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Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls

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Abstract. Flow cytometry was utilised for the first time to independently measure five sperm parameters of individual spermatozoa of bull ejaculates to differentiate between outcome successes after artificial insemination (AI). These parameters included plasma membrane and acrosome integrity, mitochondrial functionality and DNA damage measured by sperm chromatin structure assay (SCSA) and terminal deoxynucleotide transferase-mediated dUTP nick end labelling (TUNEL) assays. For each parameter, results of 142 ejaculates (30 bulls) were ranked into three groups according to their flow cytometric measures: (*1*) ejaculates with the 25% lowest measures; (*2*) the 50% middle measures; and (*3*) the 25% highest measures. In total, 20 272 first-service inseminations (18×10^6 spermatozoa per AI dose) were performed, where fertility was defined as non-return within 60 days after first insemination. While plasma membrane and acrosome integrity, and mitochondrial functionality were not significantly related to fertility, data from SCSA and TUNEL assays were significantly associated with fertility. Ejaculates in SCSA group 1 had higher odds of AI success (1.07, 95% CI = 1.02–1.12), whereas those in group 3 had lower odds of AI success (0.94, 95% CI = 0.89–0.99), compared with the average odds of all three groups. Ejaculates in group 2 did not have significantly higher odds of AI success compared with the average odds. For TUNEL-positive spermatozoa, the odds of AI success was higher in group 1 compared with the average odds (1.10, 95% CI = 1.02–1.13), whereas odds of AI success in groups 2 and 3 were not significant compared with the average odds. In conclusion, despite the high number of spermatozoa per AI dose from high-quality bulls, both SCSA and TUNEL assays were valuable measures in this study for evaluating sperm quality in relation to fertility after AI.

Extra keywords: 60 day non-return rate, sperm DNA fragmentation.

Introduction

Since approximately 1990, numerous studies have attempted to predict fertility of semen from breeding bulls based on sperm quality. Precise and accurate estimates of field fertility are very important when attempting to explain differences in the potential fertility of a particular ejaculate or bull. Successful fertilisation and normal embryo development is a function of many factors, the most important including quality of female and male gametes, physiological and genetic female factors and artificial insemination (AI) and herd management (Amann 1989; Amann and Hammerstedt 2002). Furthermore, factors such as the number of spermatozoa per AI dose and time of AI in relation to ovulation will affect the fertility outcome. The reliability of the field fertility data will depend on factors such as the number of AIs per ejaculate and how the outcome of a given AI is reported (Amann 1989; Amann and Hammerstedt 2002). A recent article by Amann (2005) found deficiencies in 51 out of 67 papers that included fertility data as an outcome measure, due to limits in and scarcity of the field fertility data.

In Norway, semen from all young bulls entering the AI progeny testing system is distributed to herds over the entire country. Registration of fertility data from the field are based on a controlled system where all insemination data are reported into an AI database by the insemination technicians and veterinarians (AI personnel). The AI personnel are

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financially credited only when the insemination data are registered into the AI database, which is controlled by the Norwegian Dairy Herd Recording System (Ranberg *et al.* 2003).

The overall sperm quality of one ejaculate is not determined by only one sperm attribute, but depends on several sperm characteristics. The complex role of spermatozoa in fertilisation and embryo development consequently complicates the prediction of a successful outcome. Hence, besides accurate and reliable fertility data, objective and reproducible laboratory methods for analysing sperm attributes are of utmost importance (Amann 1989). The introduction and utilisation of flow cytometric assays for sperm quality analyses have greatly enhanced the objectivity and reproducibility of the andrology laboratory analyses (Graham 2001), the first being reported by Evenson *et al.* (1980) on bull sperm chromatin. Attributes such as membrane integrity (Evenson *et al.* 1982; Ericsson *et al.* 1993; Anzar *et al.* 2002), mitochondrial function (Evenson *et al.* 1982; Ericsson *et al.* 1993) and DNA quality (Evenson *et al.* 1980; Ballachey *et al.* 1987, 1988; Karabinus *et al.* 1990; Januskauskas *et al.* 2001, 2003; Anzar *et al.* 2002) have been studied. Except for the sperm chromatin structure assay (SCSA), few single sperm attributes measured by flow cytometry have been useful in relating sperm quality and field fertility (Gillan *et al.* 2005). The SCSA has shown a consistent relationship with fertility in several species (for review, see Gillan *et al.* 2005; Evenson and Wixon 2006*a*). To date, few studies have included flow cytometric analyses of several sperm attributes in the same ejaculates and furthermore, limits in and scarcity of data from field fertility records are often minimised or ignored (Amann and Hammerstedt 2002; Amann 2005).

The present controlled insemination trial aimed to use established flow cytometric assays to assess variability in certain sperm attributes among ejaculates from several bulls and study how the sperm quality associates with field fertility in a commercial AI setting. We tested whether the chance of AI success differed between ejaculates with variable sperm quality.

