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Flow Cytometry in the Assessment of Fresh and Frozen-Thawed Dog Semen, and the Effects of Different Cryopreservation Methods on Post-Thaw Sperm Survival and Longevity

Ana Isabel Peña Martínez

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Ana I. Peña Martínez

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Abstract

The use of flow cytometric methods for assessing sperm viability and acrosomal status of dog spermatozoa might facilitate the improvement and development of new cryopreservation protocols and allow accurate comparisons between different freezing treatments, since flow cytometry provides means of sperm assessment that are more accurate than conventional assays. The main objectives of the present studies were a) to validate the accuracy of flow cytometry techniques to assess viability and acrosomal status of canine spermatozoa separately or simultaneously and b) to evaluate cryopreservation protocols currently used for dog spermatozoa using flow cytometry.

The plasma membrane integrity of fresh dog spermatozoa stained by carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) was evaluated in the first study. Data obtained by flow cytometry analysis were compared with those obtained by microscopic evaluation under epifluorescence illumination and by phase contast evaluation of the samples stained with eosin-nigrosin. High correlation coefficients were found between the flow cytometry procedure and the 2 microscopy techniques. The results of this study validated the use of flow cytometry as a precise method for assessing the viability of dog spermatozoa. A procedure to simultaneously assess viability and acrosomal integrity of freshly ejaculated dog sperm by flow cytometry was developed in the second study. Sperm cells were stained with fluorescein isothiocyanate (FITC)-conjugated *Pisum sativum* agglutinin (PSA) and PI. Dog ejaculates were divided in aliquots and treated with different concentrations of lysophosphatidylcholine (LPC) to artificially induce the acrosome reaction in different proportions of spermatozoa. Data obtained by flow cytometric analysis of each sample were compared with those obtained by microscopic evaluation under epifluorescence illumination and by light microscopy evaluation of smears stained with Spermac^{R} staining. The results proved flow cytometry to be a precise method for evaluating the viability and acrosomal status of fresh dog semen samples. A new triple staining procedure was developed in the third study to assess frozen-thawed dog spermatozoa, where, in addition two cryopreservation methods (Andersen and CLONE) were compared. Plasma membrane integrity and acrosomal status of

spermatozoa were evaluated simultaneously by flow cytometry using the new combination of 3 fluorescent dyes: Carboxy-SNARF-1, to identify the live spermatozoa, PI to identify the dead or dying cells and FITC-PSA to detect acrosomal damage to spermatozoa. This new triple staining method provided an efficient procedure for evaluating frozen-thawed dog semen samples when using flow cytometry or fluorescence microscopy. Motility and plasma membrane integrity of spermatozoa immediately post-thaw were similar for the two cryopreservation methods, but the proportion of damaged acrosomes after thawing was lower, and the spermatozoa had a higher thermoresistance, when using the Andersen method than when using the CLONE method.

In the two last studies, different aspects of the cryopreservation protocol for dog spermatozoa were investigated, using flow cytometry and the triple staining procedure previously described to evaluate differences between the different treatments. In the fourth study, we evaluated the effects and interactions of: 1) adding 0.5% Equex STM paste to a Tris-egg yolk-based extender, 2) diluting the semen in 1 step (adding the Tris-egg yolk-based extender containing 5% glycerol and 0.5% Equex before the equilibration period) or in 2 steps (adding a first Tris-egg yolk-based extender containing 3% glycerol and 0% Equex before the equilibration period, and a second Tris-egg yolk-based extender containing 7% glycerol and 1% Equex after the equilibration period), 3) freezing according to 2 methods (placing the 0.5-mL straws horizontally 4 cm above liquid nitrogen in a styrofoam box or lowering them vertically into a LN_2 tank in 3 steps) and 4) thawing at 2 rates (70 ^{o}C for 8 sec and 37 ^{o}C for 15 s). The best post-thaw survival and thermoresistance of spermatozoa was obtained when Equex was present in the extender, the semen dilution was performed in 2 steps, the freezing was carried out using the styrofoam box and the straws were thawed at 70 ^{o}C for 8 s instead of at 37 ^{o}C for 15 s. In the fifth study, the effects of freezing dog semen with different sperm concentrations (50 x 10^6, 100 x 10^6, 200 x 10^6 and 400 x 10^6 spermatozoa/ mL) and diluting the semen post-thawing at different rates (1:0, 1:1, 1:2 and 1:4) were assessed. The best longevity was obtained when semen packaged at a concentration of 200 x 10^6 spermatozoa/ mL was diluted after thawing at 1: 4 dilution rate.

Key words: Dog spermatozoa, fluorescent staining, flow cytometry, cryopreservation.

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Ana Isabel Peña Martínez
Department of Obsterics anf Gyneaecology
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Key words: Dog spermatozoa, fluorescent staining, flow cytometry, cryopreservation.

Author's address: Ana Isabel Peña Martínez, Department of Obstetrics and Gynaecology, SLU, Box 7039, S-750 07 UPPSALA, Sweden.
Present address: Unit of Reproduction and Obstetrics, Department of Animal Pathology, Faculty of Veterinary Medicine, University of Santiago de Compostela, 27002 Lugo, Spain.

Contents

Appendix

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

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

II. Peña, AI., Quintela, LA. and PG. Herradón, 1999: Flow cytometric assessment of acrosomal status and viability of dog spermatozoa. Reprod. Dom. Anim. 34 (6): 495-502.

III. Peña, AI., Johannisson, A. and C. Linde-Forsberg, 1999: Post-thaw evaluation of dog spermatozoa using new triple staining and flow cytometry. Theriogenology 52: 965-980.

IV. Peña, AI. and C. Linde-Forsberg: Effects of Equex, one or two step dilution and two freezing and thawing rates on post-thaw survival of dog spermatozoa. Submitted for publication.

V. Peña, AI. and C. Linde-Forsberg: Effects of spermatozoal concentration and post-thaw dilution rate on survival after thawing of dog spermatozoa. Submitted for publication.

Introduction

The first scientific report on artificial insemination (AI) dates from 1787 and was performed by the Italian Lazzarro Spallanzani, in a bitch, using fresh semen. This technique described two centuries ago is still applied nowadays in the bitch when a physical or behavioural problem prevents natural mating. Despite AI technology first being described 200 years ago, it was not until 1949 that it made a significant impact on the reproduction of zootechnical species. The discovery of the cryoprotective effect of glycerol for spermatozoa by Polge et al. (1949) was the decisive step for developing the methodology of sperm cryopreservation. Since then, the sperm freezing technique has been further improved, and in many countries the use of AI with frozen semen in dairy cattle has almost entirely replaced natural mating, having enormous importance for livestock economy through genetic improvement by using superior sires, and by preventing the spread of veneral diseases.

The interest in the use of AI with frozen semen in dogs developed many years later than in farm animals. Traditionally, the interest of canine practitioners and dog owners was focused more on how to avoid reproduction than on how to enhance it. In the last two decades, however, that situation has substantially changed and AI with frozen-thawed semen has become a subject of world-wide interest. The value of purebred pups has been steadily increasing, and dog breeding has become an important economic as well as hobby activity. Consequently, the dog breeders' interest in AI with frozen semen has increased, although it is still rarely used. The use of AI technology with frozen semen allows the breeders to use dogs of high genetic value from all over the world and after their death, but it also allows the preservation of some ancient canine breeds, nowadays represented by reduced populations, and of species of wild canids that are threatened by extinction.

Since Rowson (1954) reported the first successful freezing of dog spermatozoa, and Seager (1969) the first pregnancy obtained after AI with frozen-thawed semen in the dog, considerable improvements in the methodology of sperm cryopreservation and AI in dogs have been achieved. Systematic studies on the reproductive physiology of the bitch have led to a greater understanding of the mechanism of ovulation (Concannon et al., 1977; 1986), gamete physiology and fertilization processes in this species (Mahi and Yamaginachi, 1978; Tsutsui, 1975; 1989), which has made it possible to accurately identify the time of optimal fertility in the bitch (Concannon et al., 1989; Jeffcoate and Lindsay, 1989). Significant advances have been made with techniques of AI, the pioneering Scandinavian workers having developed a method of non-surgical intrauterine insemination (Andersen, 1975) and it has been demonstrated that intrauterine insemination with frozen-thawed dog semen produces a higher pregnancy rate

than vaginal insemination (Linde-Forsberg et al., 1999). Although advances have also been made with extenders and freezing technology, the survival time of frozen-thawed dog spermatozoa at body temperature remains short (Concannon and Battista, 1989; Rota et al., 1997). Poor spermatozoal longevity after thawing is a probable cause of lower pregnancy rates obtained with vaginally inseminated semen (Gill et al., 1970; Farstad, 1984; Linde-Forsberg and Forsberg 1989, 1993; Linde-Forsberg et al., 1999). Recent evidence (Rota et al., 1999b) supports the hypothesis that cryopreserved dog spermatozoa display a membrane reactivity similar to capacitated spermatozoa, which accounts for their reduced longevity in the female tract. As capacitation-like changes of spermatozoa result from membrane destabilizing events, it has been suggested that significant increase in conception rates might be obtained by improving and/or developing new cryopreservation methods trying to prevent or minimize the occurrence of such membrane destabilizing events (Watson, 1995).

A cryopreservation protocol involves a series of processing steps (semen dilution with a cryoprotective extender, cooling, freezing and thawing), and each of them will have an influence on the membrane structure and function and cell metabolism. Besides, an interaction exists between the different steps of cryopreservation procedures, so that changes in one aspect of the overall protocol may directly modify the effects of the other variables (Hammerstedt et al., 1990). Modification of egg yolk lipoproteins with certain detergents has been demonstrated to significantly improve the cryoprotective effect of semen extenders in some species, including the dog (Graham et al., 1971; Arriola and Foote, 1987; Thomas et al., 1992; Penfold and Moore, 1993; Rota et al., 1997; Peña et al., 1998a). The addition of Equex STM Paste, which includes the detergent sodium dodecyl sulphate, to a Tris-egg yolk based extender was found to have a significantly positive effect on both motility and membrane integrity of dog spermatozoa during incubation at 38 ^{o}C (Rota et al., 1997), as well as on their in vitro capacity to bind to the zona pellucida of homologous oocytes (Ström Holst, 1999). Potential interactions between the effect of Equex STM Paste and the semen dilution method or the freezing and thawing rates have not been evaluated.

