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FDM, RF, MS, EG and AHG designed the study. RF, EBS and TTZ performed the SCSA analysis. NHT, IAR and AHG performed statistical analysis. FDM, NHT and AHG created the manuscript. All authors contributed to writing and approval of the finale manuscript.

Sperm DNA integrity in Landrace and Duroc boar semen and its relationship to litter size

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Contents

The sperm chromatin structure assay is a method for assessment of sperm DNA fragmentation, a parameter reported to be negatively related to field fertility in several mammal species. This method calculates a DNA fragmentation index (DFI) whose high values indicate abnormal chromatin structure. In this study, running from March 2010 until June 2017, the aim was to assess sperm DFI in stored liquid extended semen from two different pig breeds, Norwegian Landrace (NL; n=693) and Norwegian Duroc (ND; n=655) and to evaluate the influence on total number of piglets born (TNB). There was a significantly higher median DFI ($p<0.0001$) in ejaculates from the 478 ND boars compared to the 452 NL boars. Data from 19496 NL litters and 3877 ND litters of the same boars were retrieved. For either breed, sow herd ($p<0.0001$), parity ($p<0.05$) as well as DFI ($p<0.05$) showed significant effects on TNB. The DFI was negatively correlated to TNB in both breeds. The boars with the 5% lowest TNB had a least square means DFI of 3.05% and 2.24% in NL and ND, respectively, compared to 1.67% and 1.23% for the boars with the 5% highest TNB ($p<0.01$). The DFI and the motility of the same semen samples were negatively correlated ($p<0.0001$), and the high and low TNB groups showed significant differences in motility. However, this difference could not be used for practical prediction of TNB group (92.1% vs. 89.7%; $p=0.0038$ and 92.3% vs 89.5%; $p=0.018$; NL and ND, respectively). In conclusion, our results indicates that sperm DNA integrity in semen with good motility and morphology may be an additional prediction parameter for fertility in pigs.

Keywords: Boar, Semen analysis, DNA integrity, Sperm chromatin structure assay, Fertility

INTRODUCTION

Examination of sperm concentration, motility and morphology are routine procedures for semen quality evaluation at AI stations (Foxcroft et al. 2008). However, *in vivo* effects of these standard semen parameters can be masked by high sperm number in the semen dose, which can explain why they only to a limited extent are directly related to field fertility. Therefore, improved quality assessment methods have been developed such as the sperm chromatin structure assay (SCSA), in which semen samples are being frozen at -80°C and then thawed as part of the protocol for assessment of sperm DNA fragmentation (Evenson and Jost 1994, Evenson and Jost 2000, Evenson et al. 1994).

During spermatogenesis, the sperm chromatin is condensed by protamines to a highly compact structure (Rathke et al. 2014). This protects the sperm genetic material against damage during transport through the male and female reproductive tracts. Defects in sperm chromatin packaging are associated with DNA damage, which can i.e. be caused by germ cell apoptosis in the testis, incomplete epididymal sperm maturation or oxidative stress (Aitken et al. 2013). Sperm DNA integrity is protected by DNA repair mechanisms during spermatogenesis (Gonzalez-Marin et al. 2012). However, upon spermatogenesis, sperm cells lack mechanisms to repair DNA damage and thus the compact chromatin structure is essential for its protection.

DNA damaged spermatozoa can fertilize oocytes, and upon fertilization, oocytes and early embryos can repair some types of DNA breakage (Gonzalez-Marin et al. 2012). The extent of this repair is, however, associated with the level and type of DNA damage and the repair capacity of the oocyte (Wdowiak et al. 2015). Both in human (Wdowiak et al. 2015) and bovine (Fatehi et al. 2006), low DNA integrity has been reported to negatively affect embryo development and it is related to early embryonic mortality.

