

## CONVENTIONAL LABORATORY TEST AND FLOW CYTOMETRY IN THE PROGNOSTIC TESTING OF BULL SEMEN FERTILITY

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**Abstract.** We aimed to determine the relationships between the results of routine AI laboratory tests and flow cytometric analysis (FCM) of the quality of frozen-thawed (FT) bovine spermatozoa. The results were compared to the field fertility data. Forty five ejaculates from fifteen (14 to 86 Mo age) Estonian Holstein (EHF) dairy bulls were examined for motility (subjectively by light microscope and objectively using a computer assisted motility analyzer (CMA)), hypo-osmotic swelling tests (HOS-1, HOS-2, HOS-3), membrane lipid architecture status (Merocyanine 540 staining) and mitochondrial membrane potential (Mitotracker Deep Reed 633 staining). Stained spermatozoa were assessed by FCM. Significant positive correlations were observed between subjectively assessed motility variables (SubMot), general motile (GMot) and progressively motile (PMot) spermatozoa and sperm with stable membrane (LSM) ( $P < 0.001$ ). Strong positive correlations with non-return rates (NRR) were obtained for HOS-2, HOS-3, SubMot, PMot, GMot and curve line velocity (VCL) ( $P < 0.01$ ). The best predictive model PNRR (predictive non-return rates), according to the results of routine laboratory tests and FCM analysis, included seven parameters ( $R^2 = 0.91$ ). The strongest positive correlation was found between PNRR and NRR on bull level ( $r = 0.96$ ;  $P < 0.001$ ) compared to that of batch level. Combinations of common AI laboratory tests (motility analysis, HOS) and FCM assays can be used for the prediction of the FT bull semen fertility.

**Keywords:** bull semen quality, flow cytometry, fertility prediction.

## ĮPRASTINIŲ SPERMATOZOIDŲ KOKYBĖS VERTINIMO METODŲ IR TĖKMĖS CITOMETRIJOS METODO PALYGINAMASIS ĮVERTINIMAS, NUSTATANT BULIAUS SPERMOS APVAISINAMĄJĄ GALIĄ

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**Santrauka.** Darbo tikslas – nustatyti ryšį tarp bulių kriokonservuotos spermos spermatozoidų kokybės tyrimų rezultatų, gautų atliekant įprastus laboratorinius testus ir analizuojant tėkmės citometrijos metodu (TCM). Rezultatus palyginome su apvaisinimo duomenimis, gautais lauko sąlygomis (NRR). 45-iuose ejakuliatuose, surinktuose iš penkiolikos (14–86 mėnesių) Estijos holšteinų pienujų bulių, iširtas spermatozoidų judrumas (subjektyviai, naudojant šviesinį mikroskopą, ir objektyviai, naudojant kompiuterinį judrumo analizatorių), plazminės membranos rezistentiškumas, naudojant hipoosmotinius testus (HOS-1, HOS-2, HOS-3), membranos lipidų struktūrinė būklė (dažymas merocianinu) ir mitochondrijų membranų potencialas (dažymas naudojant „Mitotracker Deep Reed 633“). Nudažyti spermatozoidai įvertinti TCM. Pastebėta ženkliai teigiama koreliacija tarp subjektyviai įvertintų judrumo verčių (SubMot), bendro judrumo (GMot), progresyvaus spermatozoidų judrumo (PMot) ir spermatozoidų su stabilia plazmine membrana (SSM) verčių ( $p < 0,001$ ). Aukšta teigiama koreliacija su NRR buvo būdinga HOS-2, HOS-3, SubMot, PMot, GMot ir spermatozoidų greičio vertei (VCL) ( $p < 0,01$ ). Įprastų laboratorinių testų ir TCM rezultatai parodė, kad geriausias prognostinis apvaisinimo rezultatų (PNRR) modelis apima septynis tiriamuosius rodiklius ( $r^2 = 0,91$ ). Buliaus lygmeniu koreliacija tarp PNRR ir NRR buvo aukštesnė ( $r = 0,96$ ;  $p < 0,001$ ) už koreliaciją bulių grupės lygmeniu. Nustatant buliaus kriokonservuotos spermos apvaisinimo galią, galima derinti įprastus laboratorinius metodus (judrumo analizė, HOS) su TCM.

