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In Vitro Characterisation of Cryopreserved Canine Spermatozoa

With special reference to post-thaw survival time and zona pellucida binding capacity

Bodil Ström Holst

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Abstract

Methods for cryopreservation of canine spermatozoa have mostly been developed without proper assessment of the cell functional status using *in vitro* methods. This has complicated the adequate development of new procedures and the refinement of old ones. In the present study, two methods known to give high pregnancy rates after artificial insemination in the field (the Andersen and CLONE methods), were evaluated in vitro. Sperm survival time post-thaw, assessed as motility during incubation for 3 hrs at 37°C, was short using both procedures. Additionally, a high percentage of the acrosomes were damaged, both when evaluated using light and transmission electron microscopy, indicating that acrosomal damage might be one reason for the reduced post-thaw survival time. Further, the detergent Equex STM paste was added to a Tris-based extender to evaluate its effect on sperm survival time post-thaw. Plasma membrane (PM) integrity immediately post thaw was significantly higher using Equex STM paste, and both motility and PM integrity were prolonged during incubation at 38°C when Equex STM paste was added. Moreover, frozen-thawed dog spermatozoa were evaluated for their ability to bind to homologous zona pellucida (ZP) in a ZP binding assay (ZBA). The capacity of canine spermatozoa to bind to the ZP was found to be a feature of the living sperm cell. Chilling spermatozoa for 4 days at 4°C tended to decrease ZP binding ability compared with one day's storage. The addition of Equex STM paste to the cryopreservation extender had a significantly beneficial effect on sperm ZP binding capacity. To facilitate the implementation of a ZBA in the canine, it is necessary to find a method for storage of retrieved oocytes. Deep freezing of canine ovaries and salt-storage of oocytes was seen to result in morphological changes of the ZP as detected by scanning electron microscopy, mainly manifested as a wider meshwork of the outer surface of the ZP. These changes were thought to contribute to the observed reduced sperm-binding capacity compared to fresh oocytes, but did not exclude the use of stored oocytes in a ZBA. Taken together, the addition of a ZBA to other in vitro tests can be expected to contribute to better assessments of the damage caused to dog spermatozoa when developing and refining techniques for semen chilling and cryopreservation.

Key words: Dog, sperm, cryopreservation, in vitro, evaluation, post-thaw, survival time, zona pellucida

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Fantasi är viktigare än kunskap

Albert Einstein

Abstract

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Contents

Appendix

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Ström, B., Rota, A. & Linde-Forsberg, C. 1997: In vitro characteristics of canine spermatozoa subjected to two methods of cryopreservation. Theriogenology 48: 247-256.

II. Ström Holst, B., Rota, A., Andersen Berg, K., Linde-Forsberg, C. & Rodriguez-Martinez, H. 1998: Canine sperm head damage after freezing-thawing: ultrastructural evaluation and content of selected elements. Reprod Dom Anim 33: 77-82.

III. Rota, A., Ström, B., Linde-Forsberg, C. & Rodriguez-Martinez, H. 1997: Effects of Equex STM Paste on viability of frozen-thawed dog spermatozoa during *in vitro* incubation at 38°C. Theriogenology 47: 1093-1101.

IV. Ström Holst, B., Larsson, B., Linde-Forsberg, C. & Rodriguez-Martinez, H. 1999: Sperm binding capacity and ultrastructure of the zona pellucida of stored canine oocytes. Submitted for publication.

V. Ström Holst, B., Larsson, B., Linde-Forsberg, C. & Rodriguez-Martinez, H. 1999: Evaluating chilled and frozen-thawed dog spermatozoa using a zona pellucida binding assay. Submitted for publication.

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Abbreviations

AI	Artificial insemination
CASA	Computer assisted sperm motion analysis
CCM	Canine capacitation medium
C-FDA	6-carboxyfluorescein diacetate
COCs	Cumulus-oocyte complexes
Equex	Equex STM paste
FRE	Fresh (oocytes)
FRESS	Salt-stored (oocytes)
FRO	Frozen (oocytes)
FROSS	Salt stored (oocytes) from frozen ovaries
HOS	Hypo-osmotic swelling
HZA	Hemizona binding assay
LN_2	Liquid nitrogen
OHE	Ovariohysterectomy
PBS	Phosphate buffered saline solution
PI	Propidium iodide
PM	Plasma membrane
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet
ZBA	Zona pellucida binding assay
ZP	Zona pellucida

Background

Introduction

The dog is not only considered to be "man's best friend", but was probably also the first species to become domesticated, about 14,000 years ago (Morey 1994). That domestication has been advantageous to dogs is suggested by their ubiquity, which is in contrast to several wild canids who either have become extinct (Dusicyon australis, the Falkland Island Wolf), or are threatened by extinction (Canis rufus, the red wolf, Canis simensis, the Abyssinian wolf, some populations of Canis lupus, the grey wolf, Lycaon pictus, the African wild dog, Speothos venaticus, the savannah dog, and Vulpes velox hebes, the Northern swift fox) (IUCN 1996 & CITES 1999). The domestic dog can to some extent serve as a model for the wild canids. Research on canine gametes, including their preservation, and artificial insemination (AI) can provide valuable knowledge for the ongoing work aiming at conservation of endangered canids (Goodrowe et al. 1998). Gamete preservation and AI are also valuable tools for dog breeders, especially in numerically small breeds, as they offer the possibility to transport semen all over the world, and to store semen from valuable stud dogs over time. In addition, numerous breeds are afflicted by various genetic diseases, which makes dogs valuable models in medical genetics (Galibert et al. 1998) and thus gamete preservation important.

Some reproductive features of the domestic dog

The grey wolf, Canis lupus, is the ancestor of dogs. Domestication has, among other things, changed some reproductive features. For instance, dogs reach puberty at around one year compared with wolves, who become pubertal at around two years of age. Wolves cycle once a year, while most domestic dogs cycle twice per year.

Nonetheless, compared with other domestic species, twice per year is still not very frequent, and the long inter-oestrus interval of dogs has the consequence that a negative result after breeding or AI is very disappointing for the breeder. The reproductive cycle of the dog differs from that of other domestic species also in that the pro-oestrus and oestrus periods are long, the average of each being more than one week. Furthermore, primary oocytes are ovulated, requiring 2-4 days for maturation (Tsutsui 1975). There is evidence that oocytes need to mature before they can be fertilised (Tsutsui and Ejima 1988), although conflicting results suggest that spermatozoa can penetrate immature oocytes (Mahi and Yanagimachi 1976, Hewitt and England 1997) and that penetration of the zona pellucida (ZP) or into the ooplasm by spermatozoa induces chromatin condensation of canine ova *in vitro* (Hay et al. 1994). To estimate the optimal time for mating or AI, careful examinations of the bitch are required, usually including vaginal smears and, more importantly, blood progesterone analyses (Linde-Forsberg 1995).

Benefits of gamete preservation

Not only were dogs domesticated early, but also the first described AI resulting in a pregnancy in a mammal was made in a bitch by abbé Lazzaro Spallanzani in Italy as early as 1780. The main advantage with the technique of AI in the dog is that it permits the use of stored semen. The life span of spermatozoa *in vitro* can be prolonged by dilution and/or temperature reduction (chilling or freezing). For short-term storage (1-2 days), dilution in an extender and chilling to 4°C offers a practical means for transportation of semen, both within and between countries (Linde-Forsberg 1995). Deep freezing of semen is a more complex process than chilling and transportation is more expensive. Furthermore, the insemination technique is more complicated since intrauterine deposition is recommended because of significantly improved pregnancy rates compared with when the semen is deposited in the vagina (Linde-Forsberg 1995, Linde-Forsberg et al. 1999). The great advantage of freezing semen is the prolongation of sperm longevity by storage in the frozen state, theoretically more than 2,000 years (Mazur 1984). The long storage time possible is clearly advantageous for dog breeders as well as for researchers working with endangered canids or in medical genetics. However, freezing and thawing of semen will cause a reduction in sperm survival time after thawing, possibly by causing capacitation-like changes (Watson 1995, Rota et al. 1999b). Due to the long oestrus period with subsequent difficulties in determining the optimal insemination time, a prolonged survival of the processed spermatozoa is one of the goals of cryopreservation research. Such a prolonged survival post-thaw would be expected to increase pregnancy rates and litter sizes after AI, since also sub-optimally timed inseminations could be expected to lead to conception to a larger extent. Additionally, pregnancy rates and litter sizes after intravaginal AI might be improved, as a long post-thaw survival might be even more important after intravaginal than after intrauterine insemination (Linde-Forsberg et al. 1999).

In vitro evaluation of sperm function

High fertility is the ultimate goal when working with sperm cryopreservation. However, field fertility trials, besides being expensive, have the drawback of having a low sensitivity if the insemination dosage is high or too few animals are used (Woelders 1991, Rota et al. 1999a, Tardif et al. 1999). *In vitro* assays have the advantage of showing which aspects of sperm function that are damaged or intact, which is of special interest when working with refinement of semen preservation techniques. They are also interesting, as such, in fundamental sperm research. Several *in vitro* techniques have been described for semen evaluation. The combination of different *in vitro* assays has been shown to give a more reliable result than a single test when attempting to predict *in vivo* fertility (Zhang et al. 1998, 1999).