In some of the early studies on the cryopreservation of dog semen the pellet freezing method was used (Seager, 1969; Seager and Fletcher, 1973; Seager and Platz, 1977). Although the post-thaw survival of spermatozoa was found to be similar using both the pellet and straw freezing methods (Davies, 1982; Battista et al., 1988), freezing in straws allows easier identification and handling of the frozen semen and is less susceptible to contamination. Therefore, today this is the method adopted in most of the cryopreservation protocols for dog spermatozoa, although other alternative packaging systems have also been used (Foote, 1964; Ivanova-Kicheva et al., 1997b). Freezing in 0.5-mL straws, semen is commonly packaged at concentrations varying between 40 x 10^6 and 200 x 10^6 spermatozoa/mL (Farstad and Andersen-Berg, 1989; Olar et al., 1989; Dobrinski

et al., 1993; Thomas et al., 1993; Silva and Verstegen, 1995; Ström et al., 1997; Rota et al., 1999a), so that, depending on the post-thaw survival rate, one to five 0.5 mL-straws are necessary to obtain an insemination dose with at least 100 x 10^6 progressively motile spermatozoa (Farstad and Andersen Berg, 1989). Whether the concentration of spermatozoa per 0.5 mL-straw has any influence on the post-thaw sperm survival in this species is not known.

In many studies, attempts have been made to determine the optimal freezing and thawing rates for dog spermatozoa (Davies, 1982; Olar et al., 1989; Dobrinsky et al., 1993; Hay et al., 1997; Rota et al., 1998a). Freezing rates found to be optimal have varied in the different studies and results are still not conclusive, but in contrast, it seems that fast thawing rates are more suitable for dog spermatozoa (Davies, 1982; Smith, 1984; Morton, 1988; Ivanova-Kicheva et al., 1997a; Rota et al., 1998a). Little attention has been paid, however, to the effects of using thawing diluents, which are employed in some cryopreservation protocols (Rota et al., 1997; 1998ab; 1999a; Ström et al., 1997; Linde-Forsberg et al., 1999) to reduce the glycerol and egg yolk concentrations in the thawed spermatozoa and/or to increase the volume of the insemination dose.

The most accurate method to evaluate the fertilizing capacity of a semen sample or the success of a cryopreservation protocol is, obviously, the insemination of a large number of females (Amann, 1989). Fertility trials, however, are very expensive and have a low sensitivity when the number of bitches used is small, and they can only be used with great difficulty to identify the sperm function affected by a given treatment. Besides, quantitative differences are often obscured because semen dosages exceed those that would yield a "linear" fertility response (Saacke, 1982; Amann and Hammerstedt, 1993; Saacke et al., 1994). There is little doubt that objective measures of sperm functional and morphological characteristics may be significantly correlated with their fertilizing potential (Amann, 1989), thus, it is not surprising that researchers have been attempting to use *in vitro* technologies to measure the fertilizing potential of sperm by the most objective means possible. All spermatozoa in an ejaculate or in a thawed semen sample are not functionally equivalent, and although it is not known which attributes differentiate a fertile spermatozoon from an infertile one (Amann, 1989), there are several sperm characteristics that are known to be necessary for a spermatozoon to be able to fertilize, and that can be evaluated using *in vitro* methods. Therefore, to measure the fertilizing potential of a semen sample it is essential that tests of several independent parameters are made (Hammerstedt et al., 1990).

Although motility is only one of the many important attributes of a fertile spermatozoon, it was the first and continues to be the most often used indicator of sperm function. Visual estimation of motile spermatozoa is the most simple, rapid and inexpensive method for evaluating semen quality; unfortunately, it is highly subjective and therefore, not a reliable assay for predicting fertility (Saacke and

White, 1972; Linford et al., 1976). Thus, in an attempt to eliminate the subjective nature of the visual method for the assessment of sperm motility, Computerized Assisted Sperm Analysis (CASA) systems have been developed, and are being continually refined since they were first introduced in the early 1980's (Amann, 1979; Amann and Hammerstedt, 1980; O'Connor et al., 1981). CASA systems allow us to obtain objective and precise information not only about the proportion of motile cells in a semen sample but also about the quality of their movement. As the trajectories of individual spermatozoa are determined by their flagellar function, characteristics such as sperm velocity, flagellar beat frequency and amplitude will correctly reflect the physiological status of individual cells. Large sets of data derived from hundreds of individual sperm measurements are provided by CASA systems, giving interesting information about the mean quality of the motility in a semen sample (Amann, 1988; Anzar et al., 1991; Tuli et al., 1992; Ellington et al., 1993; Günzel-Apel et al., 1993) but also the different sperm subpopulations coexisting in a semen sample can be identified based on the characteristics of their movement (Davies et al., 1995; Holt, 1996).

Reduced fertility of frozen-thawed semen is largely attributed to altered membrane structure and function during cooling, freezing and thawing (Hammersted et al., 1990; Parks and Graham, 1992). Intact plasma and acrosomal membranes are required for a spermatozoon to remain viable during its transit through the female genital tract and to penetrate across the structures surrounding the oocyte. Several methods have been developed to assess the integrity of the sperm membranes. Conventional tests for sperm membrane integrity, such as staining with eosin and nigrosin (Dott and Foster, 1972), are poorly correlated with fertility (Graham et al., 1990). Besides, supravital stains are not effective in assessing viability of cryopreserved spermatozoa because glycerol, a necessary ingredient in most cryopreservation media, interferes with differential staining. The use of different fluorescent dyes, excited by a light of an appropriate wavelength, has proved to be a reliable method for assessing sperm viability. Furthermore, the use of fluorophores has eliminated the problem of observing spermatozoa in opaque media. Fluorescence stained sperm cells can be assessed for both morphological and functional aspects without interference from fat globules or other nonstaining extraneous material (Ericsson et al., 1989). Over the last decade, fluorescence microscopy has been the primary technique used for evaluating semen quality with fluorescent stainings. Scoring such slides is laborious, time-consuming, and a subjective process because only a limited number of cells can be evaluated for each sample. Flow cytometry, however, provides an objective and accurate method to evaluate sperm cells using fluorescent probes; it allows the evaluation of multiple characteristics simultaneously in the same sample since a double or triple staining procedure can be used, and data on thousands of cells can be acquired for each sample in a short period of time (Harrison and Miller, 1998). Until recently, the cost of flow cytometry equipment has prevented many research laboratories and commercial centers from using this technique. However, with the appearance on the market of

flow cytometers with comparatively inexpensive, air-cooled lasers, and personal computer hardware and software for data accumulation and analysis, these instruments have become increasingly useful for assessment of sperm quality characteristics in several mammalian species.

Some fluorescent stainings and combinations of fluorophores have been developed and tested by flow cytometry in different species to quantify proportions of live and dead cells (Ericsson et al., 1989; Garner et al., 1986; 1988; 1994; 1999; Garner and Johnson, 1995; Maxwell et al., 1997), to assess the mitochondrial function (Evenson et al., 1982; Graham et al., 1990; Papaioannou et al., 1997; Garner et al., 1999), the acrosomal status (Graham et al., 1990; Tao et al., 1993; Uhler et al., 1993; Asworth et al., 1995; Garner et al., 1999) and the DNA content (Dresser et al., 1993; Evenson, 1989; Evenson et al., 1991). In addition, when using this technique it is possible to produce aliquots of sexed sperm for insemination or for in vitro fertilization (Garner et al., 1983; Johnson et al., 1989; Pinkel et al., 1985).

Garner et al. (1986) and Harrison and Vickers (1990) developed a sensitive fluorescence-based method for the assessment of membrane integrity in spermatozoa from several species. They used propidium iodide (PI), which stains the nuclei of dead cells or those with damaged membranes, causing them to emit red fluorescence, and carboxyfluorescein diacetate (CFDA), which is able to permeate the cell membranes where it is converted into a green fluorescent membrane-impermeable compound which is trapped intracellularly by intact membranes.

The most common method to detect the acrosomal changes using fluorescent probes is staining with fluorescein-conjugated lectins, such as *Pisum sativum* agglutinin (PSA) coupled with fluorescein isothiocyanate (FITC). *Pisum sativum* agglutinin has been proposed as an acrosomal stain based on the empirical finding that the lectin selectively binds to acrosomal proteins from mammalian spermatozoa and that a major PSA-reactive protein is human proacrosin (Mendoza et al., 1992). Cross et al. (1986) and others (Centola et al., 1990; Mendoza et al., 1992; DasGupta et al., 1993) reported the use of FITC-PSA to detect intact, rather than reacted acrosomes, in human spermatozoa, using fluorescence microscopy or flow cytometry. In these experiments, sperm cells were fixed and their membranes permeabilized to expose the acrosomal contents to the PSA lectin. The use of FITC-PSA on permeabilized cells was also reported to stain intact acrosomes of spermatozoa from a variety of species: stallion (Farlin et al., 1992), dog (Kawakami et al., 1993), ram (Sukardi et al., 1997), mouse (Tao et al., 1993). Graham et al. (1990) using bovine spermatozoa, showed that fluorescence labelled PSA could be used with flow cytometry to assess the percentage of cells undergoing the acrosome reaction, since a reacting or reacted spermatozoon with some of the matrix intact would bind PSA. This staining method was validated by electron microscopy to detect acrosome integrity in

bovine spermatozoa (Nolan et al., 1992). The same method was later used to quantify the proportion of acrosome-reacted spermatozoa by flow cytometry in boar, ram (Ashworth et al., 1995) and bovine spermatozoa (Maxwell et al., 1997). When using flow cytometry, FITC-PSA is usually combined with PI for the simultaneous assessment of cell viability and acrosomal integrity (Graham et al., 1990; Maxwell et al., 1997; Maxwell and Johnson, 1997; Sukardi et al., 1997). Other fluorescence labelled lectins, such as Concanavallin A, wheat germ agglutinin or peanut agglutinin, have been used for the acrosomal staining of human (Holden et al., 1990), bovine (Medeiros and Parrish, 1996) and mouse (Tao et al., 1993) spermatozoa, being reported to bind more specifically to the acrosomal region than the PSA.

Simultaneous staining with 3 specific stains has been used to quantify specific characteristics of sperm organelles considered to be of importance for the fertility of a semen sample. Graham et al. (1990) used the combined staining with Rhodamine 123 for mitochondrial assessment, PI for viability and Phycoerythrin (PE)-conjugated PSA for the acrosomal status, and they demonstrated that by adding three probes to sperm cells, three sperm compartments can be simultaneously analyzed by flow cytometry.

