Relationships between the results of hypo-osmotic swelling tests, sperm motility, and fertility in Estonian Holstein dairy bulls

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ABSTRACT: As an attempt to find an inexpensive and simple laboratory method for artificial insemination (AI) bull semen quality assessment, the osmotic resistance of spermatozoa was measured using the hypo-osmotic swelling (HOS) test, developed by Jeyendran et al. (1984) (labelled HOS-1), and its modifications (HOS-2, HOS-3), with decreased osmotic pressure aimed at challenging sperm survival ability. The test results were benchmarked against sperm viability measurements performed using the Computerized Motility Analyzer (CMA), and field fertility was calculated as non-return rate (NRR). Two age groups of Estonian Holstein bull sires were included in this study to test possible age effect on semen quality parameters. The HOS-1 test in fresh bull semen correlated well with sperm general motility (GMot) (r = 0.63, P < 0.001 at batch level and r = 0.77, P < 0.001 at bull level) as well as with progressive motility (PMot) in frozen-thawed (FT) semen (r = 66, P < 0.001 at batch level and r = 0.81, P < 0.001 at bull level), which makes the test suitable for the prediction of post-thaw semen quality. However, the HOS-2 and HOS-3 values in FT semen had high correlations with NRR (r = 0.65, r = 0.66, P < 0.001 at batch level and r = 0.63, r = 0.71, P < 0.01 at bull level), which was comparable to those between GMot and NRR or PMot and NRR. A combination of motility parameters and the results of the HOS-1 and HOS-3 tests provided a good model for predicting the potential fertility of bull semen. Values of sperm membrane post-thaw intactness, assessed using HOS-2, as well as of sperm motility measurements were higher in mature bulls compared to those in young bulls. Short conclusion: different modifications of the hypo-osmotic swelling test are useful for routine bovine semen quality assessment at AI stations.

Keywords: bull fertility; semen quality; sperm membrane intactness; bull age

Laboratory analyses have been used over many decades to evaluate semen quality. Despite the fact that none of the single assays developed provide results that consistently and highly correlate with fertility, the laboratory evaluation of semen samples remains an important procedure for the artificial insemination (AI) industry to eliminate low fertility bulls or semen from being used in artificial insemination programmes (Graham and Mocé, 2005).

Post-thaw viability and fertility of cryopreserved sperm are often reduced due to accumulated cellular damage during the cryopreservation phases (Muiño et al., 2008). Decrease in temperature, cold shock, and intracellular ice formation can affect sperm plasma membrane, acrosomal and mitochondrial membrane integrity (Thomas et al., 1998; Defoin et al., 2008), and cause the loss of intracellular components (Graham and Mocé, 2005), which can initiate cell death.

Sperm membrane integrity can be evaluated by several methods, e.g. light or fluorescent microscopy combined with vital stains (Brito et al., 2003), and flow cytometry (Januskauskas et al.,

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1999; Hallap et al., 2004). One of the simplest methods to evaluate the plasmalemma of sperm cells is the hypo-osmotic swelling (HOS) test. The traditional HOS test, originally presented by Jeyendran et al. (1984), enables reproduction specialists to determine the functional intactness of sperm membranes as spermatozoa "swell" under hypo-osmotic conditions due to the influx of water, and the expansion of the membranes causes the tails to coil. Several authors (Rodríguez-Martínez, 1998; Neild et al., 1999; Imam et al., 2008) have emphasized the suitability of the HOS test for assessing the quality of fresh and frozen-thawed semen in different farm animal species. A positive correlation was found between the results of the HOS test or its modifications and the non-return rate of female animals (Revell and Mrode, 1994; Correa et al., 1997a), which potentially makes it one of the most appropriate and simple methods for semen quality evaluation for the AI industry. Several authors have found a positive correlation between plasmalemmal integrity and sperm motility characteristics (Neild et al,. 1999; Mandal et al., 2003). Mandal et al. (2003) reported that in Murrah buffalo bulls ejaculates with less than 50% HOS-positive spermatozoa showed lower motility as compared with those with > 50% HOS-positive spermatozoa. Stanger et al. (2010) observed that in humans low HOS test values of neat semen samples were significantly associated with increased DNA damage identified by the sperm chromatin structure assay as well as TUNEL assay.

