In vitro sperm penetration through the zona pellucida of immature and in vitro matured oocytes using fresh, chilled and frozen canine semen

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Abstract

The aim of this study was to evaluate the effect of sperm cryopreservation and the maturation state of the oocyte on the time course of canine gamete interaction during co-culture for periods of 1–10 h. Semen samples were obtained by digital stimulation and ejaculates processed as fresh, chilled and frozen samples. Sperm were co-cultured with immature or in vitro mature bitch oocytes for up to 10 h. At hourly intervals, oocytes were evaluated for sperm penetration with epifluorescence microscopy. The results were analyzed statistically using generalized linear models. Spermatozoa treatments had a significant effect on the total percentage of oocyte penetration for both types of oocytes; fresh spermatozoa showed the highest average penetration rate, while frozen sperm showed the lowest value ($p < 0.05$). At the 1st hour of co-culture, chilled and frozen dog sperm had a higher penetration percentage ($p < 0.05$) of in vitro matured canine oocytes (43.6% and 45.7%, respectively) than the fresh sperm had (33.8%). Sperm penetration was directly proportional to the time of incubation, when fresh or chilled sperm were used ($P < 0.05$); in contrast, frozen dog sperm did not change penetration rates with either immature or in vitro matured oocytes over time. There was a significant difference in the average of penetration rate between immature (47.3%) and in vitro matured oocytes (56.6%) throughout the 10 h of culturing; irrespective of sperm treatment. The optimal incubation time in terms of maximizing penetration rates probably are dependent on how spermatozoa were processed prior to fertilization.
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1. Introduction

Interaction of gametes is a complex process that requires several sperm functions involving initial recognition, attachment followed by binding, acrosome reaction and penetration of zona pellucida (ZP) (Yanagimachi, 1994; Barros et al., 1996). Before gamete interaction, sperm must undergo the physiological changes of capacitation in the female reproductive tract (Austin, 1952), which involves hyperactivated motility and acrosome reaction (Yanagimachi, 1994; Petrunkina et al., 2003), the latter is a prerequisite for fertilization and occurs, or at least is completed, at the surface of the zona pellucida (Bleil and Wassarman, 1988). Zona components can induce acrosome reaction in several species (Florman and First, 1988) including canines (Kawakami et al., 1993; Brewis et al., 2001), although, it has been demonstrated that both acrosome intact and acrosome-reacted dog sperm are capable of binding to the ZP (Kawakami et al., 1993).

The process of capacitation is a time-dependent phenomenon and in vitro is different among species (Yanagimachi, 2003). Preliminary studies using fresh semen, have found that canine spermatozoa capacitated in vitro are able to penetrate ZP within 7 h after initiation of incubation (Mahi and Yanagimachi, 1976). However, later studies demonstrated that canine sperm penetrate ZP around 2–4 h after incubation (Shimatsu et al., 1992; Yamada et al., 1993; Peña et al., 2004). Studies utilizing the chlortetracycline (CTC) assay with cryopreserved dog semen, have demonstrated a significant increase in the number of capacitated sperm between 0 and 2 h of incubation in capacitating medium (Rota et al., 1999).

Semen cryopreservation is a useful tool for extending the availability of spermatozoa for long or short periods (frozen or chilling, respectively); nevertheless, cryopreservation has been associated with reduced fertility. It has been hypothesized that during cryopreservation, the sperm acquire capacitation-like changes (Wheeler and Seidel, 1986; Watson, 1995; Cormier et al., 1997; Bailey et al., 2000; Watson, 2000) and these changes are frequently cited as a factor associated with the reduced longevity in vivo (Bailey et al., 2000) and in vitro (Ström et al., 1997). While alterations to the acrosome and plasma membrane have been demonstrated after chilling and freezing steps (Rota et al., 1997; Ström Holst et al., 2000), damage appears to occur first during the dilution and cooling (Oettlé, 1986; Schäfer-Somi et al., 2006). Induction of cryocapacitation has been attributed to plasma membrane reorganization and to increased intracellular calcium levels (Watson, 2000; Peña et al., 2004). Cryopreservation has also been shown to change the ability of spermatozoa to regulate internal Ca^{2+} (Yanagimachi, 1994; Watson, 1995). These changes in cryopreserved sperm may not only affect the final percentage of fertilized oocytes, but also the time course of sperm penetration through the oocyte envelop as reported previously in frozen/thawed sperm from other species (Maxwell et al., 1996; Cormier et al., 1997).