The use of flow cytometry for sperm analysis is an attempt to overcome the problem of the subjective nature of the manual methods commonly used for semen analysis (Ferrara et al., 1997), since it allows thousands of cells in each sample to be assessed in few seconds. The results obtained using flow cytometric techniques suggest that this method will become more frequently used not only as a routine tool in research but also in commercial artificial insemination centers.

Aims of the study

The aims of this study were:

- To evaluate the effectiveness of flow cytometry methods to assess the plasma membrane integrity and acrosomal status of fresh and frozen-thawed dog spermatozoa.

- To apply flow cytometry methods to evaluate several aspects of cryopreservation protocols currently used for dog spermatozoa, more specifically:

  a) To compare viability parameters of dog spermatozoa cryopreserved using two methods known to give high pregnancy rates in vivo.

  b) To study the effects and interactions of: 1) adding Equex STM Paste to a Tris-egg yolk based extender, 2) diluting the semen in 1 or 2 steps, 3) freezing according to two methods and 4) thawing at two rates, on the post-thaw survival and longevity of dog spermatozoa during incubation at 38 ^{o}C.

  c) To study the effects and interactions of: 1) freezing dog semen using four different sperm concentrations and 2) diluting the semen immeditately post-thaw with Tris buffer at four dilution rates, on the post-thaw survival and longevity of dog spermatozoa during incubation at 38 ^{o}C.

Materials and Methods

Animals

The dogs in papers I and II (two Beagles, one English setter and one crossbred) belonged to the Department of Animal Pathology of the Faculty of Veterinary Medicine of Lugo, USC, Lugo. Their ages ranged between 3 and 7 years. A total of 17 ejaculates were obtained from the 4 dogs. Only ejaculates with at least 80% motile spermatozoa were included in the studies.

In papers III-V, 24 dogs of different breeds (2 Bavarian Schweisshunds, 8 Beagles, 1 Briard, 1 German Shepherd, 9 Labrador retrievers and 3 crossbreds) were used. The ages of the dogs ranged between 10 months and 8 years. Fifteen of the 24 dogs were privately owned whereas the other 9 (7 Beagles and 2

crossbreds) belonged to the Department of Small Animal Clinical Sciences at the Faculty of Veterinary Medicine, SLU, Uppsala. A total of 35 ejaculates with more than 80% morphologically normal and motile spermatozoa were obtained from the 24 dogs.

Semen processing

Ejaculates were collected by digital manipulation using glass funnels and 10 mL-calibrated tubes (papers I and II), or calibrated plastic vials (papers III-V), prewarmed at 37°C. Only sperm-rich fractions of the ejaculates were collected. The volumes and sperm concentrations were recorded. The concentration of spermatozoa was determined using a photometer (Spectronic 20D; Milton Roy Company, Philadelphia, USA, in papers I and II, or SpermaCue, Minitüb, Tiefenbach, Germany, in Papers III-V). The percentage of progressively motile spermatozoa was determined by subjective microscopic examination at a magnification of x400 on a pre-warmed slide at 37°C. Ejaculates were processed individually (papers I-III) or mixed and processed as a single pool (papers IV and V).

In papers I and II, individual ejaculates were diluted at 1:3 (v:v) ratio with a Tris-fructose-citric acid buffer, filtered through a 30 μm nylon mesh (Millipore UK Ltd, Watford, Hertfordshire, UK) to remove any large debris, and washed by centrifugation. The sperm pellets were diluted to 1 mL with Tris buffer and the concentration was adjusted to 40 x 10^6 (paper I) or to 80 x 10^6 (paper II) spermatozoa/mL.

In paper I, each sperm suspension was divided in 2 halves of equal volume. One fraction was maintained at 30°C, while the cells in the other fraction were killed by 3 cycles of plunging into liquid nitrogen (LN_2) and thawing at 37°C. Then 1 mL samples were made by combining aliquots of "live" and "dead" sperm cells at ratios of 1: 0, 1: 1, 1: 3 and 3: 1. Subsamples of 100 μL were removed for light microscopy evaluation using the eosin-nigrosin staining (WHO, 1992), and the remainder of the sperm suspension was fluorescence labelled for fluorescence microscopy and flow cytometry analysis.

In paper II, each sperm suspension was divided in five 1 mL-aliquots, each treated with a different concentration (0-288 μmol/l) of egg yolk lysophosphatidylcholine (LPC; Fluka Chemie AG, CH-9470, Switzerland), and incubated at 39°C for 20 min. Subsamples of 20 μL were removed for light microscopy evaluation using the Spermac^R staining (Oettlé and Soley, 1988) and the remaining sperm suspension was fluorescently labelled for fluorescence microscopy and flow cytometry analysis.

In papers III-V, semen was processed for cryopreservation. In paper III, each ejaculate was split in 2 equal volumes, which were centrifuged and rediluted

according to the Andersen (Andersen 1975) and CLONE freezing methods, respectively. In paper IV, each ejaculate was centrifuged and the seminal plasma discarded. The sperm pellets were rediluted with 2 mL of a first extender and pooled. The semen pool was divided into aliquots and each processed according to a 2 x 2 x 2 x 2 factorial design, with the following factors: 1) addition of 0% or 1% Equex STM Paste (Nova Chemical Sales, Scituate, Inc., MA, USA) to an Extender 2, 2) addition of the second extender before or after the equilibration period, 3) freezing the straws horizontally above LN_2 in a styrofoam box or by lowering them vertically into a LN_2 tank in 3 steps, and 4) thawing at 70°C for 8 s or at 37°C for 15 s. In paper V, ejaculates were pooled and the pool was divided in 4 aliquots containing different numbers of spermatozoa. Each sperm aliquot was centrifuged, the seminal plasma discarded and the sperm pellets rediluted in two steps (Extender 1 + Extender 2) to obtain final sperm concentrations of 400 x 10^6, 200 x 10^6, 100 x 10^6 and 50 x 10^6 spermatozoa/mL, respectively.

Semen extenders

In paper III, the semen frozen by the Andersen method (Andersen, 1975) was extended in a Tris-fructose-citric acid extender containing 6.4% (v/v) glycerol and 20% (v/v) egg yolk. The CLONE method is commercial, developed by Cryogenetic Laboratories of New England, and uses extenders of proprietary composition. In papers IV and V, semen was first diluted with a Tris-glucose-citric acid extender containing 3% (v/v) glycerol and 20% (v/v) egg yolk (Extender 1) (Rota et al., 1997). A second Tris-glucose-citric acid extender containing 7% (v/v) glycerol, 20% (v/v) egg yolk and 0% (paper IV) or 1% (v/v) Equex STM Paste (papers IV and V) (Extender 2) (Rota et al., 1997) was added before (paper IV) or after (papers IV and V) the equilibration period.

Freezing and thawing methods

For equilibration, the extended semen was placed in a cooler at room temperature, and it reached 4°C in about 45 min. The equilibration period, from the onset of cooling, varied for the different methods. In paper III, the semen frozen according to the Andersen method was equilibrated for approximately 2 h, whereas the semen frozen according to the CLONE method was equilibrated for approximately 1h. In papers IV and V, the equilibration period was approximately 1 h. Semen was frozen in 0.5-mL straws, placing the straws 4 cm above the surface of LN_2 for 10 min (Andersen method in paper III, "box method" in paper IV, paper V) in a styrofoam box, or by lowering them in 3 steps in an Apollo SX-18 LN_2-tank (Minnesota Valley Engineering, Inc., New Prague, MN, USA) (CLONE method in paper III, "tank method" in paper IV).

The semen was thawed by immersing the straws in a waterbath at 37°C for 15 s (papers III and IV) or at 70°C for 8 s (papers III-V). After thawing, the content of

each straw was emptied in 1 mL of a thawing medium (paper III) or Tris-glucose-citric acid buffer (paper IV) at 37°C. In paper V, the content of each thawed straw was divided into four aliquots and each one diluted with a Tris buffer at 1: 0, 1: 1, 1: 2 or 1: 4 (v : v) dilution rates, respectively.

Semen evaluation

Sperm morphology
Sperm morphology of individual (paper III) or pooled ejaculates (papers IV and V) was assessed by phase contrast microscopy in samples fixed in buffered formol saline (Bane, 1961), and by light microscopy, in carbol fuchsine-stained smears (Williams, 1920).

Sperm motility
The percentage of motile spermatozoa in fresh semen samples was evaluated subjectively at a magnification of x400 using a phase contrast microscope (papers I-V). Post-thaw sperm motility was estimated subjectively after thawing and after incubation post-thaw at 37°C (paper III) or 38°C (papers IV and V) by phase contrast microscopy at x400 magnification, and (paper IV) by using a CASA system (Strömberg-Mika Cell Motion Analyzer, Bad Feilnbach, Germany). The motility parameters obtained using the CASA system were: percentage of total motile sperm (mot %), percentage of progressively motile sperm (PM%), straight line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF).

Evaluation of sperm plasma membrane integrity and acrosomal status
Sperm plasma membrane integrity and acrosomal status of fresh (papers I and II) and frozen-thawed (papers III-V) spermatozoa were assessed by microscopy (papers I-III) and flow cytometry (papers I-V) methods. Sperm plasma membrane integrity and acrosomal status of cryopreserved semen samples (papers III-V), were evaluated immediately post-thaw and at time intervals after incubation post-thaw at 37°C (paper III) or at 38°C (papers IV and V).

Microscopic methods
Sperm plasma membrane integrity and acrosomal status of spermatozoa were assessed by epifluorescence microscopy in fresh (papers I and II) and frozen-thawed (paper III) dog semen samples fluorescently labelled with: Carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) (paper I), PI and fluorescein (FITC)-conjugated *Pisum sativum* agglutinin (PSA) (paper II), or Carboxy-SNARF-1 (SNARF), PI and FITC-PSA (paper III). Light microscopy was also used to assess the plasma membrane integrity of fresh semen samples (paper I) stained with the eosin-nigrosin staining (WHO, 1992) and the acrosomal morphology of fresh spermatozoa (paper II) stained with Spermac^{R} (Stain

Enterprises, P.O. Box 152, Wellington, South Africa), as described by Oettlé and Soley (1988).

Flow cytometry

In papers I and III, sperm plasma membrane integrity was assessed using the dual fluorescent staining CFDA-PI. In papers II-V, sperm plasma membrane integrity and acrosomal status of spermatozoa were simultaneously evaluated using two combinations of fluorophores: PI and PSA (paper II) and SNARF, PI and PSA (papers III-V).