Materials and methods

Semen processing

Semen from 30 young Norwegian Red (NRF) bulls entering the AI progeny testing system (Geno Breeding and AI Association, Hamar, Norway) during September–November of 2000 and 2001 in Norway was included. The bulls were kept at Geno Store Ree AI Centre (Hamar, Norway) under uniform feeding and housing conditions throughout the semen production period. At the start of semen collection, the bulls were 14–17 months old, except for two bulls that were 19 months old. Approximately 2200 AI doses were routinely used in the progeny testing of each bull and semen was collected once a week for a period of 3–7 weeks to achieve this number for the included bulls. On the day of collection, two ejaculates were routinely collected within 15 min, and then the ejaculates were pooled and given the same freeze code identification (bull ID and freezing date, hereafter referred to as 'freeze code'). The semen was diluted to a final concentration of 82×10^6 spermatozoa per mL in a skim-milk-based extender in a two-step procedure and frozen in 0.25 mL French mini-straws (IMV, L'Aigle, France), with $\sim 18 \times 10^6$ spermatozoa per straw, in liquid nitrogen (LN_2) (Kommisrud *et al.* 1996). After the first dilution step, morphology and progressive motility were determined microscopically at the AI station as part of the routine semen assessment. Only semen with ≥70% progressively motile spermatozoa and ≥80% normal morphology was further processed to freezing. Routinely, a motility control was done at the AI station after freezing–thawing (assessed ~10 min after thawing) and only freeze codes with ≥50% progressively motile spermatozoa were included in the progeny testing. In total, 149 freeze codes were collected from the included bulls. One freeze code was excluded before freezing owing to low morphology score. Six freeze codes, from five different bulls, were excluded after freezing–thawing owing to low motility score (30–45%). Mean motility of the 142 included freeze codes was 60% (range 50–70%). The mean number of included freeze codes from each bull was five, with a range of three to seven (the number depending on the total number of AI doses produced per freeze code). Frozen straws from all 142 freeze codes included in the progeny testing were systematically collected and stored at the AI station for later sperm quality assessment by flow cytometry.

Field fertility recordings

The fertility expressed by non-return (NR) 60 days after first insemination was used as a measure of fertility on freeze code level. Approximately 2200 AI doses were produced and distributed for all test bulls. On average, ~40% of the cows and heifers are bred with young test bulls each year and an average of 1500 first, second and later inseminations are reported to the Norwegian Dairy Herd Recording System per test bull (Ranberg *et al.* 2003). The freeze codes included in the present study were routinely mixed within bull (~2200 AI doses) and batches of AI doses were randomly distributed to the four semen depot stations that cover all parts of Norway. The batch size was determined by the number of animals covered by each depot. From each depot, batches of 10–20 AI doses from each bull were randomly distributed to different AI personnel, who further used these AI doses randomly among herds and animals. To be sure about the paternity, animals inseminated within 3 days after first insemination were excluded from the present study. Animals older than 5th lactation were also excluded, as well as those inseminations where information about parity, month of AI and AI personnel was not available. After exclusion, 20 272 first-service inseminations were included in the dataset, with a mean of 143 AIs per freeze code. Of the animals inseminated, 44% were heifers, 27% 1st lactation cows and 29% multiparous cows, and the overall NR rate of the AIs with the 142 freeze codes was 72.9% (range 63.0–83.5%).

Flow cytometric analyses

All 142 freeze codes included in the progeny testing were subjected to flow cytometric analyses as described below. One straw from each freeze code was used for each of the flow cytometric assays and two replicate sample runs were prepared from the same straw (except for the terminal deoxynucleotide transferase-mediated dUTP nick end labelling (TUNEL) assay, where one replicate sample was run). The semen was thawed at 37°C for 1 min. All flow cytometry-generated data were analysed using EXPO32 Analysis Software (Beckman Coulter, Fullerton, CA, USA).

Sperm chromatin structure assay

The SCSA defines abnormal chromatin structure as increased susceptibility of sperm DNA to acid-induced denaturation *in situ* (for review, see Evenson *et al.* 2002). The method utilises the unique metachromatic properties of acridine orange (AO; Polysciences Europe, Eppelheim, Germany), which fluoresces green when intercalated into double-stranded DNA and red when bound to single-stranded DNA.

Semen was diluted to 200 μL in TNE buffer (0.15 M NaCl, 0.01 M TRIS-HCl, 1 mM disodium ethylenediaminetetraacetic acid (EDTA), pH 7.4, 4°C) to ~2×10^6 spermatozoa per mL and mixed with 400 μL acid denaturation solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2, 4°C). After 30 s, 1.2 mL of staining solution (0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011 M disodium EDTA, 0.15 M NaCl, pH 6.0, 4°C), containing 6 μg mL^{-1} AO, was added. The sample was then subjected to flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA, USA) equipped with a 15 mW argon laser with excitation at 488 nm. The sample was run in setup mode until 3 min after the staining procedure had started, and then 5000 sperm events were collected, using a sample flow rate of ~200 events s^{-1} (low sample pressure). If the replicate sample or the next freeze code was not ready, the previous sample was run in setup mode to keep the sampling line saturated with AO. A bull reference sample was run for every fifth freeze code to ensure that the instrument and laser remained stable throughout the experiment, with X-mean channel value of 125 ± 5 and Y-mean channel value of 425 ± 5. Light scatter and fluorescence data were collected in linear mode. Green fluorescence was detected using a 515–545 nm band pass (BP) filter (FL1) and red fluorescence was detected using a 650 nm long pass filter (FL3). A cytogram of green versus red DNA fluorescence was used to identify spermatozoa with denatured DNA (Fig. 1). A computer-defined gate was set around the sperm signals with increased red DNA fluorescence (denatured DNA) compared with the main population to determine the percentages of spermatozoa with single-stranded (fragmented) DNA (Fig. 1, region B). This percentage was calculated as a percentage of the total sperm population and was reported as the DNA fragmentation index (% DFI).