On the other hand, early studies of canine gamete interaction in vitro, suggested that the maturation stage of dog oocytes does not affect sperm penetration (Mahi and Yanagimachi, 1976; Mahi and Yanagimachi, 1978), which has been corroborated in later reports (Hewitt and England, 1997). However, even though fresh (Mahi and Yanagimachi, 1976; Hewitt and England, 1997) or cryopreserved dog sperm (De los Reyes et al., 2006; Hay et al., 1997) are able to bind and penetrate canine oocytes in vitro regardless their maturation state, it is unclear if the dynamic of this process is modified by the maturation state of the oocyte. Thus, the aim of the present work was to compare the time course of frozen/thawed, chilled/rewarmed or fresh dog sperm in the penetration of the zona pellucida of immature and in vitro mature canine oocytes throughout different co-culture periods.

2. Materials and methods

2.1. Semen collection and evaluation

Fifteen ejaculates were collected by digital manipulation of penis from six healthy fertile dogs (aged 2–6 years) from different breeds. The sperm-rich fraction of each ejaculate was used for experiments as one experimental replicate. Semen quality estimation including evaluation of sperm concentration, sperm motility, and assessment of morphological sperm alterations were performed as previously described (Rota et al., 1997). Ejaculates with sperm motility >70% and up to 15% of abnormal spermatozoa were used.

2.2. Oocyte collection and culture

In each experimental replicate, oocytes were obtained from crossbred and purebred normal bitch ovaries following ovariohysterectomy. At the laboratory, the ovaries were washed in PBS and cumulus–oocytes complexes (COCs) were released by slicing the ovarian cortex. Oocytes with uniform ooplasm and a compact cumulus cell mass were selected. After two washes in PBS, COCs were randomly distributed in two groups; the first group of COCs were processed at immature stage, stored in 100 μL droplets of Fert-Talp medium (Parrish et al., 1988) supplemented with 6 mg/mL fatty free bovine serum albumin (Sigma) at 38.5 °C and 5% CO_2 in air, until in vitro fertilization (IVF) was used. The second group of COCs were cultured for in vitro maturation (IVM), in a 50-μL droplet of TCM 199 (Earle's salt, buffered with 25-mM Hepes; Invitrogen, Grand Island, NY, USA), as previously described (De los Reyes et al., 2005). After IVM, the COCs were washed twice in Fert-Talp medium and then placed in 100-μL droplets of Fert-Talp in 5% CO_2 in humidified air at 38.5 °C, until IVF was used.

2.3. Semen processing

The sperm-rich fraction of each ejaculate was divided into three aliquots and the semen washed by centrifugation at a $300 \times g$ for 15 min in Tris buffer (Rota et al., 1999). The pellet of one aliquot was diluted in Fert-Talp medium to a final concentration of 100×10^6 sperm/mL (fresh sample). The other two aliquots were processed for freezing and chilling. These two later samples were extended in Tris–fructose–egg yolk medium (De los Reyes et al., 2006), and placed at 4 °C. After 1 h, one extended aliquot was supplemented with 5% glycerol (v/v) and loaded into 0.25-mL straws (L'Aigle Cedex, France) for freezing at −196 °C. The second extended aliquot was stored at 4 °C in a refrigerator for 24 h.

After 24 h, chilled semen was warmed at 37 °C for 10 min. Frozen semen was thawed in a water bath at 60 °C for 8 s. Both samples were centrifuged separately in Fert-Talp at $500 \times g$ for 5 min and the sperm pellet of each sample was rediluted to 100×10^6 sperm/mL in Fert-Talp.

2.4. In vitro fertilization assay

Sperm penetration through ZP was assessed by in vitro fertilization assays with immature and in vitro matured dog oocytes. Only oocytes matured in vitro with clear signs of mucification were used for experiments. In each replicate, fresh, chilled/warmed and frozen/thawed spermatozoa were added separately to 100-μL Fert-Talp drops containing 10–15 immature or in vitro matured oocytes, at a final sperm concentration of 5×10^6 spermatozoa/mL. Sperm and oocytes were co-cultured at 38.5 °C under 5% CO_2 in air and with high humidity for up to 10 h.