Fluorescent stainings

Dual staining CFDA / PI

A 2 mM stock solution of CFDA (Sigma, St. Louis, MO, USA; or Calbiochem, La Jolla, CA, USA) in DMSO and a 1.5 mM stock solution of PI (Sigma, St. Louis, MO, USA) in PBS were prepared and stored frozen. Aliquots of fresh spermatozoa of 900 µL (paper I) and of frozen-thawed spermatozoa of 200 µL (paper III) were stained with CFDA and PI to final concentrations of 5 and 50 µM, respectively, and incubated at 37°C (paper I) or at room temperature (paper III) during 15 min before the cells were analyzed by flow cytometry. In paper III, the 200 µl-aliquots of fluorescent stained spermatozoa were further diluted with 300 µL of Tris buffer after the staining incubation. Semen samples to be analysed by epifluorescence microscopy (paper I) were stained with CFDA and PI following the method described by Harrison and Vickers (1990), with a low concentration of formaldehyde included in the staining solution to immobilize the live cells.

Dual staining FITC-PSA / PI

A 2 µM stock solution of FITC-PSA (Sigma, St. Louis, MO, USA) in PBS was prepared and stored frozen. To fresh semen aliquots of 1.080 mL (paper II) a 60 µL volume of FITC-PSA and a 20 µL volume of PI were added. Samples were incubated at 39°C for 15 min, afterwhich aliquots of 80 µL were taken, diluted in 240 µL of saline medium (Harrison and Vickers, 1990) and stored a maximum of 45 min at room temperature and in the dark until their assessment under epifluorescence illumination. The remaining samples of 1 mL were analysed by flow cytometry.

Triple staining SNARF / PI / FITC-PSA

A 10 mM solution of SNARF (Molecular Probes, Inc., Eugene, OR, USA) in DMSO was prepared just before use. The DMSO solution was diluted in PBS to a final concentration of 100 µM. To 100 or 200 µl-aliquots of thawed semen (papers III-V), SNARF was added to a final concentration of 25 µM, PI to 50 µM and FITC-PSA to a final concentration of 0.1 µM. The semen samples were incubated at room temperature (paper III) or at 38°C (papers IV-V) and in the dark for 30 min and analysed by flow cytometry. In paper III, the triple stained

samples, after the flow cytometric analysis, were evaluated by epifluorescence microscopy, without including any fixative in the sperm suspensions.

Epifluorescence microscopy
In papers I and II, fluorescent staining of spermatozoa was evaluated with an epifluorescent Olympus microscope equipped with a FITC filter set (BP 485 excitation filter, FT 510 dichromatic beam splitter and a LP 520 barrier filter). In paper III, a Diaplan Leitz microscope equipped with a filter H3-I3 block (Leitz 513673-719, blue excitation range, with a BP420-490 excitation filter) was used. A 5 µl-aliquot of fluorescent stained sperm suspension was pipetted onto a clean slide and covered with a coverglass. Two replicates were made from each sample and a minimum of 100 cells were counted in each slide.

Flow cytometry
Flow cytometric analysis were performed by using an Epics XL flow cytometer (Coulter Corporation, Miami, FL, USA) (papers I and II) or a Facstar Plus flow cytometer (Becton Dickinson, San José, CA, USA) (papers III-V). The stained cells were excited by air-cooled argon ion lasers operating at 488 nm. Fluorescence data from at least 10,000 stained spermatozoa were collected in list mode. Green fluorescence (from CFDA or FITC) was collected in the FL1 sensor using a 525 nm (papers I and II) or a 530/30 nm (papers III-V) band-pass filters while red fluorescence (from SNARF and PI) was collected in the FL3 sensor through a 620 band-pass filter. Flow cytometric data in papers I and II were analysed using the System II program (Coulter Corporation, Miami, FL, USA), and in papers III-V, they were analysed using the program Cell Quest 1.2.2. (Becton Dickinson, San José, CA, USA).

Statistical Analysis

Pearson correlations and Regression analysis were used to compare microscopic methods with Flow cytometry for the assessment of plasma and acrosomal membrane integrity of dog spermatozoa. In papers I and II, data were analysed using the JMP programme (SAS Institute Inc., Cary, North Carolina, USA), while in paper III, they were analysed using the Minitab statistical software (Minitab Inc, State College, PA). To determine differences between the Andersen and CLONE freezing methods (paper III), paired Student's t-tests were used to compare means and standard deviations at each time point after thawing. In papers IV and V, data on post-thaw sperm motility, motility parameters, sperm plasma membrane integrity and acrosomal status were analyzed using the general linear model procedure (GLM, Minitab statistical software) at each evaluation time. When significant differences were detected in the models, differences among treatments were analysed using Tukey's Studentized Range (HSD) Test using the SAS programme (Cary, North Carolina, USA) and were considered significant at the $P<0.05$ level.

Results

Effectiveness of flow cytometry methods for assessing sperm viability and acrosomal status of fresh and frozen-thawed dog spermatozoa (papers I, II and III)

High and significant ($P<0.001$) correlation coefficients were found between proportions of live ($r=0.90$) and dead ($r=0.91$) spermatozoa stained by the combination CFDA/PI when analysed by flow cytometry and epifluorescence microscopy (paper I). The eosin-nigrosin staining was also highly correlated ($P<0.001$) with the CFDA/PI staining when the semen samples were analysed by flow cytometry ($r=0.90$ and 0.84 for proportions of live and dead cells, respectively) and by fluorescence microscopy ($r=0.87$ and 0.88 for proportions of live and dead cells, respectively).

When fresh dog semen samples were stained only with PI, to detect percentages of spermatozoa with damaged sperm membranes (paper II), significant correlation coefficients ($P<0.001$) were also observed between epifluorescence microscopy and flow cytometry methods ($r=0.79$ for both proportions of live and dead cells).

For assessing sperm viability in frozen-thawed semen samples (paper III), the combination SNARF/PI was found highly and significantly ($P<0.001$) correlated with the CFDA/PI staining when paired samples stained with both combinations of fluorophores were analysed by flow cytometry ($r= 0.96$ for both percentages of live and dead cells), or when CFDA/PI stained samples analysed by flow cytometry were compared with SNARF/PI stained samples analysed by epifluorescence microscopy ($r=0.92$ and 0.91 for proportions of live and dead cells, respectively).

The assessment of the acrosomal status of fresh semen samples (paper II) stained with Spermac^R and analysed by light microscopy was significantly correlated ($P<0.001$) with that of samples stained by FITC-PSA and analysed by both fluorescent methods (fluorescence microscopy and flow cytometry). The correlation coefficient obtained between the Spermac^R staining and the FITC-PSA staining for detecting proportions of spermatozoa with reacted acrosomes was higher when the latter was analysed by flow cytometry ($r=0.72$) than when it was analysed by epifluorescence microscopy ($r=0.56$). The correlation coefficient between flow cytometry and epifluorescence microscopy for detecting proportions of acrosome-reacted spermatozoa was 0.51. Both fluorescence methods were compared in order to quantify populations of live and dead spermatozoa with intact or damaged acrosomes, stained with FITC-PSA and PI,

and the correlation coefficients ($P<0.001$) obtained were: 0.78, 0.64, 0.26 and 0.58 for subpopulations of acrosome-reacted dead spermatozoa, acrosome-reacted live spermatozoa, acrosome-intact dead spermatozoa and acrosome-intact live spermatozoa, respectively.

In paper III, acrosomal status and viability were simultaneously evaluated in frozen-thawed dog semen samples stained by the combination of SNARF, PI and FITC-PSA and analysed by flow cytometry and epifluorescence microscopy. Both fluorescent methods were significantly correlated ($P<0.001$). Correlation coefficients between the two methods for subpopulations of live spermatozoa with intact acrosomes, dead spermatozoa with intact acrosomes and dead spermatozoa with reacted acrosomes were 0.94, 0.79 and 0.88, respectively. A small population of live spermatozoa with reacted acrosomes ranging between 0 % and 2 % was detected with both methods.

Evaluation of cryopreserved dog spermatozoa processed with the Andersen and CLONE methods (paper III)

Motility immediately after thawing was not different for the Andersen (74.0±8.4%) and CLONE (69.0±12.2%) methods. After 3 h of incubation at 37°C, the difference in sperm motility between the two methods was significant ($P<0.05$), the Andersen (21.0±13.5%) being superior to the CLONE (1.5±2.0%) method.

There were no significant differences between the two methods in percentages of spermatozoa with intact plasma membrane, immediately after thawing (64.9±8.9% and 59.6±11.0% for Andersen and CLONE, respectively) or after 3 h of incubation at 37°C (20.4±10.9% and 17.1±6.3% for Andersen and CLONE methods, respectively).

The percentages of live spermatozoa with reacted acrosomes were similar in the semen samples processed with both methods, and they ranged between 0 and 2%. The proportions of spermatozoa with damaged acrosomes (including both the live and dead populations), after thawing, were significantly higher ($P<0.05$) in the samples cryopreserved with the CLONE method than in those cryopreserved with the Andersen method (25.5±8.4% and 18.4±5.0%, respectively). After 3 h of incubation, there were no significant differences in the proportions of spermatozoa with damaged acrosomes between the two cryopreservation methods (49.3±13.2% and 59.2±12.1 for the Andersen and CLONE methods, respectively).

Effects of different cryopreservation treatments on post-thaw survival and longevity of dog spermatozoa (papers IV and V)

Motility immediately post-thaw

The mean motility post-thaw, determined by subjective evaluation, ranged between treatments from 56 to 75% in paper IV and from 44 to 68% in paper V. The proportion of total motile cells (determined by CASA) as well as that of spermatozoa with progressive motility (determined subjectively and by CASA) were significantly ($P<0.001$) influenced by the thawing rate (paper IV), both being higher when the thawing was done at 70°C for 8 s instead of at 37°C for 15 s. The sperm concentration per straw ($P<0.001$) and the post-thaw dilution rate ($P=0.035$) also had significant effects on the percentage of progressively motile spermatozoa (subjective determination) immediately after thawing (paper V). Motility immediately post-thaw was higher in samples frozen with 200 x 10^6 spermatozoa/ mL than with the other sperm concentrations. Semen samples diluted post-thaw at the highest rate (1:4) showed the lowest percentage of progressively motile spermatozoa whereas there was no significant difference between undiluted samples and those diluted at rates 1: 1 or 1: 2.