Canine semen preservation methods have mostly been developed without making proper *in vitro* characterisations of the spermatozoa, which has led to a variety of cryopreservation methods being used, some resulting in high pregnancy rates, but without proper knowledge of the type and extent of damage caused to the spermatozoa by the procedures involved. Information on *in vitro* characteristics of spermatozoa frozen according to methods with known field fertility facilitates the improvement and development of new methods, and is also advantageous in that comparisons and correlations between *in vitro* characteristics and field fertility can be made. ***The first aim*** of the present thesis was to evaluate frozen-thawed canine spermatozoa using *in vitro* assays. The semen was frozen according to two methods with high field fertility (the Andersen and CLONE procedures).

Sperm morphology

Already in 1934, Lagerlöf described evidence of a relationship between sperm morphology and fertility in the bull. In dogs, evidence of this relation is scarce (Oettlé and Soley 1985, Renton et al. 1986, Plummer et al. 1987, Oettlé 1993). England and Allen (1989) showed that dogs with poor semen quality still could be fertile using natural mating. However, when working with frozen-thawed semen, it must be borne in mind that the freezing-thawing procedures usually cause extra damage to the spermatozoa. Further, since application of AI is bound to the use of a limited number of spermatozoa in the AI-dose, the threshold where visible sperm defects cause reduced fertility can be expected to be much lower. In the bull, even low frequencies (<4%) of abnormalities in fresh semen have been shown to correlate significantly with results after AI with frozen-thawed semen (Söderquist et al. 1991a). There is also evidence that dog spermatozoa with certain morphological defects do not freeze as well as morphologically normal ones (Morton and Bruce, 1989). When developing new protocols for cryopreservation, a morphological evaluation of spermatozoa is often performed to ensure that ejaculates with a high proportion of morphologically normal spermatozoa are used. However, gross structural changes are generally not caused by preservation (Watson 1979), and thus other assays are required when evaluating damage caused by the cryopreservation procedure.

Sperm motility

By far the most commonly used *in vitro* assay for semen evaluation is the estimation of sperm motility. The proportion of spermatozoa exhibiting a progressive forward motion pattern is commonly estimated either subjectively in a phase contrast microscope or more objectively using computer assisted sperm motion analysis (CASA). It is used also in the evaluation of frozen-thawed spermatozoa, as a measure of the proportion of spermatozoa that survived the procedure. It has been shown that both in fresh ejaculates (Casey et al. 1993) and cryopreserved semen (Januskauskas et al. 1996) there is a proportion of spermatozoa that are viable although being immotile, and which might regain motility after appropriate stimulation, e.g. with caffeine (Larsson et al. 1976). Thus, motility evaluation can not be used as a measure of live and dead

spermatozoa, but it gives information on a trait that is necessary for sperm fertilising capacity. Despite this, reports of relations between sperm motility and field fertility are conflicting (e.g. Söderquist et al. 1991b, Kjaestad et al. 1993, Sanchez-Partida et al. 1999, Tardif et al. 1999).

Plasma membrane integrity

An intact plasma membrane (PM) is a prerequisite for normal sperm function, including fertilisation. During the cryopreservation process, the PM is damaged by phase transitions of the lipids, efflux of water, mechanical stress, high salt solutions and possibly ice crystals (Woelders 1997). The integrity of the canine PM can be evaluated using fluorochromes, which stain the spermatozoa differently depending on the status of the PM (Rota et al. 1995, Peña et al. 1998c). Scanning electron microscopy (SEM) is another way of visualising the PM and damage caused to it. The differential live/dead staining using eosin is also a test for the integrity of the PM, as it is a dye exclusion test (Dott and Foster 1972). The status of the dog sperm PM can also be assessed using a hypo-osmotic swelling (HOS) test (e.g. Kumi-Diaka 1993). With such a test, the osmotic integrity of the PM is examined.

Acrosomal status

The acrosome plays a crucial role in sperm function. After the spermatozoon has undergone the acrosome reaction, it is capable of penetrating the ZP, allowing the PM over the equatorial segment to fuse with the oolemma (Yanagimachi 1994). The acrosome of frozen-thawed spermatozoa often shows morphological changes (Watson 1990), and evaluation of acrosomal status can thus be considered a useful parameter when evaluating cryopreservation methods. Transmission electron microscopy (TEM) allows detailed examination of the acrosomal status (Yanagimachi 1994), but it is laborious and time consuming. The acrosomal status of dog spermatozoa can be evaluated quicker and more easily by using ordinary light microscopy (Oettlé 1986, Peña et al. 1998a,b). Recently, methods have been developed for acrosomal evaluation using fluorescence microscopy (Kawakami et al. 1993a, Szasz et al. 1997, Hewitt and England 1998, Rota et al. 1999b). In addition, the release of acrosomal enzymes, e.g. acrosin, can be measured (Froman et al. 1984), or monoclonal antibodies can be used (Geussová et al. 1997) to indirectly evaluate the acrosomal integrity of canine spermatozoa.

Increase of sperm post-thaw survival time

Various substances have been added to extenders to prolong sperm post-thaw survival. The methylxanthine pentoxifylline has been shown to increase sperm motility during post-thaw incubation at 39°C when added during the process of thawing (Koutsarova et al. 1997). Addition of the amino acid proline to a Tris-based extender has also resulted in higher sperm motility immediately post-thaw as well as during incubation at 39°C, and to result in a higher live/dead ratio and proportion of spermatozoa with intact acrosomes, compared with controls (Peña

et al. 1998a). A third substance that has been associated with increased motility during incubation of dog spermatozoa is sodium dodecyl sulphate (SDS, Thomas et al. 1992, Peña et al. 1998b). SDS is a detergent that alters the physical properties of egg yolk contained in the extender (Pursel et al. 1978). Orvus ES paste or Equex STM paste, both including SDS, have been included in extenders for several species, and found beneficial (e.g. Pursel et al. 1978, Martin et al. 1979, Arriola et al. 1987). The effect of Equex STM paste on post-thaw survival time of canine spermatozoa has not been studied, and such a study was **the second aim** of the present thesis.

Sperm-oocyte interaction assays

In addition to the previously mentioned tests for evaluation of single parameters of sperm function, there are also assays that evaluate multiple sperm parameters, such as the sperm-oocyte interaction assays (ZP binding and zona penetration) and *in vitro* fertilisation. The ability of a spermatozoon to interact correctly with the oocyte is crucial and dependent on maintenance of several separate traits, including the ability to maintain motility and membrane fluidity. The sperm-oocyte interaction assays are expected to additionally evaluate damage at a molecular level, which are impossible to assess using conventional sperm analysis. In the canine, these methods are still scarcely used. Canine oocytes can be obtained from bitches subjected to ovariohysterectomy (OHE), but the number of oocytes that can be recovered from an individual bitch varies greatly and is difficult to predict (Ström Holst et al., submitted). A method for storage of oocytes would, therefore, facilitate the practical realisation of a sperm-oocyte interaction test. Different approaches have been used for canine oocyte storage, such as freezing of entire ovaries or storing of oocytes in a hypertonic salt solution or by freezing (Hay et al. 1997a, Mayenco-Aguirre and Pérez Cortés 1998, Ivanova et al. 1999). However, the extent to which these procedures affect the zona pellucida and its ability to allow sperm binding is still unclear. ***The third aim*** of the present thesis was to evaluate the effect of different methods of oocyte storage on sperm-binding capacity and ultrastructure.

In sperm–oocyte interaction tests performed in the canine, spermatozoa have been incubated together with intact oocytes surrounded by cumulus cells (ZBA) (Hay et al. 1997a,b; Hewitt and England 1997), and recently a hemizona-binding assay (HZA) has been used (Mayenco-Aguirre and Pérez Cortés 1998, Ivanova et al. 1999). In a HZA the variation in binding capacity between different oocytes is overcome, but it is a time-consuming method. One way to reduce the variation between oocytes in a ZBA is to use sufficient numbers of oocytes and replicates (Zhang et al. 1995). The use of denuded oocytes might also lower the variation in binding capacity when stored oocytes are used, since the effect of storage on the cumulus cells is unknown. Gamete interaction tests using intact, denuded oocytes have been described for several species, such as the bovine (Fazeli et al. 1993), equine (Ellington et al. 1993) and porcine (Berger et al. 1989), but hitherto not for the canine. The validation of such an assay for the canine would be advantageous.

The fourth aim of the present thesis was to evaluate a ZBA for canine spermatozoa, and ***the fifth aim*** to determine the effect of chilling (to 4°C), or cryopreservation with or without Equex STM paste (Equex), on the zona binding ability of spermatozoa.

