DNA integrity in fresh, chilled and frozen-thawed canine spermatozoa

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ABSTRACT: Sperm chromatin status in fresh dog semen and the effect of long-term storage of chilled and frozen dog semen on sperm chromatin integrity was assessed by the sperm chromatin structure assay (SCSA). In the first experiment, the chromatin integrity of fresh semen from 60 dogs with different historiy of fertility was compared with other sperm parameters (total sperm count, sperm motility, viability, acrosomal integrity and sperm morphology). Except for 15 dogs that had never mated before, all were used in breeding as semen donors. Thirty-three of them were successful breeding males while in 12 repeated fertility problems were noted. Ejaculates were assigned to groups with good and poor quality, based on determined sperm motility and percentage of morphologically normal sperm. In the second experiment, chromatin status was measured in fresh and chilled spermatozoa (on day 0, 2, 4, 6, 8 and 10 of storage). Finally, in the third experiment, the chromatin status was measured in fresh and cryopreserved spermatozoa. Evaluating fresh dog semen, we observed that DNA fragmentation index (DFI) and percentage of cells with high DNA stainability (HDS) negatively correlated with total sperm count, percentage of total and progressively motile spermatozoa, sperm viability and percentage of morphologically normal spermatozoa, even with rather low correlation indices. Lower chromatin integrity was found in the group of dog ejaculates showing poor quality in comparison with the group of good quality ejaculates. All dogs with repeated fertility problems were classed in the group showing poor quality, and even though their DFI was significantly higher than the DFI of successful breeding males, the highest DFI we obtained was only just below 9%. We can assume that the chromatin damage level in any of the evaluated dogs was not high enough to have a significant effect on their fertility. Concerning the potential cause of reduced male fertility, the assessment of chromatin integrity in fresh dog ejaculates failed to add any additional information to the results obtained by other techniques of semen analysis. Thus, the current study indicates that neither 10-day preservation of canine sperm chilled in commercial extenders nor long-term cryopreservation in extenders recommended for canine sperm preservation produce adverse effects on sperm chromatin integrity.

Keywords: dog; DNA fragmentation index; sperm chromatin structure assay (SCSA); cryopreservation; chilled semen

Recently, increasing attention has been paid to sperm chromatin structure as one of the parameters determining male fertility (Erenpreisa et al. 2003; Sakkas et al. 2003; Chohan et al. 2006). According to Zini et al. (2001), sperm DNA integrity assays may prove to be better markers of male fertility potential than the conventional semen parameters. Sperm chromatin is an organized and compact structure, which consists of DNA and heterogeneous proteins. The quality of sperm chromatin structure in eutherian mammals is strongly influenced by the presence of protamines (Evenson et al. 1989) linked together via disulfide crosslinks. In contrast to other mammals such as the mouse, stallion, hamster and also humans, dog sperm contains only protamine P1 (Lee and Cho 1999), which renders chromatin relatively stable (Jager 1990).

Various detection methods of sperm chromatin integrity exist. The sperm chromatin structure assay (SCSA) is regarded as the most suitable (Evenson

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et al. 2002) and has been widely applied not only to many animal species including dogs (Garcia-Macias et al. 2006; Koderle et al. 2009; Kim et al. 2010), but also humans, where it is important for prediction of infertility treatment outcomes (Virro et al. 2004; Check et al. 2005).

Whereas it is unarguable that DNA fragmentation index (DFI) assessment is valuable for the investigation of causes of impaired fertilizing ability in human semen, there is a lack of information as to whether or not it is of similar importance in the diagnosis of reproductive disturbances in dogs.

There are many external and internal factors influencing sperm chromatin quality (Evenson et al. 2002). One of the most commonly reported is sperm processing, especially centrifugation and incubation (Bungum et al. 2008; Koderle et al. 2009; Matsuura et al. 2010). Another potential factor is the use of different types of extenders (Shahiduzzaman and Linde-Forsberg 2007; Fernandez-Santos et al. 2009; Morrell et al. 2009). The purpose of short-term preservation of dog sperm is to maintain fertilization potential for a longer time than is the case for fresh semen. Not only good sperm motility and membrane quality should be preserved, but also DNA integrity. The commonly used extenders for cold storage of canine semen promise the maintenance of good quality of sperm for at least 5 days, some even 10 days. There exist a number of commercial extenders. Some do not require supplementation with egg yolk before use and as a result, their composition is fairly standard. Also, they are easy to use under practical conditions. Alternatively, some extenders require egg yolk, which has two disadvantages: one is possible microbial contamination, and the second is the fact that the preparation of the extender is more complicated. Sperm parameters such as motility, viability and acrosomal integrity deteriorate with increasing storage time. However, whether it is necessary to expect chromatin changes during the storage of doses of chilled canine sperm before their use for insemination, remains unclear.

It is known that cryopreservation reduces dog sperm quality and thus also negatively impacts on the fertilization outcome. Adverse effects of the cryopreservation processes on the plasma membrane, acrosomal membrane and mitochondria were observed. The process of sperm freezing in liquid nitrogen immediately after collection used to be considered by itself to be harmless to both human and animal sperm chromatin integrity (Evenson et al. 1994, 2002). However, the cryopreservation process is also associated with various physical and chemical insults to the spermatozoa, such as cold shock, osmotic stress and cryoprotectant intoxication.

