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The use of comet assay to assess DNA integrity of boar spermatozoa following liquid preservation at 5˚C and 16˚C

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Abstract: The comet assay, under neutral conditions, allows the assessment of DNA integrity influenced by sperm ageing, which is manifested in DNA double-strand breaks. Here, we attempted to use a modified neutral comet assay test (single-cell gel electrophoresis), to our knowledge for the first time, to assess DNA integrity of boar spermatozoa during liquid storage for 96 h at 5˚C and 16˚C. In this comet assay protocol we used 2% β-mercaptoethanol prior to the lysis procedure, to aid in removing nuclear proteins. Ejaculates from 3 boars (designated A, C and G) were diluted with a standard semen extender, Kortowo-3 (K-3), which was supplemented with lipoprotein fractions extracted from hen egg yolk (LPFh) or ostrich egg yolk (LPFo). Irrespective of the extender type, the percentage of comet-detected spermatozoa with damaged DNA increased gradually during prolonged storage at 5˚C and 16˚C. Spermatozoa stored in K-3 extender exhibited elevated levels of DNA damage at both storage temperatures. Significant differences in DNA damage among the boars were more pronounced during storage in LPF-based extenders at 5˚C: spermatozoa of boars A and G were less susceptible to DNA damage. The percent of tail DNA in comets was lower in LPF-based extenders, and there were individual variations among the boars. We observed that changes in DNA integrity were dependent on the extender type and storage temperature. A higher level of DNA instability was observed in K-3 extended semen compared with K-3/LPFh or K-3/LPFo extended semen during storage at 5˚C. No significant difference in the level of DNA damage between K-3/LPFh and K-3/LPFo was observed. It seems that a long-term storage can affect genomic integrity of boar spermatozoa. The modified neutral comet assay can be used to detect low levels of DNA damage in boar spermatozoa during liquid preservation. Therefore, screening for sperm DNA damage may be used as an additional test of sperm function that can have diagnostic value in practice.

Key words: Boar - Spermatozoa - Extender - Lipoprotein fractions - DNA integrity

Introduction

Sperm DNA integrity is important for the success of natural or assisted fertilization, including normal development of the embryo, foetus or offspring. Several techniques have been developed to detect DNA abnormalities in single male germ cells. The sperm chromatin structure assay (SCSA) has been used to assess the susceptibility of DNA in boar sperm chromatin to acid-induced denaturation during cryopreservation [4]. Furthermore, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) has been used to identify DNA strand breakage in abnormal human sperm cells [8]. Impaired DNA integrity was quantified using the tritium-labelled ^{3}H-actinomycin D (^{3}H-AMD) assay to analyze semen quality of intensively sexually exploited boars [19].

In somatic cells, the comet assay or the single-cell gel electrophoresis assay (SCGE) has been used under alkaline or neutral conditions for detection of DNA damage [15, 16]. This assay allows efficient determination of double and single-strand breaks and alkali-labile sites in DNA of individual cells, and has been recognised in many cell lines to be one of the most sensitive techniques available for measuring DNA strand breaks [16]. The comet assay has been used for the detection of DNA strand breaks in mammalian (human and stallion) spermatozoa [3, 12], but has not been applied for boar spermatozoa.

Conventional semen analysis including sperm motility, viability and morphology, is widely used as an endpoint to evaluate semen fertilizing capacity. How-

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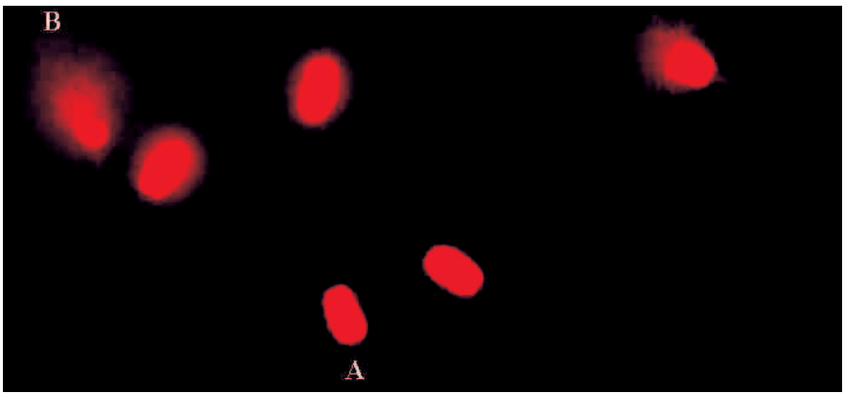