The SCSA is a flow cytometry method assessing sperm DNA fragmentation (Evenson and Jost 1994, Evenson and Jost 2000, Evenson et al. 1994). The assay evaluates the susceptibility of sperm DNA to denaturation *in situ* under acidic conditions. It utilizes the metachromatic properties of acridine orange (AO), which fluoresces green when bound to double stranded DNA (dsDNA), and red when bound to single stranded DNA (ssDNA). Flow cytometer results are used to calculate a DNA fragmentation index (DFI) for each spermatozoon. High DFI values are indicative of abnormal chromatin structure (Evenson and Jost 2000). Accumulating evidence suggests that increased levels of DFI are negatively related to field fertility (Love and Kenney 1998, Waterhouse et al. 2006, Evenson 2016). For example in swine, sperm DNA fragmentation is reported to influence the total number of piglets born (Boe-Hansen et al. 2008, Broekhuijse et al. 2012, Didion et al. 2009). Therefore, the level of DNA integrity may be a promising parameter for avoiding AI service usage of boars with potential low litter size.

The aim of the present study was to assess sperm DNA fragmentation in stored liquid extended semen from two different pig breeds, Norwegian Landrace (NL) and Norwegian Duroc (ND) and to evaluate the influence on field fertility measured as total number of piglets born.

MATERIALS AND METHODS

Animals, semen collection and preparation

During a period from March 2010 to June 2017, semen samples were collected from 452 purebred Norwegian Landrace (NL) and 478 Norwegian Duroc (ND) boars routinely used for artificial inseminations (AI), located at the AI station run by Norsvin at Hamar, Norway. The age of the boars at sampling ranged from 221-1000 days (mean = 312.1 days, SD = 86.7) for NL and from 228-829 days (mean = 297.4 days, SD = 61.9) for ND. All animals were cared for according to internationally recognized guidelines and regulations for keeping pigs in Norway (The Animal Welfare Act of June 19th, 2009 and the Regulations for keeping of pigs in Norway of February 18th, 2003). During the collection period, a total of 1346 samples (NL: n=693; ND: n=653) were analyzed upon storage. From each boar a sample was taken at the first possible semen collection after entering the AI center, and then for a number of times whenever possible until culling. The number of samples per boar had a range from 1 to 9, with an average of 1.53 for NL and 1.37 for ND.

The sperm-rich fraction of the ejaculates was collected with the “gloved hand” technique. Upon collection, the samples were diluted in Tri-X-cell® (IMV technologies, L’Aigle, France) to a concentration of 28x10^6 spermatozoa/mL. However, from July 2011 the extender used was Androstar Plus® (Minitüb GmBH, Tiefenbach, Germany). Motility and morphology evaluation was done by subjective evaluation using phase contrast microscopy (Leica DM 4000B, Leica

Microsystems, Wetzlar, Germany) until November, 2014 whereafter a CASA system (IVOS-II, Hamilton-Thorne Inc, Beverly, USA) was used.

Upon each semen collection, motility and morphology were evaluated at the AI station. According to the normal routines of the AI center, ejaculates with <70% motile and/or >20% morphologically abnormal spermatozoa were discarded and only semen accepted for AI was included in this study. Tubes with diluted semen (89 mL) were stored at 18°C until shipment in styrofoam boxes to commercial swine producers for use within the next four days after the collection date. The samples for analysis of DNA fragmentation were transported from the AI station to the laboratory. At the laboratory, semen aliquots of 1 mL were snap-frozen and stored at -80°C until analyzed for DNA fragmentation. In an initial study, aliquots from semen samples (N=75; 49 NL samples and 26 ND samples) were collected during the period from March 2010 to February 2011 and snap-frozen on the day of semen collection (Day 0) and after storage at 18°C for 96 hours (Day 4). These samples were included to evaluate the effect of liquid semen storage on sperm DNA fragmentation. In the main study, the samples were snap-frozen at -80°C upon storage for 48, 72 or 96 hours depending on the weekday the collection was performed. The snap-frozen samples were analyzed by the SCSA method within one month after collection.