Sperm longevity (subjective evaluation) during post-thaw incubation at 38 °C

The presence of Equex in the extender, the dilution of semen in 1 or 2 steps and the thawing rate exerted significant effects ($P<0.001$) on the sperm longevity (paper IV). Including Equex in the extender, performing the semen dilution in 2 steps and the thawing at 70°C for 8 s, resulted in the highest sperm longevity (45% motile spermatozoa after 6 h of incubation). The freezing method had a significant effect ($P<0.05$) at some of the intervals during incubation (between h 2 and 5), freezing in the box being preferable to freezing in the tank. The lowest sperm longevity was found when the extender did not contain Equex, the semen was diluted in 1 step, the freezing was done in the tank and the thawing was done at 37°C for 15 s (4% motile spermatozoa after 3 h of incubation). Significant effects of the interactions between Equex and dilution method ($P<0.001$), Equex and freezing method ($P<0.05$) and between dilution and freezing methods ($P<0.01$) were found, but not between freezing and thawing methods.

The sperm concentration and the post-thaw dilution of spermatozoa also had significant effects ($P<0.001$) on the sperm longevity (paper V). The highest sperm longevity was observed in samples frozen with sperm concentrations of 200 x 10^6 or 400 x 10^6 spermatozoa/ mL and diluted post-thaw at rates of 1: 4 (38% and 34% motility after 8 h of incubation, for concentrations of 400 and 200 x 10^6 spermatozoa/mL, respectively) or 1: 2 (36% motility after 8 h of incubation for both sperm concentrations) and there were no significant differences between them. The lowest sperm longevity was found in samples frozen with 50 x 10^6 spermatozoa/mL that were not diluted after thawing (0% motility after 6 h of

incubation). There was no significant effect of the interaction between sperm concentration and post-thaw dilution rate on sperm longevity. For all the post-thaw dilution rates evaluated, sperm longevity was better with the following concentrations: 200 x 10^6 > 400 x 10^6 > 100 x 10^6 > 50 x 10^6 spermatozoa/mL, although differences between them were significant only at some evaluation time points. Likewise, for all the sperm concentrations evaluated, sperm motility after 5 h of incubation was significantly ($P<0.001$) lower in undiluted samples, but it was similar for samples diluted at any of the 3 dilution rates evaluated. After 8 h of incubation, for sperm concentrations of 200 x 10^6 and 400 x 10^6 spermatozoa/mL, samples diluted at rate 1: 1 had lower motilities than those diluted at higher dilution rates.

Motility characteristics (paper IV)
The dilution method had a significant effect ($P<0.05$) on the VSL, VAP and VCL of spermatozoa immediately post-thaw, the three velocity parameters being higher when the semen dilution was done in 2 steps. The thawing rate also had a significant effect ($P<0.001$) on VSL and VAP immediately after thawing, but not on VCL.

During the incubation period, the presence of Equex in the extender, the dilution method, the thawing rate and the interactions between them had significant effects ($P<0.05$) on VSL, VAP and VCL. The freezing method also had a significant effect ($P<0.05$) on the three velocity descriptors at some of the evaluation times. When Equex was included in the extender, VSL and VAP were similar for both thawing rates, however, in the absence of Equex both parameters were significantly ($P<0.05$) higher when the thawing was done at 70°C for 8 s. BCF and ALH were significantly influenced by the presence of Equex in the extender ($P<0.005$), the dilution method ($P<0.05$) and the thawing rate ($P<0.01$). The interactions between these effects were also significant ($P<0.05$) at some intervals during the incubation period.

Plasma membrane integrity
The mean percentage of spermatozoa with intact plasma membrane after thawing, as measured by the proportion of SNARF-postitive cells, ranged between treatments from 19 % to 70 % in paper IV and from 51% to 64 % in paper V. After 6 h of incubation at 38°C, it ranged from 13 % to 49 % in paper IV and from 44 % to 63 % in paper V. After 12 and 18 h of incubation, mean percentages of sperm plasma membrane integrity (paper V, excluding samples with sperm concentrations of 50 x 10^6 spermatozoa /mL) ranged from 16 % to 56%, and from 5 % to 50 %, respectively.

The presence of Equex in the extender, the dilution method and the thawing rate (paper IV) had significant ($P<0.001$) influences on the integrity of sperm plasma membranes at 1, 3 and 6 h post-thaw. The freezing method had no significant effect on the sperm plasma membrane integrity, but the interactions between

freezing method and presence of Equex in the extender, and between freezing and dilution methods, had significant effects ($P<0.001$) at the three evaluations post-thaw.

For both freezing and thawing methods, the proportion of viable spermatozoa at the three evaluations post-thaw was the highest ($P<0.05$) when the extender contained Equex and the sperm dilution was done in 2 steps.

Thawing at 70°C yielded higher percentages of live spermatozoa than thawing at 37°C. When the extender contained Equex and the semen dilution was done in 2 steps, the sperm viability was not different during the first half of the incubation period when thawing at 70°C or at 37°C, but after 6 h of incubation the semen samples thawed at 70°C had significantly ($P<0.05$) higher proportions of spermatozoa with intact plasma membranes.

At h 0 of incubation, significant effects of sperm concentration ($P<0.05$) and post-thaw dilution rate ($P<0.005$) were seen (paper V). The highest proportion of live spermatozoa (64 %) was seen with the lowest sperm concentration (50 x 10^6 spermatozoa/mL) and the highest dilution rate (1: 4). In contrast, the lowest sperm viability (51%) was seen at the highest sperm concentration (400 x 10^6 spermatozoa/mL) and no dilution after thawing. After 18 h of incubation, the highest ($P<0.05$) proportion of viable spermatozoa was observed in samples frozen with 200 x 10^6 or 400 x 10^6 spermatozoa/mL and diluted after thawing at rate 1:4 (50% and 47%, respectively) and the lowest proportion of viable spermatozoa was observed in samples not diluted after thawing, which did not differ for the 3 sperm concentrations (12%, 6% and 9%, respectively, for samples frozen with 400 x 10^6, 200 x 10^6 and 100 x 10^6 spermatozoa/mL).

Acrosomal membrane integrity

The mean percentages of spermatozoa with damaged acrosomes after thawing, as measured by the proportion of FITC-PSA-postitive cells, ranged between treatments from 17 % to 40 % in paper IV and from 14 % to 20 % in paper V. At the first evaluation post-thaw, all the spermatozoa having damaged acrosomes were dead cells, no population of acrosome-reacted live spermatozoa was detected after thawing in the two studies. In paper IV, the proportion of dead spermatozoa with damaged acrosomes after thawing was significantly lower ($P<0.05$) when Equex was included in the extender, the dilution had been made in 2 steps and the thawing was at 70°C. The freezing method had no significant effect on the acrosomal status. In paper V, neither the sperm concentration nor the post-thaw dilution of spermatozoa exerted a significant effect on the proportion of dead spermatozoa with damaged acrosomes after thawing.

Table 1. Percentages (Means + SD) of acrosome reactions in the live sperm population for the different combinations of sperm concentration and post-thaw dilution rate

Sperm concentration (spermatozoa / mL)	Post-thaw dilution rate	Incubation time		
		6 h	12 h	18 h
400×10^6	1 : 0	8.5 ± 2.1^{ab}	25.6 ± 1.9^{abc}	48.3 ± 6.3^{ab}
400×10^6	1 : 1	6.9 ± 8.0^{ab}	29.6 ± 4.2^{abcd}	46.8 ± 2.5^{ab}
400×10^6	1 : 2	14.0 ± 7.8^{a}	25.0 ± 6.0^{abc}	45.3 ± 2.3^{ab}
400×10^6	1 : 4	13.0 ± 2.9^{a}	20.1 ± 5.5^{abc}	26.6 ± 9.6^{a}
200×10^6	1 : 0	12.0 ± 3.5^{a}	16.8 ± 15.7^{ab}	34.0 ± 20.5^{ab}
200×10^6	1 : 1	7.9 ± 1.4^{ab}	10.9 ± 15.5^{a}	30.5 ± 28.5^{ab}
200×10^6	1 : 2	4.0 ± 2.6^{ab}	14.4 ± 8.2^{ab}	60.2 ± 9.4^{b}
200×10^6	1 : 4	4.4 ± 2.8^{ab}	20.9 ± 5.1^{abc}	57.0 ± 2.3^{ab}
100×10^6	1 : 0	9.8 ± 11.0^{ab}	39.3 ± 10.9^{cd}	40.1 ± 11.8^{ab}
100×10^6	1 : 1	1.3 ± 2.1^{b}	47.8 ± 5.1^{d}	41.8 ± 15.8^{ab}
100×10^6	1 : 2	1.1 ± 2.4^{b}	34.8 ± 14.1^{bcd}	32.3 ± 20.1^{ab}
100×10^6	1 : 4	0.4 ± 0.8^{b}	30.1 ± 11.3^{abcd}	39.2 ± 14.5^{ab}
50×10^6	1 : 0	3.9 ± 6.5^{ab}	-	-
50×10^6	1 : 1	0.0 ± 0.0^{b}	-	-
50×10^6	1 : 2	0.0 ± 0.0^{b}	-	-
50×10^6	1 : 4	0.0 ± 0.0^{b}	-	-

$^{a, b, c, d}$: means with different superscript letters in the same column are significantly different ($P<0.05$).

After 6 h of incubation, the proportion of acrosome-reacted live spermatozoa ranged between 0% and 1 % in paper IV, and between 0 % and 8 % in paper V. After 18 h of incubation (paper V), the mean proportions of acrosome-reacted live spermatozoa ranged from 2 % to 28 %, and in relation to only the live sperm population (Table 1) those percentages were between 26 % and 60 %.

At h 6 of incubation, the sperm concentration (paper V) had a significant effect ($P<0.001$) on the proportion of acrosome-reacted live spermatozoa, which was higher with higher sperm concentrations. After 18 h of incubation, however, there was no difference between the different sperm concentrations, but a significant effect ($P<0.001$) of the dilution rate was seen, with higher proportions of acrosome-reacted live spermatozoa as the dilution rate increased. However, the proportion of acrosome-reacted live spermatozoa in relation to only the live sperm population (Table 1) was not correlated with the sperm concentration or the post-thaw dilution rate.

General discussion

The concept of semen preservation unequivocally implies the preservation of its functional capacity, i.e. the ability to produce live offspring, which necessarily involves the maintenance of the sperm cellular integrity, the ability for oocyte penetration and the competence to support embryo development (Harrison et al., 1996). Unfortunately, there is not a single *in vitro* test capable of measuring all these functions collectively in a semen sample, however, the reliable quantification of the relative numbers of sperm achieving these separate functions could be an important indicator of the overall functional capacity (Amann and Hammerstedt, 1993).