Fig. 1. Fluorescence microscopy displaying spermatozoa without damaged DNA (A) and with damaged DNA, as shown by a comet (B). × 400.

ever, a variety of factors, including extender type, storage temperature and storage time, have been reported to negatively affect the quality of extended boar semen, but the effect of these factors on sperm DNA integrity has not been adequately addressed. Apparently, some spermatozoa may show sustained motility and membrane integrity or unstable DNA, that limit their fertilizing ability, especially when stored for an extended period of time. Therefore, screening of sperm DNA damage, in combination with assessment of sperm motility and plasma membrane integrity, may aid in determining the quality of extended semen following storage. It should be noted that boar semen is usually stored at 16˚C to 18˚C, and most extenders are formulated to store the semen at this temperature range. When sperm cells are exposed to rapid reduction in temperature, they undergo irreversible cold-shock, which can be lethal [7]. However, the deleterious effect of cooling can be diminished when a suitable extender is added to the semen. In the study described herein, we have supplemented a standard boar extender, Kortowo-3 (K-3) with lipoprotein fractions (LPF) extracted from hen egg yolk (LPFh) or ostrich egg yolk (LPFo). The aim of this study was to test whether a modified neutral comet assay protocol may be useful to assess DNA integrity of boar spermatozoa during storage in a standard semen extender, supplemented with egg yolk lipoproteins, at 5˚C and 16˚C. Here, we sought to use the neutral comet assay, for the first time, as an additional parameter for assessment of cooled boar semen.

Materials and methods

Semen collection. Semen was collected once a week from 3 Polish Large White boars (aged 1.5 years), designated A, C, G. The boars were fed with a commercial porcine ration. Water was available *ad libitum.* Semen was collected using the gloved-hand technique, and the gel portion was removed using nylon mesh filter. Local ethics committee approval was obtained for this study.

Semen assessment and storage. Standard semen parameters were evaluated macroscopically and microscopically. Total motility was assessed under a light microscope (× 200) equipped with an attached heated stage (37˚C). Sperm concentration was determined using a haemocytometer. The percentage of morphologically abnormal spermatozoa was evaluated using the Giemsa staining method. Sperm plasma membrane integrity was assessed using a dual-fluorescent staining method, as described in a previous study [6].

Following standard semen parameter evaluation, ejaculates were diluted with a standard extender, Kortowo 3 (K-3), containing sodium citrate, fructose, EDTA, potassium acetate, and gentamycin [18]. The K-3 extender was supplemented with lipoprotein fractions extracted from hen (K-3/LPFh) or ostrich egg yolk (K-3/LPFo). The extraction procedure of the lipoprotein fractions from hen or ostrich egg yolk has been described elsewhere [2, 20]. Extraction of LPFh was performed on the day of experiment. The LPFo samples were lyophilized and stored at -20˚C, until required.

The semen samples were diluted with K-3, K-3/LPFh or K-3/LPFo extender to a final concentration of 3×10^7 spermatozoa/cm^3 and were dispersed into 100-ml plastic bottles. The diluted semen was left to equilibrate for 2 h (0 h, day 0) at room temperature, and divided into 2 equal portions, which were stored at 5˚C (refrigerator) and 16˚C (thermobox), respectively, for 96 h. For analysis of DNA integrity, sperm samples were taken at 2 h after dilution (day 0), and after 48 h (day 2) and 96 h (day 4) of storage at 5˚C and 16˚C. We also took samples for assessment of sperm motility and plasma membrane integrity prior to DNA lysing procedure.

