Animal Reproduction Science 206 (2019) 38–45

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Effect of sperm dosage transportation in stallions: Effect on sperm DNA fragmentation

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ARTICLE INFO

Keywords:
Stallion reproduction
Sperm quality
Sperm DNA fragmentation
Refrigeration
Cooled semen

ABSTRACT

Artificial insemination programs for horses usually involve *ex vivo* handling and transporting of sperm. The present experiment was designed to: (i) assess the effect of transportation on sperm DNA integrity at different time post semen collection, and (ii) evaluate if sperm DNA quality deteriorates rapidly beyond 24 h of cooled storage. After collection, the ejaculates were extended using INRA 96 and semen was prepared for prompt analysis (A0) or 24 h/48 h cooled-shipping (B24 and C48 respectively). Each sample was assessed for sperm DNA fragmentation index (SDFI) at time 0 and after incubation for 2, 6 and 24 h at 37 °C. There was very little difference in SDFI between freshly extended (A0) and 24 h/48 h cooled-transported semen samples (B24/C48) at time 0. After 2 h of incubation at 37 °C, there was an increase in SDFI ranging from 2.7% to 7.5% per hour in freshly extended semen samples (A0: 5.1 ± 1.5), while cooled-transported semen samples had a much greater increase in SDFI, ranging from 5.0% to 20.5% (B24: 14.7 ± 5.6) and from 8.2% to 26.8% (C48: 18.3 ± 7.2) respectively. There were not marked differences in the sperm DNA integrity between 24 and 48 h for transported samples, thus there is the possibility of desirable fertility with use of stallion sperm after 48 h of cooled storage.

1. Introduction

Since the late 1980s, artificial insemination (AI) of mares with cooled- transported semen is widely utilized in the horse breeding industry (Aurich and Aurich, 2006; Bedford-Guaus, 2007). The success when using AI is often related to the various advantages associated with use of this practice. By avoiding mare transport and/or direct male to female contact, there is prevention of disease transmission between farms, while accessing a wider and often superior gene pool for breeding of mares (Bedford-Guaus, 2007). At the same time, however, *ex vivo* handled gametes can undergo iatrogenic damage (Mortimer, 1991, 1994).

To ensure an acceptable sperm quality and desirable rates of pregnancy, a careful examination of semen is recommended, not only at the time of semen collection but, most importantly, at arrival of shipped semen to an insemination center (Samper et al., 2004; Heckenbichler et al., 2011). Classical routine semen evaluation includes the analysis of volume and appearance of the semen sample, sperm concentration, percentage of total and progressively motile sperm, and percent of morphologically abnormal sperm (Samper

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https://doi.org/10.1016/j.anireprosci.2019.05.005
Received 19 October 2018; Received in revised form 22 March 2019; Accepted 10 May 2019

et al., 2004; Bedford-Guaus, 2007; Gottschalk et al., 2016). Even though there is a large amount of sperm DNA fragmentation (SDF) that is associated with compromised oocyte viability, poor embryo quality, and reduced pregnancy rates (Virro et al., 2004; Zini and Libman, 2006; Fernández et al., 2009; Karoui et al., 2012; Robinson et al., 2012), SDF analysis is not generally included as a routine practice for semen analysis, and when used, is usually reported as a value that was determined at the time of semen collection. Results of different studies indicate that SDF increases from the time of sample collection to the time cryopreserved spermatozoa are prepared for utilization for AI (López-Fernández et al., 2007; Gosálvez et al., 2009; Jackson et al., 2010; Matsuura et al., 2010). It, therefore, is important to realize that semen samples that have been collected and processed for insemination purposes undergo iatrogenic sperm damage during the various aspects of semen processing and that the type of processing techniques affect the extent of this damage (Gosálvez et al., 2018). Using different experimental animals such as zebrafish (Gosálvez et al., 2014) or rabbits (Johnston et al., 2016), it has been ascertained that there is a negative effect of the increase in sperm DNA fragmentation on reproductive outcomes. In this regard, the extent of sperm DNA damage when sperm are used for *in vitro* fertilization must be considered when determining the time of AI. Subsequently, having a greater understanding of and control of how sperm DNA quality is affected by iatrogenic damage as analyzed *in vitro*, may be important for understanding pregnancy results.

