Evaluation of chilled and frozen–thawed canine spermatozoa using a zona pellucida binding assay

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Zona pellucida binding assays provide information about the fertilizing ability of spermatozoa. A zona-binding assay for canine spermatozoa using intact, denuded homologous oocytes has not been evaluated previously. In the present study, an assay using canine oocytes derived from frozen–thawed ovaries was evaluated using three types of semen: fresh untreated; killed; and a 50:50 mixture of untreated and killed spermatozoa. The assays were performed on 3×20 oocytes for each sperm treatment, using semen from pooled ejaculates (0.5×10^6 spermatozoa in each 50 μ l droplet containing five oocytes). There was a significant difference ($P < 0.001$) between all treatments. Thereafter, the same procedure was used to evaluate methods of chilling and freeze–thawing of canine semen. There was a trend ($P = 0.067$) for more sperm binding after 1 day of chilling compared with after 4 days of chilling. Semen samples frozen using an extender (with or without the addition of Equex STM paste) were evaluated. Equex had a significant ($P = 0.034$) positive effect on the capacity of the spermatozoa to bind to the zona pellucida. In conclusion, the addition of a zona pellucida binding assay to established *in vitro* tests should give a better estimate of the damage caused by the various procedures when developing new techniques for chilling and freeze–thawing. Furthermore, the present study showed that chilling for 4 days tended to reduce the zona-binding capacity of the spermatozoon, and that Equex STM paste had a beneficial effect on the capacity of the frozen–thawed spermatozoon to bind to the zona pellucida.

Introduction

When the fertility of a semen sample *in vivo* is predicted using *in vitro* methods, a combination of different tests usually gives more reliable results than one test alone (Amann, 1989; Zhang *et al.*, 1998, 1999). The reason for this is that a combination of tests measuring different aspects of sperm function provides information about several different sperm characteristics required for fertilization. These functions should be vital to the fertilizing ability of the spermatozoa. In addition, the evaluated sperm function should vary among the sperm populations evaluated, and the intra-assay variation should be smaller than the variation among the sperm populations.

The acrosome and plasma membrane are damaged after chilling (for example, see Rota *et al.*, 1995) and freeze–thawing (for example, see Rota *et al.*, 1997; Ström *et al.*, 1997; Ström Holst *et al.*, 1998) of dog semen. The addition of the detergent Equex STM paste to the freezing extender has been found to increase significantly the proportion of

spermatozoa with an intact plasma membrane after freeze–thawing (Rota *et al.*, 1997; A. Peña and C. Linde-Forsberg, *in press*). Damage to associated molecular binding structures cannot be detected using routine morphological techniques. Therefore, the ability of the spermatozoon to bind to the zona pellucida is a potentially valuable test when evaluating techniques for chilling and freeze–thawing semen. Another advantage of using gamete interaction tests is that they account for several sperm parameters. Therefore, these tests have been used to evaluate sperm function in several species. Tests to evaluate the ability of spermatozoa to bind to the zona pellucida or hemi-zona pellucida have been developed for bovine (Fazeli *et al.*, 1993a), equine (Fazeli *et al.*, 1993b) and pig (Fazeli *et al.*, 1995) species.

A sperm–oocyte interaction test for the canine species has been described (Hay *et al.*, 1997a) in which the sperm suspension is incubated with fresh, intact oocytes surrounded by cumulus cells. After 18 h, the number of spermatozoa that had penetrated the oocytes is counted. This test is useful for evaluating both chilled and frozen–thawed semen. Canine oocytes can be obtained from ovaries removed at ovariectomy, but the number of oocytes recovered varies greatly among bitches and is difficult to predict

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(B. Ström Holst, B. Larsson, H. Rodriguez-Martinez, A-S. Lagerstedt and C. Linde-Forsberg, unpublished). As a result, different regimens for storing oocytes have been investigated. The use of salt-stored oocytes surrounded by cumulus cells was unsuccessful in a study that used the zona pellucida binding–penetration assay in dogs (Hay *et al.*, 1997b). However, it was not established whether this was due to changes in the zona pellucida or in the cumulus cells.