Comet assay to assess DNA strand breaks. Normal and low melting point agarose was purchased from Gibco-BRL (Life Technologies, Paisley, Scotland). All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

The comet assay described by Marty *et al.* [15] was slightly modified for this study. Sperm cells were washed (800 × g, 10 min) with Ca^{2+} and Mg^{2+}-free PBS (Vaccine Laboratory, Warsaw) to yield a concentration of 1×10^7 spermatozoa/cm^3. Aliquots of the same sperm samples (10 µl), pre-treated with 2% β-mercaptoethanol for 1 h at 4˚C and washed with PBS, were mixed with 0.5% low-melting point agarose and placed onto frosted microscope slides pre-coated with 0.75% normal-melting point agarose. A final layer of 75 µl of 0.5% low-melting point agarose was applied, the slides were immersed in ice-cold lysing solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris-HCl, pH 10, 1.0% Triton X-100, and 1.0% sodium lauryl sarcosine) and incubated for 1 h at 4˚C. Then the slides were treated with buffer (2.5 M NaCl, 5 mM Tris-HCl, pH 7.4, 0.05% sodium lauryl sarcosine) containing 20 µg/cm^3 RNase A and incubated for 4 h at 37˚C. Thereafter, slides were transferred to another buffer (2.5 M NaCl, 5 mM Tris-HCl, pH 7.4, 0.05% sodium lauryl sarcosine) containing 1 mg/cm^3 proteinase K (DNase-free) and left overnight at 37˚C. Slides were equilibrated in an electrophoresis solution (300 mM sodium acetate and 100 mM Tris-HCl, pH 8.0) before being electrophoresed at 12 V (0.46 V/cm, 100 mA) for 1 h

Table 1. Characteristics of fresh, unextended boar semen used for liquid storage *(n=20).*

Variables	Mean ± SEM
Gel-free semen volume (cm^3)	236.25 ± 14.23
Sperm motility (%)	74.50 ± 0.80
Sperm concentration (1×10^6)	348.45 ± 26.44
Membrane-intact spermatozoa (%)	89.45 ± 0.81
Total morphologically abnormal spermatozoa (%)	6.07 ± 0.77

at room temperature. The slides were drained, flooded slowly with three changes of neutralization buffer (0.4 M Tris, pH 7.4), fixed in 70 % ethanol for 15 min and then air dried. The dried slides were stained with 40 µl of 20 µg/ml ethidium bromide.

Spermatozoa analyzed for comets were visualized under an epi-fluorescence microscope (Olympus, Tokyo, Japan). Whole sperm heads, without a comet, were not damaged (Fig. 1A), whereas spermatozoa with fragmented DNA that migrated from the sperm head, causing a "comet" pattern, were considered damaged (Fig. 1B). A total of about 100 sperm cells per slide were assessed for comets. The comets were captured with a Black &White Video camera (Sony, Tokyo, Japan) connected to the fluorescent microscope and the images were evaluated for percentage of tail DNA using special imaging analysis software (Komet System, Kinetic Imaging Ltd, Liverpool, UK).

Statistical analysis. All values obtained were expressed as means ±SEM. One-way ANOVA and Tukey's pos-hoc test were used to evaluate differences in sperm DNA damage among the storage periods and among the boars. All analyses were performed using the Statistica software (Statistica for Windows, StatSoft Incorporation, Tulsa OK). A probability of $P \leq 0.05$ was assessed to denote a significant difference in each case.

Results

The mean values of characteristics of fresh unextended boar semen were within the range of normozoospermia, as shown in Table 1. The percentage of comet-detected spermatozoa with damaged DNA was negligible and averaged 2.75 ± 0.22 (mean ± SEM) for fresh semen.