Because an intact plasma membrane is an essential requirement for general cell function, the plasma membrane integrity is an obvious parameter for assessment when evaluating fresh or frozen-thawed semen samples. The use of fluorescent probes for assessing the integrity of sperm membranes in combination with flow cytometry or fluorescence microscopy, has been successfully applied to spermatozoa of several species (Morrel, 1991). In the present study (paper I), the use of CFDA and PI in combination with flow cytometry was found to be highly correlated with microscopic methods, proving this to be a useful tool for the objective assessment of sperm membrane integrity in dog spermatozoa. A great advantage of the flow cytometer is that only particle-associated fluorescence is detected, and therefore labelled cells do not need to be washed free of unbound label prior to analysis (Harrison and Miller, 1998). As each particle passes through the flow cytometer, data with respect to four parameters (size, cellular complexity, CFDA and PI fluorescence intensities in this study) are collected simultaneously. The resultant data sets from several thousands of 'events' can be displayed in several ways, e.g. frequency distribution histograms representing the side and forward light scatter parameters of the particles present in the sample. Therefore, computer regions can be set to select only those particles or 'events' with the same light scatter parameters that represent spermatozoa, whereas data relating to small particles can be discarded. Then, the events considered to represent, as far as possible, only sperm cells can be redisplayed again as frequency distribution histograms or as dot-plots cytograms of the fluorescence intensities of the two fluorescent probes loaded into the sperm cells. Computer regions or windows can be set on the new cytograms to determine the percentages of cells exhibiting high green fluorescence intensity, which are the live spermatozoa, or high red fluorescence intensity, which are the dead spermatozoa. As Harrison and Vickers (1990) already described for boar and ram spermatozoa, and Rota et al. (1995) for dog spermatozoa dually stained by CFDA and PI, we found a small population of cells in each sample (ranging between 5% and 17%) showing both high green and red fluorescence intensities. By microscopic analysis we could identify that sperm population whose acrosomes were accumulating CFDA, and thus were intact, but whose plasma membranes

were damaged, showing the nuclei stained red. Another interesting fact is that many of the dead spermatozoa had the midpiece stained green, which means that the mitochondrial membranes were still intact, thus accumulating CFDA. It indicated, as previously observed by Harrison and Vickers (1990) and Rota et al. (1995), that the plasma membrane of dog spermatozoa is more labile than the acrosomal and mitochondrial membranes.

In paper II, the FITC-PSA staining was used in order to detect acrosomal damage in fresh spermatozoa pretreated with different LPC concentrations. This fluorescein-conjugated lectin proved to be significantly correlated with the Spermac^{R} staining when the former was analysed by flow cytometry (0.72), but the correlation coefficient was lower when it was analysed by fluorescence microscopy (0.56) or when both fluorescence methods were compared (0.51). Uhler et al. (1993), working with human spermatozoa exposed to calcium ionophore A23187, obtained a correlation coefficient of 0.61 between the method of flow cytometry to detect fluorescein-labeled acrosome-reacted spermatozoa compared with the visual method using fluorescence microscopy. The lower correlation observed in the present study between flow cytometry and fluorescence microscopy was probably due to the different sample size used for the two techniques, but also to the different 'objectivity' of the two methods. Flow cytometry objectively discriminates sperm populations showing fluorescence intensities above or below the established threshold, whereas the visual method of scoring has a level of subjectivity. In fact, in the present study, differences higher than 15% were frequently observed between the same sperm populations counted visually in two replicates from the same semen sample. However, when using flow cytometry, because a high number of cells is objectively evaluated, differences between replicates from the same sample were less than 3%. The FITC-PSA staining, in the present study, was more sensitive than the Spermac^{R} staining, revealing higher proportions of acrosomal damage when analysed both with flow cytometry and fluorescence microscopy. It was probably due to the fact that the two staining methods measure different aspects of the acrosome reaction. With the Spermac^{R} staining, altered acrosomes are seen with a decreased green staining affinity due to a loss of acrosomal contents, therefore revealing only relatively advanced acrosome reactions, whereas FITC-PSA binds to the acrosomal contents of spermatozoa whose plasma and outer acrosomal membranes are damaged. Thus, some of the initially reacting spermatozoa may have been classified as having a "normal" acrosome when evaluated using Spermac^{R} but had bound enough PSA lectin to be detected by the cytometer or under epifluorescence illumination. The use of FITC-PSA and PI in combination was useful to detect simultaneously both sperm parameters, i.e. viability and acrosomal status, and four sperm populations were identified: live spermatozoa with intact acrosomes (unstained sperm cells), live spermatozoa with damaged acrosomes (sperm cells only stained by FITC), dead spermatozoa with intact acrosomes (sperm cells only stained by PI) and dead spermatozoa with damaged acrosomes (sperm cells stained by both FITC and PI). Dead

spermatozoa that had lost the acrosomal content, in paper II, were seen showing FITC-fluorescence on the equatorial segment. It was attributed to the fact that PSA-binding sites on the equatorial segment were probably retained longer because this region does not participate in the membrane vesiculation events occurring during the acrosome reaction (Bedford, 1970), and therefore, this population was identified as dead spermatozoa with damaged acrosomes. However, in the later studies (papers IV and V), it was seen that the proportion of spermatozoa stained with both FITC and PI was smaller after 6 h of incubation post-thaw at 38°C than after 3 h, and that the increase in the proportion of spermatozoa only stained by PI was higher than the decrease in the proportion of viable cells. Therefore, it is evident that the population of spermatozoa stained by PI but not by FITC-PSA comprised two subpopulations: PI-positive spermatozoa that had entirely lost the acrosomal contents and the staining of the equatorial segment and PI-positive spermatozoa possessing intact acrosomes. The acrosomal changes induced by LPC are similar to those seen in true acrosome reactions (Parrish et al., 1988), thus, the presence of stained equatorial segments seen in paper II in the dead spermatozoa may have been due to acrosomal changes similar to physiological acrosome reactions, whereas the PSA-binding sites on the equatorial segments were not retained when degenerative acrosomal changes took place after or during the cell death.

When the same combination of fluorophores, FITC-PSA and PI, following the same methodology as described in paper II, was applied to unwashed frozen-thawed dog spermatozoa, we could not find a correlation between the proportions of PI-negative spermatozoa (live population) detected by the cytometer and those identified by epifluorescence microscopy (data not shown). It was due to the presence in the medium of a large amount of egg yolk particles and cell debris that had frontal and side light scatter parameters similar to those of spermatozoa. These particles were identified by the cytometer as unstained live cells. Therefore, to be able to accurately identify the live spermatozoa without washing the cryopreservation medium, it was necessary to stain the live cells. Selection of the fluorochromes and their means of detection is an important step in experimental planning, because the fluorochromes need to be excited simultaneously, which involves that their excitation spectrum has to be compatible with the laser used in the flow cytometer, and their individual fluorescence emissions must be clearly distinguishable. We chose the cell-permeant dye Carboxy-SNARF-1 (SNARF), in its acetoxymethylester form, which is easily loaded into cells, and combined with PI, stains the live cells orange. This stain is an intracellular pH-indicator that exhibits a significant pH-dependent emission shift from yellow-orange to deep red fluorescence under acidic and basic conditions, respectively (Haugland, 1996). Using an excitation wavelength of 488 nm it yields balanced signals for the two emissions and exhibits a pH-independent fluorescence emission at 610 nm (Haugland, 1996). When combined with PI, both fluorescent dyes were easily distinguishable because the fluorescence intensity of PI was considerably higher than that of

SNARF, and both fluorochromes in combination provided identical identification of the live and dead cells as did the combination CFDA and PI. The triple combination of SNARF, PI and FITC-PSA allowed us to identify unequivocally the live and dead spermatozoa with intact or damaged acrosomes without washing the cryopreservation medium, and with the minimal sample manipulation. When using this triple staining, high and significant correlations were obtained between flow cytometry and fluorescence microscopy methods (paper III). It suggests that when using only the two fluorochromes FITC-PSA and PI (paper II), the difficulty to count the unstained sperm population, having to change from fluorescence illumination to bright light in each microsocopic field, might also have contributed to the differences observed between both fluorescent methods (paper II) for quantifying the different sperm populations. Because the triple staining method described in paper III proved to be reliable and useful for simultaneously assessing plasma and acrosomal membrane integrity of frozen-thawed dog spermatozoa, it was used in the next studies to compare different cryopreservation treatments.

Semen cryopreserved using the Andersen or the CLONE methods (paper III) had good motility post-thaw but a low thermoresistance. After 3h of incubation at 37°C, motility and sperm plasma membrane integrity had declined abruptly. The Andersen and the CLONE cryopreservation methods are known to give high pregnancy rates when using intrauterine insemination: 80% (Andersen, 1976) and 84 % (Linde-Forsberg et al., 1999), respectively, but using vaginal insemination, pregnancy rates were reported to be lower: 25% (Farstad, 1984) and 59% (Linde-Forsberg et al., 1999), respectively. The low thermoresistance of spermatozoa, when the semen is deposited in the uterus, does not seem to be a handicap for achieving pregnancy rates similar to those obtained after well-controlled natural matings (Govette et al., 1996; Linde-Forsberg et al., 1999), but it may be the cause of the lower pregnancy rates reported when the semen is deposited intravaginally. In addition, litter sizes obtained by intravaginal insemination of frozen-thawed semen are smaller than those obtained by intrauterine insemination (Linde-Forsberg et al., 1999), probably because lower numbers of spermatozoa reach the site of fertilization.

Previous findings proving a beneficial effect of Equex STM Paste for the cryopreservation of dog spermatozoa (Rota et al., 1997; Ström-Holst, 1999) were clearly confirmed in the present study (paper IV). The proportion of viable spermatozoa recovered after thawing and the sperm longevity, as well as the quality of the motility, were significantly improved by the addition of Equex STM Paste to the extender. However, its beneficial effect was significantly dependent on the exposure time, being more pronounced when the spermatozoa were exposed to Equex during a short time before the freezing than when they were exposed during the equilibration period. It is believed that the beneficial effect of detergents for the cryopreservation of spermatozoa from different species is due to the modification or solubilization of egg yolk components

(Pursel et al., 1978), which improves their cryoprotective efficacy. Perhaps it facilitates a more complete protection of the sperm membranes by membrane-bound yolk lipoproteins, partially inhibiting the deleterious effects of hyperosmotic salt solutions upon membrane structures (Holt et al., 1992). It might also suggest that a specific interaction with membrane lipids or proteins is required for cryoprotection (Holt and North, 1994).

