Assessment of boar semen quality in relation to fertility with special reference to methanol stress

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Abstract

The relationship between various semen evaluation tests and fertility in fertile and subfertile artificial insemination (AI) boars was examined. In total, 36 boars, 19 Finnish Landrace and 17 Yorkshire, were included. The average value of three ejaculates extended in an X-cell extender from each boar was used in the analysis. Based on nonreturn results (NR60d, later referred to nonreturn rate, NR%), the boars were divided into two groups: those with poor fertility (NR% < 80, n = 19) and those with normal or above average nonreturn rates (NR% = 83, n = 17). Semen quality was determined after 1 and 7 days of storage at 17 °C. Sperm motility before and after each methanol stress was assessed both subjectively and using a computer-assisted semen analyzer (CASA). The sperm cells were stained with calcein AM and propidium iodide and evaluated for plasma membrane integrity under an epifluorescence microscope. Propidium iodide and Hoechst 33258 dyes were used in parallel to stain sperm cells for fluorometric analysis with an automatic fluorometer. Sperm morphology was evaluated in stained smears. The percentage of sows reported as not having returned to estrus within 60 days after AI (nonreturn rate, NR%) and litter size of primiparous and multiparous farrowings were used as measures of fertility. Of the parameters analyzed, only CASA-assessed total sperm motility and methanol-stressed total sperm motility correlated significantly (P < 0.05) with nonreturn rate. Those tests presenting the highest correlation with nonreturn rate were CASA-assessed total motility (r = 0.54, P < 0.01) and subjective sperm motility (r = 0.52, P < 0.01) after 7 days of storage. The highest correlation with fertility at 1 day of storage was shown by methanol-stressed total sperm motility assessed with the CASA (r = 0.46, P < 0.01). The only semen parameter that correlated significantly (r = 0.37, P < 0.05) with litter size of multiparous farrowings was viability of seven-day stored semen stained with Hoechst 33258 and analyzed with a fluorometer.
The methanol stress test described here could serve as a rapid test whose results could be used to predict NR% better than motility. © 2004 Elsevier Inc. All rights reserved.

**Keywords:** Sperm motility; Methanol stress; Plasma membrane integrity; Fertility; Boars

### 1. Introduction

When boar semen is used for artificial insemination (AI), the relationship between semen quality, pregnancy rate, and litter size are of great economic importance. Subfertile boars are typically first identified by the pregnancy rates and litter sizes of the females inseminated with their semen.

The time spent for examination of quality of single boar ejaculates (visual inspection, density, total sperm count, and motility) at AI stations is roughly 30 s in Finland. In addition, one semen smear of each boar is usually subjected to morphological studies at an external laboratory. Every second week, the total sperm count and motility on the seventh day of storage is determined for the remaining (not sold) AI doses at the external laboratory. A comprehensive, ejaculate-based quality control test that can be completed within the 30 s, available in the quality control schema is not feasible. Viability and membrane function are important factors in assessing the potential fertilization capacity [1] and are considered to be more accurate predictors of the fertilization capacity of semen than motility [2]. An array of sperm tests is available [3–5], but the results obtained in different studies are often controversial. No simple, rapid methods currently exist that are suitable for routine use in AI station laboratories to identify the majority of subfertile boars.

The aim of the study was to determine the relationship between various semen evaluation tests and fertility. To our knowledge, methanol stressing of semen has never been used for predicting fertility of semen doses. We investigated the correlation between the results of a methanol stress test and the percentage of sows reported as not having returned to estrus within 60 days of AI (NR60d, herein referred to as the nonreturn rate, NR%).