**Raktažodžiai:** bulių spermos kokybė, tėkmės citometrija, apvaisinimo nustatymas.

**Introduction.** Evaluation of frozen-thawed semen quality in the AI industry is often based on subjective evaluations by experienced professionals, and not guaranteed by objective methods. Although many AI

laboratories are nowadays equipped with computer-assisted sperm analyser (CASA), enabling objective measurement of motility, combination of multiple sperm attributes could explain more variation in fertility between the bulls than can variables of motility alone (Christensen et al. 1999). Using of fluorescent markers in combination with flow cytometry allows simultaneous assessment of several sperm parameters in thousands of sperm cells and therefore, has become an valuable tool in semen quality assessment (Gillan et al. 2005; Hua et al. 2006). Thus, the fast development of measurement techniques has provided AI industry with the variety of objective tests for the semen quality. The question is, however, if the traditional simple (and cheap) methods should be replaced by the more precise but expensive methods or could they rather be incorporated into the semen quality assessment simultaneously.

In our study, we focused on sperm plasma membrane integrity, membrane stability and mitochondrial activity along with the sperm motility characteristics. Sperm membrane integrity, which is essential for normal metabolism, capacitation and further acrosome reaction can be evaluated with several methods such as light or fluorescent microscopy combined with vital stains (Brito et al., 2003; Celeghini et al., 2008) or flow cytometry (Kasai et al., 2002; Kasimanickam et al., 2006). One of the simplest methods to evaluate the sperm plasma membrane integrity is subjection of sperm cells to the hypo-osmotic swelling test. The traditional hypo-osmotic swelling (HOS) test presented by Jeyendran (1984) enables the determination of the functional intactness of the sperm membranes as spermatozoa “swell” under hypo-osmotic conditions and the expansion of the membranes causes the tails to coil. Several authors have emphasized the suitability of the hypo-osmotic test for evaluation of the quality of fresh and frozen semen and fertilizing capacity of the spermatozoa of different farm animals (Tartaglione et al., 2004). In addition to absence of damage, the plasma membrane needs to be stable to avoid premature capacitation in the female reproduction tract (Hallap et al., 2006). One possibility for estimation of sperm plasma membrane stability is to use the hydrophobic dye Merocyanine 540 (M540) and flow cytometry to determine the level of scrambling of the phospholipids in the plasma membrane of spermatozoa (Harrison et al. 1996).

Estimation of subjective motility of spermatozoa under light microscope, and objective motility with CASA, gives a good overview of the quality of fresh and FT semen and these results often correlate well with female fertility (Rodrigues-Martinez, 1998). Assessment of ATP (adenosine triphosphate) content as an energy source for sperm, is an indirect possibility to evaluate the motility potential of spermatozoa. A positive correlation has been found between sperm motility and ATP content in FT semen (Söderquist et al., 1991). Measurement of mitochondrial membrane potential (organelles where ATP is synthesized) using FCM has also been found to be useful as another parameter related to sperm motility (Volpe et al., 2009).

The aim of this study was to estimate the relationships between the results of conventional AI laboratory tests such as motility measurement and hypo-osmotic resistance and flow cytometrically estimated mitochondrial activity and plasma membrane stability. We also studied the relationships between the test results and field fertility after AI.

#### **Materials and methods**

##### **Animals, semen collection and processing**

Semen from fifteen (age from 14 to 86 months) Estonian Holstein (EHF) bulls was collected once weekly using an artificial vagina. Two consecutive ejaculates were pooled (hereafter referred to as a “batch”), extended with a commercial extender (Triladyl<sup>®</sup>, Minitüb, Germany), packed in 0.25 ml plastic straws each containing  $\sim 25\text{-}40 \times 10^6$  spermatozoa, and frozen using a manually regulated biological freezer. The frozen straws were stored in liquid nitrogen until tested or used for the insemination. Doses of 45 frozen-thawed semen batches were used to inseminate 3,475 cows and heifers (average 77 inseminations per batch and 231 inseminations per bull) by four AI technicians in four different herds according to the breeding program. Inseminations were performed routinely within one year on heifers and cows of different parity during all seasons of the year.