Aims

As outlined in the Background, the aims, thus, were to evaluate:

- Post-thaw *in vitro* characteristics of canine spermatozoa frozen according to two procedures with documented high *in vivo* fertility (the Andersen and CLONE methods),
- The effect of adding Equex STM paste to a Tris-glucose-citric acid extender on post-thaw survival time of canine spermatozoa,
- The effects of different methods for oocyte storage on sperm-binding capacity and ultrastructure of the zona pellucida,
- The value of a zona pellucida binding assay to assess the potential fertilising ability of frozen-thawed canine spermatozoa,
- The effect of chilling or cryopreservation with or without Equex STM paste on the zona pellucida binding capacity of canine spermatozoa.

Methodological considerations

The materials and methods used in the present study are described in detail in the specific papers, the discussion below containing general comments.

Semen freezing and thawing

The Andersen method (Papers I and II):

Centrifuged semen was diluted in a Tris-fructose-citric acid extender and after 2 h of equilibration frozen in straws horizontally, 4 cm above the liquid nitrogen (LN_2) surface. Semen was thawed for 8 sec in a water bath at 70°C.

The CLONE method (Papers I and II):
Using this method, centrifuged semen was diluted in two steps: first at room temperature with extender CLONE A, and after 1 h with extender CLONE B at 4°C. Semen was frozen by lowering straws vertically into a LN_2-tank. Semen was thawed for 15 sec in a water bath at 37°C, and then re-diluted in CLONE Thaw Medium and kept at 37°C for 5 min.

The Equex method (Papers III-V):
Centrifuged semen was diluted in two steps with Tris-glucose-citric acid extenders. The second extender contained (Papers III-V) or did not contain (Paper III) Equex STM paste. Semen was thawed for 1 min in a water bath at 38°C (Papers III and V) or for 8 sec in a water bath at 70°C (Paper IV). In Paper III, thawed semen was re-diluted in a Tris-buffer.

In Papers I, II, and III, a split sample design was used. The ejaculates were divided in halves and each half was frozen according to either of two methods: the Andersen or the CLONE method (Papers I and II), or in a Tris-glucose–citric acid based extender with or without the addition of Equex STM paste at a final concentration of 0.5% (Paper III).

Evaluation of sperm motility

Evaluations of sperm motility were made by subjective assessment with a phase contrast microscope at x400 (Papers I-V). To increase objectivity, motility in Paper I was estimated from video recordings or by 2 operators together. A CASA was not found suitable because of presence of particles of the same size as spermatozoa in the extenders, interfering with the computerised measurements. To increase objectivity of motility estimations in Paper III, two aliquots from each semen sample, and at least 8 fields from each aliquot, were evaluated. To get a measure of sperm survival time post-thaw, sperm motility was also measured during prolonged incubation at room temperature (24 hrs) (Paper I) or at 37-38°C (thermoresistance test, 3 hrs) (Papers I and III).

Evaluation of plasma membrane integrity

In Papers I and III, PM integrity was assessed using the fluorochromes 6-carboxyfluorescein diacetate and propidium iodide (C-FDA/PI). As for sperm motility, PM integrity in Paper III was also evaluated during incubation for 3 h at 38°C.

In Paper II, Scanning electron microscopy (SEM) was used. The advantage of SEM is that the extent of the damage can be visualised, the drawback being that only limited areas of the sperm surface are evaluated.

Evaluation of acrosomal integrity

Acrosomal integrity was evaluated using light microscopy and the Spermac® stain (Stain Enterprises, P.O. Box 152, Wellington, South Africa) (Paper I). In Paper II, TEM was used. Using both methods, changes thought to represent different stages of the acrosomal degradation could be visualised.

X-ray microanalysis

An X-ray microanalysis of the postacrosomal region of the sperm head of fresh and frozen-thawed spermatozoa was performed in Paper II. This is a method by which it is possible to relate the elemental composition of a cell or part of a cell with ultrastructural information. An electron beam strikes the specimen, and electrons within the elements of the specimen get excited, resulting in an emitted x-ray spectrum - characteristic of the element concerned - being produced, which is collected by a detector attached to the microscope (Roomans 1988).

Sperm-zona binding

In Paper IV, where the effect of different oocyte storage methods was investigated, one ejaculate from one dog was used for ZBAs. In Paper V, semen was twice collected and pooled from three dogs. The first pool was split and used to evaluate the ZBA. Killed, untreated or a 50/50 mixture of killed and untreated spermatozoa were used for a ZBA. The second pool was divided into halves. The first half was used to test the zona-binding capacity of spermatozoa chilled for one or four days in a Tris-based extender. The second half was used in a ZBA with spermatozoa frozen in Tris-based extenders with or without Equex STM paste (as in Paper III).

Oocyte recovery and storage

Oocytes were recovered from bitches subjected to OHE. The ovaries were minced with a scalpel under a stereo microscope and the cumulus-oocyte complexes (COCs) were recovered. Cumulus cells surrounding the oocytes were removed by incubating the COCs in 75mM sodium citrate for 15 min, followed by vortexing for 15 min.

To evaluate different types of storage on ultrastructure and sperm-binding capacity, fresh oocytes (FRE), salt-stored oocytes (FRESS), oocytes from frozen ovaries (FRO) and (for the ultrastructural study) salt-stored oocytes from frozen ovaries (FROSS) were studied in Paper IV.

Sperm preparation and sperm-oocyte co-incubation

Before being used in the ZBAs, the semen samples were cleansed using a Percoll density gradient fractionation. The recovered spermatozoa were thereafter suspended in canine capacitation medium (CCM, Mahi and Yanagimachi 1978). Oocytes were added to 10-µl droplets of CCM. The sperm suspension was then

added to a final concentration of 0.5 million sperm/mL in a 50-µl droplet, resulting in 5,000-25,000 spermatozoa per oocyte (Paper IV) or 5,000 spermatozoa per oocyte (Paper V). Spermatozoa and oocytes were coincubated at 37°C for 4 (Paper IV) or 6 (Paper V) hrs. The sperm-oocyte complexes were washed using repeated pipetting, either as a gentle (Paper IV) or tough (Papers IV and V) wash, by using different inner diameters of the pipettes.

Staining and evaluation

Sperm-oocyte complexes were fixed in 1.5% glutaraldehyde solution and thereafter stored in phosphate buffered saline (PBS) until evaluation. Before evaluation, the sperm-oocyte complexes were stained with PI. The number of spermatozoa bound to the ZP was counted using epifluorescence UV-illumination, either on an excavated glass slide (Paper IV), or covered and slightly compressed by a coverslip on a glass slide (Paper V).

Statistical analyses

In Papers I – IV, most analyses were made using Minitab statistical software (Minitab Inc., State College, PA, USA). The General Linear Models procedure was used for analysis of variance (Paper I), the Wilcoxon matched pairs signed rank sum test for non-parametric comparisons of paired groups (Papers II-III) and the Mann-Whitney test for non-parametric comparisons of non-paired groups (Paper IV). The Minitab statistical software was used neither for a non-parametric analysis of variance for a randomised complete-block design (Mehra and Sarangi 1967) in Papers I and II, nor for the generalised linear mixed model in SAS GLIMMIX macro (Littell et al. 1996) in Paper IV. For the latter analysis, as for the Mixed procedure in Paper V, the SAS statistical package (SAS version 6.12, SAS Institute Inc., Cary, NC, USA) was used. Values are presented as means, and $P<0.05$ was set as the level of statistical significance, except for the data on SEM and TEM in Paper II, when $P<0.06$ was used.

Results

In vitro characteristics of spermatozoa cryopreserved with the Andersen or the CLONE procedures (Papers I and II)

Using the Andersen and the CLONE procedures, motility, although still very good, decreased significantly after freezing and thawing ($P<0.001$) (Andersen: 70 %, CLONE: 75%; $P<0.05$, immediately post-thaw). During incubation at room temperature, motility using CLONE was higher than using Andersen after 3 hrs ($P<0.05$), whereas there was no significant difference between the freezing-thawing protocols after 6 and 24 hrs. During incubation at 37°C for 3 hrs there

was a more pronounced decrease in motility, and it was consistently significantly ($P<0.05$) lower for the CLONE than for the Andersen protocol (Andersen: 22% and CLONE 2.5% after 3 hrs).

Sperm membrane integrity, evaluated using C-FDA/PI (Paper I), decreased significantly ($P<0.001$) after freezing and thawing using both cryopreservation methods, but the methods did not differ significantly. The percentage of spermatozoa with intact PM immediately post-thaw approximated 75% for both cryopreservation methods. When evaluated using SEM (Paper II), approximately 70% of the spermatozoa had an intact PM immediately post-thaw, with no significant difference between cryopreservation methods.

The proportion of spermatozoa with morphologically intact acrosomes after freezing-thawing evaluated using light microscopy (Paper I) averaged 50% for both cryopreservation methods. Using TEM (Paper II), it was visualised that only 25% of spermatozoa frozen according to the Andersen method and 6% frozen with CLONE had normally appearing acrosomes, the majority of the spermatozoa (71% with Andersen and 89% with CLONE) having swollen acrosomes. These differences were not statistically significant.

Evaluating the post-acrosomal region of the sperm head using X-ray microanalysis, concentrations of phosphorus and zinc were not found to be significantly altered by either the Andersen or the CLONE protocols (Paper II). The sulphur concentration, however, decreased significantly using CLONE ($P<0.04$). The concentration of K decreased after freezing-thawing using both methods. Using the Andersen procedure, Cl concentrations decreased and Ca concentrations increased.