Reaction of spermatozoa to the HOS solution varies depending on animal species, solution, osmolality, and time of incubation (Correa et al., 1997b; Neild et al., 1999; Amorim et al., 2009). Not all spermatozoa with intact plasma membrane react to moderate osmotic pressure, however the swelling response of spermatozoa occurred mostly during the first minute of incubation in a hypotonic medium (Correa et al., 1997a). Therefore, for the maximum osmotic challenge, along with the traditional HOS test, the usefulness of the modified versions of the test was examined using 0.2% and 0.4% NaCl solutions immediately after thawing of the semen samples, or after 6 h of incubation of the samples at 37°C.

In earlier studies, a relationship between semen quality and the age of a bull was found (Padrik and Jaakma, 2004). The increase in age from 1–2 years to 3–4 years was accompanied by an increase in the proportions of motile and progressively motile spermatozoa, whereas an opposite tendency was recorded from 3–4 to 5–7 years of age (Padrik and Jaakma, 2004). A study by Hallap et al. (2004) showed that the post-thaw sperm membrane integrity estimated using fluorophores SYBR-14 and propidium iodide (PI) was higher in the semen of four-year-old bulls compared with that of younger bulls.

The aim of this study was to assess sperm membrane intactness in young and mature Estonian Holstein AI bulls using three different modifications of the HOS test, and to reveal the relationships between the HOS test and sperm motility characteristics as well as the field fertility estimated by the NRR of cows and heifers.

MATERIAL AND METHODS

Animals, semen collection, and processing

The first experiment was carried out using fresh semen from 91 Estonian Holstein (EHF) bulls aged 14 to 72 months, kept at the AI station of the Animal Breeders' Association of Estonia at Kehtna.

An artificial vagina was used to collect semen. Two consecutive ejaculates were pooled (hereafter referred to as a batch), extended with a commercial extender (Triladyl®; Minitüb GmbH, Tiefenbach, Germany), packed in 0.25 ml plastic straws, each containing ~ 25×10^6 spermatozoa for mature bulls, and $30–40 \times 10^6$ spermatozoa for young bulls, and frozen in a manually adjustable biological freezer. The frozen straws were stored in liquid nitrogen until testing. Following preservation, post-thaw motility ≥ 50% was set up as a threshold.

A total of 683 ejaculates, collected over sixteen months, were examined for sperm membrane hypo-osmotic swelling and motility in fresh and frozen-thawed semen. During this period, 4–6 ejaculates from proven bulls and 2–4 ejaculates from young bulls were collected per month. The bulls were a part of a breeding programme; all collections as well as freezing of semen were performed at the AI station under commercial conditions.

In the second experiment, FT semen (49 ejaculates) from 10 mature (Group I) and 7 young (Group II) bulls were examined and used for test inseminations. These ejaculates were used to inseminate 3850 cows and heifers (78 inseminations per ejaculate and 226 inseminations per bull on average) by four AI technicians on four different herds according to the established breeding programme. Inseminations were performed routinely,

within one year of freezing, on heifers and cows of different parity in the course of all seasons of the year. A 60-day non-return rate was recorded for each batch, but not corrected for season, location, or parity.

Hypo-osmotic swelling test

HOS-1 test. The HOS test was conducted under three modifications. A traditional hypo-osmotic test (Jeyendran et al., 1984), labelled HOS-1, was performed by incubating 100 µl of fresh semen with 1 ml of 150 mOsm/kg hypo-osmotic solution (7.35g sodium citrate and 13.51 g fructose per 1 l of distilled water) at 37°C for 60 min. The semen straw was thawed at 35°C in a water bath for 20 s before emptying into a test tube containing the hypo-osmotic solution. After incubating at 37°C for 60 min, 0.2 ml of eosin (0.99%) (Pioneer Research Chemicals, Ltd., Colchester, UK) was added to the test tube. The wet preparation was examined under a phase contrast microscope (Olympus BX40, ×1000). The ratio of spermatozoa with swollen tails was expressed as a percentage of the total count (mean of 3 replicates). A total of 100 spermatozoa were assessed in each replicate.