Table 2 shows changes in DNA stability of spermatozoa during liquid storage at 5˚C. Low levels of DNA damage assessed in spermatozoa following 2 h equilibration (0 h) at room temperature were observed. The level of DNA instability increased ($P \leq 0.05$) with storage time and was evident in all extenders. Increased proportion of comet-detected spermatozoa with DNA damage was more evident at 96 h of storage, particularly in K-3 extender. Spermatozoa from boars A and G exhibited lower ($P \leq 0.05$) DNA damage than spermatozoa of boar C during storage in K-3/LPFh or K-3/LPFo extender at 48 h and 96 h. A higher ($P \leq 0.05$) number of comet-detected spermatozoa with damaged DNA was observed in boars A and C than that of boar G during storage in K-3 extender. At 96 h of storage, the percentage of comet-detected spermatozoa with damaged DNA was lower ($P \leq 0.05$) in boar G, regardless of the extender type.

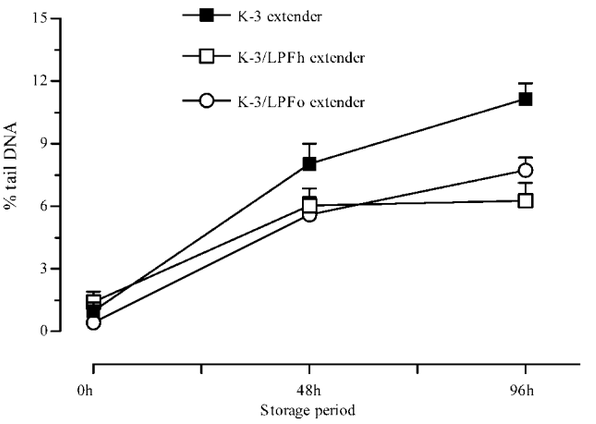

Fig. 2. Effect of liquid preservation on the percentage of tail DNA in comets of spermatozoa stored at 5˚C. We analyzed a total of 50 comets for each extender at 0 h, 48 h, and 96 h of storage, respectively. Values are expressed as means ± SEM.

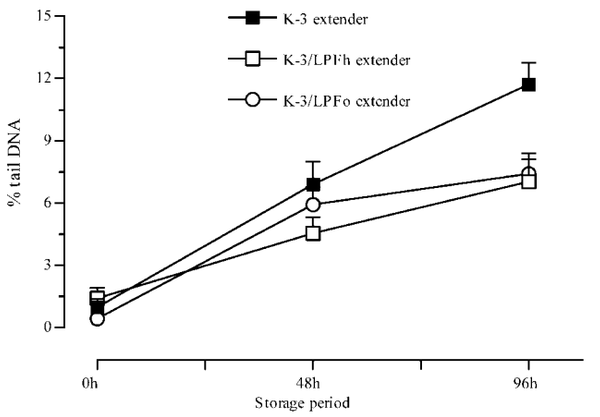

Fig. 3. Effect of liquid preservation on the percentage of tail DNA in comets of spermatozoa stored at 16˚C. We analyzed a total of 50 comets for each extender at 0 h, 48 h, and 96 h of storage, respectively. Values are expressed as means ± SEM.

Changes in DNA stability of spermatozoa during liquid storage at 16˚C are shown in Table 3. These changes in the sperm DNA stability were less pronounced compared with those of spermatozoa stored at 5˚C. Even though a high proportion of comet-detected spermatozoa with damaged DNA was observed during prolonged storage, the differences among the boars were less marked. Spermatozoa of boar G exhibited lower DNA damage than those of boars A and C during storage in K-3/LPFh extender. Furthermore, we observed that the level of DNA instability was dependent on the extender type and storage temperature. A higher percentage of comet-detected spermatozoa with damaged DNA was observed in K-3 extender compared with that of K-/LPFh or K-3/LPFo extender during storage at 5˚C. However, at 16˚C these changes were less pronounced.

Table 2. Percentage of comet-detected spermatozoa with damaged DNA during liquid storage at 5˚C.