The two freezing methods compared in paper IV had no significantly different effects on the sperm viability or acrosomal status after thawing, but concerning the percentage of motility and the motility patterns, freezing in the box seemed to be a better method than freezing in the tank. As the freezing rate obtained with the two methods mainly differed in the speed of temperature decrease in the ranges from +5°C to –10°C (-32 °C/min and -11 °C/min for the box and tank methods, respectively), and from –50 °C to -100 °C (-9 °C/min and –26.5 °C/min, for the box and tank methods, respectively) the results suggest that the faster freezing rate achieved with the box method from the beginning of the freezing operation until the crystallization temperature is more suitable for freezing dog spermatozoa than a slower rate, and that a freezing rate below -50 °C may not be critical. Very slow (-0.5 °C/min or -2 °C/min) or very fast (-99 °C /min or -75 °C/min) freezing rates were found to be detrimental for dog spermatozoa (Hay et al., 1997; Olar, 1984), whereas moderately fast freezing rates (from -10 °C/min to -50 °C/min) provided good results (Hay et al., 1997; Rota et al., 1998a).

In the present study, an evident and significant effect of the thawing rate was observed, affecting all of the sperm parameters studied. The proportion of motile cells as well as the motility characteristics, the plasma membrane integrity and the acrosomal status, were significantly better preserved during several hours of incubation when the thawing was done at 70°C for 8 s than when it was done at 37°C for 15 s. Higher post-thaw survival of dog spermatozoa when thawed at a fast rate than at a slow rate has been previously reported by several authors (Davies, 1982; Olar et al., 1989; England, 1992; Ivanova-Kicheva et al., 1997a; Rota et al., 1998a). There is evidence suggesting that frozen cells are injured during the thawing rather than during the freezing phase of the cycle (Curry and Watson, 1994). It has been thought to be due to the recrystallization of intracellular microscopic ice crystals, forming bigger ice crystals that are known to have lethal effect. Cells that have been frozen at a fast rate should be thawed rapidly to limit the time for recrystallization to occur. At the same time, the hyperosmotic conditions that the cells have to support during the freezing are suddenly reversed during the thawing. When cells have been frozen at a very slow rate, and therefore have been dehydrated to a high degree, they should be thawed slowly, since a very fast thawing rate will create a severe osmotic stress by the rapid movement of water into the cell to restore the equilibrium at both sides of the membrane (Mazur, 1984). In the present study, in contrast, we did not find any interaction between the freezing and thawing rates evaluated, the fast thawing rate being superior in combination with both the slower and the faster

freezing rates. Our results are in agreement with the findings by Olar et al. (1989) and Rota et al. (1998) who also found no interaction between freezing and thawing rates for dog semen. The difference between the two freezing rates evaluated in this study may not have been sufficient to prove such an interaction.

In human spermatozoa exposed to hyperosmotic stress, it was found (Gao et al., 1993) that the return to isosmotic conditions caused considerably more plasma membrane damage than the initial exposure and that the higher the hyperosmolarity, the more serious the cell injury. Similarly, the experimental exposure of ram spermatozoa to hyperosmotic conditions induced little membrane permeabilization, but significant damage was caused by the restoration of osmotic equilibrium (Holt and North, 1994). It is believed that membranes are initially destabilized during the freezing by the effects of both low temperatures and high salt concentrations, and the resultant post-thaw degeneration of the plasma membrane is caused by both the temperature change and the osmotic stress (Holt and North, 1994). Interacting with these effects, the lipid reorganization caused by temperature-dependent phase changes may alter the responsiveness of the sperm membranes (Watson, 1995). The mechanism responsible for the higher post-thaw survival and longevity of dog spermatozoa by thawing at a fast rate is not known, but it might be connected with a lower hyperosmotic stress on the cells when the extracellular water is thawed rapidly. Besides, the permeability of dog sperm plasma membrane to glycerol seems to be high (Peña et al., 1998b), which could also account for a better survival obtained at a fast thawing rate. If the membranes are highly permeable to glycerol, the cryoprotective agent can leave the intracellular compartment rapidly as the extracellular water enters the spermatozoa. Then, the volume changes that the sperm cells have to support during the rewarming (Hammerstedt et al., 1990) should be expected to be minor. Interestingly, when the spermatozoa were exposed to Equex during a short time before the freezing, the sperm motility and longevity were not different when thawing at 37°C or at 70°C, although samples thawed at 70°C showed a significantly higher proportion of spermatozoa with an intact plasma membrane after 6 h of incubation. It might suggest that the beneficial effect of Equex may be exerted at two levels: 1) an indirect effect through the extracellular medium, interacting with the egg-yolk lipoproteins and improving their cryoprotective efficacy, perhaps reducing the harmful effects of hyperosmotic salt solutions on the sperm membranes, and 2) through a direct effect on the sperm membranes, as suggested by Arriola and Foote (1987), increasing the fluidity of the membrane lipids. This second mechanism might explain the fact that a prolonged exposure of spermatozoa to Equex was less beneficial than a short exposure time, perhaps confering an excessive fluidity to the membrane lipids rendering them more labile. It would also explain the finding that when Equex was included in the extender and the semen was diluted in two steps, the sperm survival was similar when thawing at 37°C or at 70°C. If the sperm plasma membranes had a higher fluidity due to the effect of a short exposure to Equex, the reorganization of membrane lipids caused by

temperature-dependent phase changes during the slow rewarming might be less deleterious.

Another interesting finding was that the semen samples frozen without Equex and thawed at 37°C, which had the poorest survival and the shortest longevity, showed motility patterns characteristic of hyperactivation during a period between 1 and 3 hours post-thaw. In contrast, when the thawing was done at a fast rate or when Equex was included in the extender, the hyperactivated movement was not seen. In systems using detergent-lysed models and calcium/EGTA buffers to control free calcium, a dose-dependent relationship between calcium and flagellar wave symmetry has been found (Hyams and Borisy, 1978; Bessen et al., 1980). At very low concentrations of calcium flagella beat symmetrically. As the calcium level is elevated, the wave form becomes more asymmetric until a concentration is reached where motility is inhibited. The treatment of hamster sperm with α-agonists, which act via calcium flux, was found to cause typical capacitation-associated 'activated' motility coincident with the acrosome reaction (Cornett and Meizel 1978). Whether the hyperactivated movement seen in the present study in the semen samples rewarmed at a slow rate, but not in the same samples rewarmed at a fast rate, had any relationship with an increase of access of calcium ions to the cell interior is not known. It might indicate that the stabilizing effect of Equex on the sperm membrane lipids might reduce the modification of calcium-dependent enzymes during the reorganization of the membrane lipids caused by thermotropic phase transitions, resulting in a lower intracellular calcium concentration after thawing than in the absence of Equex.

Very few live cells binding the PSA lectin (between 0 and 2 %) could be seen at any given evaluation time, in studies III and IV, although in both studies the number of dead acrosome-reacted spermatozoa had increased after 3 h of incubation post-thaw. Because the proportion of acrosome-reacted dead spermatozoa was significantly lower in the semen samples frozen in an extender containing Equex than in the absence of Equex, we thought that one possible mechanism by which Equex increases the sperm longevity could be by delaying the changes leading to the acrosome reaction, and we deduced that dog spermatozoa that had undergone the acrosome reaction had a very short time of plasma membrane integrity. In the last study of this thesis (paper V), we found that during 6 h of incubation at 38°C, very few live spermatozoa bound the PSA lectin, but during the incubation period between hour 6 and hour 18, the proportion of acrosome-reacted live spermatozoa increased from 8% to 28 %, whereas the proportion of dead spermatozoa increased less. This finding supports the hypothesis that the beneficial effect of Equex on sperm longevity and plasma membrane integrity might be by reducing or inhibiting capacitation-like changes of thawed spermatozoa and delaying premature acrosome reactions, but the acrosome-reacted spermatozoa may not be as short-lived as we had thought. Whether the mechanism by which such capacitation-like changes are inhibited

has any relationship with preventing an uncontrolled calcium influx into the spermatozoa remains to be proved. Despite Equex seeming to prevent these capacitation-like changes in dog spermatozoa, it does not seem to block a spermatozoon's ability to respond to physiological stimuli, since the addition of Equex to the extender increased the zona pellucida binding capacity of frozen-thawed dog spermatozoa (Ström-Holst, 1999). A significant beneficial effect of the addition of Equex to the extender on the pregnancy rate could not be proved, whereas the absence of a potential negative effect of this compound on the fertilizing capacity of frozen-thawed dog spermatozoa was clearly demonstrated (Rota et al., 1999a). In the study of Rota et al. (1999a), pregnancy rates after intrauterine (5/5) or vaginal insemination (4/5) were the same when the extender contained Equex or in the absence of it, but a low number of well-timed bitches were included in each group, and a high number of spermatozoa was used (200 x 10^6) for each of the two AIs. Although the beneficial effects of Equex on the post-thaw sperm survival in vitro might not reflect on fertility results, it is probable that any difference in results of this fertility trial may have been masked by the low number of animals used, the optimal timing of the bitches, and the high number of spermatozoa used per insemination.

The best combination of processing steps for cryopreservation of dog spermatozoa, according to the results of study IV, was used in the last study presented in this thesis. Despite this, the overall motility immediately post-thaw was lower than that observed in previous studies. Possible reasons for the lower post-thaw sperm motility could have been: 1) different dogs were used, 2) ejaculates were first pooled, and then the semen pool was centrifuged and rediluted. Therefore, the ejaculates that were collected at the beginning, while waiting to be processed, were exposed to seminal plasma longer than in the previous studies, in which each ejaculate was immediately centrifuged and rediluted after collection, 3) the pool of ejaculates, divided into four aliquots, had to be centrifuged twice, whereas in previous studies only one centrifugation was done, 4) larger volumes of semen were centrifuged, the pellets obtained were larger, and probably the spermatozoa were more loosely packed in the pellets, retaining a larger volume of seminal plasma than if each ejaculate had been centrifuged separately. Whether the prolonged contact with seminal plasma had a detrimental effect on the spermatozoa in this study is not known, but Wales and White (1963) and England and Allen (1992) observed that motility of fresh dog spermatozoa significantly decreased after a relatively short incubation period (2 h) at 37°C with the first or the third fractions of the ejaculate. Rota et al. (1995) also observed that motility of fresh spermatozoa stored at 4°C was significantly lower after 24 h of storage when they were diluted in seminal plasma than in egg yolk extenders. Paradoxically, the sperm motility and the sperm plasma and acrosomal membrane integrity were maintained in higher proportions of spermatozoa in study V than in the previous study, over the same incubation periods. Although it is likely to have been due to the low detrimental effect of the cryopreservation protocol used, a potential positive effect of the prolonged

exposure to the seminal plasma, or to the seminal plasma from a particular dog, can not be excluded.