The rationale for the present investigation was that the assessment of DNA fragmentation in stallion sperm would serve the purpose of evaluating the extent of DNA damage that does not always become apparent after sperm handling and delivery and thus, cannot be observed when a single assessment of the SDF is performed immediately after semen collection. Also, the results from the present investigation provide additional information on sperm quality beyond 24 h of cooled storage.

2. Material and methods

Twelve healthy stallions of the Pure Spanish breed, from 5 to 21 years old and with known fertility were used as semen donors in the present study. The animals were unrelated and housed in individual paddocks placed at the Equine Breeding Center of The Spanish Army located in Ávila, Spain (40.66 °N, 4.70 °W). Semen samples were collected during the breeding season by allowing the stallions to mount a phantom and ejaculate into a Missouri-model artificial vagina with an in line gel filter (Nasco, Fort Atkinson, Wl) and warmed to 45–50 °C prior to semen collection. The ejaculate was extended using pre-warmed INRA 96 (IMV Technologies, L'Aigle, France) to obtain a final sperm concentration of 50×10^6/mL. Breeding doses were prepared ensuring that these contained a minimum of 500×10^6 progressively motile spermatozoa and a final volume of 20 mL. After preparation, the doses were stored in a darkened area until there was an ambient temperature of about 22 °C. Each dose was divided into three aliquots to simulate three different experimental conditions. The three aliquots were cooled slowly from 20 to 5 °C in a Neopor transport box with two cold packs (Minitube 17229/0002). One aliquot was used immediately after temperature stabilization at 5 °C and it was considered to be the freshly extended semen sample (condition A0). The other two aliquots were stored in the transport box at the environmental temperature of about 22 °C for as long as 48 h in the laboratory. After 24 h of storage, one of the sperm aliquots was removed from the container. This sample was considered to be the 24 h transportation semen sample (condition B24). After 48 h of storage, another aliquot was removed from the container. This sample was considered to be the 48 h transportation semen sample (condition C48). Temperature during storage, was controlled using a USB temperature logger (Microlite, Fourier Systems) which was placed inside the Neopor transport box (Fig. 1b).

The three aliquots (A0, B24 and C48) were processed using the same conditions to analyze the changes in sperm DNA fragmentation during the incubation time. For this purpose, each sample was assessed at time 0 (just after subsampling - T0) and after incubation at the 2 (T2), 6 (T6) and 24 (T24) h periods at 37 °C (see chart diagram in Fig. 1a). After each incubation time, samples were processed to determine the percentage of sperm cells with fragmented DNA (SDFI; Fig. 2a and b). Particular care was taken to promptly perform the second part of the experiment; thus, as soon as the subsamples were removed from the container or after the 37 °C incubation period, the semen sample was immediately processed to avoid accumulation of additional iatrogenic DNA damage. The SDFI was assessed using the Sperm Chromatin Dispersion (SCD) test, performed with Halomax® kit (Halotech DNA SL, Madrid, Spain). Spermatozoa ($n = 300$) from each treatment group and incubation time at 37 °C were counted to estimate SDFI. Differences in the patterns of SDFI increase from T0 to T2 among treatment groups (A0, B24, C48) were also studied. To accomplish this, the rate of SDFI (rSDFI) was calculated by subtracting SDFI values at the two consecutive intervals (T2-T0) and dividing the result by the 2 h elapsed time.

2.1. Statistical analysis

An exploratory data analysis was conducted to examine data structure as a function of the different treatments. When differences were observed among levels within each treatment, a paired *t*-test *post-hoc* analysis was performed by using Bonferroni correction.