There exist some reports regarding chromatin integrity in fresh semen and its changes during the storage of preserved semen in several domestic animals such as bulls (Karabinus et al. 1991), stallions (Love et al. 2002) and boars (Cordova et al. 2002; Boe-Hansen et al. 2005; Fraser and Strzezek, 2005; Hernandez et al. 2006), but little is known in dogs. Even though some papers have been describing chromatin integrity in fresh (Nunez-Martinez et al. 2005; Garcia-Masias et al. 2006; Gosk et al. 2007) and frozen dog semen (Rota et al. 2005; Eulenberger et al. 2009), only a few recently published reports have compared fresh and postthaw chromatin integrity, moreover, with varying results (Koderle et al. 2009; Kim et al. 2010).

Our aim was to focus on sperm chromatin status as a potential marker which can supplement the data obtained by other techniques of semen analysis revealing causes of reduced male fertility. Furthermore, we studied the effects of 10-day sperm chilling and the cryopreservation process on chromatin integrity.

MATERIAL AND METHODS

Animals and semen collection

In total, sixty dogs were used in this study, aged from 1.5 to 11 years, of different breeds and histories of fertility. All dogs were from private owners who kindly provided their dogs for experimental semen collection and examination. The sperm-rich fraction of ejaculates was collected by digital manipulation.

Sperm analysis

Conventional sperm parameters. The sperm-rich fraction of ejaculates was submitted to standard semen analysis encompassing semen volume, sperm concentration, sperm motility and progressive motility, plasma membrane integrity (viability) – evaluated by eosin nigrosin staining (World Health Organization 2010). Sperm concentration was measured in a Bürker chamber, sperm motil-

ity was analysed under an optical microscope at 200× to 400× magnification. Total sperm count was calculated by multiplying volume by sperm concentration. The acrosome-intact sperm rates were established by lectin *Pisum sativum* staining (Mortimer 1994). Sperm morphology was evaluated according to Tygerberg's strict criteria (Kruger et al. 1986). Samples were stained for sperm morphology analysis according to Farelly (smears were fixed in 3.5% formalin and stained with 5% aniline blue for 10 s and 0.5% crystal violet for 6 s) and evaluated with the use of the SASMO computer programme (Strict Analysis of Sperm Morphology; Veznik et al. 2001).

Sperm chromatin structure assay (SCSA). Increased susceptibility of altered DNA (strand breaks) in sperm nuclear chromatin to *in situ* denaturation was measured by flow cytometry after staining with acridine orange (AO). AO associated with single (denatured) and double (native) stranded DNA emitted red and green light, respectively. Chromatin damage of each sperm was quantified by red/(red + green) fluorescence and expressed using the DNA fragmentation index (DFI, see Figure 1). Each semen sample contained a certain percentage of mature cells with non-detectable (main population of spermatozoa in semen) and detectable DFI (percentage of mature spermatozoa with increased chromatin damage). The percentages of spermatozoa with moderate (mDFI) and high (hDFI) levels of DNA fragmentation were defined. The next evaluated parameter was the percentage of cells with defective chromatin condensation (HDS; cells with high DNA stainability).

Semen samples for Sperm chromatin structure assay were diluted with TNE buffer (0.015N NaCl, 0.01M Tris, and 0.001M EDTA, pH 6.8) to bring sperm concentrations to 1.5×10^6 spermatozoa/ml. For analysis, 200 µl of diluted samples was treated with 400 µl acid-detergent solution (0.08N HCl, 0.1% Triton-X 100; pH 1.2) for exactly 30 s to induce DNA denaturation. Then, 1.2 ml AO staining solution (6 µg/ml chromatographically purified AO in phosphate citrate buffer) were added to intercalate single stranded or double stranded DNA. Samples were then analysed using a flow cytometer (FACSCalibur flow cytometer, Becton Dickinson, Mountain View, CA, USA, operated by the CELLQuest Software).

We used one donor reference sample for each measurement to ensure comparable instrument settings throughout the measurements. Semen samples were exposed to 488 nm monochromatic laser light and red (single stranded DNA) and green (double stranded DNA) fluorescence values were collected and stored on 5000 spermatozoa per