### 2. Materials and methods

#### 2.1. Experimental design

Semen from 36 (19 Finnish Landrace and 17 Yorkshire) boars (17 of above-average fertility and 19 presumably subfertile), housed at two boar stations, was examined over a period of 2 months. The mean age of the animals was 27.1 ± 7.7 months (range 13–50 months). All boars were used for routine AI. The semen samples were collected at regular weekly intervals at each station. The sows were inseminated with fresh semen generally twice per estrus 15–24 h apart. The age of the semen at insemination varied between 6 h and 3 days. The boars were divided into two groups based on nonreturn results: those with poor fertility (NR% < 80, n = 19) and those with normal or above average nonreturn rates (NR% = 83, n = 17).
2.2. Semen analysis

Semen density was measured with a photometer (Novaspec II, Pharmacia LKB Biotechnology, Uppsala, Sweden). The semen was then diluted to approximately 35 × 10^6 spermatozoa/ml, with an X-cell (IMV Technologies, L’Aigle Cedex, France) extender and placed in 90-mL plastic tubes at the station. For the analysis, one AI dose was split into two aliquots and stored at 17 °C in a Unitron climate box (Unitron Skandinavia S/A) in closed plastic tubes until examination. Analysis of sperm motility and plasma membrane integrity was conducted 24 h after semen collection and extension and following 7 days of storage. The average value of three ejaculates extended in the X-cell extender from each boar was used for analysis.

2.3. Sperm morphology and concentration of extended semen

For morphology, the semen smears were stained with a Giemsa staining method according to Watson [6] and 200 spermatozoa were examined. These were divided into four groups: normal spermatozoa, spermatozoa with major sperm defects, spermatozoa with proximal droplets, and spermatozoa with minor sperm defects [7].

The sperm concentration in each insemination dose was determined using a Bürker counting chamber (Fortuna, Germany).

2.4. Sperm motility

Sperm motility was evaluated both subjectively and with a computer-assisted semen analyzer (CASA) (Sperm Vision Mininute™ of America, Inc., 2002). For the analysis, a 300-µL aliquot of the thoroughly but gently mixed semen sample was placed into an open 3-mL tube. The tube was kept in a 35 °C water bath (Grants Instruments Ltd., Cambridge, UK) for 5 min prior to semen analyses. A 5-µL aliquot was placed on a prewarmed 38 °C microscope slide, covered with a coverslip (24 mm × 24 mm × 1.5 mm), and the proportions of total motile and progressively motile spermatozoa recorded.

2.5. Toxic response in methanol-stressed spermatozoa

The toxic response in methanol-stressed spermatozoa was assessed by exposing 200 µL of semen to 1, 5, 10, 20, and 40 µL of methanol for 10 min at room temperature. Spermatozoa exposed to the different concentrations of methanol were assessed for motility inhibition, depolarization of mitochondria, and damage to the plasma membrane integrity barrier [8].

2.6. Methanol stress test

Sperm motility was estimated after methanol stressing with a CASA. A 200-µL aliquot of mixed semen was placed in an open 3-mL tube and supplemented with 5 µL of methanol (Mallincrodt Bacer B.V., the Netherlands). The tube was gently mixed and kept at room temperature for 10 min and thereafter in a 35 °C water bath (Grants Instruments
Ltd., Cambridge, UK) for 5 min prior to motility analyses. A 5-μL aliquot was placed on a prewarmed 38 °C microscope slide, covered with a coverslip (24 mm × 24 mm × 1.5 mm), and the proportions of total motile and progressively motile spermatozoa recorded.

2.7. Fluorescent dyes

Calcein AM (CAM), propidium iodide (PI), and Hoechst 33258 (H258) dyes were purchased from Molecular Probes Inc. (Eugene, OR, USA). One milligram of Calcein AM was diluted in 1 mL of dimethyl sulfoxide (DMSO) (Mallincrodt Bacer B.V.), mixed for 10 min, kept in the dark, and then stored in 10-μL aliquots at −20 °C. Twenty milligrams of propidium iodide (PI) were diluted in one liter of BTS (Beltsville Thawing Solution, Kubus S.A., Spain) and stored in 3-mL aliquots at −20 °C. Six milligrams of Hoechst 33258 were diluted in 200 mL of BTS, mixed for 30 min in the dark, and stored in 2-mL aliquots at −20 °C. Before use, the dyes were thawed in a dark chamber at 35 °C (Thermax, B8000, Bergen, Norway).