A mixed linear model (LMM) was used to determine how the dynamics of sperm DNA fragmentation is affected by transportation treatments, incubation time, and the interaction of the two (considered as fixed effects) as well as the stallion (considered a random factor). The *nlme* package in R was used for statistical software (Pinheiro et al., 2016; R Core Team, 2016).

Visual inspection of residual plots did not indicate there were any obvious deviations from homoscedasticity or normality. The *P*-values were obtained by conducting likelihood ratio tests using the complete model with the effect in question being compared when there was a model without this specific effect being included.

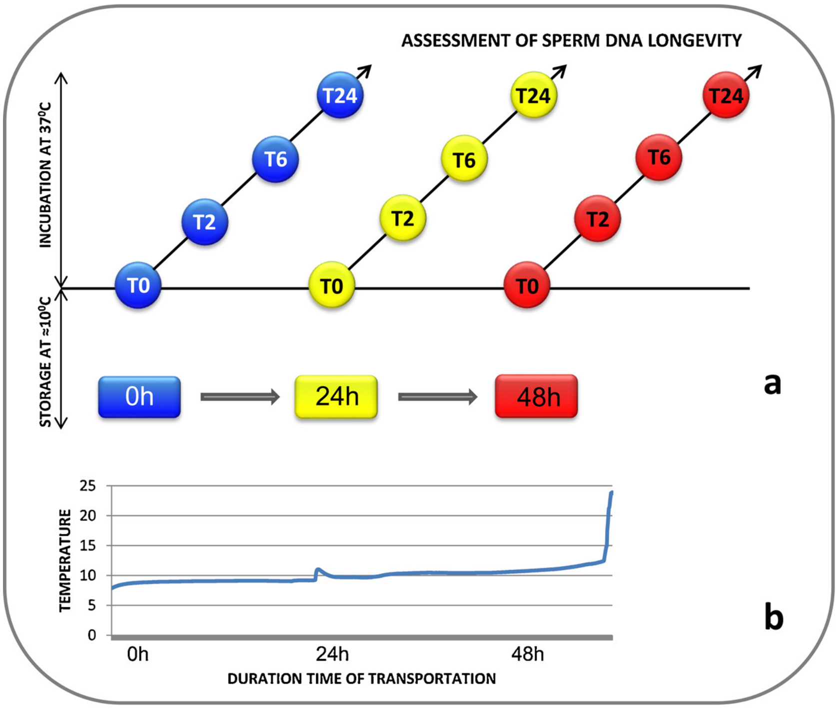

Fig. 1. (a) Experimental design used for the analysis of the effect of transportation on sperm DNA integrity at different times post collection. Semen samples were collected, diluted in INRA96, and divided into three aliquots. The first sample was used immediately after temperature stabilization at 5 °C (0 h, freshly extended sample shown in blue). The other two aliquots were stored in a Neopor transport box with cold packs, and used after 24 h (24 h transported sample depicted in yellow), and 48 h of storage (48 h transported sample depicted in red), respectively. To analyze the effect of transportation on sperm changes in DNA fragmentation post collection and sperm DNA longevity, each sample (blue, yellow or red) was analyzed at time 0 (T0) and after incubation for 2 (T2), 6 (T6) and 24 (T24) h at 37 °C. (b) Temperature changes during storage/ transport as depicted by the temperature sensor introduced inside the Neopor transport box (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3. Results

There are data in Table 1 for the results obtained in the present experiment for each stallion and transportation treatment (A0, B24 and C48) at T0, and after incubation for 2 (T2), 6 (T6) and 24 (T24) h at 37 °C. Box-and-whisker plots were used to assess the distribution of SDFI data for experimental groups (Fig. 2c, d, e). Results from exploratory data analyses indicated there were no overall differences among stallions in SDFI ($F_{11,132} = 0.62$, $P = 0.8092$; Fig. 2c). Transport effects explain 6.6% of SDFI variance ($F_{2,141} = 6.04$, $P = 0.003$) with there being differences between values in the newly extended (A0) and cooled-transported semen (either B24 or C48) samples but not between these last two experimental groups (Fig. 2d). In turn, incubation time explained 77.7% of SDFI variance ($F_{3,140} = 166.76$, $P < 0.001$) with there being differences for every pair comparison (see Fig. 2e).

