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A Scanning Electron Microscopy Study of Frozen/Thawed Dog Sperm During *In Vitro* Gamete Interaction

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Contents

The aim of this work was to study the effects of cryopreservation on the binding and penetration of dog spermatozoa to the zona pellucida (ZP) by scanning electron microscopy (SEM). The sperm-rich fraction of six ejaculates from five dogs was divided into two aliquots and washed by centrifugation. One aliquot was processed as fresh control sample and the other aliquot frozen in Tris-fructose extender. Gamete interaction was assessed using *in vitro* matured bitch oocytes, which were co-incubated for up to 3 h. At hourly intervals after the start of co-incubation, *in vitro* fertilized (IVF) oocytes were processed by SEM. The results were analysed statistically using the ANOVA test. Differences in binding and penetration of the spermatozoa to the ZP occurred; a lower proportion of oocytes with spermatozoa bound to ZP was observed using frozen sperm ($p < 0.05$) than with fresh sperm (61%, 57% and 53% vs 42%, 40% and 44% at 1, 2 and 3 h, respectively). The percentage of ZP penetration by fresh sperm was directly proportional to the time of co-incubation (9%, 25% and 34%; $p < 0.05$); in contrast, no differences were observed in the penetration rate with frozen-thawed sperm (21%, 17% and 21%). More acrosome reacted sperm were observed in frozen sperm than in fresh sperm on the surface of the ZP. The differences in the percentage of binding and penetration between fresh and frozen sperm during the co-culture could indicate that the time course of penetration is faster in frozen-thawed dog spermatozoa than in fresh sperm, but that fresh spermatozoa can penetrate more oocytes over a given period of time, which may be related to their reacted or non-reacted initial status.

Introduction

Cryopreservation of canine semen is an important tool for conserving genetic material of superior domestic dogs and/or endangered canid species; frequently, new researches are looking for methods which can properly evaluate fertilizing potential after the freezing and thawing process. It is well known that freezing procedures provoke modifications in the plasma membrane of cryopreserved mammalian spermatozoa (Parks and Graham 1992; Rota et al. 1999; Szasz et al. 2000; Watson 2000; Burgess et al. 2001; Rasul et al. 2001; Nishizono et al. 2004; Cortés et al. 2006), leading to lower fertility compared to fresh sperm. Motility, viability and acrosomal integrity have been common parameters used to evaluate frozen/thawed dog sperm (Strom et al. 1997; Rota et al. 1999; Cortés et al. 2006). Using fluorescence probes, it has been determined that near to 45% of frozen/thawed dog spermatozoa display acrosome damage, such as vesiculations and/or acrosomal rupture (Strom et al. 1997; Nöthling and Shuttleworth 2005; Cortés et al. 2006). In addition, abnormalities in acrosomal enzymes activity have been described in cryopreserved dog spermatozoa (Froman et al. 1984; Cortés et al. 2006). Structural damages, mainly in the plasma membrane over the acrosomal region, have been observed in cryopreserved canine spermatozoa evaluated with scanning and transmission electron microscopy (Rodríguez-Martínez et al. 1993; Strom-Holst et al. 1998; Burgess et al. 2001). Although acrosomal damage has been associated with a lesser ability to bind and cross the zona pellucida (ZP) compared with fresh spermatozoa, it seems that the loss of penetration of homologous oocytes by frozen dog spermatozoa is more extensive than indicated by morphological evaluation, using light microscopic assessment (Hay et al. 1997).

Scanning electron microscopical studies in other species have revealed specific aspects associated with the interaction of the spermatozoa and the oocyte investments (Barros et al. 1984; Hyttel et al. 1988; Barros et al. 1993; Crosby et al. 1998; De los Reyes and Barros 2000; Funahashi et al. 2000; Schwartz et al. 2003). To date, the effect of canine sperm cryopreservation on fertilizing ability evaluated at the ultrastructural level during gamete interaction has not been performed. The scanning electron microscope is a valid instrument to further study the process of fertilization. Thus, the aim of this study was to evaluate by scanning electron microscopy (SEM), the effects of cryopreservation on *in vitro* sperm binding and penetration to the ZP.

Materials and Methods

Semen processing

Dog semen was obtained by digital manipulation from five sexually mature dogs of different breeds and proven fertility. The sperm-rich fraction of each ejaculate was obtained and the volume was recorded, motility was estimated subjectively under a phase contrast microscope and sperm concentration was evaluated using a Neubauer counting chamber. Only samples with more than 80% of progressive motility were used. A total of one to two ejaculates per dog were collected and processed separately as one experimental replicate throughout this study.