Boar	No. of collections	Storage period								
		0 h (day 0)			48 h (day 2)			96 h (day 4)		
		K-3	K-3/ LPFh	K-3/ LPFo	K-3	K-3/ LPFh	K-3/ LPFo	K-3	K-3/ LPFh	K-3/ LPFo
A	6	3.00 ± 0.54^{a}	2.20 ± 0.49^{a}	2.60 ± 0.31^{a}	13.80 ± 2.39^{b,x}	8.20 ± 0.97^{c,x}	6.20 ± 1.82^{c,x}	16.20 ± 2.48^{d,x}	11.80 ± 1.98^{b,x}	12.80 ± 2.35^{b,x}
C	7	3.37 ± 0.54^{a}	3.62 ± 0.49^{a}	3.25 ± 0.53^{a}	12.63 ± 2.30^{b,xy}	11.13 ± 1.60^{b,y}	14.85 ± 3.09^{bd,y}	17.25 ± 1.83^{cd,x}	13.85 ± 1.10^{b,x}	15.37 ± 2.98^{cd,x}
G	7	2.29 ± 0.29^{a}	3.20 ± 0.57^{a}	2.86 ± 0.46^{a}	9.57 ± 1.95^{bd,y}	7.56 ± 2.26^{bc,x}	6.42 ± 1.04^{c,x}	12.42 ± 1.36^{d,y}	9.14 ± 0.70^{b,y}	9.13 ± 1.18^{b,y}
A+C+G	20	2.90 ± 0.28^{a}	3.05 ± 0.32^{a}	2.95 ± 0.30^{a}	11.85 ± 1.29^{b}	9.48 ± 0.96^{bc}	9.47 ± 1.56^{c}	15.30 ± 1.13^{d}	11.60 ± 0.76^{b}	12.55 ± 1.46^{bd}

Means ± SEM. K-3 = Kortowo 3 extender; K-3/LPFh = Kortowo 3 extender supplemented with lipoprotein fractions extracted from hen egg yolk; K-3/LPFo = Kortowo 3 extender supplemented with lipoprotein fractions extracted from ostrich egg yolk.
^{abcd}Values with different letters in the same row are significant ($P \leq 0.05$).
^{xy}Among boars, values with different letters in the same column are significant ($P \leq 0.05$).

Table 3. Percentage of comet-detected spermatozoa with damaged DNA during liquid storage at 16˚C.

Boar	No. of collections	Storage period					
		48 h (day 2)			96 h (day 4)		
		K-3	K-3/LPFh	K-3/LPFo	K-3	K-3/LPFh	K-3/LPFo
A	6	9.80 ± 2.56^{a}	7.80 ± 2.11^{a}	7.25 ± 1.33^{a}	13.25 ± 2.02^{b}	11.56 ± 2.69^{b,xy}	12.00 ± 2.69^{b}
C	7	9.25 ± 2.04^{a}	8.33 ± 1.50^{a}	9.85 ± 2.79^{a}	13.26 ± 1.93^{b}	12.24 ± 1.13^{b,x}	10.63 ± 1.37^{b}
G	7	7.14 ± 1.32^{a}	8.11 ± 1.15^{ac}	7.71 ± 0.77^{a}	11.14 ± 0.90^{b}	10.86 ± 0.63^{b,y}	10.29 ± 1.87^{ac}
A+C+G	20	8.65 ± 1.10^{a}	8.09 ± 0.86^{a}	8.42 ± 1.11^{a}	12.45 ± 0.94^{b}	11.65 ± 0.95^{b}	10.85 ± 0.87^{b}

Means ± SEM.
^{abc}Values with different letters in the same row are significant ($P \leq 0.05$).
^{xy}Among boars, values with different letters in the same column are significant ($P \leq 0.05$).
See Table 2 for explanation.

We did not observe any significant difference between K-3/LPFh and K3-LPFo extended semen throughout this experiment.

Figures 2 and 3 illustrate the percentage of tail DNA in the comets during storage at 5˚C and 16˚C. Irrespective of the storage period, an increase in the percentage of tail DNA was observed in all extenders, but it was more pronounced in K3 extender. No significant changes ($P \geq 0.05$) between extenders supplemented with LPFh and LPFo were observed. There were different distributions of the percentages of tail DNA during storage at both temperatures. Variations in the length of the comet tails were more evident among individual boars.