Differences between freshly extended (A0) and cooled-transported (B24 and C48) semen samples were evident in the slopes of the increase in SDFI from T0 to T2 (Table 1). Graphical analysis further supports the importance of considering the interaction between transport and incubation time as an explanatory variable of the observed variance in SDFI (Fig. 2f, g).

Taking into account the results from exploratory data analysis, there was construction of a linear mixed-effects model of sperm DNA fragmentation. For model analysis, there was an effect of stallion as a random factor ($\chi^2_{(1)} = 46.88$, $P < 0.0001$). Also, sperm DNA fragmentation was affected by transport ($\chi^2_{(2)} = 82.75$, $P < 0.0001$), incubation time at 37 ºC ($\chi^2_{(3)} = 300.03$, $P < 0.0001$), and the interaction of transport by incubation time ($\chi^2_{(6)} = 63.87$, $P < 0.0001$).

For both freshly-extended (A0) and cooled-transported semen (B24 or C48), there was an increase of SDFI after each time interval of incubation at 37 °C ($P < 0.001$; Table 1). At the baseline time (T0) there were differences only when comparing A0 and C48 ($P = 0.0057$). After incubation for 2 h (T2), however, there were SDFI differences for all paired comparisons (A0 compared with B24, A0 compared with C48, and B24 compared with C48, $P < 0.005$). After incubation for 6 h (T6), there were only SDFI differences when comparing either A0 and B24 or A0 and C48 ($P < 0.0001$). After 24 h of incubation, there were no SDFI differences for any of the paired comparisons (Table 1). To summarize, there were differences when comparing both cooled-transported semen (B24 and C48), SDFI differences after 2 h of incubation at 37 °C, when SDFI at C48 is + 0.10 ± 0.03 greater than SDFI at B24 ($P = 0.0012$).