Terminal deoxynucleotide transferase-mediated dUTP nick end labelling assay

The TUNEL assay is thought to specifically detect 3′-OH ends of DNA strand breaks (Gavrieli *et al.* 1992; Anzar *et al.* 2002). DNA fragmentation was assessed by treating permeabilised fixed spermatozoa with exogenous terminal deoxynucleotidyl transferase (TdT) enzyme (TdT-kit, Roche Diagnostics, Oslo, Norway) in the presence of biotin-16-deoxyuridine triphosphates (biotin-dUTP). The 3′-OH ends of DNA strand breaks serve as primers for the incorporation of biotin-dUTP, which is detected by the use of fluorescein isothiocyanate (FITC)-conjugated streptavidin. Thawed semen was washed in phosphate-buffered saline (PBS) and fixed in 1% paraformaldehyde for 15 min on ice. The spermatozoa were washed and stored in 100% methanol at −20°C until further processing. Approximately 3×10^6 fixed spermatozoa were washed in PBS and permeabilised with 0.1% Triton X-100 and 0.1% sodium citrate in PBS for 10 min on ice. The spermatozoa were washed again and incubated with 20 μg mL^{-1} proteinase K (to degrade chromatin-associated proteins; Roche Diagnostics, Oslo, Norway) for 20 min at room temperature. Thereafter, spermatozoa were washed and incubated at 37°C for 4 h (Anzar *et al.* 2002) in 50 μL TdT solution containing 5 units TdT, 5 μL 5× reaction buffer (supplied with TdT kit), 1.5 mM $CoCl_2$, 0.5 nM biotin-16-dUTP, 0.1 mM dithiothreitol and double-distilled water. The suspensions were agitated every 30 min. Negative staining controls received similar treatment, except that the TdT was omitted. Spermatozoa treated with 10 μg mL^{-1} DNase (DNase I, Roche Diagnostics, Oslo, Norway) for 10 min were used as positive controls. After incubation, spermatozoa were washed and incubated with 50 μL 1/50 streptavidin–FITC in PBS containing 0.1% Triton X-100 and 3% dry milk solution for 30 min at room temperature. The spermatozoa were washed again and stored at 4°C overnight. The next day, 2 μg mL^{-1} Hoechst 33258 (Amersham Pharmacia Biotech, Oslo, Norway) was added in order to discriminate and exclude doublets, aggregates and debris from the analyses, using Hoechst fluorescence pulse-width processing (Stokke *et al.* 1998). Samples were analysed on a FACSVantage SE (BD Biosciences Immunocytometry Systems) and 10 000 spermatozoa per sample were collected at low sample pressure. Hoechst 33258 was excited with a 50 mW UV laser (351–365 nm excitation) and fluorescence was collected using the FL4 detector (402–446 nm BP filter), whereas FITC was excited with a 350 mW laser (488 nm excitation) and fluorescence was collected using the FL1 detector (515–545 nm BP filter). A cytogram of Hoechst 33258 fluorescence (DNA content) versus FITC fluorescence (biotin-dUTP-positivity) was used to determine the percentages of spermatozoa with DNA strand breaks (TUNEL-positive spermatozoa) expressed as a percentage of the total sperm population (Fig. 2, region B).

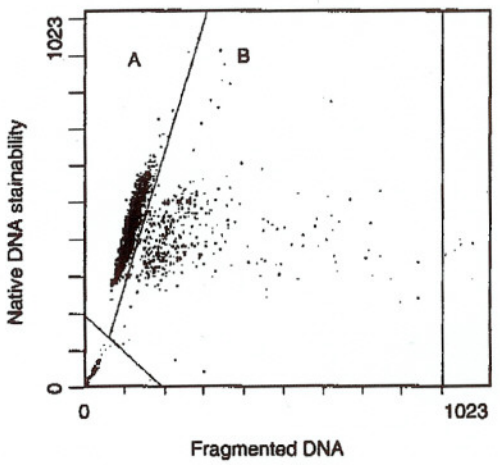

Fig. 1. A typical cytogram of red (single-stranded DNA) *v.* green (double-stranded DNA) acridine orange (AO) fluorescence of thawed bull spermatozoa following treatment with acid denaturation solution. Region A, spermatozoa having predominantly green AO fluorescence. Region B, spermatozoa having increased red AO fluorescence (fragmented DNA). The percentage of spermatozoa in B relative to the total sperm population (regions A + B) corresponds to the DNA fragmentation index (%).

Assessment of plasma membrane and acrosome integrity, and mitochondrial functionality

The membrane-impermeable DNA-binding dye Yo-Pro-1 (Molecular Probes Europe, Leiden, the Netherlands) was used to distinguish between plasma membrane-intact and -degenerated spermatozoa. Acrosome integrity was assessed with the non-lipophilic peanut agglutinin conjugated with FITC (PNA–FITC; Sigma Aldrich, Oslo, Norway), which only binds to glycoproteins in the acrosome membrane of acrosome-reacted or -damaged spermatozoa (Arya and Vanha-Perttula 1985). MitoTracker Red CMXRos (MT-RED, Molecular Probes Europe, Leiden, the Netherlands), which accumulates in functionally polarised mitochondria and leaks out of mitochondria with a depolarised potential, was used to distinguish between spermatozoa with functional and non-functional mitochondria (Gadella and Harrison 2002), respectively, within the live acrosome-intact population. Two replicate samples from each straw were diluted to ~2×10^6 spermatozoa per mL in PBS kept at 37°C, stained with 25 nM Yo-Pro-1, 0.1 μg mL^{-1} PNA–FITC and 25 nM MT-RED for 15 min in the dark and then subjected to flow cytometric analyses. Flow cytometric analyses were performed using a Coulter EPICS XL flow cytometer (Beckman Coulter) equipped with a 15 mW argon laser with a 488 nm excitation wavelength. Light scatter data were collected in linear mode, whereas fluorescence data were collected in logarithmic mode. Side and forward light scatter parameters were used to identify sperm events and 10 000 spermatozoa per