Frozen oocytes from previously frozen ovaries have been used for a hemi-zona pellucida-binding assay, and the results indicate that this test can be used to differentiate between fertile and infertile dogs (Mayenco-Aguirre and Pérez Cortés, 1998). A hemi-zona pellucida-binding assay using frozen, *in vitro* matured oocytes has also been used for evaluating the post-thaw fertilizing ability of canine spermatozoa (Ivanova *et al.*, 1999). However, the hemi-zona pellucida-binding assay is a time-consuming test and its use in humans has recently been questioned, as the sperm-binding capacity of oocytes is unevenly distributed (Magerkurth *et al.*, 1999), that is, the two halves of an oocyte do not have an equivalent sperm binding capacity (which is the basic assumption of the hemi-zona pellucida-binding assay). Deep-freezing of canine ovaries has been shown to constitute a possible source of canine oocytes when ovariectomies are performed at irregular intervals (Ström Holst *et al.*, 1999). Furthermore, deep-freezing of ovaries has the advantage that the potential number of oocytes recovered from an ovary can be predicted after removal of oocytes from the other ovary of the same bitch (B. Ström Holst, B. Larsson, H. Rodriguez-Martinez, A-S. Lagerstedt and C. Linde-Forsberg, unpublished). There is a large variation in sperm-binding capacity among oocytes (Ström Holst *et al.*, 1999), which may be attributed in part to the stickiness of the canine zona pellucida, which is why the zona pellucida-binding assay for the estimation of canine sperm function has to be evaluated.

The aim of the present study was to evaluate a zona pellucida-binding assay for canine spermatozoa using intact, homologous, denuded oocytes recovered from frozen–thawed ovaries. Comparisons were made between untreated and killed spermatozoa, and a 50:50 mixture of untreated and killed spermatozoa, between semen chilled for 1 or 4 days, and between semen frozen using one extender with Equex STM paste and one extender without.

Materials and Methods

Semen collection and evaluation

Semen was collected twice from three dogs, a 3-year-old Briard, a 4-year-old German shepherd and a 7-year-old of mixed breed, each with a sperm motility of $\geq 75\%$ and $< 20\%$ sperm defects, as estimated subjectively using a phase-contrast microscope at $\times 400$ magnification. Sperm concentration was measured using a photometer (Spermacue®, Minitüb, Tiefenbach). On both occasions, the semen was pooled. The first pooled sample was used for Expt 1 and the second pooled sample was used for Expts 2 and 3.

Oocyte recovery

Ovaries were obtained from 21 bitches subjected to ovariectomy. The bitches were of 19 different breeds and two mixed breeds and their mean age was 8.5 years ($SD = 2.1$). Three of the bitches had follicles as the only visible structure on the ovarian surface, eight had corpora lutea only, nine had follicles and corpora lutea, and one bitch had no visible structures on the ovarian surface. The ovaries were washed with PBS, frozen in 20 ml NaCl and then stored at -20°C until thawing at room temperature. After thawing, the cumulus–oocyte complexes were recovered by repeated slicing of the ovaries at intervals of approximately 1 mm, and then dicing of these slices, again with intervals of approximately 1 mm, in a Petri dish with PBS and 0.5% (w/v) BSA under a stereomicroscope. The cumulus cells were removed by incubating the oocytes in 75 mmol sodium citrate l^{-1} for 15 min, whereupon they were vortexed (Vortex-Genie 2, Labora, Sollentuna) for 15 min (Mahi-Brown, 1991). The oocytes were then stored overnight at 4°C in PBS with 0.5% (w/v) BSA, and used the following day for a zona pellucida-binding assay.