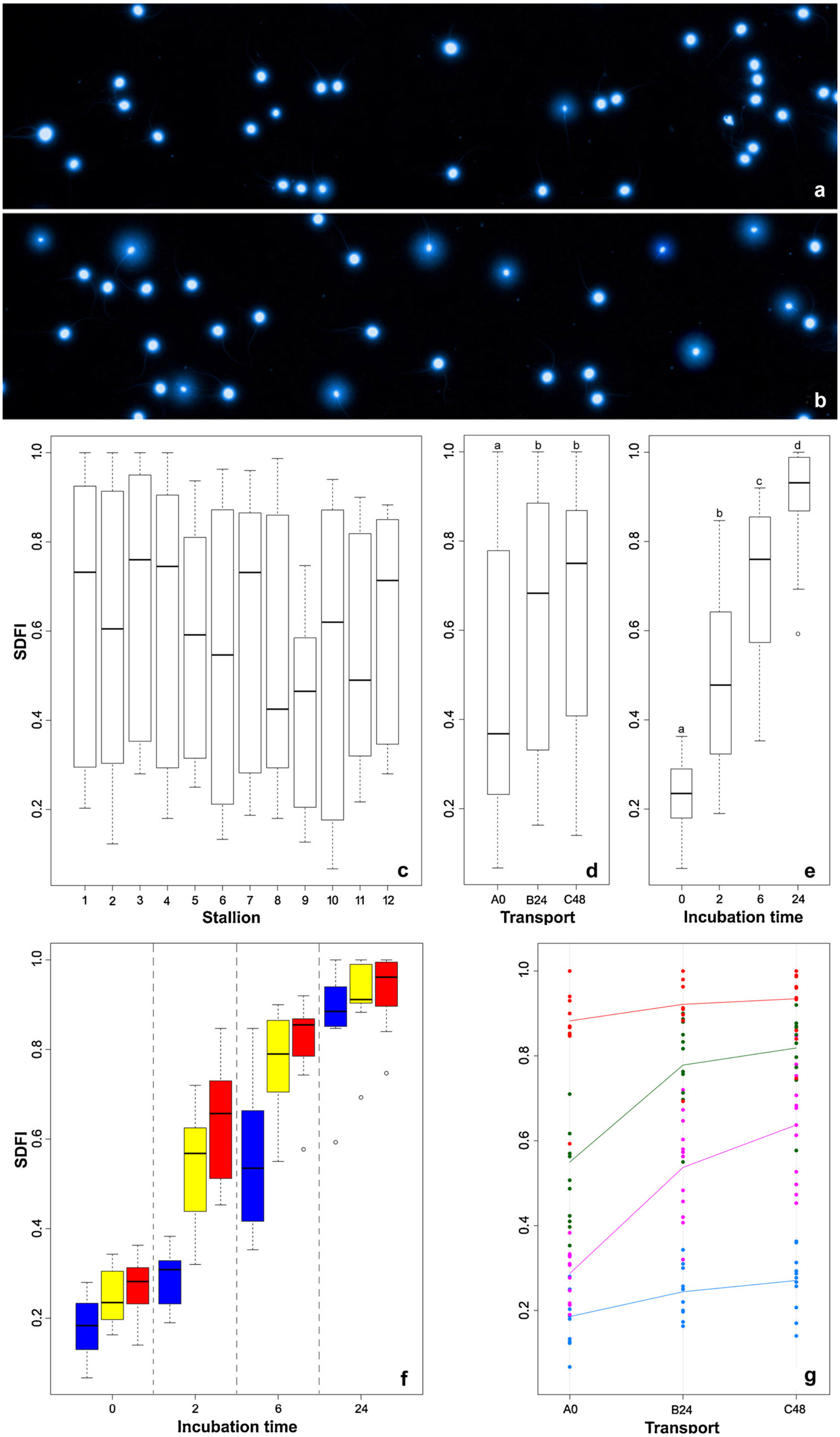

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Fig. 2. (a) Sperm chromatin dispersion (SCD) of freshly extended sperm sample after DAPI staining, showing spermatozoa with large halos of chromatin dispersion containing fragmented DNA. (b) SCD processed sperm sample after 48 h transportation, percentage of damaged sperm is greater. (c–e) Box-and-whisker plots used to compare the distribution of SDFI data among stallions (c), transportation conditions (d), and during the time of incubation at 37 °C (e). Different letters denote differences between experimental groups ($P \leq 0.05$). (f) Box-and-whisker plots used to compare the distribution of SDFI data during the time of incubation at 37 ºC among the three experimental groups (freshly extended in blue, 24 h transported in yellow, and 48 h transported in red). Note the interaction of transportation condition by incubation time. (g) SDFI represented against transportation condition at T0 (blue), T2 (pink), T6 (green) and T24 (red). Note differences between freshly extended (A0) and both transported samples (B24 and C48) after 2 h and 6 h of incubation. Differences between transported samples (B24 and C48) were only detected at T2 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1

Analysis of sperm DNA fragmentation changes post collection for the three different experimental groups: freshly extended semen sample (A0), and after 24 (B24) and 48 (C48) h subsequent to transportation. Percentage of fragmented spermatozoa (SDFI) is depicted for each stallion at the time of semen collection (T0) and after 2 (T2), 6 (T6) and 24 (T24) h of incubation at 37 ºC. Mean SDFI and standard deviation for each experimental condition are also included. The rate of SDFI increase (rSDFI) from T0 to T2 was obtained by subtracting SDFI values at the two time intervals (T2 − T0) and dividing the result by the elapsed time (2 h). Different superscripts are used to indicate differences ($P \leq 0.05$). First letter (in lower case) is used for comparisons in the same row; *i.e.* same conditions regarding transport (A0, B24 or C48) as time of incubation at 37 ºC increases. Second and third letters (in upper case) are used for comparisons in the same column; *i.e.* same conditions regarding time of incubation at 37 ºC (T0, T2, T6 and T24). The second letter indicates comparisons of either A0 and B24, or A0 and C48, while the third letter comparisons of B24 and C48.