2.8. Assessment of plasma membrane integrity

Microscopic evaluation of plasma membrane integrity was carried out with a combination of two fluorescent stains, CAM and PI, according to Januskauskas and Rodriguez-Martinez [9], but using PI instead of ethidium homodimer-1. Briefly, 10 μL of CAM (1 mg/mL) were mixed with 500 μL of BTS and 500 μL of PI (0.02 mg/mL) in BTS. For staining, 100-μL aliquots of semen were placed in 3-mL tubes and 100 μl of CAM/PI solution was added. Each sample was further incubated for 10 min in the dark at 35 °C. Subsamples 5 μL of the stained suspension were placed on clean microscope slides and overlaid carefully with coverslips. The smears were evaluated under an epifluorescence microscope (Olympus BH2 with epifluorescence optics, Olympus Optical Co., Ltd., Japan) using ×500 magnification. For each semen sample, 200 spermatozoa were differentiated into green (live) and red (dead) cell categories.

PI and Hoechst 33258 were used to measure the plasma membrane integrity in a fluorometer developed for reading the fluorescence output of 96-well plates (Fluoroscan Ascent, Thermo Labsystems Oy, Vantaa, Finland). Analysis was done in an incubation compartment at 32 °C, according to Alm et al. [10]. Briefly, 500-μL aliquots of freshly mixed semen were placed in 3-mL tubes and closed. The tubes were rapidly frozen by immersion directly into liquid nitrogen for 1 min. The tubes were then kept at room temperature for 30 s, before being placed in a 35 °C water bath for 3 min to cause disruption of plasma membranes. For the analysis, equal 50-μL aliquots of freshly mixed semen sample and PI were dispensed into the wells of the plate (Black Microtiter Plate 96 wells, Thermo Labsystems Oy, Vantaa, Finland) in three replicates. The rapidly frozen subsamples were then analyzed in the same manner as the nontreated samples. Blanks containing 50 μL of X-cell extender (IMV Technologies) and 50 μL of PI were dispensed in four replicates. The plate was gently shaken for 2 min. Before the analysis, the plate was incubated in the fluorometer for 8 min. Eleven samples and their blanks were analyzed simultaneously. The interference filter at the excitation path and the emission filter had
maximum transmissions at 544 and 590 nm for PI, and 355 and 460 nm for Hoechst 33258, respectively.

2.9. Fertility data of boars

Fertility data were obtained from the Agricultural Data-Processing Center Ltd. (Vantaa, Finland). Nonreturn rate within 60 days of the first insemination (NR%) and litter size (total number of piglets born) of primiparous and multiparous farrowings were used as fertility parameters. All boars were used for at least the first 50 inseminations in recorded herds and had ≥ 12 L of recorded farrowings. In total, 13 993 first inseminations and 3519 L were recorded from inseminations on approximately 200 commercial farms.

2.10. Statistical analysis

Statistical analyses were carried out using SPSS software (version 7.0 for Windows, SPSS Inc., Chicago, IL, USA). Summary statistics, two-sample analysis, and Spearman rank correlations were used. The Spearman rank correlations were used to calculate the relationships between sperm the evaluation parameters and fertility. The statistical values are presented as means ± S.D. and were considered statistically significant when \( P < 0.05 \).

3. Results

A summary of basic semen quality and fertility parameters is shown in Table 1. Of the parameters analyzed, only subjectively and CASA-assessed total sperm motility and methanol-stressed total sperm motility correlated significantly with NR% (Table 2).