Effects of adding Equex STM paste to a Tris-citrate-glucose extender for freezing dog spermatozoa (Papers III and V)

The addition of Equex STM paste to the freezing extender (Paper III), had no significant effect on motility immediately post-thaw (61% with and 56% without Equex), but a significantly positive effect on the proportion of spermatozoa with intact PM (63% with and 56% without Equex). The addition of Equex had a clearly beneficial influence on both motility and PM integrity when spermatozoa were incubated at 38°C (Fig 1).

The main decrease in PM integrity during post-thaw incubation was seen during the first hour. After one hour, PM integrity was 49% with and 18% without Equex. From hour 1-3 after thawing, the proportion of spermatozoa with an intact PM remained fairly constant (48% with and 16% without Equex after 3 hrs). Motility, on the other hand, decreased throughout the incubation period for both treatments (50% h1 to 35% h3 with and 20% h1 to 7% h3 without Equex), proportionally more without Equex.

A beneficial effect of the addition of Equex STM paste to a cryopreservation extender on the ZP binding capacity of spermatozoa was demonstrated (3.0 spermatozoa/oocyte vs 0.33 spermatozoa/oocyte, P=0.034).

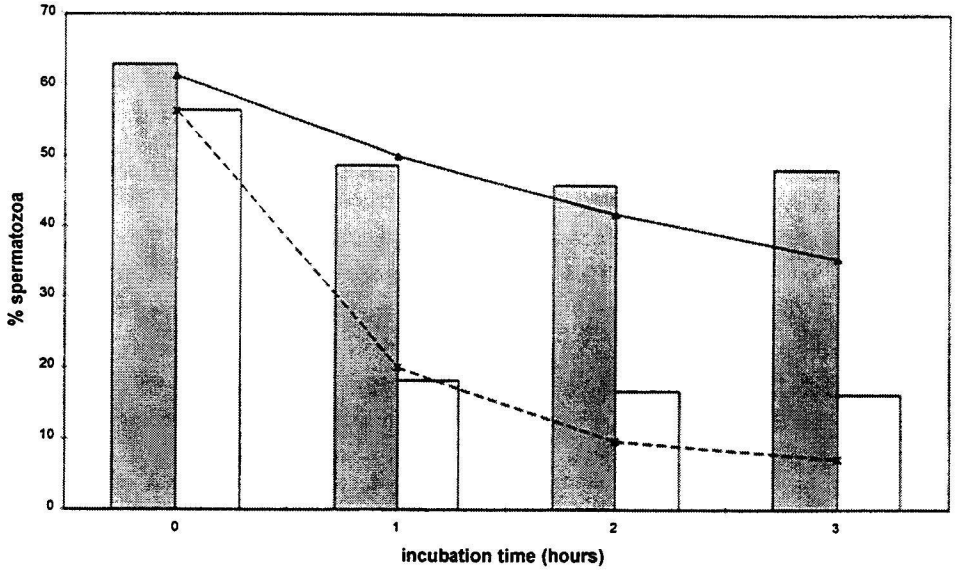

Figure 1. The effect of an extender with Equex STM paste (solid columns, solid line) or without Equex STM paste (open columns, dashed line) on the proportion of spermatozoa with an intact PM (columns) and sperm motility (lines) during incubation at 38°C (Paper III, mean values).

Effects of oocyte storage on ZP sperm-binding capacity and ultrastructure (Paper IV)

The sperm-binding capacity of stored oocytes was reduced: significantly fewer spermatozoa bound to salt-stored oocytes (FRESS) and to oocytes from frozen ovaries (FRO), than to fresh (FRE) oocytes (P<0.001). There was no significant difference in sperm-binding capacity between FRESS and FRO oocytes (P=0.3).

Using SEM, the morphology of stored oocytes was seen to differ from fresh ones in that the fibrous network that usually characterises the ZP was wider, more oocytes were malformed and the ZP was thinner. Salt-stored oocytes from frozen ovaries (FROSS) were more often broken and malformed than oocytes in the other groups, and were therefore not used for ZBA testing.

Significantly fewer spermatozoa bound after the tough wash than after the gentle wash (P<0.001), except for the FRESS oocytes (P=0.17). The proportion of oocytes that had spermatozoa bound to the ZP was significantly lower for both

the FRESS (50%) and the FRO (62%) group than for the FRE group (84%) after the tough wash.

Evaluation of a ZBA, and the effect of storage 4 days at 4°C on the capacity of spermatozoa to bind to the ZP (Paper V)

In the evaluation of the ZBA, using pooled semen that was untreated, killed or a 50/50 mixture of untreated and killed spermatozoa, there was a significant difference between all treatments on the number of spermatozoa bound. The distribution of spermatozoa over the ZP surface was usually quite even, although clusters of spermatozoa were occasionally seen.

Evaluating chilled spermatozoa, it was shown that the ZP binding capacity was reduced after 4 days of storage (0.9 spermatozoa/oocyte), compared with after 1 day (4.8 spermatozoa/oocyte), although not statistically significant (P=0.067). There was a large variation in the number of spermatozoa bound both within and between replicates.

Discussion

As described in the Background, the function of cryopreserved spermatozoa can be evaluated using several different *in vitro* tests, most of them related to the evaluation of single traits. In the present study, semen frozen according to two procedures (the Andersen and the CLONE procedure) was evaluated. Both methods have resulted in high whelping rates in clinical practice. Using intrauterine insemination, whelping rates of 80% and 67% (16/20 and 20/30 bitches) have been obtained with the Andersen method (Andersen 1976, Farstad 1984), and 84% (141/167 bitches) with the CLONE method (Linde-Forsberg et al. 1999). These results are not much lower than those obtained after well-controlled natural matings, which have been described to amount to 85 - 90% (England and Allen 1989, Daurio et al. 1987). Plasma membrane integrity of spermatozoa frozen-thawed with the Andersen or the CLONE procedure was measured both using light microscopy, using C-FDA/PI, and using SEM (Papers I and II). The light microscopy technique is less expensive and time-consuming than SEM, and would therefore be preferable, provided it is reliable enough. The similar results using C-FDA/PI and SEM suggest that light microscopy evaluation using C-FDA/PI is a reliable tool for evaluating PM integrity. Motility was checked subjectively. The proportions of motile spermatozoa and of spermatozoa with intact PM immediately post-thaw were, although significantly lower than for fresh spermatozoa, still high.

Depending on the location of the elements that are measured, an X-ray microanalysis can give information about the integrity of different parts of the cell. A decrease in phosphorus and sulphur at the head region after cryopreservation of canine spermatozoa has previously been attributed to changes in the chromatin (Rodriguez-Martinez et al. 1993), and the decrease in sulphur concentrations using the CLONE protocol might thus be a reflection of DNA damage. If the cytosol is the main location of the elements concerned, an X-ray microanalysis can give information on PM damage (Courtens et al. 1989). A decrease in K-concentrations has previously been shown after freezing-thawing of spermatozoa from dogs and other species (Quinn and White 1966) and, in the present study, was seen using both the Andersen and the CLONE method, indicating functional alterations of the PM. The decrease in Cl-concentration and increase in Ca-concentration seen in spermatozoa cryopreserved using the Andersen procedure might also be reflections of PM damage. The results of the X-ray microanalysis are thus in accordance with the results of the light and electron microscopy evaluations of the PM; the proportion of spermatozoa with an intact PM decreases significantly after cryopreservation.

In contrast to the large proportion of spermatozoa with intact PM and high motility immediately post-thaw, the proportion of spermatozoa with intact acrosomes was low. The proportion of spermatozoa with damaged acrosomes was

higher using TEM than using light microscopy, which suggests that electron microscopy, although expensive and time-consuming, might be preferable for more detailed studies of the acrosome.

The Andersen and the CLONE methods differed significantly as to their effect on motility of the cryopreserved spermatozoa during incubation at 37°C, although it was low using both methods. The relevance of the difference between methods was difficult to evaluate, as the spermatozoa frozen with the CLONE procedure were re-diluted post-thaw and incubated in a medium of unknown composition, and due to this re-dilution, were also stored in a larger volume than the spermatozoa frozen with the Andersen protocol. It has been shown that dog sperm motility decreases after the induction of the acrosome reaction (Szasz et al. 1997), and thus the high proportion of spermatozoa with swollen acrosomes might be related to the marked decrease of sperm motility during incubation at 37°C.