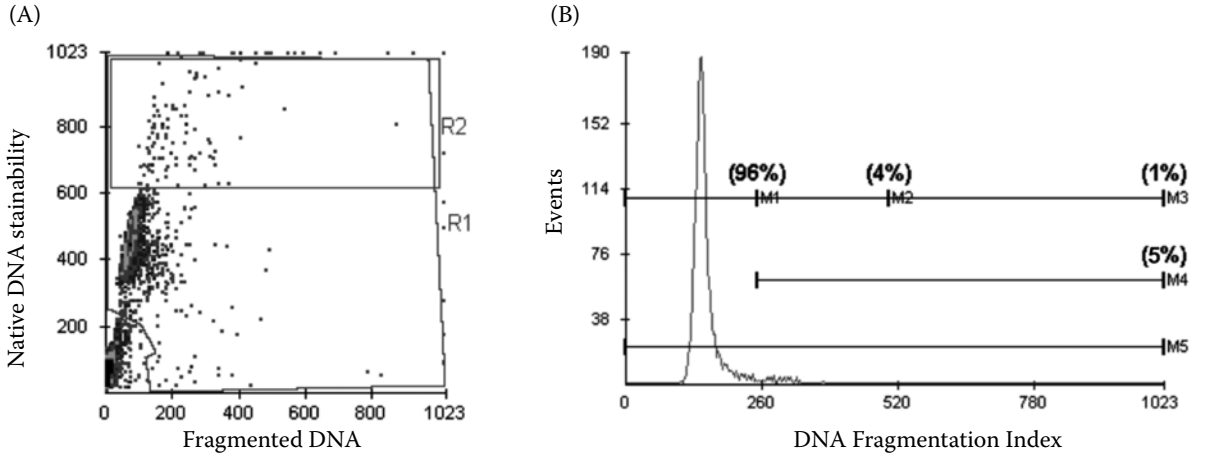

Figure 1. Examples of SCSA two-parameter cytogram (**A**) and histogram (**B**) of individual dog sperm cells. Each cell's position is based on the amount of green (native DNA stainability) and red fluorescence (fragmented DNA) emitted from that cell. (**A**) Only cells falling in Region 1 (R1) are included in the analysis. Cellular debris (lower left hand corner) is excluded by the analysis. Region 2 (R2) contains the cell population with high green fluorescence, i.e. forms with defective chromatin condensation (HDS). Cells with decreased green and increased red fluorescence, i.e. cells with denatured DNA, fall down and to the right of the main population. (**B**) Markers for calculating SCSA parameters are shown here: Marker M1 represents cells of the main population with non-detectable DNA fragmentation index, marker M2 shows population of cells with moderate DFI (m-DFI), marker M3 demonstrates cells with high DFI (h-DFI). M4 combines all cells with altered integrity of chromatin

sample after 2.5 min. In every sample, duplicate measurements were performed in succession for statistical considerations; the second sample was taken from the same thawed aliquot, diluted appropriately, processed for the SCSA and measured.

Experimental design

Experiment 1: Monitoring the chromatin status in fresh dog semen. All sixty dogs used in the study were assigned to this experiment. Except for 15 dogs that had never mated before, all were used in breeding as semen donors, although with different exploitation intensities. Thirty-three of them were successful breeding males (Group A) and in 12 repeated fertility problems had been reported (Group B). As calculated from the history given by the dog owners, the average pregnancy rate for Group A was 96.0 ± 7.03% (range 80–100%) and for males in Group B 56.6 ± 6.22% (range 40–65%).

Moreover, ejaculates were assigned to Groups 1 and 2, with good and poor quality respectively, based on determined sperm motility and percentage of morphologically normal sperm. Ejaculates from Group 1 showed sperm motilities higher than 70% together with percentages of morphologically normal sperm higher than 60%.

Besides sperm motility and morphological images, total sperm count, progressive motility, plasma membrane integrity and sperm acrosomal integrity of the sperm-rich fraction of all 60 dog ejaculates included in the study were assessed by the abovementioned methods. The chromatin integrity of fresh semen was compared with the other sperm quality parameters.

Dogs with repeated fertility problems (Group B) were compared with successful breeding males (Group A). Furthermore, differences in all evaluated sperm characteristics, including sperm chromatin status, were studied between the Groups 1 and 2.

Experiment 2: Influence of storage time on chromatin integrity of chilled dog semen. Eighteen dogs aged from 2 to 9 years, of different breeds, with a history of good fertility and without exposure to any factor that could seriously impair their spermatogenesis were selected from the whole group of dogs for this experiment. Collections from each dog were made once.

Semen parameters measured were the same as in Experiment 1 except for sperm acrosomal integrity. Sperm-rich fractions were analysed and then centrifuged at 700 g for 6 min. The supernatant was removed and sperm concentration was adjusted to 50×10^6 spermatozoa/ml with either CaniPRO^{TM} Chill 5 (Ext. 1) or CaniPRO^{TM} Chill 10 (Ext. 2; with 20% of egg yolk), i.e., commercial extenders for dog semen chilling (Minitub GmbH, Germany). Samples were divided into six aliquots and placed in a glass filled with room-temperature water before being placed in the refrigerator to ensure a low cooling rate. Aliquots were stored at 5 °C and analysed after 3 h (day 0), 48 h (day 2), 96 h (day 4), 144 h (day 6), 192 h (day 8) and 240 h (day 10).

Experiment 3: Influence of cryopreservation on chromatin integrity of dog semen. Thirty dogs aged from 2 to 8.5 years, of different breeds, with histories of good fertility and without exposure to any factor that could seriously impair their spermatogenesis were selected from the whole group of dogs for Experiment 3. Collections were made from each dog once.

The semen parameters measured were the same as in Experiment 2. The sperm-rich fraction was cryopreserved after sperm analysis. Samples were centrifuged at 700 g for 6 min and the supernatant was removed. Each sperm pellet was re-diluted with Tris-citric acid fructose extender with egg yolk and glycerol (3.025 g TRIS, 1.7 g citric acid, 1.25 g fructose, 25 mg gentamicin, 20 ml egg yolk, 6 ml glycerol and distilled water to 100 ml; pH 6.7, 1.318 mOsm; modified according to Andersen, 1975) to produce a final concentration of 300×10^6 spermatozoa/ml. The diluted semen was then poured into 0.5 ml straws and sealed with polyvinylalcohol. The straws were equilibrated at 4 °C for 2 h and then frozen in a liquid nitrogen stream in a styrofoam box. After 10 min, they were plunged into liquid nitrogen and stored for at least for two weeks before subsequent assessment. After thawing in a water bath at a temperature of 70 °C for 8 s, the semen samples were emptied into 1 ml of a Tris-citric acid-fructose buffer (3.025 g TRIS, 1.7 g citric acid, 1.25 g fructose and distilled water to 100 ml; pH 6.8, 314 mOsm; filtered through a 0.2 μm filter) to reach an approximate concentration of 100×10^6 spermatozoa/ml.