Sperm chromatin structure assay

The SCSA protocol was performed according to the procedure described by Evenson and Jost (2001) and later modified by Boe-Hansen et al. (2005). For sample analysis, a Cell Lab QuantaTM SC MPL flow cytometer equipped with an argon laser with excitation at 488 nm and 22 mW power (Beckman Coulter, Fullerton, CA, USA) was used. Snap-frozen semen samples were thawed in water bath at 37°C and diluted to $2x10^6$ sperm cells/mL in TNE buffer (10 mM Tris-HCL, 0.15 M NaCl, 1 mM EDTA, pH 7.4) to a final volume of 200 µL. Thereafter, 400 µL acid detergent solution (0.38 M NaCl, 80 mM HCL, 0.1 % (w/v) Triton X-100, pH 1.2) was added to the sample. A stopwatch was started and upon exactly 30 seconds incubation, 1.2 mL AO staining solution (6 µg/mL AO (A3568, Life Technologies, OR, USA) in a buffer containing 37 mM citric acid, 0.126 M Na_2HPO4, 1.1 µM EDTA, 0.15 M NaCl, pH 6) was added. Further, the sample was placed in the flow cytometer and run in setup mode for 3 minutes. Then the data acquisition started and 5000 events were collected for each sample. Signals were first separated by 550 nm dichroic long pass mirror. Subsequently, fluorescence was collected through a 525 nm band pass filter (green) and a 670 nm long pass filter (red). Prior to sample analysis, the flow cytometry instrument was AO saturated by running AO equilibration solution (1.2 mL AO staining solution and 400 µL acid detergent solution) through the system for 5 minutes. At the start and after analyzing every fifth sample, mean green and red fluorescence signals were set to 425±5 and 125±5, respectively, using reference boar semen of known DFI to control laser stability. The percentage of red (ssDNA) and green (dsDNA) fluorescence was determined using Cell Lab QuantaTM SC MPL Analysis software (Beckman Coulter, Software Version 1.0 A). Based on the fluorescence ratio red/(red + green), percentage DFI was calculated and mean of two replicates was used for further statistical analysis.

Fertility records

Insemination data from gilts and sows were retrieved from "Ingris", the national litter recording system. Farrowing records from all litters having one of the boars sampled for DFI assessment as sire were collected. Type of insemination as an effect in the models was classified as either single or double with the same sire. For double insemination dates, the latest insemination date was used as the valid date. Storage time of semen doses before use was determined based on corresponding insemination dates and semen collection dates of any given boar. Only herds situated geographically close enough for courier car or self-service would be able to use doses on the day of semen collection. Insemination records indicating that doses had been used later than the fifth day post collection were omitted from the data set. Only purebred litters were included in the dataset (gestation lengths between 109 and 125 days). The total number of piglets born (TNB) for each litter was calculated as the sum of liveborn and stillborn piglets and mummified foetuses. Litter records with zero TNB or with >29 TNB were deleted from the dataset as well as litter data from NL and ND boar with less than 15 and 10 litters from each boar, respectively. The full litter data set included 19496 litters from 312 NL boars and 3877 litters from 217 ND boars with analyses of DFI. Among these, 75.2% and 74.3% of the NL and ND litters, respectively, were from semen stored from 48 to 96 hours prior to AI.

Statistical analyses

Statistical analyses were performed using the software package Statistical Analysis Software (SAS) version 9.4 for Microsoft Windows (SAS Institute Inc., Cary, NC, USA). A Shapiro-Wilk test showed that the DFI data were not normally distributed. Therefore, a log transformation of the DFI data was performed prior to further statistical analysis. The log-transformed DFI data (log DFI) were analyzed using paired t-test for testing the effect of storage (Day 0 and Day 4). Statistical analyses were performed separately for the two breeds.