2.5. *Examination for oocyte penetration*

At hourly intervals of gamete co-incubation, groups of oocytes of each experimental condition were removed from the drops and denuded by agitating in a tube containing sodium citrate (5 mg/mL in PBS), fixed in acetic acid/methanol/chlorophorm (3:6:2 v/v) (Otoi et al., 2000) for 3 min and then in acetic acid/methanol (1:3 v/v) fixative for 3 days. The oocytes were stained with 200 μg/mL of propidium iodide (PI; Molecular Probes, Eugene, OR, USA) and then examined with an epifluorescence microscope (UV emission 480 nm; Nikon Optiphot 2) (Nikon, Kawasaki, Japan). Penetrated oocytes were defined as those having sperm heads in the perivitelline space or in the oocyte cytoplasm.

2.6. *Statistical analysis*

To establish whether there was a difference between spermatozoa treatment (fresh, chilled or frozen) in oocyte penetration during each co-culture time (1–10 h), and the effect of oocyte maturation stage on spermatozoa penetration (and their interactions), the data were analyzed using generalized linear models using SAS (Statistical Analysis System, 1996, SAS Institute, Cary, NC, USA). The transformation of the percentages had little effect on the significance levels and, therefore, the analysis of percentages will be presented throughout, for easiness of interpretation. Differences were considered statistically significant for a p-value ≤ 0.05.

3. Results

A total of 5072 COCs (2660 immature and 2412 in vitro mature) were obtained throughout 15 experimental replicates.

Considering the average penetration percentage throughout all co-incubation periods (10 h) (Fig. 1), a significant effect of spermatozoa treatment (fresh, chilled or frozen) ($p < 0.001$), on the percentage of oocyte penetration, was observed. Fresh dog spermatozoa showed the highest average of penetration rate ($p < 0.05$) with both immature and in vitro mature canine oocytes

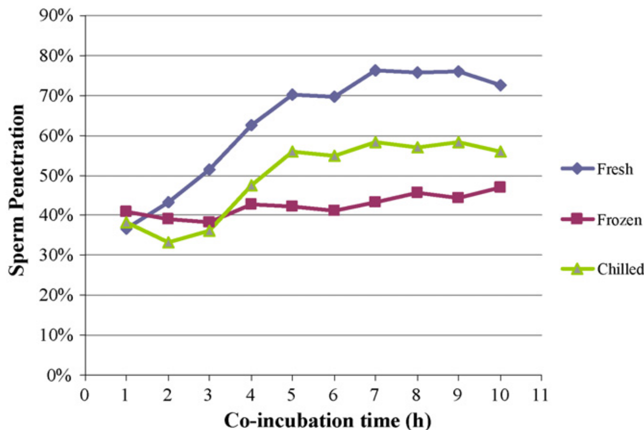

Fig. 1. Differences between types of sperm across time. The effects of sperm treatment and time of culture and their interaction were significant ($p < 0.001$). No significant trend across time was observed for frozen sperm.

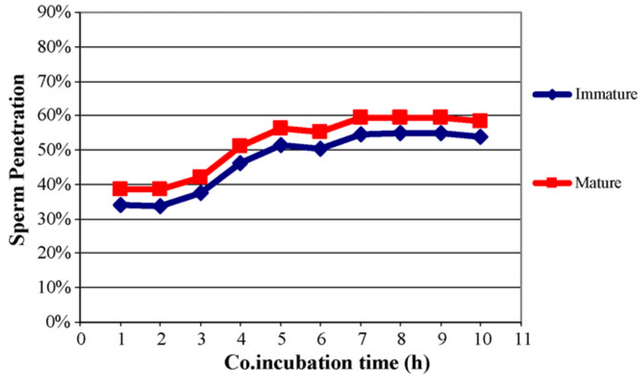

Fig. 2. Differences between types of oocytes across time. The effects of oocytes maturity and time of culture were significant ($p<0.001$), while their interaction was not significant.

in comparison to chilled or frozen sperm, while frozen sperm manifested the lowest ($p<0.05$) average of penetration rate with both oocytes types.