Statistical analysis

Descriptive statistics were expressed as the mean values ± SD, and minimum and maximum values. The statistical analysis was performed with SPSS

Table 1. Experiment 1: Conventional parameters and sperm chromatin integrity in fresh dog semen (n = 60)

		Total sperm count (10^6/ml)	M	PM	Viability	Intact acrosomes	Normal sperm	DFI)	m-DFI	h-DFI	HDS
			(%)								
Group 1 (n = 40)	mean ± S.D.	1125.8 ± 769.1*	85.1 ± 4.9*	83.1 ± 7.4*	91.5 ± 3.8*	88.0 ± 10.3	75.0 ± 8.6*	1.43 ± 0.99*	0.76 ± 0.57*	0.67 ± 0.61	1.06 ± 0.50*
	min	95.2	70.0	55.0	82.5	50.0	60.0	0.26	0.14	0.09	0.33
	max	3846.4	91.0	90.0	96.5	97.0	91.5	4.20	2.48	2.79	2.65
Group 2 (n = 20)	mean ± S.D.	584.3 ± 431.3*	68.9 ± 12.5*	65.1 ± 15.2*	81.9 ± 9.5*	84.1 ± 10.0	44.4 ± 16.5*	2.90 ± 2.36*	2.05 ± 1.94*	0.85 ± 0.70	2.32 ± 2.09*
	min	49.0	40.0	25.0	65.5	65.0	14.0	0.82	0.63	0.19	0.78
	max	1478.4	90.0	90.0	94.0	94.5	67.0	8.98	7.23	2.87	9.29

Group 1 = good quality ejaculates; Group 2 = poor quality ejaculates. Ejaculates from Group 1 showed sperm motility higher than 70% together with percentage of morphologically normal sperm higher than 60%
M = sperm motility; PM = progressive sperm motility; DFI = DNA fragmentation index; m-DFI = moderate DFI; h-DFI = high DFI; HDS = high DNA stainability
*(P < 0.01)

software (Version 18.0 for Windows, SPSS Inc., Chicago, IL, USA). If the data were normally distributed according to the Kolmogorov-Smirnov test Student's t-test (in Experiment 1) and the paired t-test (in Experiments 2 and 3) were used for comparison between groups. The nonparametric Mann-Whitney U test and Wilcoxon signed rank test were used in the case of violation of normal distribution. Spearman's correlation was used for assessment of the relationship between sperm parameters. P-values of < 0.05 and < 0.01 were considered statistically significant.

RESULTS

Experiment 1

The overall DFI negatively correlated with the following sperm parameters: total sperm count (r = –0.564, P < 0.01), percentage of total and progressively motile spermatozoa (r = –0.285 and –0.299, respectively, P < 0.05), sperm viability (r = –0.297, P < 0.05) and the percentage of morphologically normal spermatozoa (r = –0.284, P < 0.05). HDS also negatively correlated with total sperm count

Table 2. Experiment 1: Differences in parameters of conventional sperm analysis and sperm chromatin status between successful breeding males (Group A) and dogs with repeated fertility problems (Groupe B

		Total sperm count (10^6/ml)	M	PM	Viability	Intact acrosomes	Normal sperm	DFI	m-DFI	h-DFI	HDS
			(%)								
Group A (n = 33)	mean ± S.D.	920.2 ± 707.5	80.3 ± 11.9^b	77.8 ± 14.9^b	89.2 ± 7.1^a	87.6 ± 8.6	69.5 ± 14.0^b	1.60 ± 1.01^a	0.89 ± 0.61^b	0.71 ± 0.60	1.15 ± 0.49^b
	min	93.1	40.0	30.0	65.5	65.0	31.0	0.26	0.17	0.09	0.61
	max	3846.4	91.0	90.0	96.5	96.5	90.5	4.20	3.20	2.79	2.65
Group B (n = 12)	mean ± S.D.	603.2 ± 454.7	71.3 ± 9.2^b	67.6 ± 10.9^b	83.2 ± 9.1^a	88.1 ± 8.4	41.2 ± 18.7^b	3.48 ± 2.83^a	2.51 ± 2.34^b	0.97 ± 0.83	2.89 ± 2.53^b
	min	49.0	58.0	50.0	69.0	70.5	14.0	0.82	0.63	0.19	0.94
	max	1478.4	90.0	89.0	94.0	94.5	66.5	8.98	7.23	2.87	9.29

M = sperm motility; PM = progressive sperm motility; DFI = DNA fragmentation index; m-DFI = moderate DFI; h-DFI = high DFI; HDS = high DNA stainability
$^a P$ < 0.05, $^b P$ < 0.01