Semen freezing

The sperm-rich fraction of each ejaculate was divided into two aliquots and washed by centrifugation in TRIS buffer medium (Rota et al. 1999) at $700 \times \boldsymbol{g}$ for 5 min, the supernatant was discarded, the pellet of one aliquot was resuspended in Fert-Talp medium (Parrish et al.

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1988) and processed as fresh control sample. The pellet of the second aliquot was rediluted in a freezing medium (Tris 249 mM; citric acid 88.4 mM; fructose 69.3 mM; penicillin G 100 IU/ml, egg yolk 20% and 5% of glycerol, pH 7), at a final sperm concentration of 200×10^6 spermatozoa/ml. The samples were loaded into 0.25 ml straws (L'Aigle Cedex, France) and frozen at a rate of 30°C/min to −70°C, and then plunged into liquid Nitrogen at −196°C. Thawing was performed in a water bath at 60ºC for 8 s.

Oocytes processing

Ovaries were obtained from 16 normal adult pure and cross-breed bitches following routine ovariohysterectomy, at random stages of the oestrus cycle. In the laboratory, ovaries were washed in PBS at room temperature and cumulus-oocytes complexes (COCs) were released by slicing the ovaries. After two washes in PBS supplemented with 2.5 μl/ml pyruvic solution (11.2 mg/ml pyruvic acid), 10% fetal calf serum (FCS) and 50 μg/ml streptomycin (all from Sigma, St Louis, MO, USA), the COCs were selected with criteria of their size (> 110 μm in diameter; measured with a micrometer disc) (Leitz, Wetzlar, Germany), three or more compact cumulus cell layers and darkly granulated cytoplasm (Fujii et al. 2000; Otoi et al. 2000). The selected COCs were placed into 100 μl droples (approximately 8–10 oocytes/droplet) of maturation medium: TCM-199 (Earle's salt, buffered with 25 mM Hepes; Invitrogen, Grand Island, NY, USA), supplemented with 2.5 μl/ml pyruvic solution, 10% FCS, 5 μl/ml antibiotic solution (12.2 mg/ml penicillin and 20 mg/ml streptomycin) and 10 IU/ml of human chorionic gonadotrophin (hCG), and cultured for 48 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air, and then for an extra 24 h in the same medium without hCG (De los Reyes et al. 2005). Only oocytes matured *in vitro* with clear signs of cumulus cell expansion and normal appearance were used for experiments.

Gamete interaction

Frozen-thawed samples were washed by centrifugation at $700 \times g$ for 5 min and the pellet of spermatozoa rediluted in Fert-Talp medium, as previously performed in fresh semen, at a final concentration of 5×10^6 sperm/ml. Fresh and frozen/thawed samples were maintained in Fert-Talp medium for 30 min for capacitation. In each replicate, gamete co-incubation was performed in 100 μl droples of diluted fresh or frozen/thawed spermatozoa by mixing 8–12 *in vitro* matured oocytes randomly distributed in each drop before culturing in high humidity at 38.5ºC, 5% CO_2 in air for 1–3 h.

Scanning electron microscopic evaluation

At hourly intervals after the start of co-culture with both fresh and frozen/thawed sperm, the oocytes were washed and denuded by agitating in a tube containing sodium citrate (5 mg/ml in PBS), and fixed for 1 h in 2.5 % (v/v) gluteraldehyde in a 0.1 M sodium cacodylate buffer, pH 7.4 (Barros et al. 1984). The oocytes were dehydrated in increasing concentration of acetones, critical point dried, mounted and sputtered with palladium-gold target using a Pelco 91.000 sputter. Sperm binding and penetration to canine ZP were evaluated with Leo 1420vp Scanning Electron Microscope.

Statistical analysis

A total of 435 *in vitro* matured bitch oocytes inseminated with either fresh (228) or frozen-thawed (207) dog sperm were evaluated in six replicates.

To establish whether there was a difference between spermatozoa treatment (fresh or frozen) in zona binding/penetration during each co-culture time (1–3 h), the percentages were arc-sine transformed and evaluated by ANOVA test (Statistical Analysis System, SAS Institute, Cary, NC, USA). The model included the main effects of spermatozoa treatment, duration of culture and their interaction. The means were compared through Tukey test and differences were considered statistically significant when $p \leq 0.05$.