		SPERM DNA FRAGMENTATION DYNAMICS (37 °C)				
CONDITIONS	STALLIONS	T0	T2	T6	T24	rSDF (T0-T2)
A0	1	20.5	27.7	61.6	93.0	3.6
	2	12.5	24.7	48.6	94.0	6.1
	3	27.9	33.3	84.5	100.0	2.7
	4	18.1	30.8	71.0	100.0	6.4
	5	25.0	31.0	57.0	84.7	3.0
	6	13.3	21.7	41.1	86.7	4.2
	7	18.7	30.7	71.0	87.0	6.0
	8	18.0	33.0	39.7	85.3	7.5
	9	12.6	21.3	35.3	59.3	4.4
	10	6.7	19.0	56.3	94.0	6.2
	11	21.7	32.7	42.3	90.0	5.5
	12	28.0	38.3	50.7	85.0	5.2
	MEAN ± ST DEV	$18.6 \pm 6.6^{a,A}$	$28.7 \pm 5.9^{b,A}$	$54.9 \pm 15.0^{c,A}$	$88.3 \pm 10.6^{d,A}$	5.1 ± 1.5
B24	1	25.7	58.0	88.0	100.0	16.2
	2	22.0	57.3	88.7	100.0	17.7
	3	34.3	67.6	90.0	100.0	16.7
	4	20.0	60.3	81.7	98.0	20.2
	5	32.0	42.0	75.7	91.0	5.0
	6	17.3	40.7	71.3	91.3	11.7
	7	25.0	64.7	85.0	91.0	19.8
	8	19.7	32.0	69.3	96.3	6.2
	9	19.7	45.7	55.0	69.3	13.0
	10	16.3	56.3	83.3	91.0	20.0
	11	30.0	48.3	69.7	89.7	9.2
	12	31.0	72.0	76.3	88.3	20.5
	MEAN ± ST DEV	$24.4 \pm 6.2^{a,A,A}$	$53.7 \pm 12.1^{b,B,A}$	$77.8 \pm 10.3^{c,B,A}$	$92.2 \pm 8.4^{d,A,A}$	14.7 ± 5.6
C48	1	31.3	84.7	92.0	100.0	26.7
	2	36.0	63.7	87.0	100.0	13.8
	3	36.3	52.7	86.0	100.0	8.2
	4	27.7	78.0	83.0	99.0	25.2
	5	29.3	61.3	77.3	93.7	16.0
	6	20.7	68.3	87.7	96.3	23.8
	7	25.7	75.3	86.0	96.0	24.8
	8	26.7	45.3	86.7	98.7	9.3
	9	17.0	47.5	57.7	74.7	15.2
	10	14.0	67.7	74.3	93.3	26.8
	11	31.3	49.7	79.7	84.0	9.2
	12	28.7	70.7	85.0	86.0	21.0
	MEAN ± ST DEV	$27.1 \pm 6.9^{a,B,A}$	$63.7 \pm 12.8^{b,B,B}$	$81.9 \pm 9.0^{c,B,A}$	$93.5 \pm 8.0^{d,A,A}$	18.3 ± 7.2

4. Discussion

Results in the present study indicate there is sperm DNA fragmentation in stallions that is not directly apparent at the time of receiving cooled-shipped semen to the insemination center, and that this damage will remain undetected if there is only a single assessment of the SDFI performed at the time semen is received (T0 in the present study). In this regard, although there was very little difference in SDFI at T0 when comparing effects of transportation treatments, differences became evident after 2 or 6 h of incubation at 37 °C. The time when changes can be detected in DNA fragmentation of sperm is different depending on whether the semen has been transported or used freshly extended as it was evidenced by the different slopes of the increase in SDFI from T0 to T2 (see reported rSDFI values in Table 1).