The direct influence of the acrosomal changes detected in the present study on the whelping rates is not known. Although the changes most likely indicate acrosome degradation, the high field fertility obtained using both cryopreservation methods indicates that a high proportion of spermatozoa with swollen acrosomes might not have a large impact on whelping rates. Swelling of the acrosomal matrix has been seen at the early stages of the acrosome reaction (Yanagimachi 1994), and it might be that, as long as the outer acrosomal membrane is intact, the spermatozoa retain their fertilising capacity. The possible negative relationship between the acrosomal changes and sperm survival time might be of greater importance. The reduced survival time of frozen-thawed dog spermatozoa, compared with fresh, has previously been described, and believed to be a major concern when working with techniques for cryopreservation of canine spermatozoa (Concannon and Battista 1989, England 1992). Due to the prolonged oestrus period in bitches, with subsequent difficulties in determining the optimal insemination time, cryopreservation methods resulting in an increased long-term viability of the thawed spermatozoa would be expected to result in higher whelping rates and larger litter sizes. In addition, a longer post-thaw survival of spermatozoa is probably needed when using intravaginal than when using intrauterine AI (Linde-Forsberg et al. 1999). Whelping rates after intravaginal insemination with the Andersen and the CLONE protocols are clearly lower than those obtained after intrauterine insemination. No pregnancies were obtained after inseminating two bitches intravaginally with semen frozen with the Andersen procedure (Andersen 1972), and with CLONE, the whelping rate after intravaginal insemination was 59% (83/141 bitches) (Linde-Forsberg et al. 1999). Thus a better cryopreservation method, leading to a prolonged survival of the thawed spermatozoa, might also lead to higher whelping rates and larger litter sizes after intravaginal AI.

A ZBA using oocytes from frozen-stored ovaries was evaluated in Paper V. The ZP of the canine oocyte is quite sticky (Ström Holst, unpublished), but as there was a significant difference in ZP binding capacity between untreated, killed, or a

50/50 mixture of untreated and killed spermatozoa, it could be concluded that the ZP binding capacity of spermatozoa is a feature of the living cell, not influenced to any larger extent by the stickiness of the ZP surface. The occasional presence of clusters is in contrast to the findings in the human by, e.g. Magerkurth et al. (1999), who reported a high degree of oocytes with a heterogeneous sperm-binding pattern. A heterogeneous sperm-binding pattern does thus not seem to be a reason to advise against a HZA in the canine species, although the greater need for time and labour with a HZA than a ZBA, as mentioned in the Background section, favours the use of a ZBA.

After having been evaluated, the ZBA was used for the evaluation of semen chilled for 1 or 4 days. The reduced ZP binding capacity of spermatozoa chilled for 4 days was clear, but not statistically significant, and there was a large variation in the number of spermatozoa bound between oocytes. The Tris-based extender that was used for chilling in the present study has previously been evaluated for its effect on sperm motility, PM integrity and acrosomal status by Rota et al. (1995). The decreases in sperm motility (74%-54%) and PM integrity (80%-72%) between Day 1 and Day 4 were proportionally smaller than the decrease in ZP binding capacity in the present study (4.8–0.9 spermatozoa per oocyte). Thus, the decrease in ZP binding capacity is likely a reflection of a reduced fertilising capacity of spermatozoa stored for 4d at 4°C.

For a ZBA to be practical, a reliable source of oocytes is necessary. Usually this means that oocytes need to be stored. Methods for long-term storage of oocytes to be used in gamete interaction assays include deep-freezing of ovaries or recovered oocytes, and salt-storage of oocytes. In Paper IV, the effects of deep-freezing of the ovaries and salt-storage of the oocytes on sperm-binding capacity and morphology of the ZP were evaluated using ZBA and SEM. It was found that fewer spermatozoa bound to stored than to freshly obtained oocytes, and that the fibrous network of the ZP of stored oocytes was wider than that of fresh ones.

In humans, mice, and goats, the meshwork of the ZP has been shown to become wider during oocyte maturation (Familiari et al. 1988, Calafell et al. 1992, Villamediana et al. 1998). In humans and bovines (Nikas et al. 1994, Suzuki et al. 1994), the network has been shown to get more compact after fertilisation, a change thought to represent the zona reaction. Thus, there seems to be a relationship between ZP ultrastructure and sperm-binding capacity, supported also by the findings in the present study, but recently questioned in the human (Magerkurth et al. 1999). The proportion of FRESS denuded oocytes binding spermatozoa in the present study is higher (50%) than the proportion of salt-stored cumulus enclosed oocytes (COCs) that bound spermatozoa in a previous study (36.5%) (Hay et al. 1997a). Hay et al. (1997a) speculated that either ZP hardening or changes of the cumulus cells caused this reduction in sperm-binding capacity. The results of the present study suggest that part of the reduced sperm-

binding capacity of stored COCs can be attributed to changes of the cumulus cells.

Although a larger ZP meshwork under physiological conditions has been related to an increased sperm-binding capacity, the morphological changes of the ZP described in Paper IV are probably reflections of damage caused by the process of storing. It thus seems likely that they do contribute to the reduced sperm-binding capacity of the stored oocytes. The reduced sperm-binding capacity of stored canine oocytes does not, however, exclude their use in a ZBA. An advantage of using oocytes from frozen-stored ovaries is that, after the oocytes from one ovary of a bitch have been recovered, the number of oocytes that can be recovered from the other ovary can be roughly estimated (Ström Holst et al., submitted). This information is of importance when a certain amount of oocytes must be obtained, e.g. for a ZBA.

In the canine, it has been suggested that both acrosome-intact and acrosome-reacted spermatozoa are capable of binding to the ZP (Kawakami et al. 1993b). It has also been reported that acrosome-intact spermatozoa adhere loosely to the zona surface and can be removed by pipetting (Mahi-Brown 1991). No clear correlation has been found between the percentage of capacitated or acrosome-reacted spermatozoa and the percentage of oocytes penetrated (Hewitt and England 1997). In the human, it has been speculated that acrosome-reacted spermatozoa have the capacity to loosely associate with the zona by a reversible attachment, whereas acrosome-intact spermatozoa will achieve irreversible binding (Franken 1998). In the bovine, *in vitro* capacitation leads to an increased capacity of spermatozoa to bind to the ZP (Topper et al. 1999). In the porcine, observations indicate that acrosome-intact spermatozoa initiate binding to the ZP (Fazeli et al. 1997). However, no relationship has been detected between acrosomal status and strength of sperm-zona binding in the porcine, which is why the relevance of a gentle washing procedure to remove loosely attached spermatozoa has been questioned (Lynham and Harrison 1998). In the porcine, it has been suggested that it is the physical orientation of spermatozoa that influences the strength of sperm-binding to the ZP (Lynham and Harrison 1998). The path of the canine spermatozoon through the ZP is angular (Mahi-Brown 1991), and it is possible that the wider ZP–meshwork of stored oocytes affects the orientation and strength of sperm-binding. Even if generally fewer spermatozoa bound after the tough than after the gentle wash, this difference was not statistically significant for the FRESS oocytes, suggesting that physical orientation alone does not determine the strength of the sperm-ZP binding in the canine species.

The ZBA is an assay that can be performed using oocytes from canine ovaries stored at -20°C, constituting a constant source of oocytes without involving complicated or expensive equipment. The sperm-binding capacity of oocytes stored this way is reduced, compared with fresh ones, but this does not exclude

their use in a ZBA. A drawback with the ZBA is the large variation in sperm-binding capacity among oocytes. As in the bovine (Zhang et al. 1995), several replicates should therefore be evaluated; three, as in the present study, being an absolute minimum. However, as the sperm-oocyte complexes can be stored before evaluation, this does not complicate the practical implementation of a ZBA to any particular extent. The ZBA is consequently an assay that can be recommended for evaluating frozen-thawed canine spermatozoa.

As the low thermoresistance of spermatozoa frozen-thawed with the Andersen and the CLONE method was one of the major findings when making *in vitro* evaluations of these methods, an attempt was made to improve this parameter by the addition of Equex STM paste to a cryopreservation extender. When evaluating the effect of the addition of Equex on the post-thaw survival time of dog spermatozoa, in contrast to the study of the Andersen and the CLONE procedures, not only motility but also PM integrity was evaluated both immediately post-thaw and during 3 hrs incubation at 38°C. In addition, they were both incubated in equal volumes of a Tris-buffer of known composition. One explanation for the clearly beneficial effect of adding Equex to the extender, on motility as well as PM integrity during incubation, is that it is caused by a delay of the changes leading to acrosome exocytosis. This is supported by the finding that the addition of Equex to canine semen has resulted in a higher number of spermatozoa with intact acrosomes after freezing-thawing and incubation at 38°C compared with controls (Peña and Linde-Forsberg, personal communication).

The fact that the PM integrity of spermatozoa frozen both with and without the addition of Equex was fairly constant from 1-3 hrs after thawing is interesting, and suggests that the changes apparent after one hour most likely were caused by latent damage caused by the cryopreservation process, rather than related to ageing. Evaluation of PM integrity immediately post-thaw and after 1h incubation at 38°C thus seems to be advantageous when evaluating cryopreservation techniques. As a consequence of the fact that motility decreased throughout the incubation period, the proportion of spermatozoa with intact PM was generally higher than the proportion of motile spermatozoa, and it is not excluded that some of these immotile spermatozoa would have been able to regain motility if inseminated into the female tract.

A beneficial effect of the addition of Equex STM paste to a cryopreservation extender on the molecular structures involved in ZP binding was demonstrated in Paper V, as significantly more spermatozoa frozen with than without Equex bound to the ZP. One explanation of this is likely to the protective effect exerted by Equex on PM integrity, as previously described.