Results

Spermatozoa bound to the ZP (Fig. 1a) and spermatozoa at different zona penetration stages were observed with both types of sperm (Fig. 1b–d). Nevertheless, a lower ($p < 0.05$) proportion of oocytes with spermatozoa bound to ZP was observed using frozen/thawed sperm than with fresh spermatozoa at 3 h of co-culture. However, penetration rates were higher using frozen/thawed sperm than fresh sperm at the first hour of co-culture and lower than those fresh spermatozoa at the second and third hours after the start of co-culture ($p < 0.05$) (Tables 1 and 2).

Gametes co-incubated for 1, 2 and 3 h, did not show a different proportion ($p > 0.05$) of oocytes with sperm bound to the ZP over the time when fresh dog sperm was used. Whereas, ZP penetration was directly proportional to the time of co-incubation, increasing proportions of oocytes with spermatozoa crossing the ZP was found throughout the time of co-culture up to 3 h (Table 1; $p < 0.05$). In contrast, using frozen-thawed sperm, there were no changes in binding or penetration rates up to 3 h of co-culture (Table 2).

On the surface of the ZP, intact acrosome and acrosome reacted sperm were observed during each hour of co-cultured using either fresh or frozen/thawed sperm (Fig. 2). However, the proportion of acrosomal reacted or abnormal acrosomes (characterized by a fenestration of the membranes covering the acrosome region of the sperm head), were more often seen in frozen/thawed sperm, especially at the first hour, in comparison with fresh samples.

Discussion

The present study analyzed the process of *in vitro* fertilization in canines, with both fresh and frozen/thawed sperm by means of scanning electron microscopy. Sperm binding and penetration to the ZP represent critical steps of gamete interaction, and this

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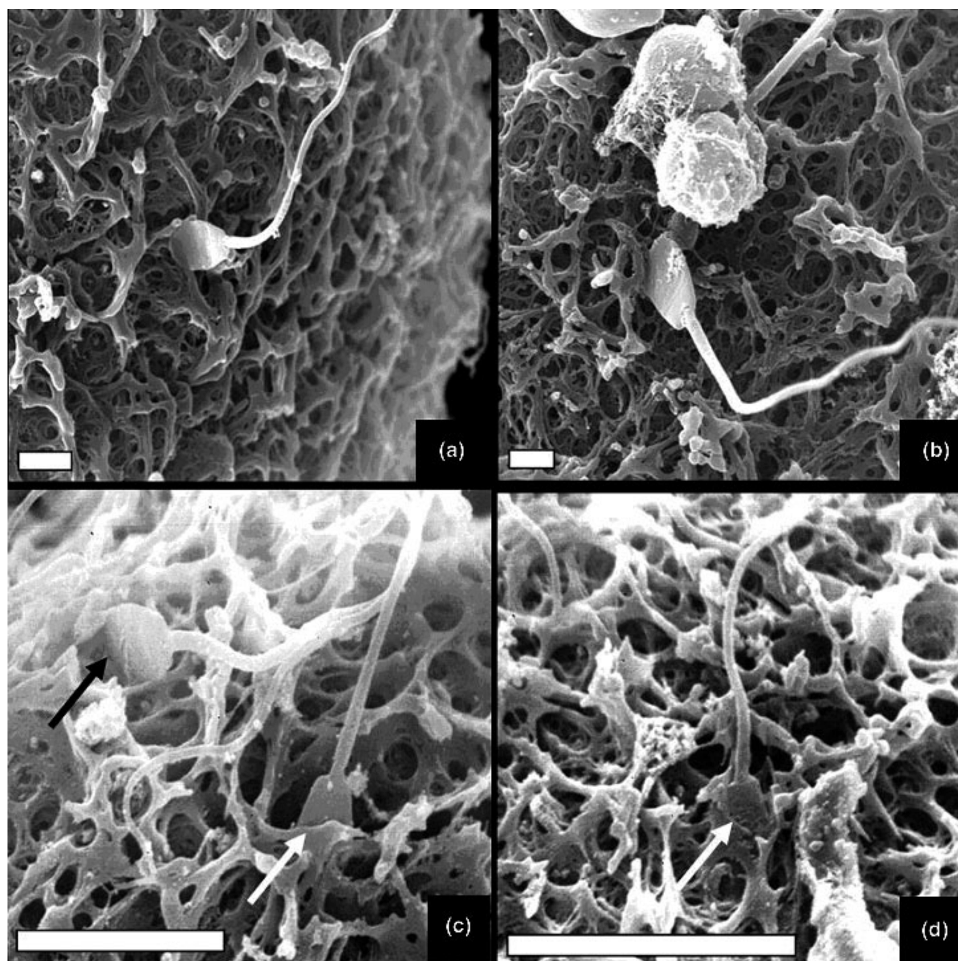