In regard to the penetration rate at each hour of co-culture, it was observed that chilled and frozen dog sperm could penetrate a higher ($p<0.05$) percentage of in vitro mature canine oocytes at the 1st hour than did fresh sperm. An increased proportion of penetration was observed up to the 7th hour after the start of co-incubation, when fresh semen was used in conjunction with either immature or in vitro mature oocytes ($p<0.05$). Chilled dog sperm also increased penetration rates over time, peaking at the 5 h after co-incubation with immature oocytes and at 4 h when co-incubated with in vitro matured oocytes. In contrast, frozen dog sperm showed no tendency to modify penetration rates when co-cultured with immature or in vitro matured oocytes for up to 10 h.

Considering the effect of oocyte maturation upon penetration by spermatozoa, the oocyte nuclear stage was not always observed when sperm penetration was evaluated, however, oocytes matured in vitro that showed immature stages of meiotic development were discarded. There was no interaction between sperm and oocyte maturity across time. However, there was a significant effect ($p<0.001$) of oocyte maturity and time of culture throughout the 10 h. In vitro matured oocytes showed higher penetration rate than did immature oocytes irrespective of sperm treatment (Fig. 2).

4. Discussion

This study demonstrates significant differences in sperm penetration rates when using fresh or cryopreserved dog sperm, and furthermore when oocytes of differing maturation states were used.

Studies in vitro have reported that canine sperm pre-incubated for 4 h can begin to penetrate ZP within 1 h after insemination (Yamada et al., 1992); for the purpose of this study we excluded pre-incubation period in capacitating medium previous to co-culture with oocytes, even though, fresh, chilled and freeze–thawed sperm were able to penetrate both immature and in vitro matured canine oocytes as early as 1 h after co-culture, indicating that some of the sperm could undergo capacitation while they were interacting with the oocytes. The media used herein for co-culture (Fert-Talp) is very similar to canine capacitation medium (CCM) (Mahi and Yanagimachi, 1978).

In this regard, these data suggest that it is possible to bypass or reduce the pre-incubation period for capacitation, especially in those cryopreserved canine sperm.

Considering the average penetration values of all co-culture periods (10 h), the lower ability of chilled and frozen dog sperm to penetrate ZP compared with fresh spermatozoa was expected. The deleterious effects of cryopreservation on different sperm parameters in vitro are well studied in canines (Froman et al., 1984; Peña et al., 2004; Cortes et al., 2006). An impaired fertilizing capacity of cryopreserved dog sperm has also been evaluated in vitro (Hay et al., 1997; Ström Holst et al., 2000). However, at the 1st hour of co-culture, cryopreserved sperm (chilled and frozen) had higher penetration rate with in vitro matured oocytes than the fresh sperm had. And the penetration rate using fresh and chilled/rewarmed sperm increased with time. These results agree with a previous report using human spermatozoa, where penetration rates using frozen–thawed sperm were highest at the beginning of co-culture, whereas penetration rates by fresh spermatozoa were lowest at the beginning and increased over the time (Critser et al., 1987). These differences in chronology of fertilizing ability between cryopreserved and fresh spermatozoa have been associated with the respective capacitation times required for those sperm (Wheeler and Seidel, 1986; Watson, 1995). Previous studies have shown that frozen–thawed bull spermatozoa were able to fertilize oocytes even in the absence of the necessary agents required for in vitro capacitation (Cormier et al., 1997). According to the CTC assay, more capacitated dog spermatozoa were present earlier in chilled/rewarmed and frozen/thawed sperm than in fresh sperm (Rota et al., 1999). The major ability of cryopreserved sperm to penetrate oocytes at the 1st hour of co-culture indicates that these sperm can undergo the events associated to fertilization earlier or faster than fresh sperm. Furthermore, in previous studies we were able to demonstrate that the proportion of dog spermatozoa with loss of the acrosomal enzyme acrosin, is significantly higher in frozen/thawed in comparison to fresh spermatozoa (Cortes et al., 2006). Acrosin is released during acrosome reaction and has been associated with binding and penetration through the ZP in a variety of mammalian species (Bleil and Wassarman, 1988; Barros et al., 1996; Moreno et al., 2002), therefore, it is possible, that the early activation and release of this acrosomal enzyme in those cryopreserved sperm, could facilitate the interaction and penetration of the ZP at the beginning of co-culture.

