

Determination of membrane integrity of canine spermatozoa

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ABSTRACT

Semen samples from 9 sexually mature dogs of different breeds, aged from 1 to 5 years were used. Quality of sperm was evaluated using the following parameters: progressive motility (subjective), then structural and functional integrity of spermal membrane (supravital colouring (eosine/nigrosine) and HOS test). The majority of HOS reactive or swollen spermatozoa was placed in a hypoosmolar solution of 100 mosmol and incubated for 30 minutes. Results of research are elaborated by the ANOVA statistical method and Tukey HSD tests post-hoc analysis. We also determined correlations between semen parameters. Between middle values for HOS reactive (swollen) spermatozoa in hypoosmolar solutions with different osmolarity significant differences were determined ($P < 0.0001$). Using a hypoosmotic solution with osmolarity 100 mosmol, we determined significantly strong positive correlations between HOS test and progressive motility ($r = 0.82$; $P < 0.05$), between HOS test and supravital staining eosine/nigrosine ($r = 0.98$; $P < 0.05$), and between progressive motility and supravital staining eosine/nigrosine ($r = 0.89$; $P < 0.05$). These positive correlations were anticipated because sperm motility partially depends on the functional integrity of the sperm membrane and other events included in the metabolism of the sperm. Results of our research have shown that HOS test can be an easy method for routine evaluation of sperm quality in dogs.

Key words: canine semen, progressive motility, HOS-test, membrane integrity

Introduction

Standard diagnostic evaluation of sperm is primarily based on physical parameters such as progressive motility, sperm morphology and sperm concentration in the semen (KEEL and WEBSTER, 1990). In any case, those standard parameters as used in evaluation of fertility in males have a limited capacity for prediction of their fertilizing potential (AMANN, 1989).

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Sperm motility has been the most common examined parameter for spermatozoa quality and viability, although its relation to fertilization capacity of sperm is often contradictory (KJAESTAD et al., 1993). 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). Due to the great importance of sperm membrane in fertilization, considerable attention is given to membrane integrity in spermiogram evaluation. To evaluate plasma membrane integrity two simple basic tests are used: supravital staining (eosin/nygrosin) and hypoosmotic swelling test (HOS) assay (CURRY and WATSON, 1994).

Some physiological processes during fertilization (capacitation, acrosomal reaction, fusion of sperm and ovum) demand an active membrane, and it is impossible to have fertilization with a physically inactive membrane (JEYENDRAN et al., 1984). In the present study we used the HOS test to evaluate the functional integrity of sperm membrane (CURRY et al., 1995). In hypoosmolar solution, fluid is transferred into the cell through the plasma membrane of spermatozoa. In attempting to achieve a balance between intracellular and extracellular spaces, a functionally intact membrane begins to swell starting at the tail of the spermatozoa. The swelling of the membrane leads to curling and invagination. The tail fibres changes are clearly visible under a phase-contrast microscope. Such spermatozoa are denoted as swollen 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 lysing the sperm membrane (ROTA et al., 1999). Fertilization of oocyte will not occur if the sperm membrane is biochemically inactive, even if it remains structurally intact. The HOS test is therefore a better indicator of fertilization potential than supravital staining (TAMULI and WATSON, 1992). Supravital staining is based on the fact that the membrane of dead spermatozoa permits the passage of the red stain into the cytoplasm, but the membranes of live spermatozoa does not permit that. This means that all dead spermatozoa in ejaculate will be coloured, while live spermatozoa will remain colourless (HERAK, 1991). CHECK and CHECK (1991) concluded that evaluation of structural and functional integrity of membrane is a better indicator of fertilization than motility evaluation alone. The object of this study was to discover the relevance of the HOS assay in determining sperm membrane integrity and correlations between HOS assay and sperm motility.

Material and methods

In our research we used semen from 9 sexually mature dogs of different breeds aged from 1 to 5 years. The dogs are owned by private citizens. The one-year-old dog had not been previously used for reproduction purposes. However, we evaluated the sperm for the purpose of buying and selling. Semen was collected using manual fixation of the penis.