Non-return rates (NRRs) 60 days after AI were recorded for each semen batch and used for the analyses without correction for season, area, and parity. The fertility of an individual bull was calculated using non-return rates for all his semen batches and ranged from 37.5 to 71.5%. The fertility of semen batches ranged from 22.8 to 80.0%. Semen evaluation was performed immediately after the thawing. The semen from two straws of the same batch was thawed by immersion in water at +35°C for 20 seconds, pooled and used for testing. Following preservation, a post-thaw motility  $\geq 50\%$  was set as the threshold limit, and batches of semen that did not comply with this threshold were discarded from further use both for AI and for laboratory testing.

##### **Hypo-osmotic swelling (HOS) test**

The HOS-test was performed with three modifications. In HOS-1, the thawed semen straw was emptied into the test tube with 1ml hypoosmotic solution (150mOsm/kg; 7.35g sodium citrate and 13.51g fructose per litre of distilled water). After incubating at +37°C for 60 min, 0.2 ml of eosin (0.99%, Pioneer Research Chemicals, Ltd. England) was added. Wet preparation (5  $\mu\text{L}$  semen suspension per slide) was evaluated under the phase contrast microscope ( $\times 1000$ ) and the ratio of spermatozoa with swollen tails was expressed as a percentage from a mean of three replicates. One hundred spermatozoa were assessed in each replicate.

In 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). After incubating spermatozoa in 1 ml of solution at room temperature (+20...22°C) for 2 minutes, 0.2 ml of eosin was added.

Wet preparations of each sample were evaluated under phase contrast microscope ( $\times 1000$ ). One hundred

spermatozoa were assessed in each replicate and the ratio of spermatozoa with swollen tails was expressed as a percentage from a mean of three replicates.  $\Delta$ HOS-2 was estimated by subtracting the ratio of the spermatozoa with intact membranes in 0.2 % NaCl solution from the similar value in 0.4% NaCl solution.

In HOS-3 test, described by Padrik and Jaakma (2000), three straws of frozen semen were thawed, emptied into a test tube with 3 ml of 2.9% sodium citrate solution (Tallinn Pharmacy Ltd.), mixed and incubated for six hours at 37°C. Thereafter, 100  $\mu$ l of sperm suspension were pipetted into tubes containing 1 ml 0.2 and 0.4 % NaCl. After 2 minutes incubation at room temperature 0.2 ml of eosin was added into each NaCl solution and wet preparations were made. One hundred spermatozoa were assessed in each preparation and the ratio of the spermatozoa with swollen tails was given as a percentage from a mean of three replicates.  $\Delta$ HOS-3 was estimated as described in HOS-2 test.

#### **Sperm motility**

Sperm motility characteristics were determined with a computer-assisted motility analyzer (Computer Assisted Cell Motion Analyzer (CMA), Sperm Vision, Minitüb GmbH&Co, Germany).

5 $\mu$ l samples were placed in Makler chamber (Sefi-Medical Instruments, Ltd.) where ~400 post-thaw spermatozoa were tracked and assessed ( $\times$ 400) at +38°C. The following parameters were determined: percentages of general motile (GMot) and progressively motile (PMot) spermatozoa, curve line velocity (VCL,  $\mu$ m/sec), linearity (LIN, %) and amplitude of lateral head displacement (ALH,  $\mu$ m). Subjective sperm motility (Submot) was estimated under a phase contrast microscope ( $\times$ 400) equipped with a warm stage (+38°C). The mean value from evaluations of four fields was recorded in % from total.

#### **Sperm plasma membrane stability**

The following working solutions were prepared: Merocyanine 540 (M-540; Molecular Probes, M24571, Leiden, The Netherlands) 1mM in dimethyl sulfoxide (DMSO); Yo-PRO 1 (Molecular Probes, Y3603) 25  $\mu$ M in DMSO. Washed spermatozoa were stained with 25 nM Yo-PRO 1 and further incubated at 38°C for 9 min in the dark as previously described (Harrison et al.1996).