When the toxic response in methanol-stressed spermatozoa was assessed, it as found that motility was inhibited and the plasma membrane integrity barrier was damaged at the same concentrations: 20 \( \mu \)L of methanol in 200 \( \mu \)L semen. The same concentration depolarized

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Descriptive statistics for semen quality parameters and fertility (( n = 36 ) boars)</td>
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<tr>
<td>Min–max</td>
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<tr>
<td>DENS</td>
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<tr>
<td>NORM</td>
</tr>
<tr>
<td>PROX</td>
</tr>
<tr>
<td>MAJOR</td>
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<tr>
<td>MINOR</td>
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<tr>
<td>NR%</td>
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<tr>
<td>LS.PRIM</td>
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<td>LS.MULT</td>
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</tbody>
</table>

DENS = sperm density (Bürker counting chamber); NORM = morphologically normal spermatozoa (%); PROX = proximal droplets (%); MAJOR = major sperm defects (%); MINOR = minor sperm defects (%); NR% = nonreturn rate within 60 days of first insemination; LS.PRIM = litter size of primiparous farrowings; LS.MULT = litter size of multiparous farrowings.
the mitochondria, indicating that methanol inhibited sperm motility by damaging plasma membrane functioning. Decreased motility in response to methanol stress may thus indicate minor damage in the plasma membrane.

Several semen quality parameters differed significantly between the fertile and subfertile boars (Table 3): subjective and total motility for seven-day stored semen, total and total motility for methanol-stressed semen stored for one day, and major sperm defects. Fertile boars had significantly higher ($P < 0.001$) fertility (NR%) results (84.59 ± 0.71) than subfertile boars (75.32 ± 3.62). The only semen parameter that correlated significantly ($r = 0.37, P < 0.05$) with litter size of multiparous farrowings was the viability of seven-day stored semen stained with Hoechst 33258 and analyzed using a fluorometer.

### 4. Discussion

The three most significant sperm quality parameters correlating with nonreturn rate were computer-assessed total sperm motility in semen after 7 days of storage, subjective motility in seven-day stored semen, and computer-assessed total motility of methanol-stressed spermatozoa in one-day stored semen. The only parameter to correlate with litter size was sperm viability assessed in seven-day stored semen with a fluorometer using a Hoechst 33258 probe. The correlation coefficients between these sperm quality parameters and

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>NR%</th>
<th>LS.PRIM</th>
<th>LS.MULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUBJECTIVE</td>
<td>0.360*</td>
<td>0.172</td>
<td>0.231</td>
</tr>
<tr>
<td>SUBJECTIVE7</td>
<td>0.518**</td>
<td>0.198</td>
<td>0.114</td>
</tr>
<tr>
<td>C.TOTAL</td>
<td>0.384*</td>
<td>0.189</td>
<td>0.171</td>
</tr>
<tr>
<td>C.TOTAL7</td>
<td>0.542**</td>
<td>0.190</td>
<td>0.104</td>
</tr>
<tr>
<td>C.PROG</td>
<td>−0.059</td>
<td>0.092</td>
<td>−0.130</td>
</tr>
<tr>
<td>C.PROG7</td>
<td>0.063</td>
<td>0.126</td>
<td>−0.068</td>
</tr>
<tr>
<td>M.SUBJECTIVE</td>
<td>0.445*</td>
<td>0.144</td>
<td>0.166</td>
</tr>
<tr>
<td>M.SUBJECTIVE7</td>
<td>0.424*</td>
<td>0.174</td>
<td>0.064</td>
</tr>
<tr>
<td>C.M.TOTAL</td>
<td>0.464**</td>
<td>0.160</td>
<td>0.155</td>
</tr>
<tr>
<td>C.M.TOTAL7</td>
<td>0.427**</td>
<td>0.198</td>
<td>0.072</td>
</tr>
<tr>
<td>C.M.PROG</td>
<td>0.150</td>
<td>0.154</td>
<td>−0.029</td>
</tr>
<tr>
<td>C.M.PROG7</td>
<td>0.127</td>
<td>0.153</td>
<td>−0.075</td>
</tr>
</tbody>
</table>