The second and a part of the third fraction of the ejaculates were collected for evaluation by the spermogram.

Sperm evaluation. The parameters evaluated immediately after collection included: volume, progressive motility, concentration and evaluation of plasma membrane integrity. Progressive motility was determined by placing a drop of semen on a warmed glass slide (37 °C) and using a light microscope. Total sperm concentration was determined with Thoma's chamber according to standard methods (SAMARDŽIJA, 2003).

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

Evaluation of the functional integrity of sperm membrane was determined by HOS test. Before the test we checked the efficacy of the HOS test using a fructose solution and a Na-citrate solution with different osmolarity (100, 150, 200, 250 and 300 mosmol). This enabled us to obtain a hypoosmolar solution with optimal osmolarity which would give us the most HOS-reactive (HOS+) spermatozoa. We then incubated mixtures of sperm and hypoosmolar solution at 35°C and 37 °C at 15-minute intervals for a total of 120 minutes 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. On the basis of the preliminary results we selected a hypoosmolar solution of 100 mosmol 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 placed on a glass slide, covered with coverslip and examined at under a microscope at 30 x, 40 x magnification. At least 200 sperm cells on each slide were counted.

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

Results

The results of this study are summarized in Tables 1. and 2. and in Figs. 1. and 2. In evaluating the sperm parameters we focused first on evaluation of progressive motility, sperm concentration and semen volume and then determined structural membrane integrity by supravital staining by Bloom (HERAK, 1991), and the functional integrity of membrane

using the HOS test. The value of progressive motility was between 50-90%, averaging 74.44%. The method eosine/nygrosine supravital staining method yielded an average of 73.19% spermatozoa with structurally intact plasma membrane. The largest number of swollen spermatozoa (HOS-reactive) was observed with a hypoosmolar solution of fructose and Na-citrate with an osmolarity of 100 mosmol. Between the middle values of swollen spermatozoa in hypoosmolar solution with differing osmolarity we determined significant variances ($P < 0.05$). Using a hypoosmolar solution with an osmolarity of 100 mosmol in the HOS test we obtained values ranging from 20.55% till 86.64% with functionally intact plasma membrane; middle value was 67.66%. Correlations between the HOS test, eosine/nygrosine supravital staining and progressive motility are shown in Figs 1. and 2. The data revealed very high positive correlations between the HOS test and progressive motility ($r = 0.82$; $P < 0.05$) and between progressive motility and supravital staining eosine/nygrosine ($r = 0.89$, $P < 0.05$) than between HOS test and supravital staining eosine/nygrosine ($r = 0.98$; $P < 0.05$).

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

Dog number 2 mated with 4 bitches before sperm evaluation tests during a 3-month

		Osmolarity (mosmol)			
Hypoosmotic	100	150	200	250	300
Solution	79.89% (± 1.64)	77.57% (± 1.74)	77.90% (± 0.91)	76.25% (± 1.66)	75.72% (± 1.32)

period; none of the mated bitches conceived. Dog number 8 mated with 3 bitches before sperm evaluation tests over a period of 1 year; none of the mated bitches conceived. The remaining dogs mated with many bitches, which mostly conceived. Sperm evaluation tests were performed at the request of the owners.

Discussion and conclusion

Comparing spermatozoa morphology and capability for fertilization produced many conflicting data (SAMARDŽIJA, 2003). 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 epididimidis that capsule covers the 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

Table 2. Correlation of HOS test and differing sperm quality parameters

Dog	Progressive motility %	Eozin/Nigrozin % live	HOS % live
1	65	72.76	75.11
2	50	16.55	20.55
3	90	95.59	78.04
4	90	93.18	86.64
5	85	92.87	81.76
6	70	83.33	80.87
7	75	88.54	79.76
8	85	94.73	82.67
9	60	21.15	23.56
Mean	74.44	73.19	67.66
SD	14.24	31.64	26.06