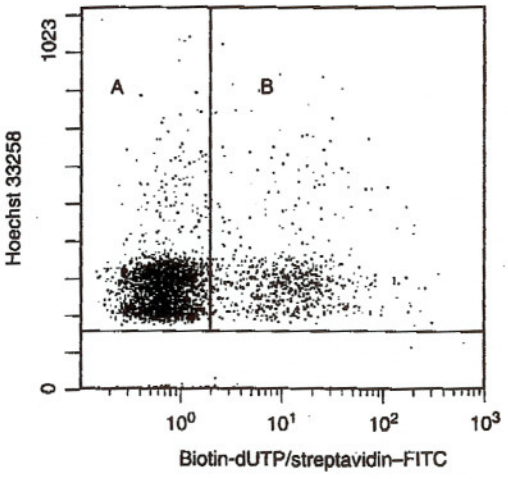

Fig. 2. A typical cytogram of fluorescein isothiocyanate (FITC) (corresponds to biotin-dUTP-positive spermatozoa) *v.* Hoechst 33258 fluorescence (DNA content). Events in regions A and B are spermatozoa, i.e. positively labelled with Hoechst 33258. Region A, spermatozoa with negative FITC-fluorescence intensities. Region B, spermatozoa that are TUNEL-positive, i.e. those spermatozoa with fragmented DNA that have been end-labelled by TdT with biotin-dUTP/streptavidin–FITC.

sample were collected at low sample pressure. Yo-Pro-1 and PNA–FITC fluorescence were detected simultaneously using a 505–545 nm BP filter (FL1) (Harrison *et al.* 1996), and MT-RED fluorescence was detected using a 605–635 nm BP filter (FL3). Unstained samples were used as negative fluorescence controls. The double negative population in FL1 represents the live acrosome-intact (LAI) spermatozoa (Fig. 3*a*, region A) and the positive populations represent dead, acrosome-reacted or -damaged spermatozoa or both (Fig. 3*a*, region B). A cytogram of MT-RED fluorescence versus Yo-Pro-1 and PNA–FITC fluorescence was used to determine the percentages of LAI spermatozoa with functional mitochondria (LAI-MT-RED-positive) (Fig. 3*b*, region A). Populations of dead, acrosome-reacted or -damaged spermatozoa, or both, with high MT-RED fluorescence (region B), live acrosome-intact spermatozoa with low MT-RED fluorescence (region C) and dead, acrosome-reacted or -damaged spermatozoa, or both, with low MT-RED fluorescence (region D) are also shown in the cytogram.

Statistical analyses

The mean value of the two replicate sample runs per freeze code (except for the TUNEL assay, where one replicate sample was run), for each of the flow cytometric assays, was used in the analysis of the data. All sperm parameters are presented as mean values of the 142 freeze codes with standard deviations in brackets. Statistical analyses were conducted using SAS version 8.01 for Windows (SAS Institute 1999). Spearman rank correlations were used to test the relationships between the sperm parameters. Logistic regression models were fit using the proc logistic procedure in SAS to assess the relationship between each of the sperm quality attributes and field fertility. Results from the flow cytometric analyses of each sperm quality attribute were ranked into three groups in the logistic regression models: (*1*) freeze codes with the lowest 25% measures; (*2*) freeze codes with the middle 50% measures; and (*3*) freeze codes with the highest 25% measures. In the logistic models, the three ranked groups were coded by deviation from means coding (Hosmer and Lemeshow 2000). The three ranked freeze code groups were tested against the average of all three freeze code groups for each sperm parameter. Results are presented as odds ratio of AI

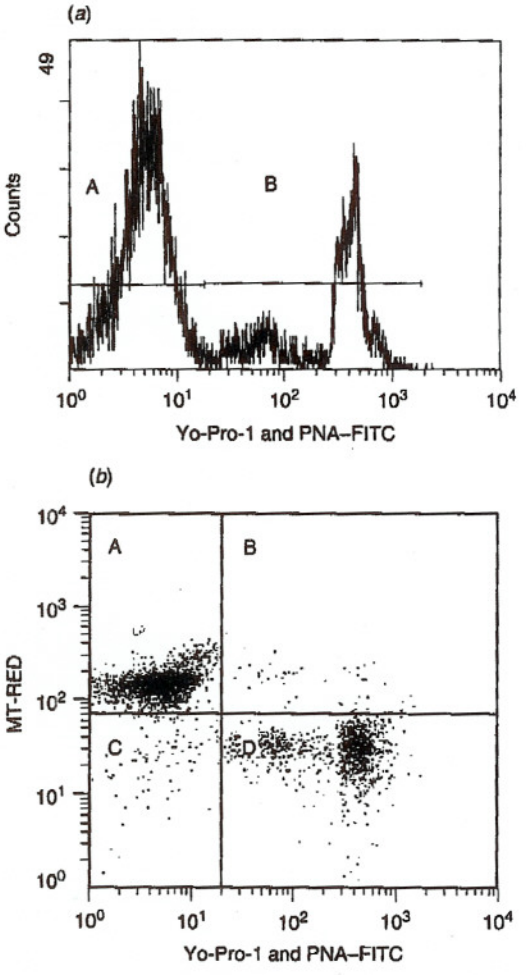

Fig. 3. (*a*) A typical histogram of Yo-Pro-1 and peanut agglutinin (PNA)–fluorescein isothiocyanate (FITC) fluorescence intensities in post-thawed bull spermatozoa. Region A, live acrosome-intact (LAI) spermatozoa, negative for Yo-Pro-1 and PNA–FITC. Region B, dead, acrosome-reacted or -damaged spermatozoa or both, positive for Yo-Pro-1 and/or positively labelled with PNA–FITC. (*b*) A typical cytogram of Yo-Pro-1 and PNA–FITC *v.* MT-RED fluorescence in post-thawed bull spermatozoa. Region A, LAI spermatozoa with high MitoTracker Red CMXRos (MT-RED) fluorescence. Region B, dead, acrosome-reacted or -damaged spermatozoa, or both, with high MT-RED fluorescence. Region C, LAI spermatozoa with low MT-RED fluorescence. Region D, dead, acrosome-reacted or -damaged spermatozoa, or both, with low MT-RED fluorescence.

success defined as the change in odds between each ranked group and the average odds of all the three groups.