Least Square Means (LS-means) for log DFI of each boar was calculated using a General Linear Model (GLM) procedure. First, possible correlations (Pearson test) between log DFI and age of the boar, sperm cell concentration, motility, collection interval (the interval in days since previous collection from each boar) and storage time of the semen when analyzed (2, 3, 4 days) were tested using PROC CORR (significance: $p<0.05$). Further, a GLM analysis was performed by evaluating the effect of the boar and other specific boar parameters on DFI values. The effect of the two extenders and of the motility assessment method were included as class variables. The collection interval was divided into four classes; "A" (a boar's first semen collection hence no interval), and "B", "C" and "D" representing 1-3 days, 4-5 days and >5 days, respectively. The season of semen collection was divided into four classes according to the quarters of the year, and the age of each boar (in days) at each sampling date was calculated. A backwards selection approach was used where all the variables of interest were fitted into a model. The variable with the highest p-value was excluded, if the variable had no significant effect. This re-fitting of the model was continued until all the effects in the model were statistically significant ($p<0.05$). In the final NL model for log DFI, boar, age at collection was integrated as an effect, while in the ND model, boar, motility and liquid storage time were integrated in the model. Based on the GLM results, LS-means for log DFI per boar were calculated and included in the model for TNB.

In addition to LS-mean log DFI per boar, the following variables were tested in the model for TNB: sow herd, sow parity (divided into three classes; 1, 2 and >2), type of insemination (single/double), storage time of the semen when inseminated (0, 1, 2, 3, or 4 days) and semen collection month (month/year) were evaluated by using the GLM procedure with the backwards selection approach. The following model 1) for NL and model 2) for ND were used:

Model 1): TNB = herd + parity + type of insemination + LS-means log DFI

Model 2): TNB = herd + parity + semen storage time + LS-means log DFI

In order to investigate threshold values for DFI of the boars, GLM models for each breed were constructed as in model 1 and model 2, with the exception that the effect of log DFI per boar was changed into the effect of the boar. The LS-means for TNB per boar were then aligned with the LS-means for DFI per boar, backtransformed from log DFI. The percentiles of LS-means TNB were used as limits to classify boars as lowest 5%, medium 90% and highest 5% with regards to litter size. The LS-means for DFI, motility and TNB per percentile group were calculated.

RESULTS

Semen storage and sperm DNA fragmentation

Initially, the effect of liquid semen storage on sperm DNA fragmentation was evaluated by analyzing 75 of the ejaculates on the collection day and upon 96 hours' storage. Descriptive data from the analysis are presented in Table 1. On the day of semen collection, the median of DFI was higher in ND compared to NL. In NL the median of DFI was, however, higher upon storage at 18°C for 96 hours (Day 4). In addition, NL showed a wider range of DFI both on Day 0 and Day 4.

Results from a paired t-test with log-transformed DFI values showed a significant increase in log DFI from Day 0 to Day 4 in both breeds (NL p=0.002, ND p=0.04). Norsvin recommends to use the semen within 96 hours upon collection. Thus, in order to mimic the status on the day of use in the herds, DFI was screened in liquid semen samples stored at 18°C for 48, 72 or 96 hours depending on the weekday the collection was performed.

Sperm DNA fragmentation in liquid stored semen samples

DNA fragmentation was analyzed in all the stored samples (NL n=695, ND n=655) and the result showed that median DFI for NL was 1.38% (range 0.19 - 28.39%) and for ND 1.61% (range 0.26 - 36.58%). DFI values above 10% was observed for 1.7% and 0.5% of the NL and ND ejaculates, respectively. Results from a t-test with log-transformed DFI data showed a significant effect of breed ($p<0.0001$). Therefore, further statistical analyses were performed separately for the two breeds.

In the study, factors that contributed to the differences in measured log DFI were evaluated. First, the Pearson correlation coefficient and p-values for the correlations between the log DFI and parameters linked to the boar were calculated (Table 2). For NL, log DFI was negatively correlated to collection motility and collection interval, and positively correlated to storage time of semen. However, for ND log DFI was negatively correlated both to motility at collection and to the age of the boar.