Fig. 1. Scanning electron photomicrographs of *in vitro* gamete interaction in canines. (a) Oocyte with spermatozoa bound to zona pellucida (bar = 3 μm) (×2000). (b) Acrosome reacted spermatozoa at the beginning of penetration (bar = 2 μm) (×4000). (c) Spermatozoa penetrating zona pellucida (bar = 10 μm) (×6000). (d) Spermatozoa with its head into the zona pellucida (bar = 10 μm) (×5000)

Table 1. Number and percentage of *in vitro* matured bitch oocytes co-cultured with fresh dog spermatozoa for three different time periods. Evaluation assessed by scanning electron microscopy

Co-incubation time (h)	Number (%) of oocytes with sperm bound to ZP	Number (%) of oocytes with sperm penetration	Total oocytes
1	42 (61)[a]	6 (9)[a]	69
2	45 (57)[a]	20 (25)[b]	79
3	42 (53)[a]	27 (34)[c]	80

Different superscripts within the same column are significantly different ($p < 0.05$). Interaction between penetration and duration of culture was significant ($p < 0.05$).

Table 2. Number and percentage of *in vitro* matured bitch oocytes co-cultured with frozen/thawed dog spermatozoa for three different time periods. Evaluation assessed by scanning electron microscopy

Coincubation time (h)	Number of oocytes with sperm bound to ZP (%)	Number of oocytes with sperm penetration (%)	Total oocytes
1	30 (42)	14 (21)	72
2	29 (40)	12 (17)	72
3	28 (44)	13 (21)	63

There were no effect of binding/penetration and duration of culture ($p > 0.05$).

work demonstrates significant differences in these processes when either fresh or frozen/thawed dog sperm is used. It is worth noting that the sperm-oocyte interaction evaluated by scanning electron microscopy allows better discrimination between spermatozoa bound to the ZP surface and those at the beginning of ZP penetration than do other microscopy techniques (Hyttel et al. 1988).

The percentages of oocytes with frozen/thawed spermatozoa bound at 1, 2 or 3 h of co-culture, were lower than the 57.5% described in cryopreserved dog spermatozoa by Silva et al. (2006) after 18 h of incubation; however, within the range reported by these authors (26.3–89.4%). The SEM analysis showed a minor proportion of oocytes with frozen/thawed spermatozoa bound to ZP as compared to fresh sperm at the three times points; this agrees with previous studies of ZP binding activity with canine semen, where the differences between fresh and frozen/thawed samples in respect to ZP binding capacity were highly significant (Ivanova et al. 1999; Peña et al. 2004). In the same way, binding rates of oocytes incubated with chilled spermatozoa were lower using dog spermatozoa chilled for 4 days than those chilled for only one day (Strom-Holst et al. 2000). The process of binding to the ZP is a specific feature of viable sperm (Zhang et al. 1998; Strom-Holst et al. 2000); the cryopreservation process produces death of some sperm and reduces the life span in the

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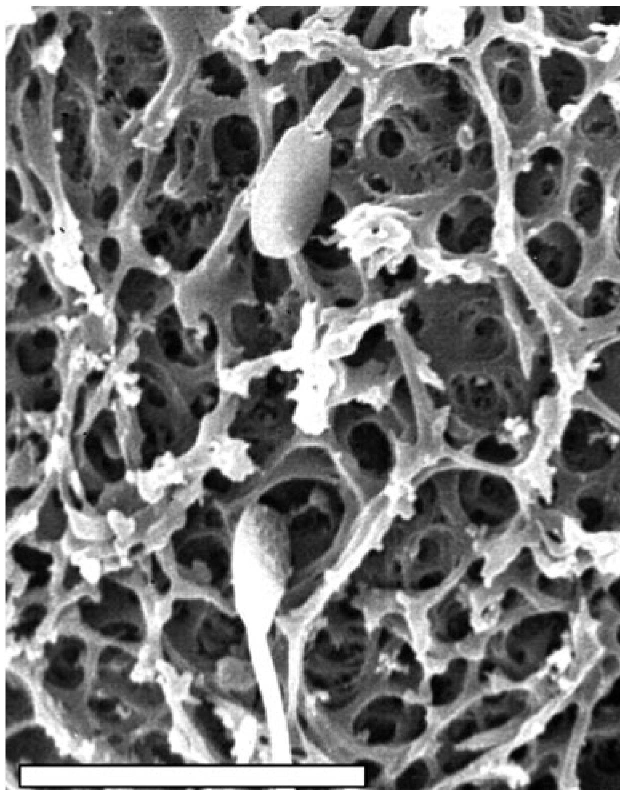