There was a gradual reduction in sperm motility (Fig. 4) and the proportions of spermatozoa with intact plasma membrane (Fig. 5) during storage in K-3, K-3/LPFh or K-3/LPFo extender at 5˚C and 16˚C. These changes were less marked in LPF-based extenders when compared with K-3 extender. However, we found that they were accompanied by increased DNA instability.

Discussion

In this study we show for the first time that the comet assay, under neutral conditions, can be used to detect the level of DNA damage in boar spermatozoa during preservation. The highly compact structure of DNA in spermatozoa due to disulphide bonding between DNA and protamines makes them more resistant to damage agents. Here, we describe a modification of the comet assay which was used for analyzing DNA fragmentation in mouse spermatozoa [15]. During the preparation of the comet assay, spermatozoa were treated with β-mercaptoethanol, which reduced disulphide bonds in protamines, prior to the lysis procedure. This step was necessary in order to reduce the protamine disulphide, so that the high salt solution with detergents could facilitate protamine extraction. We also applied an extended period of enzymatic digestion with proteinase K to ensure effective removal of the DNA-associated proteins. Moreover, the conditions used for the neutral comet assay prevent the DNA from unwinding, thus any dam-

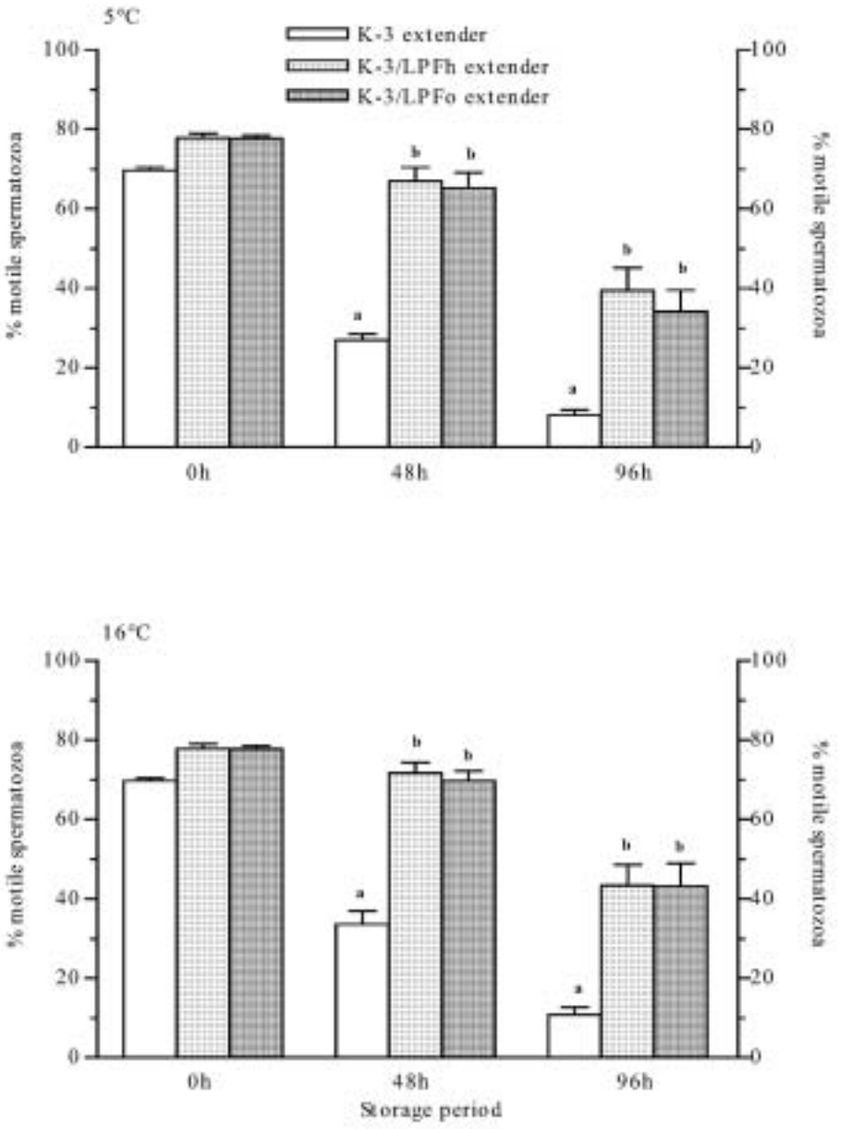

Fig. 4. Effect of liquid preservation on the motility (%) of boar spermatozoa stored in K3 extender, supplemented with or without egg yolk lipoproteins, at 5˚C and 16˚C. Values are expressed as means ± SEM. [ab]Values within similar storage period with different letters are significantly different ($P \leq 0.05$).