The effect of the parameters included in the correlation analysis on log DFI, were in addition to herd, interval class, extender and motility evaluation method further evaluated by construction of separate GLM for each breed. In both models boar had a significant effect on log DFI ($p<0.0001$). In addition, for NL boars, age had a significant negative effect on log DFI ($p<0.0001$). Thus, for NL boars included in this study log DFI decreases with increased boar age. For ND boars, both storage time and motility at collection had significant negative effects on log DFI ($p=0.0025$ and $p<0.0001$, respectively).

The effect of DNA fragmentation on field fertility

Based on the results from the GLM analyses (NL: model 1 and ND: model 2), LS-means for log DFI of each boar were calculated and these values were used to evaluate the effect of DNA fragmentation on field fertility in form of TNB. The GLM results are shown in Table 3 and 4 and for both NL and ND litters, herd, parity and LS-means log DFI showed a significant effect on TNB. In addition, for NL, type of insemination (single/double) and semen collection month had a significant effect on TNB (Table 3), while for ND, semen storage time was an additional parameter showing a significant effect on TNB (Table 4). For both breeds, the effect of LS-means log DFI on TNB was found to be negative indicating that lower log DFI values give higher TNB. In the ND model (Table 4), LS-means log DFI had the highest impact (F value=28.64). However, in the NL model (Table 3) insemination type had the highest impact (F value=38.67) and the LS-means log DFI showed a lower impact (F value=5.53) than both herd and type of insemination.

For both breeds, the LS-means percentage DFI values within the 5% of boars with the lowest litter size were higher than the DFI values of boars within the other groups, and the DFI of the boars with the 5% highest litter size were the lowest, as represented in Table 5. The DFI and the motility of the same semen samples were negatively correlated ($p<0.0001$) (Table 2), and the high and low TNB groups showed significant differences in motility (92.1% vs. 89.7%; $p=0.0038$ and 92.3% vs 89.5%; $p=0.018$; NL and ND, respectively).

DISCUSSION

In swine, sperm DNA fragmentation has previously been reported to influence litter size. However, it is shown that the genetic line has an impact on the fertility outcome (Boe-Hansen et al. 2008, Broekhuijse et al. 2012). Therefore, in order to take in account the difference between genetic lines, the objective of the present study was to determine the level of DNA fragmentation in stored liquid extended semen from Norwegian Landrace (NL) and Norwegian Duroc (ND) boars and to evaluate the effect of DNA fragmentation on TNB.

In Norway, most of the semen is used for AI two or more days after collection and in the present study, around 75% of the litters were from semen stored from 48 to 96 hours prior to AI. Data from our initial work showed a small but significant increase in the DNA fragmentation index (DFI) upon 96 hours liquid storage. This is in agreement with several previous studies reporting that in liquid preserved boar semen, spermatozoa show increased DNA fragmentation upon storage (Bielas et al. 2017, Boe-Hansen et al. 2008, Boe-Hansen et al. 2005, Broekhuijse et al. 2012). In contrast to this, De Ambrogi et al. (2006) reports that liquid storage of boar semen for up to 96 hours does not cause loss of DNA integrity. However, only four ejaculates from four different boars were evaluated in this study. In addition, the boars represented three different breeds. Thus in the current study, screening of DFI from samples stored for 48 to 96 hours was performed in order to mimic the status on the day of use in the herds. In the studies by Bielas et al. (2017) and Broekhuijse et al. (2012) the greatest increase of DFI was observed between the day of collection and 24 hours storage. This supports our assumption that samples stored for 48 to 96 hours will adopt the "worst case" DFI value of the samples used for AI.