HOS-2 test. In the HOS-2 test (Padrik, 1999), the proportion of FT spermatozoa with swollen tails was determined in 0.2% and 0.4% NaCl solutions (osmotic pressure 66 and 130 mOsm/kg, respectively). The semen straws were thawed in a water bath at 35°C for 20 s, and emptied into a test tube containing 1 ml of 0.2% and 0.4% NaCl solution. After incubating at room temperature (20–22°C) for 2 min, 0.2 ml of eosin was added to each test tube. A wet preparation of each concentration was examined under a phase contrast microscope. The ratio of spermatozoa with swollen tails was expressed as a percentage of the total count (mean of 3 replicates). A total of 100 spermatozoa were assessed in each replicate. ΔHOS-2 was estimated by subtracting the ratio of spermatozoa with intact membranes in the 0.2% NaCl solution from the similar value in the 0.4% NaCl solution.

HOS-3 test. In the HOS-3 test (Padrik and Jaakma, 2000), three straws of frozen semen were thawed at 35°C for 20 s, emptied into a test tube containing 3 ml of 2.9% sodium citrate solution (Tallinn Pharmacy Ltd., Tallinn, Estonia), mixed and incubated at 37°C for 6 h. A 100 µl fraction of the sperm suspension was pipetted into each of two solutions, 1 ml of 0.2% and 1 ml of 0.4% NaCl. After 2 min incubation at room temperature (20–22°C), 0.2 ml of eosin was added into each test tube and wet preparations were made. A total of 100 spermatozoa were assessed in each preparation, and the ratio of the spermatozoa with swollen tails was given as a percentage of the total count (mean of 3 replicates). ΔHOS-3 was estimated by subtracting the ratio of spermatozoa with intact membranes in the 0.2% NaCl solution from the similar value in the 0.4% NaCl solution.

Sperm motility

Sperm motility characteristics were determined using Computer Assisted Cell Motion Analyzer, Sperm Vision (Minitüb GmbH, Tiefenbach, Germany). Samples of 5 µl were placed in the Makler chamber (Makler Counting Chamber, Sefi-Medical Instruments, Haifa, Israel) to track and assess ~ 400 fresh or post-thaw sperm (×400). The following parameters were determined: percentage of general motile (GMot) and progressively motile (PMot) sperms, curve line velocity (VCL, µm/s), linearity (LIN, straight line velocity (VSL)/VCL), and amplitude of lateral head displacement (ALH, µm).

Statistical analysis

The characteristics of the observed traits were expressed as means ± SD. The Pearson's correlation test was used to calculate correlations between different sperm parameters in fresh and FT semen, and between the measured sperm parameters and field fertility (60-day NRR). The bulls were divided into two age groups: mature bulls (Group I, aged 36–72 months, n = 10) and young bulls (Group II, aged 14–22 months, n = 7). General linear model analyses were performed using SAS® software (Version 9.1.3, 1999) to assess the influence of different sperm quality characteristics and bull age on the pregnancy rate, and to develop a predictive model for non-return rates.

RESULTS

Experiment 1

HOS-1 in fresh semen. The percentage of swollen spermatozoa was 57.9 ± 13.6% (range

7.0–85.0%) in batches, and 57.6 ± 10.2% (range 31.0–77.4%) in bulls. A medium correlation was found between the results of the HOS-1 test and GMot and PMot at both the bull and batch levels in fresh semen (Table 1). A stronger correlation was recorded between the HOS-1 results in fresh semen, and GMot and PMot in FT semen at both the batch and bull levels.

HOS-1 in frozen-thawed semen. The mean HOS-1 test scores in FT semen were 35.2 ± 6.8% (range 22.0–45.5%) at bull, and 33.3 ± 9.7% (range 13–50%) at batch level. A significant positive correlation was determined between the HOS-1 and PMot (r = 0.55, P < 0.05) at bull level, between the HOS-1 and PMot (r = 0.47, P < 0.01), and the HOS-1 and GMot (r = 0.55, P < 0.01) at batch level.

HOS-2 in frozen-thawed semen. The mean ΔHOS-2 for bulls was 7.1 ± 6.5% (range –4.6 to 22.0%) and for batches 6.0 ± 8.9% (range –15.0–25.0%). Strong correlations were found between ΔHOS-2 and GMot, and ΔHOS-2 and PMot (r = 0.66, 0.55, respectively; P < 0.05) at bull level. Significant positive correlations were also observed between ΔHOS-2 and GMot, PMot, VCL, ALH at batch level (r = 0.42–0.64, P < 0.01).