The sperm variables were entered one at a time in the logistic regression with 60 days NR (0 = return, 1 = non-return) as the binary outcome variable. The model contained the following explanatory variables: AI personnel (divided into veterinarian and AI technician); lactation number (divided into heifers, 1st lactation and multiparous); month of AI; and the sperm parameter (divided into 25% lowest, 50% middle and 25% highest freeze code measures from the flow cytometric analyses). Odds ratio estimates for AI success (return *v.* non-return) in the three ranked groups compared with the average odds of all the three groups

Table 1. Correlation coefficients between sperm quality parameters assessed by flow cytometry

LAI, percentage of live acrosome-intact spermatozoa; LAI-MitoTracker Red CMXRos (MT-RED)-positive, percentage of live acrosome-intact spermatozoa with functional MT-RED fluorescence intensity; DFI, DNA fragmentation index measured by the sperm chromatin structure assay; DNA strand breaks, percentage of TUNEL-positive spermatozoa

Sperm parameters	LAI	LAI-MT-RED-positive	DNA strand breaks
LAI-MT-RED-positive	0.99*		
DFI	−0.29*	−0.31*	0.82*
DNA strand breaks	−0.40*	−0.41*	

*$P < 0.001$.

are presented for significant sperm parameters. *P*-values less than 0.05 were considered statistically significant.

The same type of model as described above was used to test the relationship between multiple sperm parameters and 60 days NR with stepwise backward selection procedure. Sperm parameters with high correlation coefficients were tested in separate models.

From the logistic regression models that were significantly related to fertility, the 60 days NR rate (independent of parity) was also estimated for the three ranked groups of significant sperm parameters.

Results

Post-thaw sperm quality

Some spermatozoa with abnormal chromatin structure were observed in all included freeze codes, with a mean percentage DFI of 6.1 (s.d. = 3.1). Spermatozoa with fragmented, single-stranded DNA assessed as having increased red AO-fluorescence compared with the main sperm population are shown in Fig. 1.

A typical cytogram of spermatozoa demonstrating biotin-dUTP-positivity (FITC fluorescence), representing spermatozoa with DNA strand breaks, is presented in Fig. 2. The TUNEL assay detected some spermatozoa with DNA strand breaks in all the included freeze codes, with a mean percentage of 7.6 (s.d. = 3.5) of TUNEL-positive spermatozoa. The percentage of spermatozoa having DNA strand breaks and % DFI were highly correlated (0.82, $P < 0.001$).

A typical histogram with populations of LAI spermatozoa and dead, acrosome-reacted or -damaged spermatozoa or both are shown in Fig. 3*a* and a typical cytogram of MT-RED staining in LAI spermatozoa is shown in Fig. 3*b*. After freezing, the mean percentage of LAI spermatozoa was 54.7 (s.d. = 10.2), whereas the mean percentage of LAI spermatozoa with positive MT-RED staining was 51.3 (s.d. = 10.2). Almost all spermatozoa in the LAI population had distinctly higher MT-RED staining intensity compared with the same parameter in the dead, acrosome-reacted or -damaged spermatozoa, thus we were only able to detect a few LAI spermatozoa with depolarised potential over the inner mitochondrial membrane (mean percentage of 3.0 (s.d. = 1.1), region C, Fig. 3*b*). Hence, the percentage of LAI spermatozoa and the percentage of LAI-MT-RED-positive spermatozoa were highly correlated (Table 1).

Significant negative correlations were found between both percentages of DFI and DNA strand breaks and the percentage of LAI and LAI-MT-RED-positive spermatozoa (Table 1).

Relationship between sperm parameters and field fertility

Percentages of DFI and TUNEL-positive spermatozoa at freeze code level were significantly associated with field fertility ($P = 0.019$ and $P = 0.024$ respectively). Compared with the average of all freeze code groups, group 1 with DFI of 1.6–3.8% had an estimated 7% increase in odds of AI success (60 days NR, $P = 0.010$), whereas group 3 with DFI of 7.5–21.6% had an estimated 6% reduction in odds of AI success ($P = 0.011$) (Table 2). The odds of AI success for group 2 with 3.8–7.5% DFI was not significantly different from the average of all freeze code groups ($P = 0.848$) (Table 2). For group 1 with 2.2–4.8% TUNEL-positive spermatozoa, the odds of AI success was 10% ($P = 0.006$) higher compared with the average of all freeze code groups, whereas the odds of AI success for group 3 with 9.4–21.1% TUNEL-positive spermatozoa or group 2 with 4.8–9.4% TUNEL-positive spermatozoa were not significantly different from the average ($P = 0.089$ and $P = 0.202$ respectively; Table 2).

Neither percentages of LAI nor MT-RED LAI at freeze code level were significantly associated with field fertility when entered one at a time in the logistic regression models ($P = 0.734$ and $P = 0.870$ respectively).

The backward stepwise selection procedure did not give any significant multiple models where other sperm parameters than DFI or DNA strand breaks were included.

Estimated 60 days NR rate values based on the three ranked groups of DFI and DNA strand breaks measures are presented in Table 2.