Thereafter 10  $\mu$ l of a 40  $\mu$ M solution of M-540 in SP-TALP was added to give a final M-540 concentration of 2.7  $\mu$ M and vortexed for 10 s before analysis on a flow cytometer (FacsCalibur, Becton Dickinson, San Jose, USA). Data collection was started at 60 s after M-540 addition. Measurements were made with a flow cytometer, equipped with standard optical lasers as excitation sources. The M-540 and Yo-PRO 1 dyes were excited by an Argon ion 488 nm laser running at 15 mW. Forward and side scatter values were recorded on a linear scale; while fluorescent values were recorded on a logarithmic scale. Obscuration bars were set for maximum sensitivity in order to obtain L-shaped forward light – scatter/sideways light scatter distribution of sperm cells. Fluorescence of Yo-PRO 1 was detected on detector FL 1 (530/28 nm), while M-540 fluorescence was

detected on detector FL 2 (585/2 nm). From each sample, a total of 10,000 events were measured with flow rate of approx. 200 cells/s. Acquisitions were made using CellQuest Pro software (Becton Dickinson, San Jose, USA). Dot plots for offline analyses were drawn by WinMDI, version 2.8. Events accumulated in the lower left corner correspond to sample debris and were excluded from the analysis by gating. On FL 1/FL 2 (Yo-PRO 1/M-540) dot plots regions were set to differentiate viable, stable plasma membrane LSM (Yo-PRO 1 negative and M-540 negative); viable, scrambled plasma membrane (Yo-PRO 1 negative and M-540 positive); and dead (Yo-PRO 1 positive) events.

#### **Sperm mitochondrial activity**

The staining protocol was identical to that described by Hallap et al. (2005). The measurements were made using a FacsCalibur flow cytometer (Becton Dickinson, San Jose, USA). The SYBR-14 dye was excited by a 15 mW Ar ion 488 nm laser while MitoTracker Deep Red was excited by a 17 mW HeNe 633 nm laser. The SYBR-14 fluorescence (cells with intact plasma membrane) was detected on detector FL 1 (530/28 nm) while MitoTracker Deep Red fluorescence was detected by a detector FL 3 (670 LP). Forward and side scatter (FSC and SSC) values were recorded on a linear scale while fluorescent values were recorded on a logarithmic scale. Compensations were set according to Roederer (2000). Acquisitions were made using the CellQuest Pro software (Becton Dickinson, San Jose, USA).

Non-sperm events were gated out based on SYBR-14 fluorescence (DNA content). The FC was used at a low flow rate (6-24  $\mu$ L/min). Acquisitions were stopped after recording 10 000 SYBR-14-positive events and the data stored in list mode for further analysis. On SYBR-14 (FL 1/FL 2) dot plots, regions were drawn around the SYBR-14-positive cluster, and these events were classified as spermatozoa. In SYBR-14/ MitoTracker Deep Red dot plots sperm cells with low MTDR-L) and high (MTDR-H) Deep Red fluorescence were specified.

#### **Statistical Analyses**

The statistical analyses based on two datasets composed on batch level (analyses of 45 semen batches), and bull level (analyses the data of 15 bulls). In the last case the values for each bull were calculated by pooling the data of the different batches. The results were expressed as means  $\pm$  S.D.

The Pearson correlation test was used to calculate the correlations between different sperm parameters in fresh and FT semen and between the sperm parameters measured and field fertility (60-days NRR). The analysis of variance with SAS software (version 9.1.3; SAS Institute Inc., Cary, NC) was used to assess the influence of different sperm quality characteristics and bulls' age on the fertility and to find the predictive model for non-return rates. On batch level analyses the effect of repeated measurements of the bulls was considered.

#### **Results**

##### **Relationships between conventional AI laboratory tests, flow cytometric analysis and *in vivo* fertility**

In general, sperm motility parameters estimated either

subjectively or by CASA and the results of the hypo-osmotic tests correlated to percentage of viable sperm with stable membrane (LSM) and sperm with high mitochondrial activity (MTDR-H) (Table 1 and 2). The strongest correlations were found between PMot and LSM on batch level ( $P<0.001$ ) (Table 1) and between PMot and (MTDR-H) on bull level ( $P<0.001$ ) (Table 2).