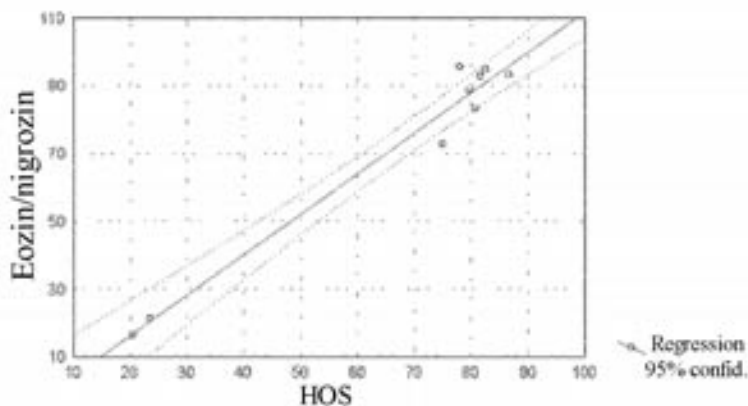


Fig. 1. Correlation of HOS test and supravital staining (eozin/nigrozin)

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 the functional integrity of plasma membrane of spermatozoa. The HOS test has much better efficacy than supravital staining. The evaluation

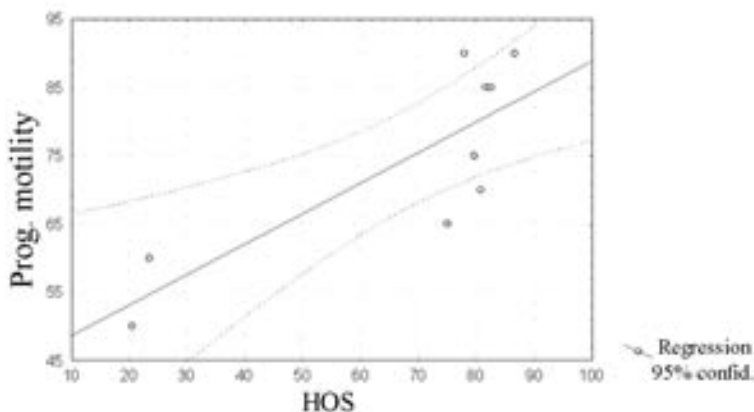


Fig. 2. Correlation of HOS test and progressive motility

of the functional status of sperm membrane by the HOS test is a better indicator of their fertilization capacity (ZAVOS, 1990). In hypoosmolar solution, liquid is transferred into the cell through the plasma membrane of spermatozoa. Attempting to achieve a balance between intra- and extra-cellular space, functionally intact membrane swells, beginning with the fibres in the sperm tail. As the membrane swells, 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 osmolarities 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). Between the middle values of swollen spermatozoa in hypoosmolar solutions of different osmolarity we determined significant differences ($P < 0.0001$); which agrees with the results given by Kumi-Diaka for dog sperm, and also for the results given by Neild et al., for stallion and buck sperm. Very high positive correlations were achieved in our research between the HOS test and progressive motility ($r = 0.82$; $P < 0.05$), and between progressive motility and supravital staining eosine/nygrosine ($r = 0.89$; $P < 0.05$) than between the HOS test and supravital staining eosine/nygrosine ($r = 0.98$; $P < 0.05$). Those very high correlations between HOS-reactive, morphologically normal and progressively motile spermatozoa are 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). Results congruent with results of our research were given by (KUMI-DIAKA, 1993) who determined the efficacy of the HOS test for dog sperm together with supravital staining and found a high correlation between the percentage of motile spermatozoa and HOS-reactive spermatozoa ($r = 0.94$).

Comparing correlations between HOS-reactive spermatozoa and morphologically defected spermatozoa. KUMI-DIAKA (1993) determined weak, negative correlations ($r = -0.22$; $r = -0.38$) which also accords with our results. Determining the efficacy of HOS test for bull sperm together with supravital staining CORREA and ZAVOS (1994) found a high correlation between the percentage of spermatozoa with structurally a intact membrane and HOS-reactive spermatozoa ($r = 0.81$). The results of our studies are in agreement with these observations. It is concluded from our data that the HOS assay can accurately determine sperm membrane integrity which correlates significantly with motility.