In the current study, the DNA fragmentation level in the analyzed samples was relatively low with DFI median values below 2% Individual variation was, however, observed and a few samples showed DFI values above 10% and even up to values around 30%. The general low level of DNA fragmentation detected is in accordance to previous studies reporting mean DFI values from around 2 - 4% in liquid stored boar semen (Bielas et al. 2017, Boe-Hansen et al. 2008, Broekhuijse et al. 2012). However, these studies do also report on individual variation between boars and ejaculates regarding the DNA fragmentation level on the day of collection and upon storage.

The results of this study showed that DFI level improved with increasing age for the NL boars. At the first semen collection, the age of the boars was around 7-10 months and this result could indicate immaturity of sperm structures in the young boars. This finding is supported by the observation that for NL boars, the level of DNA fragmentation decreases by increased boar age. A recent study has reported a lower incidence of chromatin instability in semen from mature boars (18-33 months) compared to young boars (7-10 months) and old boars (51-61 months) (Tsakmakidis et al. 2012). In comparison, our samples are limited to the young and mature age groups, and for NL boars our results are in accordance to the observation by Tsakmakidis et al. (2012). Sampling of Duroc semen in this study was stopped at an even lower age due to more frequent change of boars in the breeding system used, which may have influenced a lack of significant age effect on the DFI levels.

The correlation analyses showed that DFI after storage was negatively correlated to motility at collection, however only for Duroc samples motility was included in the GLM model. The litter size groups defined in Table 5 showed significant differences in motility, but these differences were so small that they could not be practically implemented for prediction of potential litter size in the field. A positive correlation between DNA fragmentation and motility has previously been reported. However as discussed above, this study include only four boars which in addition represents three different breeds (De Ambrogi et al. 2006).

Optimally, the relationship between DNA fragmentation and TNB should have been evaluated by linking litters from the exact same ejaculate as DFI samples. However, in pigs the number of inseminations performed for each ejaculate is low and therefore, in the present study all

known litters from the boars with a DFI value for one or more ejaculates were included for calculation of TNB. The models of TNB showed that for both NL and ND, the DNA fragmentation had a significant effect on TNB. In the ND model, DNA fragmentation was found to have the highest impact on TNB of the effects in the model. For both breeds, it appears that litter size is negatively affected by increased DFI. Thus, our findings confirm previous observations about the relationship between DNA fragmentation and field fertility (Boe-Hansen et al. 2008, Broekhuijse et al. 2012, Didion et al. 2009). Some studies have suggested threshold values for DFI in boar semen around 2-6% or between 2-18% (Boe-Hansen et al. 2008, Didion et al. 2009). The current study showed that the 5% boars with the lowest litter size had a LS means DFI of 3.05% and 2.24% in NL and ND, respectively, compared to 1.67% and 1.23% for the 5% boars with the highest litter size. This gives a difference in litter size above three piglets for NL litters and four piglets for ND litters indicating that TNB is markedly reduced by increased DFI values. In a commercial herd such litter size differences would be economically noticeable. Therefore, identification of candidate genes and polymorphisms related to sperm DNA integrity can be of interest for the pig breeding industry (van Son et al. 2017).

In conclusion, the present study shows that the sperm DNA fragmentation parameter measured in liquid stored semen provides important information regarding fertility of NL and ND boars. This parameter should therefore be taken into consideration for evaluation of NL and ND boars entering an AI center. In the context of sustainable breeding, the use of boars with high degree of DNA fragmentation may have a deleterious effect in a longer perspective.

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CONFLICT OF INTEREST

The authors have no known conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

FDM, RF, MS, EG and AHG designed the study. RF, EBS and TTZ performed the SCSA analysis. NHT, IAR and AHG performed statistical analysis. FDM, NHT and AHG created the manuscript. All authors contributed to writing and approval of the finale manuscript.

REFERENCES

Aitken R. J.; Bronson R.; Smith T. B.; De Iuliis G. N., 2013: The source and significance of DNA damage in human spermatozoa; a commentary on diagnostic strategies and straw man fallacies. MHR: Basic science of reproductive medicine, *19* 475-485.