The increasing penetration rate observed using fresh sperm with either immature or with in vitro mature oocytes up to 7 h after co-culture, is coincident with previous studies where the percentage of acrosome-reacted fresh dog sperm was higher at 7 h of incubation in capacitating medium than at 4 h, suggesting that more sperm were able to respond to induction of the acrosome reaction as the incubation time increased (Kawakami et al., 1993). Moreover, the major activity of acrosomal enzymes (hyaluronidase, *N*-acetylhexosaminidase and acrosin), were significantly higher after 7 h of incubation in capacitating medium in fresh dog spermatozoa (Kawakami et al., 1999).

The penetration rates using chilled–rewarmed sperm also increased with time in both immature and in vitro matured oocytes, whereas using frozen–thawed sperm with immature or in vitro matured oocytes, no differences were present in penetration values throughout the time. Although cooling procedures might also promote premature capacitation (Cormier et al., 1997; Rota et al., 1999), and chilled sperm could in fact penetrate earlier than fresh sperm, as judged by the major percentage of penetration occurring at the 1st hour of co-culture, the most significant effects on capacitation-like changes are, therefore, probably sustained during the freezing and thawing process, drawing upon the differences in penetration patterns observed in this study over time between chilled and frozen dog sperm. It is possible, that the penetration rates observed with frozen dog sperm on the following hours of co-culture were mainly achieved at the beginning of co-culture.

These findings suggest that sperm penetration is more critically compromised during the freeze–thaw process than during the cooling process. Possibly, special acrosomal mechanisms or membrane damage to specific Ca^{2+} channels, are disrupted during major temperature changes. These observations support the concept that cooling and freezing injuries are distinct and should not be confounded in cryopreservation studies (Froman et al., 1984). In this regard, the use of separate protocols must be considered when using fresh, chilled or frozen dog semen.

Even though fresh, chilled and frozen dog sperm could penetrate immature or in vitro matured canine oocytes as previously reported (Mahi and Yanagimachi, 1976; Hay et al., 1997; Hewitt and England, 1997), the penetration rates throughout the time period were not equal in either type of oocyte. In contrast to other studies (Hewitt and England, 1997), the maturation state of oocytes affected the final percentage of penetration irrespective of sperm treatment, as no interaction between sperm treatment and oocyte maturity was found. During maturation, important changes at nuclear, cytoplasmatic and oocyte envelops occur (Saint-Dizier et al., 2004; Luvoni et al., 2005; Rodrigues and Rodrigues, 2006; De los Reyes et al., 2007), and many of these changes are known to favor fertilization. Cumulus cell expansion is a sign of oocyte maturation, which facilitates sperm capacitation, acrosome reaction and penetration (Thange et al., 2002; Otoi et al., 2007). Furthermore, it has been shown that as the oocyte grows to the pre-ovulatory stage, gradual granulose cell-ZP dissociation occurs (Motta et al., 1988), suggesting that mesh-like arrangement of the ZP after the process of maturation could have biological significance, assisting spermatozoa in becoming adequately oriented to fertilize the oocyte. Ongoing studies at our laboratory, have also revealed ultra structural differences in ZP between immature and in vitro matured canine oocytes (data not published). In fact, studies have demonstrated that fertilization in the bitch occurs in vivo only at the MII stage (Reynaud et al., 2005). The higher percentage of penetration, registered in cryopreserved sperm versus fresh sperm with in vitro matured oocytes at the 1st hour of co-culture, was not observed with immature oocytes, and thus this difference may be related to the changes in oocyte development.

In conclusion, the differences in penetration rates with time, between frozen/thawed, chilled/rewarmed and fresh sperm, suggest that the optimal incubation time in terms of maximizing penetration rates probably are dependent on how spermatozoa were processed prior to fertilization. Furthermore, the use of in vitro matured oocytes may be a more physiological approach to evaluate in vitro fertilization in canines.

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