SUBJECTIVE = subjective motility (%); SUBJECTIVE7 = subjective motility (%) after 7 days of storage; C.TOTAL = CASA-assessed total motility (%); C.TOTAL7 = CASA-assessed total motility (%) after 7 days of storage; C.PROG = CASA-assessed progressive motility (%); C.PROG7 = CASA-assessed progressive motility (%) after 7 days of storage; M.SUBJECTIVE = methanol-stressed subjective motility (%); M.SUBJECTIVE7 = methanol-stressed subjective motility (%) after 7 days of storage; C.M.TOTAL = CASA-assessed methanol-stressed total motility (%); C.M.TOTAL7 = CASA-assessed methanol-stressed total motility (%) after 7 days of storage; C.M.PROG = CASA-assessed methanol-stressed progressive motility (%); C.M.PROG7 = CASA-assessed methanol-stressed progressive motility (%) after 7 days of storage; NR% = nonreturn rate within 60 days of first insemination; LS.PRIM = litter size of primiparous farrowings; LS.MULT = litter size of multiparous farrowings.

* $P < 0.05$.
** $P < 0.01$. 

The correlation coefficients are significant at the 0.05 level for the table.
nonreturn rate and litter size are, however, not very high. New sperm quality parameters that correlate better with fertility should nevertheless be sought.

The methanol stress test described here was an approach aimed at finding a new parameter correlating with fertility. Methanol was used for in vitro toxicology studies in connection with boar spermatozoa [8]. While spermatozoa from some boars have been shown to be more sensitive to methanol than the spermatozoa of other boars, the relationship between the results of the methanol stress test and fertility has thus far not been explored (MA Andersson, personal communication). In addition, the methanol-stressed sperm motility parameters had a higher correlation coefficient with fertility than did the sperm motility parameters that were not stressed. In our preliminary trials, we showed that methanol damaged the plasma membrane integrity and depolarized the mitochondria. Since proper functioning of the plasma membrane and the mitochondria are essential for fertilization, resistance to methanol could serve as a semen quality indicator. This test is rapid and easy to perform and our results with it correlated significantly with nonreturn rates. This test would be suitable for routine semen quality control at AI stations.

Significant correlations between semen quality parameters were well documented in many studies [11,12], although the results are often controversial [13,14]. While some

Table 3
Means ± S.D. for semen quality and fertility parameters in two boar groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Subfertile (Mean ± S.D.)</th>
<th>Fertile (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUBJ</td>
<td>75.37 ± 5.27</td>
<td>78.06 ± 3.47</td>
</tr>
<tr>
<td>SUBJ7</td>
<td>65.59 ± 8.38</td>
<td>73.21 ± 5.31**</td>
</tr>
<tr>
<td>C.TOTAL</td>
<td>89.52 ± 6.00</td>
<td>93.72 ± 5.00**</td>
</tr>
<tr>
<td>C.TOTAL7</td>
<td>78.31 ± 9.62</td>
<td>87.36 ± 5.84**</td>
</tr>
<tr>
<td>M.SUBJ</td>
<td>56.21 ± 14.89</td>
<td>64.71 ± 10.61</td>
</tr>
<tr>
<td>M.SUBJ7</td>
<td>41.12 ± 17.87</td>
<td>51.23 ± 12.82</td>
</tr>
<tr>
<td>C.M.TOTAL</td>
<td>67.18 ± 15.35</td>
<td>77.25 ± 11.47</td>
</tr>
<tr>
<td>C.M.TOTAL7</td>
<td>52.73 ± 18.41</td>
<td>62.7 ± 13.59</td>
</tr>
<tr>
<td>NORM</td>
<td>80.91 ± 13.51</td>
<td>87.85 ± 8.59</td>
</tr>
<tr>
<td>PROX</td>
<td>0.76 ± 0.91</td>
<td>0.67 ± 0.58</td>
</tr>
<tr>
<td>MAJOR</td>
<td>3.84 ± 3.11</td>
<td>2.02 ± 1.16*</td>
</tr>
<tr>
<td>MINOR</td>
<td>13.97 ± 10.8</td>
<td>9.65 ± 7.68</td>
</tr>
<tr>
<td>NR%</td>
<td>75.32 ± 3.62</td>
<td>84.59 ± 0.71***</td>
</tr>
<tr>
<td>LS.PRIM</td>
<td>10.15 ± 0.92</td>
<td>10.38 ± 0.83</td>
</tr>
<tr>
<td>LS.MULT</td>
<td>12.02 ± 0.69</td>
<td>12.21 ± 0.48</td>
</tr>
<tr>
<td>n</td>
<td>19</td>
<td>17</td>
</tr>
</tbody>
</table>