Discussion

Despite the high number of spermatozoa per AI dose and the overall high field fertility of the included freeze codes, significant relationships between sperm quality attributes and

Table 2. Odds ratio estimates of artificial insemination (AI) success and estimated 60 days non-return (NR) rate of the three ranked freeze code groups of DNA fragmentation assessed by the sperm chromatin structure assay (SCSA) and TUNEL assay

DFI, DNA fragmentation index measured by the SCSA; DNA strand breaks, percentage of TUNEL-positive spermatozoa

Sperm parameters	Odds ratio	95% CI	No. AIs	Estimated 60 days NR rate (%)[D]
DFI (%)				
Lower quarter (1.6–3.8[A])	1.07*	1.02–1.12	4846	73.9
Middle half (3.8–7.5[B])	1.00	0.96–1.04	10 180	72.8
Upper quarter (7.5–21.6[C])	0.94*	0.89–0.99	5246	71.6
DNA strand breaks (%)				
Lower quarter (2.2–4.8[A])	1.07*	1.02–1.13	5018	74.2
Middle half (4.8–9.4[B])	0.97	0.93–1.01	9787	72.9
Upper quarter (9.4–21.1[C])	0.96	0.99–1.01	5467	72.1

[A]Range of the 25% lowest freeze code measures (group 1). [B]Range of the 50% middle freeze code measures (group 2). [C]Range of the 25% highest freeze code measures (group 3). [D]Sixty days NR rate (independent of parity) for the three ranked groups were estimated from the logistic regression models. *Significantly different from the average of all three freeze code groups, $P < 0.05$.

field fertility were revealed in the present study. In particular, the SCSA-derived % DFI and % TUNEL-positive spermatozoa were significantly related to field fertility. The range in % DFI and % TUNEL-positive spermatozoa were not at the level to separate freeze codes with an unacceptably low fertility potential from those with a high fertility potential. However, our findings are of significant practical and economical consequence for the AI industry (Economy and Fertility in Cattle Software; personal communication with A. O. Refsdal, Geno Breeding and AI Association).

The SCSA has proven valuable as a way to distinguish between fertile, subfertile or infertile men (Evenson *et al.* 1999, 2002; Evenson and Wixon 2006*b*). Bulls with poor semen quality and potential sub fertility are removed from the breeding population and are therefore not the main issue here. The question is rather if the SCSA can distinguish between semen from fertile bulls in the high range and whose spermatozoa have been pre-selected before AI (≥50% motile spermatozoa in the present study). Previous studies in bulls with a wide range in fertility of 46–83% (Januskauskas *et al.* 2001, 2003) and 53–80% in NR rate (Karabinus *et al.* 1990) have reported significant correlations between % DFI and field fertility. Even though the range in NR for the AIs of the freeze codes in the present study was rather narrow, a significant association between % DFI and field fertility was found. Thus, the SCSA does seem able to distinguish between fertility levels of freeze codes with proven fertility in the high range.

Sailer *et al.* (1995) found a high correlation between % DFI and % TUNEL-positive spermatozoa in bull semen ($r = 0.78$, $P < 0.001$). To our knowledge, our study is one of the first to confirm these findings. The high correlation indicates that DNA strand breaks most likely are responsible for the increase in susceptibility of sperm chromatin to *in situ* denaturation (Sailer *et al.* 1995; Evenson and Wixon 2006*a*). Our data suggest that the two assays are measuring the same sites of DNA damage, and thus the high correlation between SCSA and TUNEL measures justifies using only one of the methods for evaluating sperm DNA quality. The less expensive and time consuming method would be preferable, which in this case would be the SCSA.

As with the DFI, the percentage of spermatozoa with DNA strand breaks assessed by the TUNEL assay was significantly associated with fertility. Only a limited number of studies have looked at the relationship between DNA strand breaks measured by the TUNEL assay and bull fertility, and to our knowledge, the present study is the first to demonstrate a significant relationship between fertility of bull semen and DNA strand breaks of frozen–thawed spermatozoa. Anzar *et al.* (2002) found a significant correlation between TUNEL-positive spermatozoa in fresh semen and fertility ($r = -0.90$, $P < 0.05$), but no significant correlation was found for frozen–thawed spermatozoa. It has been speculated that DNA fragmentation in mature spermatozoa is a result of one of the following: defects in the reorganisation of chromatin during sperm maturation; insufficient protection against reactive oxygen species (ROS) in the epididymis; or the presence of an 'abortive apoptosis' mechanism (Sakkas *et al.* 1999; Shen *et al.* 2002). Recently, it has been shown that DNA fragmentation of human spermatozoa was not related to early apoptotic markers, thus ROS might be a stronger candidate (Henkel *et al.* 2004). Spermatozoa with fragmented DNA may in fact have normal motility and morphology and thus may still be able to fertilise an oocyte (Ahmadi and Ng 1999; Fatehi *et al.* 2006). A recent study of bull spermatozoa showed that DNA fragmentation does not impair *in vitro* fertilisation or completion of the first cleavage stages, but blocks further embryonic development when the blastocyst

formation is reached, by inducing apoptosis (Fatehi *et al.* 2006).