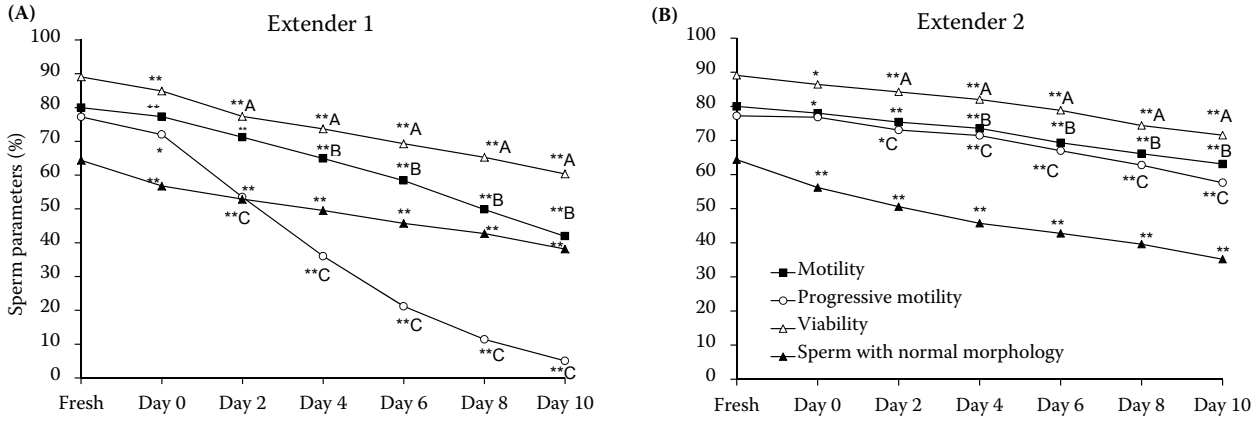

Figure 2. Experiment 2: The effect of long-term preservation of chilled canine semen on conventional sperm quality parameters (n = 18). Graphs show the values obtained during semen storage in Ext. 1 (CaniPROTM Chill 5; (**A**) and Ext. 2 (CaniPROTM Chill 10; (**B**) All sperm characteristics were significantly altered during 10 days of storage. Values marked with * show significant differences between chilled and fresh semen (*P < 0.05, **P < 0.01). Values with the same superscripts differ significantly between extenders in particular experimental days (P < 0.01)

(r = –0.451, P < 0.01), sperm motility and progressive motility (r = –0.427, -0.391, respectively, P < 0.01), viability (r = –0.389, P < 0.01) and morphology (r = –0.437, P < 0.01).

DFI, m-DFI and HDS were significantly lower (P < 0.01) in the good quality ejaculates (Group 1) than in ejaculates showing pathospermia or low motility (Group 2, Table 1).

All dogs with repeated fertility problems (Group B) were classed in Group 2. Their DFI and HDS values were significantly higher than those of successful breeding males (Group A). Moreover, most of their parameters in conventional sperm analysis displayed significantly more alterations (Table 2).

Experiment 2

As was expected, all sperm characteristics as revealed by conventional analysis were significantly altered in response to 10 days of storage (Figure 2). All examined sperm characteristics, except for sperm morphology, were significantly better protected against the negative effects of storage in

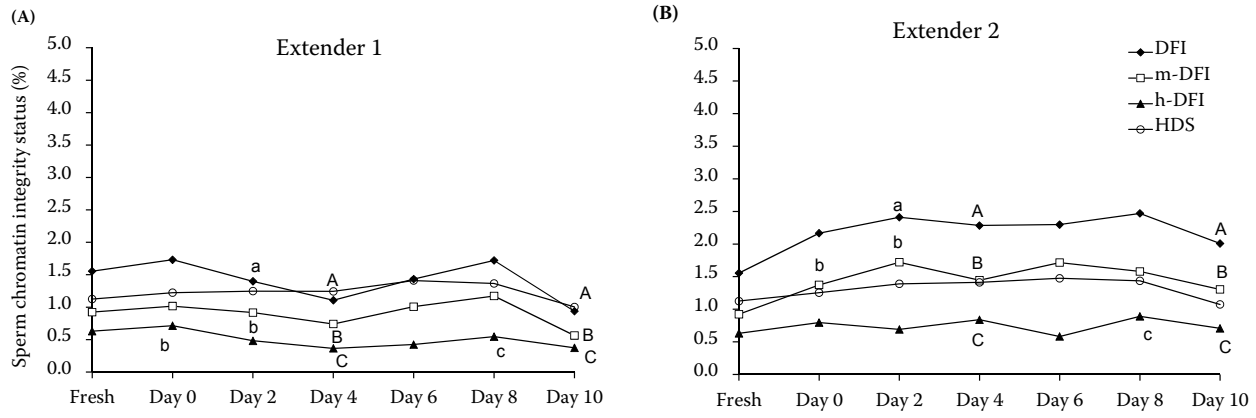

Figure 3. Experiment 2: The effect of long-term preservation of chilled canine semen on sperm chromatin integrity (n = 18). Graphs show the values obtained during semen storage in Ext. 1 (CaniPROTM Chill 5; (**A**) and Ext. 2 (CaniPROTM Chill 10; (**B**). Sperm chromatin integrity did not change significantly during 10 days of semen storage either in Ext. 1 or in Ext. 2 in comparison with fresh samples. Values with the same superscripts differ significantly between extenders in particular experimental days (a = P < 0.05, A = P < 0.01)

Table 3. Experiment 3: The effects of cryopreservation on sperm quality parameters (n = 30)