Because of the prolonged oestrus of the bitch, with subsequent difficulties in determining the optimal time for mating, the prolonged post-thaw sperm survival obtained by adding Equex to a Tris- glucose-citrate extender would be expected

to result in higher pregnancy rates and larger litter sizes, especially after intravaginal AI, than when spermatozoa are frozen without the addition of Equex. The extenders used in Paper III have been used for an insemination trial, resulting in an overall pregnancy rate of 84% (Rota et al. 1999a). The addition of Equex had no significantly beneficial effect on the pregnancy rate, either after intravaginal or after intrauterine insemination, when an insemination dose of 200 million spermatozoa was used twice on 5 well-timed (by clinical examinations, vaginal smears and plasma progesterone analyses) bitches per group. The good timing and the relatively low number of animals used is likely to have contributed to mask the beneficial effect of Equex STM paste seen in *in vitro* studies, although, of course, it cannot be excluded that this beneficial effect does not have a correspondence with *in vivo* results. Further, the insemination dose used in the insemination trial is relatively high, especially considering that sub-optimal sperm numbers should be used to detect relationships between *in vitro* and *in vivo* fertility (Tardif et al. 1999), and is likely to have masked any difference between the groups. The total number of frozen-thawed spermatozoa per insemination dose that has been used to obtain high pregnancy rates after transcervical intrauterine insemination varies between $50x10^6$ (Wilson 1993) and $200x10^6$ (Farstad and Andersen Berg 1989). Usually, $150\text{-}200x10^6$ spermatozoa per insemination dose is recommended (Andersen 1980, Linde-Forsberg 1995), but the minimum sperm number needed to obtain both a high whelping rate and a large litter size after frozen-thawed semen AI in the canine remains to be determined. It is likely that the insemination dose needed will be reduced if sperm post-thaw survival is prolonged. The results of the above-mentioned study (Rota et al. 1999a) further stress the difficulties with insemination trials, and the importance of developing and evaluating new *in vitro* tests and combinations of *in vitro* tests, in order to be able to evaluate semen samples properly.

In species like the bovine, it has been demonstrated that a combination of *in vitro* tests will offer a better means of predicting fertility than a single test (Zhang et al. 1998, 1999). In the canine, because of the long oestrus period, the difficulties in determining the optimal time for AI might constitute a problem that is more pronounced than in other species. One consequence of this is that the post-thaw sperm survival time might be of greater importance than in other species. Another might be that single tests are even less predictive of field fertility than in other species, as the basis of the majority of *in vitro* tests is to measure an aspect of sperm function at a given time point, thus, in a way, measuring the homogeneity of the sperm population, when in clinical practice many inseminations will be performed at a sub-optimal time in the oestrous cycle, thus demanding a high degree of heterogeneity of the sperm population. However, even if heterogeneity is demanded, this does not exclude that sperm function at any given time point should admit the fertilising capacity at that certain point. Therefore, *in vitro* tests still can be expected to give information on the fertilising capacity of the spermatozoa, and, as a consequence, it also seems likely that a combination of different *in vitro* assays is clearly advantageous when evaluating canine sperm

function. In the present study, a technique for performing a new test for *in vitro* evaluation of canine spermatozoa; the ZBA using intact denuded oocytes, was developed, and the test was evaluated. The ZBA, in combination with assessments of motility and acrosome integrity immediately post-thaw, and PM integrity immediately post-thaw and after incubation 1h at 38°C, is likely to result in a better measure of damage inflicted on dog spermatozoa when developing new techniques for sperm storage.

Conclusions

- For *in vitro* evaluation of cryopreserved canine spermatozoa, motility and acrosome integrity immediately post-thaw, PM integrity immediately post-thaw and after incubation 1h at 38°C and a ZBA seem to be valuable assays.

- Using oocytes from canine ovaries that have been stored at -20°C, the ZBA is a test that is practical to perform.

- The addition of Equex STM paste to a cryopreservation extender had a significantly positive effect on zona pellucida binding capacity, membrane integrity immediately post-thaw, and on both motility and membrane integrity during incubation at 38 °C.

References

Andersen K (1972) Fertility of frozen dog semen. Acta vet scand, 13, 128-130.

Andersen K (1976) Artificial uterine insemination in dogs. Proc 8th Int Cong Anim Reprod AI, IV, 960-963.

Andersen K (1980) Artificial insemination and storage of canine semen. In: Morrow DA (ed): Current Therapy in Theriogenology: Diagnosis, Treatment and Prevention of Reproductive Diseases in Animals. Philadelphia, PA, Saunders, 661-556.

Arriola J and Foote RH (1987) Glycerolation and thawing effects on bull spermatozoa frozen in detergent-treated egg yolk and whole egg extenders. J Dairy Sci, 70, 1664-1670.

Berger T, Davis A, Wardrip NJ and Hedrick J (1989) Sperm binding to the pig zona pellucida and inhibition of binding by solubilized components of the zona pellucida. J Reprod Fertil, 86, 559-565.

Calafell JM, Nogués C, Ponsà M, Santaló J and Egozcue J (1992) Zona pellucida surface of immature and in vitro matured mouse oocytes: analysis by scanning electron microscopy. J Assist Reprod Genet, 9, 365-372.

Casey PJ, Hillman RB, Robertson KR, Yudin AI, Liu IKM and Drobnis EZ (1993) Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. J Androl, 14, 289-297.

CITES (1999) Convention on international trade in endangered species of wild fauna and flora. CITES-listed species database: fauna. Châtelaine-Genève, Switzerland.

Concannon PW and Battista M (1989) Canine semen freezing and artificial insemination. In: Kirk RW (ed) Current Veterinary Therapy X. WB Saunders Company, Philadelphia, 1247-1258.

Courtens JL, Ekwall H, Paquignon M and Plöen L (1989) Preliminary study of water and some element contents in boar spermatozoa, before, during, and after freezing. J Reprod Fertil, 87, 613-626.

Daurio CP, Gilman MR, Pulliam JD and Seward L (1987) Reproductive evaluation of male Beagles and the safety of ivermectin. Am J Vet Res, 48, 1755-1760.

Dott HM and Foster GC (1972) A technique for studying the morphology of mammalian spermatozoa which are eosinophilic in a differential live/dead stain. J Reprod Fertil, 29, 443-445.

Ellington JE, Ball BA and Yang X (1993) Binding of stallion spermatozoa to the equine zona pellucida after coculture with oviductal epithelial cells. J Reprod Fertil, 98, 203-208.

England GCW (1992) The cryopreservation of canine semen. Thesis, University of London.

England GCW and Allen WE (1989) Seminal characteristics and fertility in dogs. Vet Rec, 125:339.

Familiari G, Nottola SA, Micara G, Aragona C and Motta PM (1988) Is the sperm-binding capability of the zona pellucida linked to its surface structure? A scanning electron microscopic study of human in vitro fertilization. J In Vitro Fert Embryo Transf, 5, 134-143.

Farstad W (1984) Bitch fertility after natural mating and after artificial insemination with fresh or frozen semen. J Small Anim Pract, 25, 561-565.

Farstad W and Andersen Berg K (1989) Factors influencing the success rate of artificial insemination with frozen semen in the dog. J Reprod Fertil, 39 (Suppl), 289-292.

Fazeli AR, Steenweg W, Bevers MM, de Loos FAM, van den Broek J and Colenbrander B (1993) Development of a sperm zona pellucida binding assay for bull semen. Vet Rec, 132, 14-16.

Fazeli AR, Hage WJ, Cheng F-P, Voorhout WF, Marks A, Bevers MM and Colenbrander B (1997) Acrosome-intact boar spermatozoa initiate binding to the homologous zona pellucida in vitro. Biol Reprod, 56, 430-438.

Franken DR (1998) New aspects of sperm-zona binding. Andrologia, 30, 263-268.

Froman DP, Amann RP, Riek PM and Olar TT (1984) Acrosin activity of canine spermatozoa as an index of cellular damage. J Reprod Fertil, 70, 301-308.

Galibert F, Andre C, Cheron A, Chuat JC, Hitte C, Jiang Z, Jouquand S, Priat C, Renier C and Vignaux F (1998) The importance of the canine model in medical genetics. Bull Acad Natl Med, 182, 811-821.

Geussová G, Peknicová J, Capková J, Kaláb P, Moos J, Philimenko VV and Hozák P (1997) Monoclonal antibodies to canine intra-acrosomal sperm proteins recognizing acrosomal status during capacitation and acrosome reaction. Andrologia, 29, 261-128.

Goodrowe KL, Hay MA, Platz CC, Behrns SK, Jones MH and Waddell WT (1998) Characteristics of fresh and frozen-thawed red wolf (Canis rufus) spermatozoa. Anim Reprod Sci, 53, 299-308.

Hay MA, King WA, Gartley CJ and Goodrowe KL (1994) Influence of spermatozoa on in vitro nuclear maturation of canine ova. Biol Reprod, 50, Suppl 1, 362 (abstract).