A negative correlation was found between DNA fragmentation, assessed by SCSA or TUNEL assay, and the percentage of LAI spermatozoa, which may indicate that factors affecting DNA during sperm maturation in the epididymis also act at the plasma- and acrosome-membrane level and make the spermatozoa more vulnerable to the freezing and thawing process. The high level of polyunsaturated fatty acids in mammalian sperm membranes makes them especially vulnerable to ROS, which may be the common factor that damages both membranes and DNA (Sikka 2004). A rather high variation between the freeze codes in the percentage of LAI spermatozoa was observed. However, the percentage of LAI spermatozoa showed no significant relation with field fertility. The literature is somewhat contradictory on this point. Anzar *et al.* (2002) showed a significant correlation ($r = 0.87$, $P < 0.05$) between the percentage of plasma membrane-intact spermatozoa and 56 days NR rate for fresh bull semen, but not for frozen–thawed semen. Although Januskauskas *et al.* (2003) found a significant correlation between fertility and the percentage of plasma membrane-intact spermatozoa assessed by fluorometry, a significant relationship was not found between the percentage of plasma membrane-intact spermatozoa assessed by flow cytometry and fertility.

To our knowledge, the present study is the first in which mitochondrial membrane functionality has been assessed simultaneously with plasma membrane and acrosome integrity, and related to field fertility. In all freeze codes, most LAI spermatozoa had staining corresponding to a functional potential over the inner mitochondrial membrane, reflected in the high correlation coefficient between the percentage of LAI and LAI-MT-RED-positive spermatozoa. Variation between freeze codes in percentage of LAI-MT-RED-positive spermatozoa was observed, but no significant relation to fertility was found. These findings are in agreement with Ericsson *et al.* (1993), who found no significant correlation between fertility and the percentage of live spermatozoa with functional mitochondria.

It is crucial to have accurate and reliable fertility data in studies as described here. Otherwise it will be difficult to draw valid conclusions concerning relations between sperm quality and fertility (Amann and Hammerstedt 2002; Amann 2005). The Norwegian Dairy Herd Recording System, providing fertility data for the present study, made it possible to record accurate and reliable fertility data. The fertility data used in the present study were based on AIs with a sperm number per AI dose of $\sim 18 \times 10^6$, which is a number commonly used in an AI setting, as well as a number that is likely to fall on the asymptote of the spermatozoa per AI dose–response curve (Amann and Hammerstedt 2002). While DNA fragmentation is considered to be an uncompensable trait, plasma membrane and acrosome integrity and motility of spermatozoa are considered to be compensable attributes (Pace *et al.* 1981; Saacke *et al.* 2000). We suggest that the observed freeze code variation in % LAI and % LAI-MT-RED-positive spermatozoa is camouflaged by the relatively high number of spermatozoa per AI dose. Below a certain threshold we would expect a negative effect on fertility with use of AI doses with lowered sperm number, i.e. with reduced number of LAI spermatozoa in the AI dose. It would be interesting to use much lower sperm numbers for obtaining additional research data and knowledge on the relationships between compensable sperm attributes and fertility. However, carrying out field trials with lowered sperm numbers, i.e. on the dose–response portion of the curve, would be far too expensive for the farmers and AI industry and would not reflect the present commercial AI setting in Norway. Consequently, defects in compensable sperm attributes may be masked.

To conclude, the relatively high number of spermatozoa per AI dose may partially be responsible for the lack of significant relationships between some of the sperm parameters and field fertility. Despite the high number of spermatozoa per AI dose, pre-selection of AI doses and the high field fertility of the included freeze codes, the SCSA and TUNEL assays were associated with field fertility. This is the first study using the powerful technique of flow cytometry to assess five sperm parameters.

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References

Ahmadi, A., and Ng, S. C. (1999). Fertilizing ability of DNA-damaged spermatozoa. *J. Exp. Zool.* **284**, 696–704. doi:10.1002/(SICI)1097-010X(19991101)284:6<696::AID-JEZ11>3.0.CO;2-E

Amann, R. P. (1989). Can the fertility potential of a seminal sample be predicted accurately? *J. Androl.* **10**, 89–98.

Amann, R. P. (2005). Weaknesses in reports of "fertility" for horses and other species. *Theriogenology* **63**, 698–715. doi:10.1016/J.THERIOGENOLOGY.2004.04.010

Amann, R. P., and Hammerstedt, R. H. (2002). Detection of differences in fertility. *J. Androl.* **23**, 317–325.

Anzar, M., He, L., Buhr, M. M., Kroetsch, T. G., and Pauls, K. P. (2002). Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biol. Reprod.* **66**, 354–360. doi:10.1095/BIOLREPROD66.2.354

Arya, M., and Vanha-Perttula, T. (1985). Lectin-binding pattern of bull testis and epididymis. *J. Androl.* **6**, 230–242.

Ballachey, B. E., Hohenboken, W. D., and Evenson, D. P. (1987). Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. *Biol. Reprod.* **36**, 915–925. doi:10.1095/BIOLREPROD36.4.915

Ballachey, B. E., Saacke, R. G., and Evenson, D. P. (1988). The sperm chromatin structure assay: Relationship with alternate tests of sperm quality and heterospermic performance of bulls. *J. Androl.* **9**, 109–115.

Ericsson, S. A., Garner, D. L., Thomas, C. A., Downing, T. W., and Marshall, C. E. (1993). Interrelationships among fluorometric

analyses of spermatozoal function, classical semen quality parameters and the fertility of frozen–thawed bovine spermatozoa. *Theriogenology* **39**, 1009–1024. doi:10.1016/0093-691X(93)90002-M

Evenson, D. P., and Wixon, R. (2006*a*). Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology* **65**, 979–991. doi:10.1016/J.THERIOGENOLOGY.2005.09.011

Evenson, D. P., and Wixon, R. (2006*b*). Meta-analysis of sperm DNA fragmentation using the Sperm Chromatin Structure Assay. *Reprod. Biomed. Online* **12**, 466–472.

Evenson, D. P., Darzynkiewicz, Z., and Melamed, M. R. (1980). Relation of mammalian sperm chromatin heterogeneity to fertility. *Science* **210**, 1131–1133.