Zona pellucida binding assay

For the sperm–zona binding, 10 μl droplets of canine capacitation medium (CCM) (Mahi and Yanagimachi, 1978), without antibiotics were prepared in Petri dishes (35×10 mm), and covered with 3 ml mineral oil. Five oocytes were added to each droplet, whereupon the oocytes were incubated in 5% CO_2 in humidified air at 37°C for a minimum of 1 h for equilibration. Thereafter, 40 μl of the sperm suspension was added to each droplet, making the final concentration 0.5×10^6 spermatozoa ml^{-1} in a 50 μl droplet. The droplets containing oocytes and spermatozoa were co-incubated in 5% CO_2 in humidified air at 37°C for 6 h. Thereafter, the oocytes were removed from the incubation medium, fixed for 15 min in 1.5% (v/v) glutaraldehyde in 0.067 mol sodium cacodylate buffer l^{-1} , and stored in PBS with 0.5% (w/v) BSA until evaluation. The sperm–oocyte complexes were washed after storing by pipetting ten times in a 100 μl droplet of PBS using a pipette with a diameter only slightly bigger than the oocyte (0.1–0.2 mm) to remove loosely attached spermatozoa. The sperm–oocyte complexes were stained for 15 min with a solution of 20 μl propidium iodide in 1000 μl PBS with BSA. For evaluation, oocytes were placed on a slide and overlaid with a coverslip supported by four droplets of a vaseline–paraffin mixture (1:3). Pressure was applied on each droplet to secure the oocyte and to lightly compress it onto the slide. The number of spermatozoa that had bound to the zona was counted using epifluorescence UV illumination on a Leitz Dialux 20 (Wetzlar) microscope. Observations were made at a magnification of $\times 400$.

Experiment 1. Control: untreated-killed spermatozoa

Semen was collected and ejaculates were pooled as described above. The pooled semen had a volume of 6 ml,

80% sperm motility and a total of 1980×10^6 spermatozoa. The semen was washed using Percoll density gradient fractionation and 1 ml semen was gently poured on top of a Percoll column (1 ml 80% (v/v) and 1 ml 40% (v/v) iso-osmotic Percoll in CCM), and centrifuged for 30 min at 400 g. After centrifugation, the pellets were resuspended in CCM to make a total volume of 10 ml, and the suspension was then divided into halves. The motility after washing with Percoll was 50%. Half of the spermatozoa were killed by plunging the test tube directly into liquid nitrogen three times and each time thawing the suspension in a 90°C waterbath. No spermatozoa were motile after this treatment. Half of the killed sperm suspension was then mixed with half of the untreated suspension, making three groups: untreated spermatozoa; a 50:50 mixture of killed and untreated spermatozoa; and killed spermatozoa. The sperm concentration was determined for each group by manual counting using a haemocytometer (Bürker chamber; Bane, 1952) and the suspensions were diluted with CCM to a final concentration of 0.625×10^6 spermatozoa ml⁻¹. A zona pellucida-binding assay was carried out as described above, using 3×20 oocytes derived and pooled from nine bitches for each group.

Experiment 2. Semen chilled for 1 or 4 days

Semen was collected and ejaculates were pooled as described above. The pooled semen had a volume of 12 ml, 85% sperm motility and a total of 1890×10^6 spermatozoa. Half of the pooled sample was used as chilled semen in Expt 2, and half as frozen semen in Expt 3.

For chilling, the semen was first centrifuged for 6 min at 700 g. The pellet was resuspended in a tris-citric acid-fructose extender with 20% (v/v) egg yolk (Rota *et al.*, 1995) to a concentration of 100×10^6 spermatozoa ml⁻¹. The extended semen was placed in a cooler, where it reached 4°C in about 45 min, and was then stored at 4°C for 1 or 4 days.

On day 1 and day 4, 1 ml extended semen was removed and warmed to room temperature. It was then cleansed using Percoll density-gradient fractionation, as for Expt 1. Subjective motility on day 1 was 75% before and 60% after washing with Percoll; on day 4 motility was 60% before and 55% after Percoll centrifugation. The pellet was resuspended in CCM to a concentration of 0.625×10^6 spermatozoa ml⁻¹. A zona pellucida-binding assay was carried out as described above, using 3×20 oocytes for each semen treatment group. The oocytes were derived and pooled from six bitches so that, from the same bitch, oocytes from one ovary were used to test semen on day 1, and oocytes from the other ovary were used for testing semen on day 4, making a total of 120 oocytes evaluated.