HOS-3 in frozen-thawed semen. The mean ΔHOS-3 score for bulls was –0.3 ± 3.7% (range –6.5–7.3%), and for batches –0.7 ± 6.1% (range –11.0–10.0%). The ΔHOS-3 was positively correlated with GMot and PMot at bull level (r = 0.61, 0.52 respectively; P < 0.05). Medium correlations were also found between ΔHOS-3 and GMot, PMot, VCL, ALH at batch level (r = 0.36–0.55, P < 0.01).

Relationships between HOS-1, HOS-2, and HOS-3 in frozen-thawed semen. A positive correlation was found between HOS-1 and ΔHOS-2 both at bull (r = 0.28, P > 0.05) and batch (r = 0.49, P < 0.05) level, and also between HOS-1 and ΔHOS-3 at bull (r = 0.28, P > 0.05) and batch (r = 0.38, P < 0.05) level. ΔHOS-2 related to ΔHOS-3 both at bull (r = 0.81, P < 0.001) and batch (r = 0.72, P < 0.001) level.

Experiment 2

Effect of bull age on the results of hypo-osmotic swelling test and sperm motility in frozen-thawed semen. The results of the HOS-1 and HOS-3 tests on FT semen were not different between mature and young bulls, either at bull or batch level, whereas the HOS-2 showed an improvement in semen quality of mature bulls compared to semen quality of young bulls at batch level (Table 2). The results also showed a significant influence of the age of the bulls on sperm motility parameters.

Relationships between laboratory tests and *in vivo* fertility. Fertility, expressed as 60-days NRR, ranged 37.5–57.0% for young, and 60.0–71.5% for mature bulls. A medium positive correlation was found between the HOS-1 results in FT semen and NRR at batch level (r = 0.37, P < 0.01), but not at bull level. A strong positive correlation was detected between both ΔHOS-2 and NRR, and ΔHOS-3 and NRR at batch (r = 0.65, 0.66, respectively; P < 0.001) and bull (r = 0.63, 0.71, respectively, P < 0.01) level, which was comparable to that between GMot and NRR, PMot and NRR at batch (r = 0.69, 0.66, respectively; P < 0.001) and bull (r = 0.71, 0.63, respectively; P < 0.01) level. Positive correlations were also observed between VCL and ALH in FT semen and NRR at batch (r = 0.59, 0.50, respectively; P < 0.001) and bull (r = 0.59, 0.56, respectively; P < 0.05) level.

Table 1. Correlations (r) between HOS-1 test in fresh semen and sperm motility characteristics in fresh and frozen-thawed semen (batch and bull level)

Sperm motility parameters, fresh semen	HOS-1 in fresh semen		Sperm motility parameters, post-thaw	HOS-1 in fresh semen	
	683 batches	91 bulls		683 batches	91 bulls
GMot (%)	0.22*	0.33**	GMot (%)	0.63***	0.77***
PMot (%)	0.20*	0.32**	PMot (%)	0.66***	0.81***
VCL (μm/s)	0.09	–0.04	VCL (μm/s)	0.23*	0.22*
LIN	0.02	0.10	LIN	0.04	0.26*
ALH (μm)	0.04	–0.01	ALH (μm)	0.13	0.14

HOS = hypo-osmotic swelling, GMot = general motile, PMot = progressively motile, VCL = curve line velocity, LIN = linearity, ALH = amplitude of lateral head displacement

*P < 0.05, **P < 0.01, ***P < 0.001

Table 2. Influence of age of bulls on hypo-osmotic swelling and motility of spermatozoa in frozen-thawed semen, bull and batch level (means ± SD)