Bielas W.; Nizanski W.; Partyka A.; Rzasa A.; Mordak R., 2017: Effect of long-term storage in Safe Cell+ extender on boar sperm DNA integrity and other key sperm parameters. Acta Veterinaria Scandinavica, *59* 58.

Boe-Hansen G. B.; Christensen P.; Vibjerg D.; Nielsen M. B. F.; Hedeboe A. M., 2008: Sperm chromatin structure integrity in liquid stored boar semen and its relationships with field fertility. Theriogenology, *69* 728-736.

Boe-Hansen G. B.; Ersboll A. K.; Greve T.; Christensen P., 2005: Increasing storage time of extended boar semen reduces sperm DNA integrity. Theriogenology, *63* 2006-2019.

Broekhuijse M. L. W. J.; Sostaric E.; Feitsma H.; Gadella B. M., 2012: Relation of flow cytometric sperm integrity assessments with boar fertility performance under optimized field conditions. J Anim Sci, *90* 4327-4336.

De Ambrogi M.; Ballester J.; Saravia F.; Caballero I.; Johannisson A.; Wallgren M.; Andersson M.; Rodriguez-Martinez H., 2006: Effect of storage in short- and long-term commercial semen extenders on the motility, plasma membrane and chromatin integrity of boar spermatozoa. Int J Androl, *29* 543-552.

Didion B. A.; Kasperson K. M.; Wixon R. L.; Evenson D. P., 2009: Boar fertility and sperm chromatin structure status: a retrospective report. J Androl, *30* 655-660.

Evenson D., 2016: The Sperm Chromatin Structure Assay (SCSA) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Animal Reproduction Science, *169* 56-75.

Evenson D.; Jost L., 1994: Sperm chromatin structure assay: DNA denaturability. Methods in cell biology, *42 Pt B* 159-176.

Evenson D.; Jost L., 2000: Sperm chromatin structure assay is useful for fertility assessment. Methods in Cell Science, *22* 169-189.

Evenson D.; Jost L., 2001: Sperm chromatin structure assay for fertility assessment. Current Protocols in Cytometry, *Chapter 7* Unit 7 13.

Evenson D. P.; Thompson L.; Jost L., 1994: Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. Theriogenology, *41* 637-651.

Fatehi A. N.; Bevers M. M.; Schoevers E.; Roelen B. A. J.; Colenbrander B.; 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.

Foxcroft G. R.; Dyck M. K.; Ruiz-Sanchez A.; Novak S.; Dixon W. T., 2008: Identifying useable semen. Theriogenology, *70* 1324-1336.

Gonzalez-Marin C.; Gosalvez J.; Roy R., 2012: Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. International Journal of Molecular Sciences, *13* 14026-14052.

Love C. C.; Kenney R. M., 1998: The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. Theriogenology, *50* 955-972.

Rathke C.; Baarends W. M.; Awe S.; Renkawitz-Pohl R., 2014: Chromatin dynamics during spermiogenesis. Biochimica et Biophysica Acta, *1839* 155-168.

Tsakmakidis I. A.; Khalifa T. A.; Boscos C. M., 2012: Age-related changes in quality and fertility of porcine semen. Biological Research, *45* 381-386.

van Son M.; Tremoen N. H.; Gaustad A. H.; Myromslien F. D.; Vage D. I.; Stenseth E. B.; Zeremichael T. T.; Grindflek E., 2017: RNA sequencing reveals candidate genes and polymorphisms related to sperm DNA integrity in testis tissue from boars. BMC Vet Res, *13* 362.

Waterhouse K. E.; Haugan T.; Kommisrud E.; Tverdal A.; Flatberg G.; Farstad W.; Evenson D. P.; De Angelis P. M., 2006: Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. Reprod Fertil Dev, *18* 781-788.