Experiment 3. Semen frozen using a tris-citric acid-glucose extender, with or without Equex STM paste

Half of the pool collected for Expt 2 was divided into two parts and centrifuged for 6 min at 700 g. The two pellets were

resuspended in a tris-citric acid-glucose extender containing 20% (v/v) egg yolk and 3% (v/v) glycerol (Rota *et al.*, 1997) (EYT-G) to a concentration of 200×10^6 spermatozoa ml⁻¹, placed in a cooler and chilled to 4°C. After 1 h, an equal amount of a second extender was added to each of the diluted semen samples, with the same composition as described above, except for the glycerol concentration, which was now 7% (v/v). Equex STM paste (1% (v/v); Nova Chemical Sales, Scituate, Inc., MA) was added to one of the two samples. The extended semen was frozen in 0.5 ml straws by lowering them in three steps into a liquid nitrogen tank (Rota *et al.*, 1997).

For the zona pellucida-binding assay, three straws from each treatment were thawed in a waterbath at 38°C for 1 min. Each straw was then emptied into a separate test tube and kept at 38°C for 5 min. Motility was 65, 70 and 65% for the three straws containing extender with Equex STM paste (EYT-GE) and 65, 65 and 65% for the three straws containing extender without Equex STM paste (EYT-G). The spermatozoa were washed using Percoll density-gradient fractionation, as for Expt 1, except that smaller volumes were used (0.4 ml semen poured on top of 0.4 ml 80% and 0.4 ml 40% iso-osmotic Percoll in CCM). The pellets were resuspended in CCM, motility was reassessed and found to be 70, 65 and 50% with EYT-GE and 70, 70 and 65% with EYT-G. The samples were diluted to a final concentration of 0.625×10^6 spermatozoa ml⁻¹, whereupon they were subjected to a zona pellucida-binding assay as described above. Each semen sample was evaluated using 20 oocytes from a pool from six bitches. The same bitches were used for both treatments, with the oocytes from one ovary included in a pool for the evaluation of semen frozen with EYT-GE, and the oocytes from the other ovary were used for semen frozen with EYT-G. A total of 120 oocytes was evaluated.

Statistical analysis

The effect of the treatment on the number of spermatozoa bound to each oocyte was evaluated with a generalized least squares analysis using the mixed procedure in the SAS statistical package (SAS version 6.12, SAS Institute Inc., Cary, NC). The square root of the number of spermatozoa bound was used as the response variable so that the random components would be normally distributed. The fixed part of the model was treatment (α) with three or two levels, and the random part was replicates within treatments (r) and residuals (e). The random components were assumed to be independent and normally distributed, and this assumption was checked by box-plots and an extension of Shapiro and Wilk's test (Royston, 1982). The model was:

$$y_{ijk} = \mu + \alpha_i + r_{ij} + e_{ijk}$$

Where i = index for treatments (1,2 or 1,2,3); j = replicate within treatment; and k = measurements within replicate. Values were given in the transformed scale that belongs to the analysis. Back-transformed values on the mean number of bound spermatozoa were also presented for clarity. The level of statistical significance was set at $P < 0.05$.

Results

Experiment 1

The spermatozoa bound to zona pellucida usually were quite evenly distributed, although both in the untreated group and, in the 50:50 killed:untreated group, they occasionally bound in clusters. The variation between replicates within treatment was small (Table 1) and there was a significant difference among all treatments in the number of bound spermatozoa (untreated group: 19.1, 50:50 killed:untreated group: 7.7, killed group: 0.06) (Table 2).

Experiment 2

The number of spermatozoa bound on day 1 (4.8) and day 4 (0.9) did not differ significantly, although there was a trend ($P = 0.067$) for decreased sperm binding after 4 days. Both

the variation between and within replicates was higher than in Expts 1 and 3 (Table 3).

Experiment 3

Significantly more spermatozoa bound to the zona pellucida when treated with EYT-GE than with EYT-G ($P < 0.05$) (Table 3). The spermatozoa were evenly bound to the zona pellucida, except for one oocyte incubated with semen treated with EYT-GE, on which clusters were observed.

Discussion

Evaluation of sperm function using zona pellucida-binding assay has been developed for several species (for example, see Fazeli *et al.*, 1993a,b, 1995; Franken *et al.*, 1993; Goodrowe and Hay, 1993). The zona pellucida-binding assay measures

Table 1. Effect of canine sperm treatment (Expt 1) on the capacity to bind to the zona pellucida (ZP)

Sperm treatment	Number of spermatozoa bound to the zona pellucida		SEM	Variation between replicates within treatment*	Variation within replicates*
	Back-transformed from square roots (LSM)	Square roots (LSM)			
Untreated	19.1	4.4	0.18	0.0	1.8
50% killed	7.7	2.3	0.18		
100% killed	0.056	0.24	0.17		

*Calculated from square roots.
LSM: least squares means.