Sperm parameters	Bull level		Batch level	
	mature (n = 10)	young (n = 7)	mature (n = 30)	young (n = 19)
HOS-1	34.3 ± 6.6	36.4 ± 7.5	32.3 ± 9.6	34.4 ± 10.1
Δ HOS-2	9.1 ± 6.1	4.2 ± 6.5	8.3 ± 8.7[a]	2.6 ± 8.5[b]
Δ HOS-3	0.6 ± 3.3	−1.5 ± 4.1	0.3 ± 6.3	−2.2 ± 5.4
GMot (%)	79.1 ± 4.9	74.0 ± 9.1	79.1 ± 7.6[a]	71.5 ± 12.3[b]
PMot (%)	63.1 ± 4.9	58.4 ± 11.6	62.8 ± 7.4[a]	55.1 ± 15.0[b]
VCL (μm/s)	92.9 ± 10.1[a]	89.7 ± 5.6[b]	99.1 ± 9.2[c]	88.3 ± 5.6[d]
LIN	0.4 ± 0[c]	0.5 ± 0[d]	0.4 ± 0[c]	0.5 ± 0[d]
ALH(μm)	3.0 ± 0.2[c]	2.5 ± 0.3[d]	3.1 ± 0.3[c]	2.7 ± 0.3[d]

HOS = hypo-osmotic swelling, GMot = general motile, PMot = progressively motile, VCL = curve line velocity, LIN = linearity, ALH = amplitude of lateral head displacement

[a,b]values with different superscripts in a row are significantly different ($P < 0.05$)

[c,d]values with different superscripts in a row are significantly different ($P < 0.001$)

Relationship between predicted (PNRR) and observed non-return rates (NRR) of mature and young bulls. Based on the results, the predictive model for NRR (PNRRage) included five parameters: HOS-1, ΔHOS-3, general motility (GMot, in %), progressive motility (PMot, in %), and linearity (LIN).

R^2 = 0.80 in mature bulls at batch level

PNRRage = 36.423 + 0.174 HOS-1 + 0.634 ΔHOS-3 – 0.388 GMot + 0.652 PMot + 16.32 LIN

R^2 = 0.79 in young bulls at batch level

PNRRage = –0.88 + 0.648 HOS-1 + 0.466 ΔHOS-3 + 1.291 GMot – 0.887 PMot + 135.3 LIN

A strong positive correlation between PNRR (predicted NRR in %) and NRR (60-day non-return rate in %) was found at batch level (r = 0.89, P < 0.001) and at bull level (r = 0.92, P < 0.001) in mature bulls. A medium positive correlation between PNRRage and NRR was observed at batch level (r = 0.64, P < 0.001), and a strong positive correlation was found between PNRRage and non-return rates at bull level (r = 0.95, P < 0.001) in young bulls.

DISCUSSION

The objective of this investigation was to study the hypo-osmotic swelling of spermatozoa in fresh and FT semen of young and mature Estonian Holstein bulls, and assess relationships between the HOS test results, sperm motility characteristics, and fertility of bulls expressed as 60-days NRR.

Several authors have emphasized the suitability of the HOS test for assessing the quality of human semen (Moskovtsev et al., 2005; Cincik et al., 2007) and also that of various domestic animals including cattle (Correa and Zavos, 1994; Mandal et al., 2003; Hu et al., 2010), horses (Neild et al., 1999), and pigs (Gadea et al., 1998). As the test gives a consistent estimate of the percentage of spermatozoa with a physiologically active membrane, it can be used to predict the fertilizing capacity of spermatozoa in animals (Rota et al., 2000; Brito et al., 2003).

Correa et al. (1997b) and Neild et al. (1999) found that spermatozoa show different swelling patterns and not all spermatozoa with intact plasma membrane react to moderate osmotic pressure. Therefore, the traditional HOS test was modified by shortening the incubation time of sperm cells in hypotonic solution, using hypo-osmotic concentrations of 0.2% and 0.4% NaCl (HOS-2), and adding sperm survival (HOS-3). The decrease in osmotic pressure from 130 to 66 mOsm/kg resulted in a significant difference in sperm behaviour in different semen batches. The percentage of spermatozoa with swollen tails increased in some batches, while it remained stable or decreased in others. The latter can be explained by the fast breakage of the sperm membrane due to the influx of water under highly hypo-osmotic con-

ditions. Contrarily, the increase in the percentage of spermatozoa with swollen tails after short-term exposure to low osmotic pressure showed the maintenance of sperm membrane functional integrity.