SUBJ = subjective motility (%); SUBJ7 = subjective motility (%) after 7 days of storage; C.TOTAL = CASA-assessed total motility (%); C.TOTAL7 = CASA-assessed total motility (%) after 7 days of storage; M.SUBJ = methanol-stressed subjective motility (%); M.SUBJ7 = methanol-stressed subjective motility (%) after 7 days of storage; C.M.TOTAL = CASA-assessed methanol-stressed total motility (%); C.M.TOTAL7 = CASA-assessed methanol-stressed total motility (%) after 7 days of storage; NORM = morphologically normal spermatozoa (%); PROX = proximal droplets (%); MAJOR = major sperm defects (%); MINOR = minor sperm defects (%); NR% = nonreturn rate within 60 days of first insemination; LS.PRIM = litter size of primiparous farrowings; LS.MULT = litter size of multiparous farrowings; n = number of boars.

* P < 0.05.
** P < 0.01.
*** P < 0.001.
studies found a significant association between motility and nonreturn rates [4,15,16],
others did not [17,18]. In our study, only the sperm motility and methanol-stressed sperm
motility parameters correlated significantly with nonreturn rate. However, none of the
sperm motility parameters correlated significantly with litter size. We observed that the
level of significance was higher for seven-day stored semen, confirming a previous report in
which none of the initial semen parameters showed a significant correlation with fertility,
except motility and plasma membrane integrity parameters of semen stored for 7 days [11].

In our study, progressive motility did not correlate with fertility or with any other
motility parameters. The likely reason was that we used ordinary object glasses without
agar coating; boar spermatozoa tend to adhere to glass.

Various fluorescence stains with different properties give slightly varying results. We
used two such DNA stains: Propidium iodide is impermeant through sperm cells with an
intact plasma membrane, while Hoechst 33258 is slowly permeant. The sperm viability
results obtained with the slowly permeant Hoechst 33258 were the only ones that
correlated significantly with the litter size of multiparous farrowings.

Significant correlations between viability and fertility have been documented recently in
fluorescence studies [10–12,16], with some reports suggesting that assessment of viability
would be a more accurate predictor of fertilizing capacity than motility [2,12]. Although
assessment of viability in fluorescence studies is essential, the methods used are not
suitable for routine assays at AI stations since they are time-consuming (when using a
microscope) and/or have a high price and running costs (flow cytometry) [10]. Moreover,
motility assessments of preserved sperm cells can never be replaced entirely by fluoro-
metric measurements of viability because spermatozoa stored in different conditions, such
as in improper diluents, can also show good fluorescence in plasma membrane integrity
tests, even though the spermatozoa are immotile in microscopic examination [2]. Thus,
poor motility does not necessarily indicate cellular damage [19]. Our motility estimates in
seven-day stored semen correlated significantly with fertility.

Our work confirms that sperm motility and sperm motility after methanol stress are
valuable semen quality parameters and should not be underestimated when selecting
optimal methods for semen analysis. They are useful indicators of semen quality and can
serve to eliminate at least some subfertile boars. We conclude that the novel methanol
stress test described here could serve as a rapid test whose results could be used to predict
NR% better than motility.

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maan Jalostuskeskus for providing the semen doses.

References