age observed is of a double-stranded nature [5], which is associated with sperm ageing.

Sperm ageing occurring during prolonged liquid storage results in changes that are significantly associated with DNA instability [22]. The modification of the neutral comet assay simplifies and facilitates its use for assessment of DNA integrity of boar spermatozoa during liquid storage at 5˚C and 16˚C. A significant increase in the number of comet-detected spermatozoa with damaged DNA was observed during storage at both temperatures, regardless of the extender type. These results are in accordance with those of Linfor and Meyers [12], who demonstrated that DNA damage in equine spermatozoa was higher after storage for 48 h at 5˚C when compared with fresh semen values. In the present study, the increased susceptibility of boar spermatozoa to DNA damage during liquid storage at 5˚C might be associated with marked changes in the chromatin packaging. This strengthens the argument that chromatin packaging was altered during a long-term storage and this in turn reflected a higher level of DNA damage, as assessed by the neutral comet assay.

Evidence is accumulating on the importance of sperm DNA integrity during the fertilization and embryonic development. Correlation was found between poor semen quality and higher levels of damaged DNA detected as DNA fragmentation using the comet assay [9, 17]. The ability of the sperm cells to fuse with the oocyte and the genetic material itself undergo gradual detrimental changes during *in vitro* storage at room temperature [11]. The results of this study show that spermatozoa in LPF-based extenders exhibited a lower