Evenson, D. P., Darzynkiewicz, Z., and Melamed, M. R. (1982). Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. *J. Histochem. Cytochem.* **30**, 279–280.

Evenson, D. P., Jost, L. K., Marshall, D., Zinaman, M. J., Clegg, E., Purvis, K., De Angelis, P., and Claussen, O. P. (1999). Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum. Reprod.* **14**, 1039–1049. doi:10.1093/HUMREP/14.4.1039

Evenson, D. P., Larson, K. L., and Jost, L. K. (2002). Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J. Androl.* **23**, 25–43.

Fatehi, A. N., Bevers, M. M., Schoevers, E., Roelen, B. A. J., Colenbrander, B., and Gadella, B. M. (2006). DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages. *J. Androl.* **27**, 176–188. doi:10.2164/JANDROL.04152

Gadella, B. M., and Harrison, R. A. P. (2002). Capacitation induces cyclic adenosine 3′,5′-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. *Biol. Reprod.* **67**, 340–350. doi:10.1095/BIOLREPROD67.1.340

Gavrieli, Y., Sherman, Y., and Ben Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493–501. doi:10.1083/JCB.119.3.493

Gillan, L., Evans, G., and Maxwell, W. M. (2005). Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology* **63**, 445–457. doi:10.1016/J.THERIOGENOLOGY.2004.09.024

Graham, J. K. (2001). Assessment of sperm quality: a flow cytometric approach. *Anim. Reprod. Sci.* **68**, 239–247. doi:10.1016/S0378-4320(01)00160-9

Harrison, R. A., Ashworth, P. J., and Miller, N. G. (1996). Bicarbonate/CO_2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Mol. Reprod. Dev.* **45**, 378–391. doi:10.1002/(SICI)1098-2795(199611)45:3<378::AID-MRD16>3.0.CO;2-V

Henkel, R., Hajimohammad, M., Stalf, T., Hoogendijk, C., Mehnert, C., Menkveld, R., Gips, H., Schill, W. B., and Kruger, T. F. (2004). Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil. Steril.* **81**, 965–972. doi:10.1016/J.FERTNSTERT.2003.09.044

Hosmer, D. W., and Lemeshow, S., Jr (2000). Interpretation of the fitted logistic regression model. In 'Applied Logistic Regression'. (Eds N. A. C. Cressie, N. I. Fisher, I. M. Johnstone, J. B. Kadane, D. W. Scott *et al.*) pp. 47–90. (John Wiley & Sons: New York, USA.)

Januskauskas, A., Johannisson, A., and Rodriguez-Martinez, H. (2001). Assessment of sperm quality through fluorometry and sperm chromatin structure assay to field fertility of frozen–thawed semen form Swedish AI bulls. *Theriogenology* **55**, 947–961. doi:10.1016/S0093-691X(01)00456-3

Januskauskas, A., Johannisson, A., and Rodriguez-Martinez, H. (2003). Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. *Theriogenology* **60**, 743–758. doi:10.1016/S0093-691X(03)00050-5

Karabinus, D. S., Evenson, D. P., Jost, L. K., Baer, R. K., and Kaproth, M. T. (1990). Comparison of semen quality in young and mature Holstein bulls measured by light microscopy and flow cytometry. *J. Dairy Sci.* **73**, 2364–2371.

Kommisrud, E., Steine, T., and Graffer, T. (1996). Comparison of fertility rates following insemination with different numbers of spermatozoa per insemination dose of frozen bovine semen. *Reprod. Domest. Anim.* **31**, 359–362.

Pace, M. M., Sullivan, J. J., Elliott, F. I., Graham, E. F., and Coulter, G. H. (1981). Effects of thawing temperature, number of spermatozoa and spermatozoal quality on fertility of bovine spermatozoa packed in 5-mL French straws. *J. Anim. Sci.* **53**, 693–701.

Ranberg, I. M., Heringstad, B., Klemetsdal, G., Svendsen, M., and Steine, T. (2003). Heifer fertility in Norwegian dairy cattle: variance components and genetic change. *J. Dairy Sci.* **86**, 2706–2714.

Saacke, R. G., Dalton, J. C., Nadir, S., Nebel, R. L., and Bame, J. H. (2000). Relationship of seminal traits and insemination time to fertilization rate and embryo quality. *Anim. Reprod. Sci.* **60–61**, 663–677. doi:10.1016/S0378-4320(00)00137-8

Sailer, B. L., Jost, L. K., and Evenson, D. P. (1995). Mammalian sperm DNA susceptibility to *in situ* denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *J. Androl.* **16**, 80–87.

Sakkas, D., Mariethoz, E., Manicardi, G., Bizzaro, D., Bianchi, P. G., and Bianchi, U. (1999). Origin of DNA damage in ejaculated human spermatozoa. *Rev. Reprod.* **4**, 31–37. doi:10.1530/ROR.0.0040031

SAS Institute (1999) 'SAS OnlineDoc Version 8.' (SAS Institute: Cary, NC, USA.)

Shen, H. M., Dai, J., Chia, S. E., Lim, A., and Ong, C. N. (2002). Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. *Hum. Reprod.* **17**, 1266–1273. doi:10.1093/HUMREP/17.5.1266

Sikka, S. C. (2004). Role of oxidative stress and antioxidants in andrology and assisted reproductive technology. *J. Androl.* **25**, 5–18.

Stokke, T., Solberg, K., De Angelis, P., and Steen, H. B. (1998). Propidium iodide quenches the fluorescence of TdT-incorporated FITC-labeled dUTP in apoptotic cells. *Cytometry* **33**, 428–434. doi:10.1002/(SICI)1097-0320(19981201)33:4<428::AID-CYTO6>3.0.CO;2-4

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