	M	PM	Viability	Normal sperm	DFI	m-DFI	h-DFI	HDS
	(%)							
Fresh samples	81.0 ± 8.6*	78.7 ± 10.5*	89.0 ± 6.0*	67.5 ± 18.2*	1.96 ± 1.92	1.12 ± 1.31	0.85 ± 0.94	1.59 ± 1.63
Frozen samples	59.9 ± 11.8*	57.7 ± 12.5*	62.6 ± 11.2*	36.4 ± 8.9*	1.84 ± 1.69	1.11 ± 1.17	0.73 ± 0.79	1.60 ± 1.55

M = sperm motility; PM = progressive sperm motility; DFI = DNA fragmentation index; m-DFI = moderate DFI; h-DFI = high DFI; HDS = high DNA stainability
*$P < 0.01$

Ext. 2 from day 2 (Figure 2). The percentage of morphological defects did not differ between extenders during the whole study. DFI and m-DFI values were shown to be higher in Ext. 2 compared to Ext. 1 on all days of the study. However, sperm chromatin integrity did not change significantly during 10 days of semen storage either in Ext. 1 or in Ext. 2 in comparison with fresh samples (Figure 3).

Experiment 3

Whereas all sperm characteristics measured by standard semen analysis were seriously injured by cryopreservation, neither sperm chromatin integrity nor number of cells with defective chromatin condensation changed during sperm freezing/thawing (see Table 3).

DISCUSSION

Evaluating fresh dog semen in this study, we observed that some sperm characteristics significantly correlated with sperm chromatin integrity, even with rather low correlation indices. Lower chromatin integrity was found in the group of dog ejaculates showing poor quality in comparison with the group of good quality ejaculates. The relationship between ejaculate quality and chromatin integrity is in accordance with the conclusions of Gosk et al. (2007). A certain relationship to standard sperm analysis was also documented in human (Larson et al. 2001) as well as bull (Boe-Hansen et al. 2005; Khalifa et al. 2008) and boar studies (Rybar et al. 2004; Perez-Llano et al. 2006). Sailer et al. (1995) reported that human sperm anomalies such as loosely packaged chromatin and damaged DNA are associated with poor quality semen samples. However, other previous studies of human males indicated that any association of sperm chromatin damage with standard semen parameters is ambiguous (Host et al. 1999; Saleh et al. 2002; Zini et al. 2002).

All evaluated dogs with repeated fertility problems were classed in the group showing poor semen quality, and even though their DFI was significantly higher than the DFI of successful breeding males, the highest DFI we obtained was only just under 9%. In canine semen, no DFI and HDS thresholds indicating decreased fertilizing potential have yet been established. Human studies have gradually established the thresholds for a significant decrease in fertility (DFI > 30%, HDS > 15%; Evenson et al. 1999; Larson-Cook et al. 2003; Virro et al. 2004; Evenson and Wixon 2006). Studies performed in bulls and stallions showed that their thresholds are similar (Bochenek et al. 2001; Love 2005). Even though another study (Rybar et al. 2004) suggested that the threshold for decreased fertility potential in bulls may be much lower than is reported for humans, we can assume that chromatin damage level in any evaluated dog was not high enough to have a significant effect on their fertility.

Sperm chromatin integrity did not change significantly during semen storage either in CaniPRO^{TM} Chill 5 or CaniPRO^{TM} Chill 10 medium for dog sperm chilling. Love et al. (2002) did not find any changes in chromatin values in response to up to 46 h of storage at 5 °C among the investigated stallions. However, when stallions were categorised, based on fertility status, 25% of the subfertile animals showed a rise in the percentage of DFI after 20 to 30 h. In case of semen storage at 20 °C, chromatin integrity was altered already during the first seven hours of incubation. It was found that the storage of extended boar semen at 18 °C for 72 h significantly decreased the integrity of sperm DNA (Boe-Hansen et al. 2005). It seems that the storage temperature of 5 °C can protect sperm chromatin integrity of semen samples character-

ized by good quality, but that higher temperatures have detrimental effects. Due to the fact that we used semen samples of good quality, we did not observe a significant increase in chromatin damage during 10 days of storage of chilled semen in either of the used extenders. Adverse effects of extenders on stallion sperm chromatin integrity were reported by Morrell et al. (2009) who found less chromatin damage in spermatozoa stored at 5 °C in INRA96, when compared to spermatozoa cooled in Kenney's extender. Our results obtained with the samples preserved in a medium with egg yolk showed slightly higher levels of DFI compared to medium without egg yolk during the whole time of incubation. As described by Khalifa et al. (2008), the dilution of bovine sperm, particularly in egg yolk-based extenders, compromises the antioxidant defence resulting in the exposure of spermatozoa to superphysiological concentrations of reactive oxygen species, which contribute to a loss of chromatin stability. However, the fact that chromatin integrity parameters obtained after 10 days of incubation in Ext. 2 (which contains egg yolk) did not significantly differ from values in fresh samples argues against this possibility.

According to the presented results, cryopreservation did not cause any significant changes in chromatin integrity. This conclusion is supported by the study of Anglada et al. (2009) who suggested that the cryopreservation process does not affect DNA fragmentation in stallion spermatozoa. Furthermore, it was shown that human sperm DNA integrity was unaffected by cryopreservation procedures (Isachenko et al. 2004). Koderle et al. (2009) detected even lower DFI values after thawing than in fresh dog semen. However, these reports stand in contrast to the results of Khalifa et al. (2008) who ascertained that cryopreservation of bull semen induced a pronounced decrease in sperm chromatin stability. Also, in boars Fraser and Strzezek (2007) using the Comet assay, confirmed that freezing-thawing facilitates destabilization of sperm chromatin structures, rendering the spermatozoa more vulnerable to DNA fragmentation. In a study of 8 semen samples from five dogs performed by Kim et al. (2010) significantly higher DFI was detected in frozen-thawed semen than in fresh semen. On the other hand, the unchanged HDS values are in accordance with our results.