Several conventional and flow cytometric parameters were positively related to fertility:  $\Delta$ HOS-2 ( $P<0.001$ ),  $\Delta$ HOS-3 ( $P<0.05$ ), SubMot ( $P<0.01$ ), GMot ( $P<0.001$ ), PMot ( $P<0.01$ ), VCL ( $P<0.001$ ), ALH ( $P<0.001$ ), and MTDR-H ( $P<0.05$ ) on bull level and the same parameters plus LSM (all  $P<0.001$ ) on batch level.

Table 1. Correlation between laboratory tests and flow-cytometric analysis of bull semen and 60-days NRR (batch level)

Parameters	Batches (n=45)				
	means±S.D.	Range	LSM	MTDR-H	NRR
			r	r	r
HOS-1 (%)	33.9±9.7	17...50	0.24	0.21	0.15
$\Delta$ HOS-2 (%)	5.4±8.9	-12 ...+25	0.44**	0.38**	0.63***
$\Delta$ HOS-3 (%)	-1.1±9.2	-11...+12	0.44**	0.30*	0.65***
SubMot (%)	57.8±9.9	35.0...75.0	0.67***	0.62***	0.69***
GMot (%)	75.6±10.6	49.0...87.5	0.66***	0.59***	0.68***
PMot (%)	59.3±12.0	27.8...73.6	0.72***	0.67***	0.65***
VCL ( $\mu$ m/sec)	91.4±9.8	75.0...115.5	0.33*	0.38*	0.61***
LIN	0.47±0.04	0.42...0.60	-0.28	-0.24	-0.52
ALH( $\mu$ m)	2.9±0.3	2.2...3.5	0.13	0.14	0.57***
LSM (%)	55.7±15.7	16.8...80.3	-	0.81***	0.52***
MTDR-H (%)	74.4±18.6	24.7...92.7	0.81***	-	0.51***

SubMot- Subjective motility; GMot - general motility; PMot - progressively motile; VCL - curve line velocity; LIN - linearity; ALH - amplitude of lateral head displacement; LSM- Live stable membrane; MTDR-H - high mitochondrial activity; \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$

Table 2. Correlation between laboratory tests and flow cytometric analysis of bull semen and 60-days NRR (bull level)

Parameters	Bulls (n=15)				
	means±S.D.	range	LSM	MTDR-H	NRR
			r	r	r
HOS-1 (%)	34.9±6.1	22.0...44.5	0.41	0.25	-0.001
$\Delta$ HOS-2 (%)	6.2±5.7	-4.6...+14.0	0.42	0.44	0.73***
$\Delta$ HOS-3 (%)	-0.7±3.7	-6.8...+8.6	0.34	0.34	0.57*
SubMot (%)	58.1±7.2	39.0...66.3	0.71**	0.71**	0.71**
GMot (%)	76.6±7.5	55.7...83.6	0.63**	0.66**	0.72***
PMot (%)	60.8±8.7	34.1...68.7	0.73***	0.75***	0.63**
VCL ( $\mu$ m/sec)	94.4±8.6	80.4...106.2	0.21	0.27	0.74***
LIN	0.48±0.04	0.42...0.53	-0.06	-0.11	-0.69
ALH ( $\mu$ m)	2.9±0.3	2.5...3.2	-0.03	0.004	0.76***
LSM (%)	57.6±13.3	21.9...79.8	-	0.86***	0.36
MTDR-H (%)	77.2±14.3	29.6...86.9	0.86***	-	0.53*

SubMot- Subjective motility; GMot - general motility; PMot - progressively motile; VCL - curve line velocity; LIN - linearity; ALH - amplitude of lateral head displacement; LSM- Live stable membrane; MTDR-H - high mitochondrial activity; \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ .

The strongest positive correlation was found between ALH and NRR on bull level ( $P<0.001$ ) and SubMot and NRR on batch level ( $P<0.001$ ).

#### Relationships between predicted and observed non-return rates

The NRR prediction equation I (PNRR-I) included

five parameters from conventional laboratory tests: HOS-2, SubMot, GMot, PMot and ALH.