This study found a strong correlation between the results of the HOS test in fresh semen and GMot and PMot of spermatozoa in FT semen. Thus, there is a high probability that spermatozoa with intact membranes before extension and cryopreservation maintain good motility after the FT procedure. Consequently, it is possible to apply an individual semen processing method to different semen batches to obtain an optimal quantity of semen doses while taking into account the proportion of spermatozoa with functionally intact membranes when diluting the semen.

Furthermore, positive correlations between the HOS-tests and sperm GMot and PMot, all measured in FT semen, were observed, which is similar to earlier findings (Neild et al., 1999; Mandal et al., 2003). With regard to the relationships between the spermatozoa with functionally intact membranes and other traits in FT semen, Januskauskas et al. (1996) have observed associations between ATP content and sperm membrane integrity, assessed using fluorophore probes. Zuge et al. (2008) have reported a high positive correlation between the proportion of sperm cells with high mitochondrial activity and that with intact membranes, determined using the HOS test.

The results of this study found that the age of bulls had a significant effect on the HOS-2, GMot, and PMot ($P < 0.05$), VCL, LIN, and ALH ($P < 0.001$) values at batch level, and VCL, LIN, and ALH ($P < 0.05$) values at bull level in FT semen. The better semen quality in mature bulls can be associated with an increase in testosterone levels which has an effect on the volume of the ejaculate, sperm concentration, and motility (Kastelic et al., 2001; Silva et al., 2008). An increase in testosterone levels in blood plasma is related to an increase in scrotal circumference (SC) (Andrade et al., 2008) which in turn is strongly correlated with the weight and age of bulls (Chacón-Calderón et al., 2002).

Evaluation of sperm motility characteristics has traditionally been the most frequently used semen quality test in the AI industry. In this study, as expected, a strong correlation was observed between the GMot and PMot of FT spermatozoa and NRR ($P < 0.01$), which confirms previous findings (Holt et al., 1997; Farell et al., 1998; Januskauskas et al., 2003; Hallap et al., 2006). Similarly to Revell and Mrode (1994) and Correa et al. (1997a), a significant positive correlation was found between the results of the HOS-1 test in FT semen and NRR (batch level, $P < 0.01$). The correlation coefficients were even higher between the results of the HOS-2, HOS-3, and NRR at both batch ($P < 0.001$) and bull ($P < 0.01$) level, which evidences that HOS-2 and HOS-3 are more suitable for semen quality testing. The HOS-2 test appears to be especially attractive for the AI industry as it is neither expensive nor time-consuming.

It was found earlier that the results of post-thaw hypo-osmotic swelling tests can be used to predict potential fertility of bovine semen samples used for IVF (Tartaglione and Ritta, 2004). According to Brito et al. (2003), the HOS test was the only plasmalemma evaluation method that significantly contributed to conventional sperm quality tests in predicting *in vitro* fertilization rate.

A high positive correlation was found between predicted and actual NRRs, where, in addition to the HOS tests and sperm kinematics, the age of the bulls was taken into consideration in developing the model. The highest correlation coefficient observed was between PNRRage and NRR for young bulls ($P < 0.001$). Attempts have previously been made to include the HOS test in the regression equation for fertility. Tartaglione and Ritta (2004) included an eosin-nigrosin supravital stain combined with the HOS test into a regression equation as a predictor of *in vitro* fertility of FT bull semen. Pérez-Llano et al. (2001) also included the HOS test in a regression equation along with other semen parameters such as morphology, motility, and acrosome integrity to predict the fertility of boar semen. Contrarily, also working with boars, Gadea and Matas (2000) found that the results of the HOS test were not significant when regression models for fertilization rate were evaluated. In the current study, using a regression equation with five parameters (HOS-1, ΔHOS-3, GMot, PMot, and LIN), the predicted NRRs were highly correlated with the actual NRRs.

It can be concluded that the HOS-1 test on fresh bull semen was correlated well with sperm motility in FT semen, which makes the test suitable for the prediction of post-thaw semen quality. Furthermore, the modification of the original HOS test, named HOS-2, was found to be an even better method for the assessment of the quality of FT semen samples, as it is quicker and less expensive. A combination of motility parameters and the results of the HOS-1 and HOS-3 tests is a good model for predicting the potential fertility of bull semen for the AI industry. Values of sperm membrane intact-

ness post-thaw assessed using HOS-2 and of sperm motility measurements were higher in mature bulls when compared to those in young bulls.

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