Table 2. Comparisons of canine sperm treatments (Expt 1)

Treatments compared	Difference between LSMs*	SD	P value
Untreated versus 50% killed	1.6	0.25	0.0007
50% killed versus 100% killed	2.5	0.25	0.0001
Untreated versus 100% killed	4.1	0.25	0.0001

*Calculated from square roots.
LSM: least squares means.

Table 3. Number of canine spermatozoa bound to the zona pellucida, and comparisons between treatments, between chilled (Expt 2) and frozen-thawed (Expt 3) spermatozoa

Sperm treatment	Number of spermatozoa bound to the zona pellucida			Difference between LSMs*	SD	P value	Variation between replicates within treatment*	Variation within replicates*
	Back-transformed from square roots (LSM)	Square roots (LSM)	SEM					
Chilled day 1	4.8	2.2	0.35	1.24	0.50	0.067	0.26	2.0
Chilled day 4	0.90	0.95	0.35					
Frozen EYT-GE	3.0	1.7	0.26	1.15	0.37	0.034	0.14	1.2
Frozen EYT-G	0.33	0.57	0.26					

*Calculated from square roots.
LSM: least squares means; EYT-GE: extender with Equex STM paste; EYT-G: extender without Equex STM paste.

the ability of spermatozoa to bind to oocytes. This binding is mediated through receptors at the sperm plasma membrane, and one or more zona pellucida glycoproteins (Yanagimachi, 1994). Therefore, a zona pellucida-binding assay would be expected to reveal damage that cannot be evaluated using routine microscopic techniques. The significant differences between untreated and killed spermatozoa demonstrated in the present study indicate that the binding of dog spermatozoa to the zona pellucida is a specific feature of living cells, and is not influenced to any great extent by, for example, the stickiness of the zona pellucida. In cattle, a correlation has been demonstrated between zona pellucida binding ability and fertility *in vivo* (Fazeli *et al.*, 1997a; Zhang *et al.*, 1998). Nevertheless, a lack of correlation between fertility *in vivo* and the ability of spermatozoa to bind to the zona pellucida indicates that this interaction is neither a limiting step in fertile boars (Berger *et al.*, 1996) nor a useful estimator for assessing the fertility of cryopreserved ram spermatozoa (Codde and Berger, 1995). Therefore, although interaction between the spermatozoon and the zona pellucida is essential, the usefulness of a zona pellucida-binding assay cannot be taken for granted, and depends both on the species and on the type of sperm population to be evaluated. Differences in the sperm–zona pellucida binding ability between spermatozoa subjected to different chilling procedures (Hay *et al.*, 1997a), and between the spermatozoa of fertile and infertile dogs (Mayenco-Aguirre and Pérez Cortés, 1998) indicate that, in dogs, a zona pellucida-binding assay can be used to evaluate not only damage caused by sperm storage, but also malfunctions in untreated spermatozoa causing decreased fertility.

In cattle, capacitation *in vitro* leads to an increased capacity of spermatozoa to bind to the zona pellucida (Topper *et al.*, 1999). In humans, acrosome-reacted spermatozoa may have the capacity to associate loosely with the zona pellucida by a reversible attachment, whereas acrosome-intact spermatozoa may achieve irreversible binding (Franken, 1998). In pigs, acrosome-intact spermatozoa initiate binding to the zona pellucida (Fazeli *et al.*, 1997b) but, as yet, no relationship between acrosomal status and strength of sperm–zona pellucida binding has been detected (Lynham and Harrison, 1998). The specificity of sperm binding to the zona pellucida in boars has been demonstrated by the blocking of sperm–zona pellucida binding with antibodies against two zona pellucida glycoproteins (Blase *et al.*, 1998). Monoclonal antibodies raised against pig zona pellucida have also been shown to inhibit complete penetration, but not binding, of the canine zona pellucida by canine spermatozoa in an assay *in vitro* (Bamezai *et al.*, 1988). The acrosomal status of dog spermatozoa bound to the zona pellucida is not known, but data indicate that both acrosome-intact and acrosome-reacted dog spermatozoa are capable of binding to the zona pellucida (Kawakami *et al.*, 1993), and no correlation has been found between acrosomal status and sperm penetration of the oocyte (Hewitt and England, 1998).