Bull level:

$$\text{PNRR-I}_{\text{bull}} = 26.78 + 0.70 \times \Delta\text{HOS-2} + 0.46 \times \text{Submot} + 1.51 \times \text{PMot} - 0.30 \times \text{GMot} + 25.52 \times \text{ALH}$$

$$R^2 = 0.77; \text{Adj. } R^2 = 0.65$$

Batch level:

$$\text{PNRR-Ibatch} = 4.43 + 0.35 \times \Delta\text{HOS} - 2 + 0.39 \times \text{Submot} + 0.61 \times \text{PMot} - 0.58 \times \text{GMot} + 12.06 \times \text{ALH}$$

$$R^2 = 0.62; \text{Adj. } R^2 = 0.57$$

Another prediction equations, PNRR-II, were obtained by including seven parameters from both conventional laboratory tests and flow cytometric analysis.

Bull level:

$$\text{PNRR-IIbull} = -7.13 + 0.49 \times \Delta\text{HOS} - 2 + 0.22 \times \text{Submot} + 0.77 \times \text{PMot} - 1.65 \times \text{GMot} + 33.23 \times \text{ALH} - 0.21 \times \text{LSM} + 0.58 \times \text{MTDR-H};$$

$$R^2 = 0.91; \text{Adj. } R^2 = 0.83$$

Batch level:

$$\text{PNRR-IIbatch} = 1.46 + 0.35 \times \Delta\text{HOS} - 2 + 0.31 \times \text{Submot} + 0.51 \times \text{PMot} - 0.57 \times \text{GMot} + 13.96 \times \text{ALH} - 0.07 \times \text{LSM} + 0.06 \times \text{MTDR-H};$$

$$R^2 = 0.63; \text{Adj. } R^2 = 0.56$$

These models were used to calculate the predicted fertility outcomes on bull and batch level. The highest positive correlation was found between PNRR-II and NRR on bull level ( $r=0.96$ ;  $P<0.001$ ). Positive correlation was also found between PNRR-I and NRR on batch level ( $r=0.84$ ;  $P<0.001$ ) and on bull level ( $r=0.87$ ;  $P<0.001$ ) and PNRR-II and NRR on batch level ( $r=0.78$ ;  $P<0.001$ ).

**Discussion.** The aim was to study the relationships between the results of conventional laboratory tests such as sperm motility measurement or HOS and flow cytometrically estimated mitochondrial activity and plasma membrane stability. We also studied the relationships between the test results and field fertility after AI and determined the best fertility predictive model as combination of these tests.

The study showed a positive correlation between the results of conventional laboratory tests (SubMot, PMot, GMot, VCL,  $\Delta\text{HOS-2}$ ,  $\Delta\text{and HOS-3}$ ) and sperm quality variables LSM and MTDR-H obtained by FCM analysis.

In our study, strong positive correlation was found between subjectively assessed motility (SubMot) and proportion of spermatozoa with stable plasma membrane (LSM) ( $r=0.71$ ;  $P<0.01$ ) on bull level, however good correlations between PMot and LSM and/or between PMot and MTDR-H ( $r=0.73$  and  $r=0.75$ , respectively) must be considered even more important because of the lack of subjectivity.

The results of our study also showed significant positive correlation between percentage of cells with intact membrane (estimated by the simple HOS-tests) and MTDR-H ( $r=0.39$ ;  $P<0.01$ ). Similarly, Zuge et al. (2008) observed a significant positive correlation between sperm cells with full mitochondrial activity and percentage of cells with intact membrane assessed by the HOS test. Hallap et al. (2006) and Hua et al. (2006) reported a positive correlation between sperm motility and high mitochondrial activity and between percentage of cells with stable membranes and motility in fresh and FT

semen. Some earlier studies (Januskauskas et al., 1996) described significant association between ATP content in FT semen and sperm motility. In our study, strong positive correlation was found between PMot and MTDR-H ( $r=0.75$ ,  $P<0.001$ ) which indirectly indicates importance of ATP energy produced by the intact mitochondria (Vishwanath et al., 1986; Rajender et al., 2010). The high positive correlation in our study was found between LSM and MTDR-H ( $r=0.86$ ,  $P<0.001$ ). These results confirm earlier findings that stable sperm plasma membrane is the prerequisite for mitochondrial functioning and that this in turn makes possible sperm motion (Kasai et al., 2002; Hua et al., 2006; Zuge et al., 2008).