Wdowiak A.; Bakalczuk S.; Bakalczuk G., 2015: The effect of sperm DNA fragmentation on the dynamics of the embryonic development in intracytoplasmatic sperm injection.line Reprod Biol, *15* 94-100.

Table 1: Median, minimum and maximum (range) and storage difference (Day 4-Day 0) (± SD) values for the sperm DNA fragmentation index (DFI) in ejaculates from Norwegian Landrace (n=49) and Norwegian Duroc (n=26) boars on the day of semen collection (Day 0) and after 96 hours storage at 18°C (Day 4).

	Norwegian Landrace (n=49)	**Norwegian Duroc (n=26)**
Median DFI Day 0 (%)	2.22	2.61
Range DFI Day 0 (%)	0.9 - 26.4	1.33 - 7.07
Median DFI Day 4 (%)	4.01	2.88
Range DFI Day 4 (%)	0.75 - 27.4	1.36 - 17.36
Difference mean DFI Day 4 - Day 0 (%)	0.85 (±2.02)	0.85 (±2.73)
Range mean DFI Day 4 - Day 0 (%)	-2.56 - 9.38	-0.69 - 13.57

Table 2: The Pearson correlation coefficient (corr) and p-values for the correlations between the log-transformed sperm DNA fragmentation index (log DFI) and selected boar parameters in ejaculates from Norwegian Landrace (n=693) and Norwegian Duroc (n=655).

	Norwegian Landrace		**Norwegian Duroc**	
	Corr	**p-value**	**Corr**	**p-value**
Boar age	-0.021	0.58 [b]	-0.129	0.0009 [a]
Collection interval	-0.085	0.04[a]	0.049	0.27
Sperm cell motility	-0.361	<0.0001[a]	-0.385	<0.0001[a]
Sperm cell concentration	0.058	0.24	0.050	0.29
Semen storage time	0.091	0.016 [a]	0.033	0.40

Significance of correlation ($p<0.05$) is indicated by [a] and parameters with a significant effect ($p<0.05$) in general linear models are indicated by [b].

Table 3: The degrees of freedom (DF), sum of squares (SS), mean squares, F values and p-values (p > F) for the parameters with significant effect in Norwegian Landrace on the total number of piglets born.

Source	DF	SS	Mean Square	F Value	p > F
Herd	172	12966.00	75.38	7.28	<.0001
Parity	2	614.40	307.20	29.66	<.0001
Type of insemination	1	400.53	400.53	38.67	<.0001
Semen collection month	89	1410.37	15.85	1.53	0.0010
LS-means log DFI	1	57.28	57.28	5.53	0.0187

Table 4: The degrees of freedom (DF), sum of squares (SS), mean squares, F values and p-values (p > F) for the parameters with significant effect in Norwegian Duroc on the total number of piglets born.

Source	DF	SS	Mean Square	F Value	p > F
Herd	12	1017.50	84.79	10.06	<.0001
Parity	1	49.06	49.06	5.82	0.0159
Semen storage time	4	191.85	47.96	5.69	0.0001
LS-means log DFI	1	241.40	241.40	28.64	<.0001

Table 5: The relationship between the estimated total number of piglets born (TNB) and the DNA fragmentation index (DFI) in Norwegian Landrace (NL) and in Norwegian Duroc (ND).

Percentiles LS-means TNB	Number of boars	LS-means motility per boar group (%)	LS-means DFI per boar group (%)	Mean of LS-means TNB per boar
NL				
<5%	16	89.7^{y}	3.05^{b}	11.95
5-95%	280	91.1^{x}	1.46^{a}	14.03
>95%	16	92.1^{x}	1.67^{a}	15.19
ND				
<5%	11	89.5^{y}	2.24^{b}	6.67
5-95%	195	89.9^{y}	1.86^{b}	9.22
>95%	11	92.3^{x}	1.23^{a}	10.83

For motility differences within breed, superscript x and y indicate significance (p value <0.05). For DFI differences within breed, superscript a and b indicate significance (p value <0.01).