For AI with cooled-shipped semen, the sperm is ejaculated and extended at 38 °C and then, once the semen doses are prepared, there is stabilization of samples for 10–12 min, until the samples are at an ambient temperature of about 22 °C. Every dose to be shipped is then subjected to refrigeration, at a slow cooling rate, until the temperature of the samples is 4–6 °C. According to the USB temperature logger which was placed inside the transport box, the cooled semen samples in the present study were stored at a temperature of about 10 °C. Temperature oscillations might cause some significant structural changes in the sperm. Sperm membranes might be transformed after a thermal stress (Fiser et al., 1991) and phosphatidyl serine externalization might occur as a consequence (Duru et al., 2001). These changes in sperm structure may not be evident due to low temperatures that restrict the metabolic rate of these cells during storage. After the sperm are reactivated at 37 °C, a series of mechanisms that are associated with the presence of ROS and linked to changes in mitochondrial activities and/or the massive release of enzymes such as topoisomerases (Sotolongo et al., 2005; Ortega et al., 2011) occurs as a result of the loss of membrane stability and consequently rapid expansion occurs resulting in DNA damage.

For correct timing of AI, there needs to be frequent veterinary observations of the mare and also an appropriate decision needs to occur as to when to order the semen. Sieme et al. (2003) reported that the optimal timing for mare AI was between 24 h before and 12 h after ovulation. These authors also reported that 97.5% of the mares properly treated with human chorionic gonadotrophin (hCG) had ovulations within 48 h after treatment. Results of the present study indicate prolonged cooled-storage for as long as 48 h does not result in a deterioration of sperm DNA beyond that observed after 24 h. These results indicate that there is the opportunity to include two semen doses in one shipment providing for the opportunity to re-inseminate the mare 24 h after the first AI if ovulation has not occurred by this time and thus, to increase the likelihood that AI is performed at an optimal time for increasing the probability of a pregnancy. This could be a desirable alternative when intense veterinary management is not possible for determination of the optimal time to AI mares. Squires et al. (1998) reported that there was a greater pregnancy rate in mares inseminated twice with 1×10^9 progressively motile spermatozoa on each of two consecutive days than in mares inseminated once either with 1×10^9 or 2×10^9 progressively motile spermatozoa. Shore et al. (1998), however, reported that there were no pregnancy rate differences when comparing mares inseminated either once with 500×10^6 total spermatozoa or twice on two consecutive days with 250×10^6 sperm, thus in this study there was no advantage in retaining half the seminal dose to re-inseminate the subsequent day. Considering the results of changes in amount of sperm DNA fragmentation and consequently DNA integrity ascertained in the present study, the sequential approach to inseminating mares appears to be a viable alternative. As previously described in this manuscript, there were no SDFI differences between the B24 and C48 samples at T0 (*i.e.*, after 48 h of storage, the SDFI was similar to that after 24 h of storage). Although there was an apparent more rapid increase in DNA fragmentation in the C48 than B24 samples and thus, after 2 h incubation at 37 °C (T2) there were SDFI differences when there was comparison of transported semen samples (B24 and C48), however, these differences were no longer apparent after 6 h incubation at 37 °C (T6). From these results, it would be less desirable to store the semen for use the next day (C48) than using it upon arrival (B24). There, however, also needs to be consideration that if the two seminal doses were used for AI of the mare at the time the semen was received (B24) the expectation would be that more than 90% of the inseminated sperm would be fragmented 24 h later. With use a sequential AI approach, however, the profile for DNA fragmentation cycle would be reset 24 h later so that the SDFI would be that at time 0 when the mare is inseminated with the second dose of semen (C48). There is still inconsistency of thought, however, as to whether the mare´s tract has more optimal conditions for storage of sperm than any other transport container or if, the longer duration of presence of the sperm in the uterus would have a harmful effect by being a continuous stimulus for inflammation (Watson and Nikolakopoulos, 1996).