In conclusion, with regard to a potential cause of reduced male fertility, the current study shows that the assessment of chromatin integrity in fresh dog ejaculates failed to add any further information to the results obtained by other techniques of semen analysis. Neither the short-term storage of canine sperm chilled in commercial extenders nor long-term cryopreservation in extenders recommended for the preservation of canine sperm had any adverse effects on sperm chromatin integrity.

REFERENCES

Andersen K (1975): Insemination with frozen dog semen based on a new insemination technique. Zuchthygiene 10, 1.

Anglada O, Taberner E, Pena A, Miro J (2009): DNA fragmentation in fresh and frozen-thawed stallion spermatozoa. In: 13th Annual Conference of the ESDAR. Reproduction in Domestic Animals 44 (Suppl. 3), 81.

Bochenek M, Smorag Z, Pilch J (2001): Sperm chromatin structure assay of bulls qualified for artificial insemination. Theriogenology 56, 557–567.

Boe-Hansen GB, Ersboll AK, Greve T, Christensen P (2005): Increasing storage time of extended boar semen reduces sperm DNA integrity. Theriogenology 63, 2006–2019.

Bungum M, Spano M, Humaidan P, Eleuteri P, Rescia M, Giwercman A (2008): Sperm chromatin structure assay parameters measured after density gradient centrifugation are not predictive for the outcome of ART. Human Reproduction 23, 4–10.

Check JH, Graziano V, Cohen R, Krotec J, Check ML (2005): Effect of an abnormal sperm chromatin structural assay (SCSA) on pregnancy outcome following (IVF) with ICSI in previous IVF failures. Archives of Andrology 51, 121–124.

Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ, Carrell DT (2006): Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. Journal of Andrology 27, 53–59.

Cordova A, Perez-Gutierre JF, Lleo B, Garcia-Artiga C, Alvarez A, Drobchak V, Martin-Rillo S (2002): In vitro fertilizing capacity and chromatin condensation of deep frozen boar semen packaged in 0.5 and 5 ml straws. Theriogenology 57, 2119–2128.

Erenpreisa J, Erenpreiss J, Feivalds T, Saidina M, Krampe R, Butikova J, Ivanov A, Pjanova D (2003): Toluidine blue test for sperm DNA integrity and elaboration of image cytometry algorithm. Cytometry A 52, 19–27.

Eulenberger K, Schafer-Somi S, Aurich C (2009): Effect of different concentrations of ascorbic acid on motility, membrane integrity and chromatin status of frozen-thawed canine spermatozoa within six hours of

storage at 37 °C. Reproduction in Domestic Animals 44, 354–358.

Evenson D, Wixon D (2006): Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. Reproductive Biomedicine Online 12, 466–472.

Evenson DP, Baer RK, Jost LK (1989): Flow cytometric analysis of rodent epididymal spermatozoal chromatin condensation and loss of free sulfhydryl groups. Molecular Reproduction and Development 1, 283–288.

Evenson DP, 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.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP (1999): Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Human Reproduction 14, 1039–1049.

Evenson DP, Larson KL, Jost LK (2002): Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. Journal of Andrology 23, 25–43.

Fernandez-Santos MR, Dominguez-Rebolledo AE, Esteso MC, Garde JJ, Martinez-Pastor F (2009): Refrigerated storage of red deer epididymal spermatozoa in the epididymis, diluted and with vitamin C supplementation. Reproduction in Domestic Animals 44, 212–220.

Fraser L, Strzezek J (2005): Effects of freezing-thawing on DNA integrity of boar spermatozoa assessed by the neutral comet assay. Reproduction in Domestic Animals 40, 530–536.

Fraser L, Strzezek J (2007): Is there a relationship between the chromatin status and DNA fragmentation of boar spermatozoa following freezing-thawing? Theriogenology 68, 248–257.

Garcia-Macias V, Martinez-Pastor F, Alvarez M, Garde JJ, Anel L, de Paz P (2006): Assessment of chromatin status (SCSA) in epididymal and ejaculated sperm in Iberian red deer, ram and domestic dog. Theriogenology 66, 1921–1930.

Gosk M, Eschricht F, Knoche I, Waberski D, Bollwein H, Gunzel-Apel AR (2007): Chromatin status of dog spermazotoa with regard to semen quality. In: 11th Annual Conference of the ESDAR. Reproduction in Domestic Animals 42 (Suppl. 2), 70.

Hernandez M, Roca J, Ballester J, Vazquez JM, Martinez EA, Johanisson A, Saravia F, Rodriguez-Martinez H (2006): Differences in SCSA outcome among boars with different sperm freezability. International Journal of Andrology 29, 583–591.

Host E, Lindenberg S, Ernst E, Christensen F (1999): DNA strand breaks in human spermatozoa: a possible factor, to be considered in couples suffering from unexplained infertility. Acta Obstetricia et Gynecologica Scandinavica 78, 622–625.