Hay MA, King WA, Gartley CJ, Leibo SP and Goodrowe KL (1997a) Canine spermatozoa – cryopreservation and evaluation of gamete interaction. Theriogenology, 48, 1329-1342.

Hay MA, King WA, Gartley CJ, Leibo SP and Goodrowe KL (1997b) Effects of cooling, freezing and glycerol on penetration of oocytes by spermatozoa in dogs. J Reprod Fertil, Suppl 51, 99-108.

Hewitt DA and England GC (1997) The canine oocyte penetration assay; its use as an indicator of dog spermatozoal performance in vitro. Anim Reprod Sci, 50, 123-139.

Hewitt DA and England GC (1998) An investigation of capacitation and the acrosome reaction in dog spermatozoa using a dual fluorescent staining technique. Anim Reprod Sci, 51, 321-332.

IUCN (1996) The 1996 IUCN Red list of threatened animals in: The world conservation monitoring centre database, World Conservation Monitoring Centre, Cambridge, UK.

Ivanova M, Mollova M, Ivanova-Kicheva MG, Petrov M, Djarkova Ts and Somlev B (1999) Effect of cryopreservation on zona-binding capacity of canine spermatozoa in vitro. Theriogenology, 52, 163-170.

Januskauskas A, Håård MG, Håård M Ch, Söderquist L, Lundeheim N and Rodriguez-Martinez H (1996) Estimation of sperm viability in frozen-thawed semen from Swedish AI bulls. J Vet Med A, 43, 281-287.

Kawakami E, Vandevoort C, Mahi-Brown CA, Tollner TL and Overstreet JW (1993a) Comparison of a fluoresceinated lectin stain with triple staining for evaluating acrosome reactions of dog sperm. J Exp Zool, 265, 599-603.

Kawakami E, Vandevoort C, Mahi-Brown CA and Overstreet JW (1993b) Induction of acrosome reactions of canine sperm by homologous zona pellucida. Biol Reprod, 48, 841-845.

Kjaestad H, Ropstad E and Andersen Berg K (1993) Evaluation of spermatological parameters used to predict the fertility of frozen bull semen. Acta vet scand, 34, 299-303.

Koutsarova N, Todorov P and Koutsarov G (1997) Effect of pentoxifylline on motility and longevity of fresh and thawed dog spermatozoa. J Reprod Fertil, Suppl 51, 117-121.

Kumi-Diaka J (1993) Subjecting canine semen to the hypo-osmotic test. Theriogenology, 39, 1279-1289.

Lagerlöf N (1934) Morphologishe Untersuchungen über Veränderungen im Spermabild und in den Hoden bei Bullen mit verminderter oder aufgehobener Fertilität. Acta Path Microbiolog Scand, Suppl 19, Thesis, Stockholm.

Larsson K, Einarsson S and Nicander L (1976) Influence of thawing diluents on vitality, acrosome morphology, ultrastructure and enzyme release of deep frozen boar spermatozoa. Acta vet scand, 17, 83-100.

Linde-Forsberg C (1995) Artificial insemination with fresh, chilled extended, and frozen-thawed semen in the dog. Sem Vet Med Surg (Small Anim), 10, 48-58.

Linde-Forsberg C, Ström Holst B and Govette G (1999) Comparison of fertility data from vaginal vs intrauterine insemination of frozen-thawed dog semen: a retrospective study. Theriogenology, 52, 11-23.

Littell R, Milliken G, Stroup W, and Wolfinger R (1996) SAS® System for Mixed Models, SAS® Institute Inc., Cary, NC, USA.

Lynham JA and Harrison RAP (1998) Use of stored pig eggs to assess boar sperm fertilizing functions in vitro. Biol Reprod, 58, 539-550.

Magerkurth C, Töpfer-Petersen E, Schwartz P and Michelmann HW (1999) Scanning electron microscopy analysis of the human zona pellucida: influence of maturity and fertilization on morphology and sperm binding pattern. Hum Reprod, 14, 1057-1066.

Mahi-Brown CA (1991) Fertilization in dogs. In: Dunbar BS and O'Rand MG (eds) A comparative overview of mammalian fertilization, Plenum Press, New York, 281-297.

Mahi CA and Yanagimachi R (1976) Maturation and sperm penetration of canine ovarian oocytes in vitro. J Exp Zool, 196, 189-196.

Mahi CA and Yanagimachi R (1978) Capacitation, acrosome reaction, and egg penetration by canine spermatozoa in a simple defined medium. Gamete Research, 1, 101-109.

Martin JC, Klug E and Gunzel AR (1979) Centrifugation of stallion semen and its storage in large volume straws. J Reprod Fertil, 27 (Suppl), 47-51.

Mayenco-Aguirre and Pérez Cortés AB (1998) Preliminary results of hemizona assay (HZA) as a fertility test for canine spermatozoa. Theriogenology, 50, 195-204.

Mazur P (1984) Freezing of living cells: mechanisms and implications. Am J Physiol, 247, (Cell Physiol 16): C125-C142.

Mehra KL and Sarangi J (1967) Asymptotic efficiency of certain rank tests for comparative experiments. Ann Math Stats, 38, 90-107.

Morey DF (1994) The early evolution of the domestic dog. Am Scient, 82, 336-347.

Morton DB and Bruce SG (1989) Semen evaluation, cryopreservation and factors relevant to the use of frozen semen in dogs. J Reprod Fertil, 39 (Suppl), 311-316.

Nikas G, Paraschos T, Psychoyos A and Handyside AH (1994) The zona reaction in human oocytes as seen with scanning electron microscopy. Hum Reprod, 11, 2135-8.

Oettlé EE and Soley JT (1985) Infertility in a Maltese poodle as a result of a sperm midpiece defect. J S Afr Vet Ass, 56, 103-106.

Oettlé EE (1993) Sperm morphology and fertility in the dog. J Reprod Fert, 47 (Suppl), 257-260.

Oettlé EE (1986) Using a new acrosome stain to evaluate sperm morphology. Vet Med, 81, 263-266.

Peña AI, Barrio F, Quintela LA and Herradón PG (1998a) Proline and glycine betaine in a diluent for freezing canine spermatozoa. Reprod Dom Anim, 33, 5-9.

Peña AI, Barrio F, Quintela LA and Herradón PG (1998b) Effects of sodium dodecyl sulphate on post-thaw dog semen quality during in vitro incubation at 39°C and 22°C. Reprod Dom Anim, 33, 393-398.

Peña AI, Quintela LA and Herradón PG (1998c) Viability assessment of dog spermatozoa using flow cytometry. Theriogenology, 50, 1211-1220.

Peña A and Linde-Forsberg C: Personal communication, Dep OG, Faculty of Veterinary Medicine, Centre for Reproductive Biology, SLU, SE-750 07 Uppsala, Sweden.

Plummer JM, Watson PF and Allen WE (1987) A spermatozoal midpiece abnormality associated with infertility in a Lhasa Apso dog. J Small Anim Pract, 28, 743-751.

Pursel VG, Schulman LL and Johnson LA (1978) Effect of Orvus ES Paste on acrosomal morphology, motility and fertilizing capacity of frozen-thawed boar sperm. J Anim Sci, 47, 198-202.

Quinn PJ and White IG (1966) The effect of cold shock and deep-freezing on the concentration of major cations in spermatozoa. J Reprod Fertil, 12, 263-270.

Renton JP, Harvey MJA and Harker S (1986) A spermatozoal abnormality in dogs related to infertility. Vet Rec, 118, 429-430.

Rodriguez-Martinez H, Ekwall H and Linde-Forsberg C (1993) Fine structure and elemental composition of fresh and frozen dog spermatozoa. J Reprod Fertil, 47 (Suppl) 279-285.

Roomans GM (1988) Introduction to X-ray microanalysis in biology. J Electron Microsc Techn, 9, 3-17.

Rota A, Ström B and Linde-Forsberg C (1995) Effects of seminal plasma and three different extenders on canine semen stored at 4°C. Theriogenology, 44, 885-900.

Rota A, Iguer-Ouada M, Verstegen J and Linde-Forsberg C (1999a) Fertility after vaginal or uterine deposition of dog semen frozen in a Tris extender with or without Equex STM paste. Theriogenology, 51, 1045-1058.

Rota A, Peña AI, Linde-Forsberg C and Rodriguez-Martinez H (1999b) In vitro capacitation of fresh, chilled and frozen-thawed dog spermatozoa as assessed by the chlortetracycline assay and changes in motility patterns. Anim Reprod Sci, in press.

Sanchez-Partida LG, Windsor DP, Eppleston J, Setchell BP and Maxwell WM (1999) Fertility and its relationship to motility characteristics of spermatozoa in ewes after cervical, transcervical, and intrauterine insemination with frozen-thawed ram semen. J Androl, 20, 280-288

Spallanzani L (1780) Dissertazione di Fisica Animale e Vegetabile. Modena. English ed 1784: Dissertations relative to the natural history of animals and vegetables, translated from the Italian of the Abbé Spallanzani. Murray, London, vol II.

Ström Holst B, Larsson B, Rodriguez-Martinez H, Lagerstedt A-S and Linde-Forsberg C: Oocyte recovery from ovariohysterectomised bitches. Submitted for publication.