Relations between the laboratory measurements of bull semen quality and their field fertility have been under discussion for many years. In our study, strong correlation was recorded between the SubMot, GMot, PMot, VCL and ALH of FT spermatozoa and NRR ( $r=0.63 \dots 0.76$ ;  $P<0.01$ ) on bull level, similarly to Zhang et al. (1998) and Januskauskas et al. (2003).

A significant positive correlation was also found between the results of different HOS tests in FT semen and NRR on batch level ( $r=0.15$ ;  $P>0.05$ ;  $r=0.63$  and  $0.65$ ;  $P<0.001$ ; for the HOS-1, HOS-2 and HOS-3, respectively). HOS-3 included 6 hours incubation of spermatozoa before performing a hypo-osmotic test and therefore, the results were directly dependent on sperm survival which is limiting factor also *in vivo* in the female reproductive tract. However, HOS-2 test gave similar results and from the practical point of view was more suitable as less time consuming. Usefulness of HOS tests in semen quality evaluation has been demonstrated earlier by Revell and Mrode (1994); Correa et al. (1997). It was found (Brito et al. 2003; Tartaglione and Ritta 2004) that results of hypoosmotic swelling test post-thaw could be used for prognosis of the potential fertility of bovine semen samples used for A.I.

In our study, a significant positive correlation was also obtained between the LSM, MTDR-H in FT semen and NRR on batch level (correspondingly  $r=0.51$  and  $r=0.52$ ;  $P<0.001$ ) and between the MTDR-H and NRR on the bull level ( $r=0.53$ ;  $P<0.05$ ).

A positive correlation was found between several single parameters of common AI laboratory tests and NRR similar to the earlier findings (Correa et al. 1997; Zhang et al. 1998, Verbeckmoes et al. 2002). The single tests have not been sufficiently discriminative (Christensen et al. 1999) because each of them measures only a single attribute necessary for the fertilization. Therefore, it would be rational to combine different single parameters into a prediction model (Rodriguez-Martinez, 2006).

We tested two models, one of them (PNRR-I) was based on conventional laboratory tests only and another also included flow cytometrical measurements of sperm membrane stability and mitochondrial activity (PNRR-II). The highest positive correlation was found between PNRR-II and NRR on bull level ( $r=0.96$ ;  $P<0.001$ ). Similarly, motility parameters and membrane integrity of

FT spermatozoa were included into models proposed earlier by Januskauskas et al. (2003), Tartaglione and Ritta (2004), Phillips et al. (2004). They also have reported a strong correlation between predicted and actual non-return rates. Phillips et al. (2004) found that post-thaw sperm parameters such as morphologically normal sperms, the proportion of intact sperms, and cleavage of embryos, can be used to predict field fertility of dairy sires. In the study of Tartaglione and Ritta (2004) eosin-nigrosin supravital stain combined with the HOS-test has been included in a regression equation as predictors of *in vitro* fertility of FT bull semen.

In conclusion, the results of common laboratory tests (subjectively assessed motility and the parameters measured by the CASA, hypo-osmotic resistance tests) correlated well with flow cytometrically measured membrane stability and mitochondrial activity. Strong correlations were found between single semen quality parameters GMot, PMot, HOS-2, HOS-3 and NRR. Medium correlations were found between FCM analysis and NRR. However, a combination of conventional laboratory tests and flow cytometric analysis of membrane stability and mitochondrial activity in FT spermatozoa allowed better prediction of the potential fertility of bull semen in comparison to the conventional AI laboratory tests alone.

#### Acknowledgements

This study was supported by the Estonian Science Foundation (Grant 6089, Grant 7814), and SF 1080045s07. We would like to thank General Manager of the Animal Breeders Association of Estonia, Mr. Tanel Bulitko, for the contribution of bull semen, Ms. Niina Haasmaa for technical assistance and Dr. David Arney for the linguistic revision.

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Received 2 February 2012

Accepted 21 September 2012