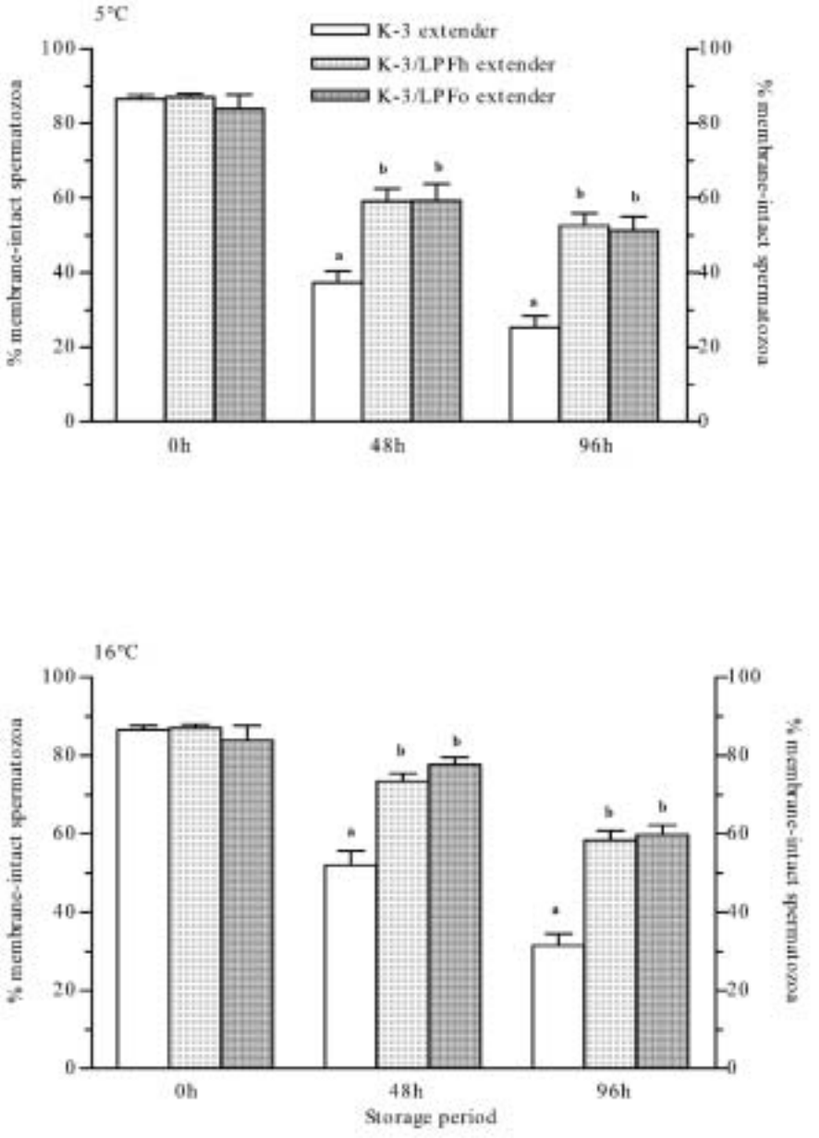

Fig. 5. Effect of liquid preservation on plasma membrane integrity of boar spermatozoa stored in K3 extender, supplemented with or without egg yolk lipoproteins, at 5˚C and 16˚C. Values are expressed as means ± SEM. [ab]Values within similar storage period with different letters are significantly different (P≤0.05).

level of DNA damage, which was partly dependent on the storage temperature. This seems to indicate that the addition of egg yolk lipoprotein fractions to boar semen extender has helped to slow down the sperm ageing changes associated with genome alterations during storage. In a previous study, the use of the sperm chromatin structure assay (SCSA) to evaluate the susceptibility of stallion DNA to denaturation showed that storage temperature and extender type affected the DNA status [13, 14].

Our results indicate that the measurements of the percentages of tail DNA give additional information about the extent of DNA damage in boar spermatozoa. Thus, spermatozoa with high levels of DNA double-strand breaks showed increased comet tail fluorescent intensity with ethidium bromide staining. In the present study, we found significant differences among boars in the percentage of DNA damage in spermatozoa. The reason for individual variations in nuclear DNA damage is not clear, but it could be related to the inherent chromatin packaging of the spermatozoa. Similar results were reported in previous studies for bull and stallion spermatozoa [10, 14]. Increased DNA damage may be, at least in part, responsible for low fertility in boars used for artificial inseminations. Even though the mechanisms responsible for increased nuclear DNA damage in spermatozoa are poorly understood, a potential explanation could be the oxidative stress. Oxidative damage by reactive oxygen species (ROS) producing ageing spermatozoa has been shown to affect DNA integrity to a different extent [1, 3]. It should be noted that spermatozoa have only two defence mechanisms against oxidative attack on their DNA, namely the packaging

arrangement of their DNA and seminal plasma [1]. However, the ability of boar spermatozoa to withstand oxidative stress is associated with seminal plasma concentration. According to Love *et al.* [13], seminal plasma concentration may affect the DNA stability of stallion spermatozoa. In a previous study, changes in the proportions of seminal plasma components, particularly zinc ions, resulted in DNA instability of boar spermatozoa [21]. Moreover, through dilution procedure the spermatozoa are deprived of their antioxidant protection, thus making them more susceptible to oxidative stress.

In summary, the present study demonstrates that DNA instability in spermatozoa may increase over time depending on the storage temperature and extender type. Since the ageing of the male genome during liquid storage plays a major role in AI practice, increased damage of sperm nuclear DNA may play a significant role in the failure of fertilization and embryonic foetal development. Evidence is mounting to suggest that sperm DNA integrity may prove to be a better marker of male infertility potential than conventional semen parameters. However, further studies are warranted to establish the effect of egg yolk lipoproteins, storage temperatures and reactive oxygen species on the integrity of nuclear DNA of boar spermatozoa during liquid preservation. Our results show that the modified neutral comet assay can be applied to detect DNA double-stranded breaks in preserved spermatozoa and may be used as an additional parameter for the quality assessment of cooled boar semen.

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