Isachenko E, Isachenko V, Katkov II, Rahimi G, Schondorf T, Mallmann P, Dessole S, Nawroth F (2004): DNA integrity and human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. Human Reproduction 19, 932–939.

Jager S (1990): Sperm nuclear stability and male infertility. Archives of Andrology 25, 253–259.

Karabinus DS, Evenson DP, Kaproth MT (1991): Effects of egg yolk-citrate and milk extenders on chromatin structure and viability of cryopreserved bull sperm. Journal of Dairy Science 74, 3836–3848.

Khalifa TAA, Rekkas CA, Lymberopoulos AG, Sioga A, Dimitriadis I, Papanikolaou T (2008): Factors affecting chromatin stability of bovine spermatozoa. Animal Reproduction Science 104, 143–163.

Kim SH, Yu DH, Kim YJ (2010): Effects of cryopreservation on phosphatidylserine translocation, intracellular hydrogen peroxide, and DNA integrity in canine sperm. Theriogenology 73, 282–292.

Koderle M, Aurich C, Schafer-Somi S (2009): The influence of cryopreservation and seminal plasma on the chromatin structure of dog spermatozoa. Theriogenology 72, 1215–1220.

Kruger TF, Menkveld R, Stander FSH, Lombard CJ, Van der Merwe JP, Van Zyl JA, Smith K (1986): Sperm morphologic features as a prognostic factor in vitro fertilization outcome. Fertility and Sterility 46, 1118–1123.

Larson KL, Brannian JD, Singh NP, Burbach JA, Jost LK, Hansen KP, Kreger DO, Evenson DP (2001): Chromatin structure in globozoospermia: a case report. Journal of Andrology 22, 424–431.

Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET, Evenson DP (2003): Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. Fertility and Sterility 80, 895–902.

Lee CH, Cho YH (1999): Aspects of mammalian spermatogenesis: electrophoretical analysis of protamines in mammalian species. Molecules and Cells 9, 556–559.

Love CC (2005): The sperm chromatin structure assay: a review of clinical applications. Animal Reproduction Science 89, 39–45.

Love CC, Thompson JA, Lowry VK, Varner DD (2002): Effect of storage time and temperature on stallion sperm DNA and fertility. Theriogenology 57, 1135–1142.

Matsuura R, Takeuchi T, Yoshida A (2010): Preparation and incubation conditions affect the DNA integrity of ejaculated human spermatozoa. Asian Journal of Andrology 12, 753–759.

Morrell JM, Meurling S, Lewin E, Johannisson A, Dalin AM, Rodriguez-Martinez H (2009): The chromatin integrity of stallion spermatozoa is maintained better in INRA96 than in Kenney's extender during cool storage. In: 11th Annual conference of the ESDAR. Reproduction in Domestic Animals 44 (Suppl. 3), 116.

Mortimer D (1994): Practical Laboratory Andrology. Oxford University Press, New York. 393 pp.

Nunez-Martinez I, Moran JM, Pena FJ (2005): Do computer-assisted, morphometric-derived sperm characteristics reflect DNA status in canine spermatozoa? Reproduction in Domestic Animals 40, 537–543.

Perez-Llano B, Enciso M, Garsia-Casado P, Sala R, Gosalvez J (2006): Sperm DNA fragmentation in boars is delayed or abolished by using sperm extenders. Theriogenology 66, 2137–2143.

Rota A, Rota A, Martini M, Milani C, Romagnoli S (2005): Evaluation of dog semen after slow (biological freezer) or rapid (nitrogen vapours) freezing. Reproduction Nutrition Development 45, 29–37.

Rybar R, Faldikova L, Faldyna J, Machatkova M, Rubes J (2004): Bull and boar sperm DNA integrity evaluated by sperm chromatin structure assay in the Czech Republic. Veterinarni Medicina 49, 1–8.

Sailer BL, Jost LK, Evenson DP (1995): Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks and measured by the terminal deoxynukleotidyl transferase assay. Journal of Andrology 16, 80–87.

Sakkas D, Manicardi DC, Bizzaro D (2003): Sperm nuclear DNA damage in the human. Advances in Experimental Medicine and Biology 518, 73–84.

Saleh RA, Agarwal A, Nelson DR, Nada EA, El-Tonsy MH, Alvarez JG, Thomas AJ Jr, Sharma RK (2002): Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study. Fertility and Sterility 78, 313–318.

Shahiduzzaman AKM, Linde-Forsberg C (2007): Induced immotility during long-term storage at +5 °C does not prolong survival of dog spermatozoa. Theriogenology 68, 920–933.

Veznik Z, Matouskova O, Svecova D, Zajicova A (2001): The use of the computer technology for the evaluation of the strict morphological sperm analysis. Veterinarni Medicina 46, 35–40.

Virro MR, Larson-Cook KL, Evenson DP (2004): Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. Fertility and Sterility 81, 1289–1295.

World Health Organization (2010): WHO laboratory manual for the Examination of human semen. 5th ed. WHO Press, Geneva, Switzerland. 271 pp.

Zini A, Kamal K, Phang D, Willis J, Jarvi K (2001): Biologic variability of sperm DNA denaturation in infertile men. Urology 58, 258–261.

Zini A, Fischer MA, Sharir S, Shayegan B, Phang D, Jarvi K (2002): Prevalence of abnormal sperm DNA denaturation in fertile and infertile men. Urology 60, 1069–1072.

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