The zona pellucida-binding assay protocol used in the present study was the same as that used by Ström Holst *et al.* (1999), except that the co-incubation time of spermatozoa and oocytes was prolonged from 4 to 6 h. This alteration was made because of the longer capacitation time for fresh

semen, compared with for frozen semen, demonstrated by Rota *et al.* (1999a) using the chlortetracycline assay and CCM. In the present study, the spermatozoa were separated using Percoll density-gradient fractionation. With this method, spermatozoa are separated from foreign material, such as extender particles, cells and bacteria (Rodriguez-Martinez *et al.*, 1997). Washing is important, especially when evaluating extended semen, as, in humans, egg yolk has been shown to have a positive effect on the sperm–zona pellucida-binding capacity (Gamzu *et al.*, 1994) and on *in vitro* fertility and sperm ultrastructure (Barak *et al.*, 1995).

The egg yolk–Tris extender used in Expt 2 was evaluated for its effect on sperm motility, plasma membrane integrity and acrosomal status by Rota *et al.* (1995), using a combination of tests *in vitro*. Rota *et al.* (1995) report that the mean motility decreased from $73.6 \pm 15.5\%$ on day 1 to $53.6 \pm 20.1\%$ on day 4, and that the percentage of spermatozoa with intact plasma membranes, evaluated using carboxyfluorescein diacetate–propidium iodide (C-FDA/PI), decreased from 80.2 ± 6.4 to $72.1 \pm 10.4\%$. In the present study, the mean number of spermatozoa bound to the zona pellucida was 4.8 on day 1 and 0.9 on day 4 (back-transformed values), which is a decrease of a proportionally greater magnitude than that described for motility and plasma membrane integrity. The results of the present study demonstrate a negative effect of prolonged chilling on the zona pellucida-binding capacity of canine spermatozoa. The large variation within and between replicates demonstrates the diversity in sperm-binding capacity among oocytes and makes it necessary that several replicates are evaluated, as is the case in cattle (Zhang *et al.*, 1995).

In the present study, there were no differences in immediate post-thaw motility between the samples using the extender in the presence or absence of Equex STM paste. This result was in agreement with previous evaluations of the same two extenders (Rota *et al.*, 1997, A. Peña and C. Linde-Forsberg, *in press*). However, Rota *et al.* (1997) and A. Peña and C. Linde-Forsberg (*in press*) demonstrated a significantly higher motility and a higher proportion of spermatozoa with intact plasma membranes during incubation at 38°C with EYT-GE than with EYT-G. In the present study, there was a significant beneficial effect of the addition of Equex STM paste on sperm-binding capacity, indicating a beneficial effect also on the molecular structures involved in binding to the zona pellucida. When the fertility *in vivo* of semen frozen using the same two extenders was evaluated, no significant difference was demonstrated, either after intra-uterine or after vaginal deposition, using five bitches from each group (Rota *et al.*, 1999b). This lack of agreement between fertility characteristics of spermatozoa *in vivo* and *in vitro* may reflect the lack of difference in the fertility *in vivo* of semen frozen using the two types of extenders. However, it is more likely that a difference in fertility *in vivo* is not detected in a small number of bitches with well-timed inseminations and a relatively large number of spermatozoa (two inseminations of 200 million).

In conclusion, there was a non-significant trend for fewer spermatozoa to bind after storage for 4 days than after storage for 1 day at 4°C. The addition of Equex STM paste to an egg yolk–Tris extender had a significant positive effect on

sperm–zona pellucida-binding after freeze–thawing. As the zona pellucida-binding assay evaluates damage that cannot be detected using other techniques, its addition to other established tests *in vitro* should give a better estimate of the damage caused by various preservation procedures. This procedure will be of use in the development of new techniques for the chilling and freeze–thawing of dog spermatozoa.

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