References

- AMANN, R. P. (1989): Can the fertility potential of a semen sample be predicted accurately? *J. Androl.* 10, 89-98.
- CHECK, M. L., J. H. CHECK (1991): Poor hypo-osmotic swelling test results from cryopreserved sperm despite preservation of sperm motility. *Arch. Androl.* 26, 37-41.
- 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. *Theriogenology* 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.
- CURRY, M. R., J. D. MILLAR, P. F. WATSON (1995): The presence of water channel proteins in ram and human sperm membranes. *J. Reprod. Fertil.* 104, 297-303.
- 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; Reprodukcijska domaćih životinja. U: Veterinarski priručnik, str. 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.
- KEEL, B. K., B. W. WEBSTER (1990): Handbook of the laboratory diagnosis and treatment of infertility. CRC Press, Boston MA, pp. 80-96.
- 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. *Theriogenology* 39, 1279-1289.
- NEILD, D., G. CHAVES, M. FLORES, N. MORA, M. BECONI, A. AGUERO (1999): Hypoosmotic test in equine spermatozoa. *Theriogenology* 51, 721-727.

- ROTA, A., N. PENZO, L. VINCENTI, R. MANTOVANI (1999): Hypoosmotic swelling (HOS) as a screening assay for testing *in vitro* fertility of bovine spermatozoa. *Theriogenology* 53, 1415-1420.
- SAMARDŽIJA, M. (2003): Priprema bičje sperme u postupcima oplodnje *in vitro*. Magistarski rad, Veterinarski fakultet Sveučilišta u Zagrebu, Zagreb.
- 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.
- ZAVOS, P. M. (1990): Hypoosmotic swelling test (HOS)/ functional integrity of sperm membrane. *J. Ass. Reprod. Technol. Androl.* 2, 215-216.

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SAŽETAK

Rabljeni su ejakulati devet spolno zrelih pasa, različitih pasmina u dobi od jedne do pet godina. Kvaliteta sperme ocjenjivana je pomoću progresivne pokretljivosti (subjektivno), te strukturalne i funkcionalne cjelovitosti membrane spermija (supravitalno bojenje eozin/nigrozin i HOS testom). Najveći broj HOS reaktivnih tj. nabubrenih spermija ustanovljen je u hipoosmotskoj otopini od 100 mosmol pri inkubaciji od 30 minuta. Rezultati istraživanja obrađeni su statistički metodom ANOVA, te Tukey HSD testovima post-hoc analize, a također je određena korelacija između promatranih obilježja. Između srednjih vrijednosti HOS reaktivnih (nabubrenih) spermija u hipoosmotskim otopinama različite osmolarnosti ustanovljene su signifikantne razlike ($P < 0,0001$). Korištenjem hipoosmotske otopine osmolarnosti 100 mosmol, ustanovljene su signifikantne, vrlo jake, pozitivne korelacije između HOS testa i progresivne pokretljivosti ($r = 0,82$; $P < 0,05$), zatim između HOS testa i supravitalnog bojenja eozin/nigrozin ($r = 0,98$; $P < 0,05$), te između progresivne pokretljivosti i supravitalnog bojenja eozin/nigrozin ($r = 0,89$; $P < 0,05$). To je očekivano jer pokretljivost spermija djelomično ovisi o funkcionalnoj cjelovitosti membrane, a djelomično i o drugim biokemijskim aktivnostima kao što je metabolizam spermija. Rezultati ovog istraživanja pokazali su da je HOS jednostavna metoda koja se može rutinski primjenjivati za ocjenu kvalitete sperme u pasa. Ustanovljene su pozitivne i signifikantne korelacije između HOS reaktivnih i progresivno pokretljivih spermija te spermija s cjelovitom plazminom membranom.

Ključne riječi: pas, spermiji, progresivna pokretljivost, HOS-test, cjelovitost membrane
