTA, A., N. PENZO, L. VINCENTI, R. MANTOVANI (1999): Hypoosmotic swelling (HOS) as a screening assay for testing *in vitro* fertility of bovine spermatozoa. Theriogenol. 53, 1415-1420.

SAMARDŽIJA, M., M. KARADJOLE, M. CERGOLJ, A. TOMAŠKOVIĆ, T. DOBRANIĆ, I. GETZ, M. MATKOVIĆ, J. PETRIĆ, J. ŠURINA, N. PRVANOVIĆ, T. KARADJOLE, D. GRAČNER, V. DOBRANIĆ (2005): Vergleich zweier Aufbereitungsmethoden des Bullenspermas zur In-vitro-fertilization. Tierärztl. Umschau 60, 193-199.

SAMARDŽIJA, M., M. KARADJOLE, I. GETZ, Z. MAKEK, M. CERGOLJ, T. DOBRANIĆ (2006): Effects of bovine spermatozoa preparation on embryonic development *in vitro*. Reprod. Biol. Endocrinol. 4, 58.

TAMULI, M. K., P. F. WATSON (1992): Effect of temperature of incubation on the development of resistance to cold stress and hypoosmotic stress in boar spermatozoa incubated for up to 24 hours. Proc. 12th Int. Cong. Anim. Reprod. 1484-1486.

VAZQUEZ, J. M., E. A. MARTINEZ, P. MARTINEZ, C. GARCIA-ARTIGA, J. ROCA (1997): Hypoosmotic swelling of boar spermatozoa compared to other methods for analysing the sperm membrane. Theriogenol. 47, 913-922.

VIDAMENT, M., E. COGNARD, J.-M. YVON, M. SATTLER, E. PALMER, M. MAGISTRINI (1998): Evaluation of stallion semen before and after freezing. Reprod. Dom. Anim. 33, 271-277.

WATSON, P. F., A. E. DUNCAN (1988): Effect of salt concentration and unfrozen water fraction on the viability of slowly frozen ram spermatozoa. Cryobiol. 25, 131-142.

ZAVOS, P. M. (1990): Hypoosmotic swelling test (HOS) / functional integrity of sperm membrane. J. Ass. Reprod. Technol. Androl. 2, 215-216.

ZOU, CHUN-XIA, ZENG-MING, YANG (2000): Evaluation on sperm quality of freshly ejaculated boar semen during *in vitro* storage under different temperatures. Theriogenol. 53, 1477-1488.

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SAŽETAK

U istraživanju su rabljeni ejakulati deset nerasta Yorkshir i Pietren pasmine, u dobi od dvije do šest godina koji su imali nizak indeks oplodnje. Kakvoća sperme ocjenjivana je na osnovi pokretljivosti (subjektivno) te strukturnog i funkcionalnog integriteta membrane spermija (supravitalno bojenje eozin/nigrozinom i HOS testom). Najveći broj HOS reaktivnih, tj. nabubrenih spermija ustanovljen je u hipoosmotskoj otopini od 100 mOsm/kg pri inkubaciji od 30 minuta. Između srednjih vrijednosti HOS reaktivnih (nabubrenih) spermija u hipoosmotskim otopinama različitih osmolarnosti ustanovljene su značajne razlike ($P<0,05$). Korištenjem hipoosmotske otopine osmolarnosti 100 mOsm/kg, ustanovljene su značajne, vrlo jake, pozitivne korelacije između HOS testa i progresivne pokretljivosti ($r = 0.98$; $P<0,05$), zatim između HOS testa i supravitalnog bojenja eozin/nigrozinom ($r = 0,99$; $P<0,05$), te između progresivne pokretljivosti i supravitalnog bojenja eozin/nigrozinom ($r = 0,98$; $P<0,05$). Rezultati ovog istraživanja pokazali su da su HOS test i supravitalno bojenje eozin/nigrozinom jednostavne metode koje se mogu rutinski primjenjivati za ocjenu funkcionalnog i strukturnog integriteta sperme nerasta.

Ključne riječi: sperma, nerast, hipoosmotski test, eozin/nigrozin bojenje, kakvoća sperme