Suzuki H, Yang X and Foote RH (1994) Surface alterations of the bovine oocyte and its investments during and after maturation in vitro. Mol Reprod Fertil, 38, 421-30.

Szasz F, Cheng FP, Marks A, Colenbrander B and Solti L (1997) Induction of the acrosome reaction in dog sperm by calcium ionophore. Acta Vet Hung, 45, 177-187.

Söderquist L, Janson L, Larsson K, and Einarsson S (1991a) Sperm morphology and fertility in AI bulls. J Vet Med A, 38, 534-543.

Söderquist L, Rodriguez-Martinez H, and Janson L (1991b) Post-thaw motility, ATP content and cytochrome C oxidase activity of AI bull spermatozoa in relation to fertility. J Vet Med A, 38, 165-174.

Tardif S, Laforest J-P, Cormier N and Bailey JL (1999) The importance of porcine sperm parameters on fertility in vivo. Theriogenology, 52, 447-459.

Thomas PGA, Surman V, Myers-Wallen VN and Concannon PW (1992) Addition of sodium dodecyl sulphate to Tris-citrate extender improves motility and longevity of frozen-thawed canine spermatozoa. 12th Int Cong Anim Reprod AI, 4, 1823-1825.

Topper EK, Killian GJ, Way A, Engel B and Woelders H (1999) Influence of capacitation and fluids from the male and female genital tract on the zona binding ability of bull spermatozoa. J Reprod Fertil, 115, 175-183.

Tsutsui T (1975) Studies on the physiology of reproduction in the dog. V. On cleavage and transport of fertilized ova in the oviduct. Jap J Anim Reprod, 21, 70-75.

Tsutsui T and Ejima H (1988) Experimental induction of superfecundation in the dog. Jpn J Vet Sci, 50, 581-583.

Villamediana P, Ruttlán J, Lopez M, Martino A and Paramio MT (1998) Change in zona pellucida surface after in vivo and in vitro maturation of caprine oocytes. 50th anniversary of ICAR, Milano, Gametes: Development and Function, 612 (abstract).

Watson PF (1979) The preservation of semen in mammals. Oxford Rev Reprod Biol, 1, 283-350.

Watson PF (1990) Artificial insemination and the preservation of semen. In: Lamming GE (ed) Marshall's Physiology of Reproduction, Churchill Livingstone, London, Vol 2, 747-869.

Watson PF (1995) Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev, 7, 781-891.

Wilson MS (1993) Non-surgical intrauterine artificial insemination in bitches using frozen semen. J Reprod Fertil, 47 (Suppl), 307-311.

Woelders H (1991) Overview of in vitro methods for evaluation of semen quality. Rep Dom Anim, 1 (Suppl), 145-164.

Woelders H (1997) Fundamentals and recent development in cryopreservation of bull and boar semen. Vet Quart, 19, 135-138.

Yanagimachi R (1994) Mammalian fertilization. In: Knobil E and Neill JD (eds) The physiology of reproduction, 2nd ed, Raven Press, Ltd, New York, 189-315.

Zhang BR, Larsson B and Rodriguez-Martinez H (1995) Influence of batches of bovine oocytes on the outcome of an intact zona pellucida binding assay and in vitro fertilisation. Int J Androl, 18, 213-220.

Zhang BR, Larsson B, Lundeheim N and Rodriguez-Martinez H (1998) Sperm characteristics and zona pellucida binding in relation to field fertility of frozen-thawed semen from dairy AI bulls. Int J Androl, 21, 207-216.

Zhang BR, Larsson B, Lundeheim N, Håård MGH and Rodriguez-Martinez H (1999) Prediction of bull fertility by combined in vitro assessments of frozen-thawed semen from young dairy bulls entering an AI-program. Int J Androl, 22, 253-260.

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Populärvetenskaplig sammanfattning

Orsakerna till att man bedriver forskning om hundägg och –spermier är flera. Hunduppfödare, speciellt de med numerärt små raser, har nytta av metoder för spermalagring i sitt arbete med att bredda avelsbasen och hålla raserna sunda. Genom att frysa spermier och sedan lagra dem i flytande kväve kan dessa bevaras mycket länge, teoretiskt i mer än tusen år, för att därefter insemineras i tiken. Den lagrade sperman kan både lagras inom landet och skickas på export, och kan på så sätt bidra med nytt genetiskt material. Detta är mycket värdefullt också för arbetet med utrotningshotade vilda hunddjur (såsom till exempel röd varg och afrikansk vildhund), för vilka tamhunden kan användas som modelldjur.

Vid artificiell (konstgjord) insemination är det framför allt tre faktorer som påverkar dräktighetsresultatet: spermans kvalité, vid vilken tidpunkt i löpningen tiken insemineras, och hur inseminationen utförs. I arbetet med att utveckla metoder för att frysa hundsperma har man tidigare inte studerat metodernas påverkan på spermierna med hjälp av laboratorietester i någon nämnvärd utsträckning. Det har försvårat utvecklingen av nya metoder, eftersom man då inte har kunnat säga på vilket sätt spermien är påverkad av frysningen. I de två första delarbetena i denna avhandling studerades två olika frysmetoder, som båda ger bra dräktighetsresultat i klinisk verksamhet, med hjälp av olika laboratorietester. Man såg att det framför allt var en del av spermiehuvudet, akrosomen, som var förändrad. Dessutom såg man att spermiernas rörlighet snabbt avtog när de förvarades vid kroppstemperatur, och det är möjligt att det finns ett samband mellan dessa två fynd.

Att spermierna så snabbt förlorade rörligheten vid kroppstemperatur är sannolikt negativt, och skulle kunna innebära att tidpunkten när tiken ska insemineras är mycket viktig – och kort. Tikens löpningscykel är utsträckt i tiden, och för att bestämma när äggen är avlossade och har mognat krävs det ofta flera blodprov för hormonanalys. Det vore alltså sannolikt fördelaktigt om man kunde förlänga spermiernas överlevnadstid efter upptining, så att den tidsrymd under vilken tiken kan bli dräktig efter insemination blir längre. Dessutom skulle man kanske kunna få bättre dräktighetsresultat efter insemination i vagina om spermierna levde längre. Som det nu är krävs det att man inseminerar direkt in i livmodern för att få riktigt bra dräktighetsresultat med fryst sperma, och det är relativt komplicerat och kräver mycket träning. Om man kunde utveckla metoder för spermafrysning som fungerade även med insemination i vagina, skulle fler veterinärer kunna inseminera med fryst hundsperma. Dessutom skulle man med en bättre överlevnad på spermierna kanske kunna minska antalet spermier/dos, vilket vore särskilt bra för mindre hundraser, som inte ger så många spermier per ejakulat. I det tredje delarbetet i denna avhandling studerades effekten av en detergent, Equex, på spermiernas överlevnad efter upptining vid kroppstemperatur. Det visade sig att både spermiernas rörlighet och deras membran påverkades positivt

av Equex. Dessutom framkom att om man utöver att mäta proportionen spermier med intakta membran omedelbart efter upptining också gör det efter 1 timmes förvaring vid 38°C, så får man ett ytterligare mått på skador som tillfogats spermierna under frysningsprocessen.

För att så säkert som möjligt kunna avgöra spermiernas befruktningsduglighet utgående från laboratorietester, vill man göra flera tester, så att många aspekter av spermiens funktion utvärderas. Ett test som hos flera andra djurslag har visat sig värdefullt är spermiens förmåga att binda till ägget. Det är en egenskap som spermien måste besitta för att vara befruktningsduglig, och testet kan alltså vara ett bra komplement till andra tester (av till exempel spermiernas rörlighet, akrosomstatus och membranstatus). För att praktiskt kunna använda ett sådant test krävs en jämn tillgång på hundägg, och då är det bra om äggen kan lagras så att de finns tillgängliga när de behövs. I delarbete fyra studerades olika sätt att lagra hundägg, och hur lagringen påverkade äggens utseende och förmåga att binda spermier. Det framkom att skalet (zona pellucida) som omger äggen förändrades av lagringen; nätstrukturen som skalet har blev mer grovmaskig, och den spermiebindande förmågan försämrades. Försämringen uteslöt dock inte att lagrade ägg kunde användas i ett spermiebindningstest. I det femte och sista delarbetet undersöktes kylda och frysta spermier med avseende på deras förmåga att binda till ägget. Kylda spermier utvärderades efter en och fyra dagar vid 4°C, och man såg en tendens till att förmågan att binda till äggen försämrades. När man studerade spermier frysta med eller utan detergent, Equex, såg man att spermiens förmåga att binda till ägget påverkades positivt av Equex. Equex har alltså inte bara en positiv effekt på spermiernas rörlighet och membranintegritet, som sågs i delarbete tre, utan också på spermiens förmåga att binda till ägget. Ett test som mäter spermiens förmåga att binda till ägget kan således förmodas utgöra ett värdefullt tillskott till existerande laboratorietester för att mäta spermiens befruktningsförmåga, i arbetet med att utveckla nya och bättre metoder för hundspermafrysning.

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