In all mammalian species studied, including farm species, spermatozoa are transported to the oviduct where there is attachment to and interaction with ciliated epithelial cells in the caudal isthmus, thus resulting in the formation of the oviductal sperm reservoir (Hunter, 1981; Thomas et al., 1994a; Suarez, 2002). This reservoir is important for the transport of sperm in adequate numbers, and in the proper physiological state necessary for normal fertilization (reviewed by Bosch and Wright, 2005); thus, this transportation scenario may result in selection of a greater quality sperm subpopulation for fertilization (Thomas et al., 1994b). Results from transmission electron microscopy studies performed by Esponda and Moreno (1998) indicate 81% of mouse spermatozoa attached to the oviductal epithelium have intact acrosomes, while most of the free spermatozoa have swelled or disrupted acrosomes. Also, by using the flow cytometric sperm chromatin structure assay (SCSA), it has been ascertained that human sperm bound to oviductal cells have greater chromatin quality than unbound sperm (Ellington et al., 1999).

Membrane and DNA integrity are required for attachment of spermatozoa to oviductal epithelium, but cooling and shipping of semen can induce changes, damaging sperm, resulting in the need for greater synchrony in timing between when ovulation and AI to increase the likelihood that fertilization occurs. In this regard, sperm cells of fresh semen may remain viable for 3–4 days or even 1 week in the oviducts of mares inseminated by mating with a stallion (Woods et al., 1990; Newcombe, 1994). In contrast, Sieme et al. (2003) reported that there was a reduction in viability of cooled transported semen after 24 h relative to the timing of AI and, as expected, there were increased pregnancy rates when the timing of ovulation and AI were more synchronous in mares that were administered with hCG for ovulation induction (Sieme et al., 2003; Newcombe and Cuervo-Arango, 2011). The results from the present study indicate that SDFI increases with time of incubation at 37 °C, thus gradually reducing the number of sperm that have the potential capacity for anchoring to the oviduct of epithelial cells. If spermatozoa that had been stored by cooling do not remain attached to the oviductal wall for 48 h prior to ovulation, the availability of viable spermatozoa in the reservoir attached to the oviduct wall will be gradually reduced, and thus, the number of viable and functionally competent spermatozoa might not be sufficient for fertilization at the time when ovulation occurs. By using a second semen dose, the profile for DNA fragmentation would be reset to SDFI values found at time 0, and so that replenishment of the reservoir will be favoured, thus increasing the probability for fertilization. From an operative viewpoint, a second AI 24 h after the initial AI may be contraindicative in the case of older mares, with poor uterine clearance, in which a second AI may induce a greater inflammatory response of the uterus.

In conclusion, the results of the present study indicate that there are changes in the amount of sperm DNA fragmentation which may be informative about pattern of sperm DNA integrity after ejaculation depending on procedures of sperm storage. These findings may be useful for determining the pattern of latent DNA damage that is not perceived by determining the amount of DNA damage in fresh semen samples at the time of collection. Prolonged cooled-storage for up to 48 h does not result in a deterioration of sperm DNA beyond that observed after 24 h of cooled-transport. This observation supports the possibility of shipment of two semen doses and re-insemination of mares 24 h after the time of the first AI if ovulation has not occurred by the time the second AI would occur.

Conflicts of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

Acknowledgements

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. The authors thank Juan Carlos Blázquez for technical assistance. This research was supported with public funding from the Spanish Agency for International Cooperation and Development (AECID: AP/039620/11) and the Spanish Ministry of Economy and Competitiveness (MINECO: BFU-2013-44290-R).

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