Fig. 2. Scanning electron photomicrographs of dog spermatozoa interacting to the zona pellucida. One acrosomal reacted sperm is seen with clear fenestration covering the acrosomal region and above, another sperm with an acrosomal intact appearance (bar = 10 μm) (×6000)

surviving population. This process is associated with membrane damage (De Leeuw et al. 1990; Januskauskas et al. 1999; Watson 2000; Eilts 2005), which in turns affects molecular binding structures, such as the location at the sperm plasma membrane of specific receptors required for sperm-zona interaction, correct binding and thus fertilization (Florman and Wassarman 1985; Leyton and Saling 1989; Barros et al. 1996).

Zona pellucida binding assays using frozen/thawed dog sperm, as assessed by a confocal microscopy, have shown that the mean number of spermatozoa bound to the ZP was reduced by more than 50% when sperm capacitation was increased from 2 to 8 h (Peña et al. 2004). Likewise, a 23% decrease in the number of fresh dog spermatozoa bound to the homologous ZP, has been reported when increasing the capacitation time from 4 to 7 h, suggesting a detachment of spermatozoa during this time (Kawakami et al. 1993). It is known that cryopreservation induces capacitation-like changes (Watson 1995; Sirivaidyapong et al. 2000; Peña et al. 2004). More capacitated and acrosomal reacted dog spermatozoa are present earlier when sperm is cryopreserved than in fresh sperm during *in vitro* incubation (Rota et al. 1999; Peña et al. 2004); although it is still unclear whether spermatozoa are actually capacitated or merely proceed directly to acrosomal exocitosis (Watson 2000). In this study, the acrosomal status of frozen and fresh spermatozoa bound, indicated that both acrosomal intact and acrosomal reacted dog sperm, as judged by membranes fenestration and disintegration over the acrosome region, were capable of binding to the ZP. In contrast to other species (Barros et al. 1992; Yanagimachi 1994), this phenomenon has been previously described in dogs (Kawakami et al. 1993) and guinea pig (Fleming and Yanagimachi 1982). Reacted and non-reacted spermatozoa were presented at three times of culturing; however, the predominant proportion of acrosomal reacted spermatozoa was observed at the first hour in frozen samples. Whether the binding in the acrosomal intact and acrosomal reacted sperm occur by the same mechanism is unclear, however, the retention of the fertilizing capacity is different, with the acrosome reacted spermatozoa losing their fertilizing ability more rapidly than intact sperm (Barros et al. 1992).

Binding rates using frozen/thawed and fresh samples did not show differences between the three co-culture periods, whereas the penetration percentages increased throughout the time periods tested when fresh sperm were used, in contrast, with frozen/thawed sperm there was no change on penetration rate during 3 h of co-culturing. The percentages of oocytes with spermatozoa penetrating the ZP were higher in fresh than frozen/thawed samples at the second and third hour of co-culturing. However, at the first hour, frozen/thawed sperm had higher penetration percentages than fresh sperm. These results assessed by SEM, are in concordance with those previously observed using fluorescence microscopy for the evaluation of sperm penetration in canines (De los Reyes et al. 2008), and with those in human spermatozoa (Critser et al. 1987), which describe the highest penetration rates using frozen-thawed sperm at the beginning of co-culture, while with fresh spermatozoa, the penetration rates were lowest at the beginning and increased over the time. The higher rate of penetration at the first hour in cryopreserved sperm as compared with those fresh sperm, and the lack of significance in the rate of penetration in the following hours, agrees with the mayor proportion of acrosome reaction occurring at the beginning of co-culture. Possibly, this initial acrosome status influenced the different patterns of penetration observed between fresh and frozen/thawed sperm.

In conclusion, the difference in the percentage of binding and penetration between fresh and frozen sperm during the co-culture with IVM oocytes, indicates that the time course of penetration through the ZP appears to be faster in frozen-thawed dog spermatozoa than in fresh sperm, but fresh spermatozoa can retain their fertilizing ability and thus, penetrate more oocytes over time, which could be related to their previous acrosome status (reacted or non-reacted).

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