In study V, sperm longevity and membrane integrity after prolonged incubation at body temperature were higher at the higher sperm concentrations and post-thaw dilution rates evaluated. A positive effect of post-thaw dilution can be explained by the additional source of metabolic substrate and buffering capacity for the spermatozoa and by the dilution of the toxic products present in the cryopreservation medium, such as glycerol, egg yolk or Equex. However, the cause of a higher sperm longevity at the higher sperm concentrations evaluated in the present study is not known, and it needs to be studied further in greater detail to confirm or refute the present results. Several studies in the equine species (Palacios et al., 1992; Parlevliet et al., 1992; Leipold et al., 1998) also suggest that post-thaw sperm survival increases when stallion spermatozoa are frozen in 0.5-ml straws at high concentrations (800-1600 x 10^6 spermatozoa /mL) compared with low sperm concentrations (100-200 x 10^6 spermatozoa /mL), but the cause of such an effect is unknown.

A common finding in the cryopreservation studies presented here, except for the CLONE method, was the fact that after prolonged incubation, the proportion of spermatozoa showing an intact plasma membrane was higher than the proportion of motile spermatozoa, and the difference between percentages of motile and 'live' spermatozoa tended to be higher with the cryopreservation treatments which gave shorter sperm longevity. This phenomenon has been previously observed in dog spermatozoa after chilling and after cryopreservation (Rota, 1998b), in diluted boar spermatozoa after incubation (Johnson et al., 1996) or in frozen-thawed bovine spermatozoa (Januskaukas, 1995). Whether this population of non-motile but apparently viable spermatozoa could regain motility within the female reproductive tract is not known. In the boar, immotile spermatozoa could be rendered motile by stimulation with caffeine after 3 h of storage at 37°C (Larsson et al., 1976), however, in this study (paper V) stimulation with caffeine after 12 h of incubation had no effect (data not shown). The stimulatory effect of compounds such as caffeine and theophylline on sperm motility is thought to be due to the inhibition of the cyclic nucleotide phosphodiesterase (PDE), which breaks down the cAMP to form 5'-AMP, causing an increase in the sperm cAMP concentrations (Tash and Means 1982, 1983). The elevation of cAMP levels in the sperm is known to be involved in the sperm cell's ability to achieve normal motility during epididymal transit by activating cAMP-dependent protein kinases and catalyzing the phosphorylation of sperm proteins such as dynein, tubulin or miosin (Hoskins et al., 1974). But methylxantines are known to affect other enzymes in addition to the PDE, so it could not be conclusively demonstrated that the stimulation of sperm motility by caffeine is caused directly by increasing the cAMP levels (Slaughter et al., 1982). Calcium, on the other hand, when elevated intracellularly, inhibits sperm motility and stimulates the occurrence of the acrosome reaction. Dog spermatozoa are known to become quiescent in the

presence of μM levels of intracellular calcium (Tash and Means, 1983), therefore, a small increase in the intracellular calcium concentration may be sufficient to inhibit sperm motility and response to caffeine stimulation. In fact, the ability of caffeine to influence human sperm motility was found to be counteracted by the presence of calcium in the external media at the concentrations used in capacitating media (Aitken et al., 1983, 1986), and Goltz et al. (1988), using detergent-treated bull sperm models observed that, at neutral or slightly acid pH, motility is strongly cAMP-dependent and strongly calcium-inhibited, whereas at alkaline pH, motility is less dependent on cAMP and less sensitive to the calcium antagonism. The immotile but viable sperm population seen in the present study might have an intracellular calcium concentration sufficiently high to inhibit the sperm motility and to antagonize the effect of caffeine as a consequence of a progressively lower efficiency of the sperm membrane ATPases and ion exchangers to pump calcium out of the flagellum due to latent damage induced by cryopreservation, until an intracellular calcium concentration is reached, which leads to the acrosomal exocytosis that precedes cell death. According to this theory, it seems unlikely that those sperm cells can be rendered motile within the female tract, since, if the loss of motility is an indication of increased permeability to calcium ions which induce capacitation and acrosome reaction, their survival is limited because they are prepared to meet the oocyte, and if this does not occur within a short time, they will die (Watson, 1995). Besides, spermatozoa have only a simple degradative metabolism, they have no biosynthetic capacity and, therefore, they have no ability to repair membrane damage induced by an adverse environment (Hammerstedt, 1993). The better sperm longevity seen in study V compared with that in study IV, and the smaller increase in the proportions of PI-stained spermatozoa over the same incubation periods, indicate that the less detrimental the cryopreservation procedure is, the slower are the biochemical changes causing the loss of sperm membrane integrity after rewarming and thus, the longer the fertile life of thawed spermatozoa.

The prologed sperm survival obtained when dog spermatozoa were cryopreserved according to the best combination of processing steps evaluated in the presented studies, i.e. adding Equex to a Tris-glucose-citrate-egg yolk extender, diluting the semen in two steps to a final sperm concentration of 200 x 10^6 spermatozoa/mL, freezing and thawing at the faster rates described, and finally, diluting the spermatozoa immediately after thawing at rate 1: 4 (semen volume: diluent volume) with a plain Tris buffer, would be expected to result in higher pregnancy rates and litter sizes than when spermatozoa are frozen using the Andersen or CLONE methods. Because of the long oestrus period, the identification of the optimal time for the insemination of the bitch will remain a critical point when using frozen semen. However, the intrauterine deposition of semen and the use of a high number of spermatozoa per insemination dose might not be such critical points if sperm survival was to be considerably prolonged by improved cryopreservation methods. Whether the *in vitro* results presented here

also apply *in vivo*, allowing us to obtain higher pregnancy rates after intrauterine and especially after vaginal deposition of frozen-thawed semen, and whether the number of spermatozoa per insemination dose could be reduced, needs to be investigated further.

Conclusions

- The use of flow cytometry and fluorescent stainings for *in vitro* evaluation of fresh and frozen-thawed canine spermatozoa is a valuable tool because of its objectivity. It allows the accurate detection of small differences between cryopreservation treatments, with a high repeatability. Because thousands of cells are analysed in each sample, variability between samples within-treatment is very small. The triple combination of the fluorochromes Carboxy-SNARF-1, PI and FITC-PSA is a useful method to simultaneously assess the plasma membrane integrity and acrosomal status of dog spermatozoa when using flow cytometry or epifluorescence microscopy.

- The addition of Equex STM paste to a cryopreservation extender, performing the semen dilution in two steps in order to add the extender containing Equex immediately before the freezing operation, and using fast freezing and thawing rates, improves post-thaw sperm survival and longevity of dog spermatozoa.

- Freezing dog spermatozoa in 0.5-ml straws at concentrations of 200 x 10^6 spermatozoa/mL and diluting the semen immediately post-thaw with a plain Tris buffer at rates of 1: 4 or 1: 2, seems to improve the post-thaw sperm longevity and to maintain the integrity of plasma and acrosomal membrane of spermatozoa during prolonged incubation at 38°C.

References

Aitken RJ, Best F, Richardson DW, Schats R and Simm G (1983) Influence of caffeine on movement characteristics, fertilizing capacity and ability to penetrate cervical mucus of human spermatozoa. J Reprod Fertil, 67, 19-27.

Aitken RJ, Mattei A and Irvine S (1986) Paradoxical stimulation of human sperm motility by 2-deoxyadenosine. J Reprod Fertil, 78, 515-527.

Amann RP (1979) Computerized measurements of sperm velocity and percentage of motile sperm. In: Fawcett DW and Bedford JM (eds): The Spermatozoon. Urban and Schwarzenberg, Baltimore, 431-435.

Amann RP (1988) Relationships between computerized evaluations of spermatozoal motion and competitive index. Proc 12th Technical Confer Artif Insem Reprod, 38-44.

Amann RP (1989) Can the fertility potential of a seminal sample be predicted accurately? J Androl, 10, 89-98.

Amann RP and Hammerstedt RH (1980) Validation of a system for computerized measurements of spermatozoal velocity and percentage of motile sperm. Biol Reprod, 23, 647-656.

Amann RP and Hammerstedt RH (1993) In vitro evaluation of sperm quality: an opinion. J Androl, 14, 397-406.

Andersen K (1975) Insemination with frozen dog semen based on a new insemination technique. Zuchthygiene 10, 1.

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

Anzar M, Hassan MM, Graham EF, Deyo RCM and Singh G (1991) Efficacy of the Hamilton Thorn Motility Analyzer (HTM-2030) for the evaluation of bovine semen. Theriogenology, 36, 307-317.

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.

Asworth PJC, Harrison RAP, Miller NGA, Plummer JM and Watson PF (1995) Flow cytometric detection of bicarbonate-induced changes in lectin binding in boar and ram sperm populations. Mol Reprod Dev, 40, 164-176.

Bane A (1961) Acrosomal abnormality associated with sterility in boar. Proc 4th Int Congr Anim Reprod (ICAR), 810-817.

Battista M, Parks J and Concannon PW (1988) Canine sperm post-thaw survival following freezing in straws or pellets using pipes, lactose, tris or test extenders. Proc 11th Int Cong Anim Reprod (ICAR), 3, 229.

Bedford JM (1970) Sperm capacitation and fertilization in mammals. Biol Reprod, 2 (Suppl), 128-158.

Bessen M, Fay RB and Witman GB (1980) Calcium control of waveform in isolated flagellar axonemes of Chlamydomonas, J Cell Biol, 86, 446-455.

Centola GM, Mattox JH, Burde S and Leary JF (1990) Assessment of the viability and acrosome status of fresh and frozen-thawed human spermatozoa using single wavelength fluorescence microscopy. Mol Reprod Dev, 27, 130-135.

Concannon PW, Hansel W and McEntee K (1977) Changes in LH, progesterone and sexual behaviour associated with preovulatory luteinization in the bitch. Biol Reprod, 17, 604-613.

Concannon PW (1986) Canine physiology of reproduction. In: Burke T (ed): Small Animal Reproduction and Infertility. Philadelphia, Lea and Febiger, 23-27.

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.

Concannon PW, McCann JP and Temple M (1989) Biology and endocrinology of ovulation, pregnancy and parturition in the dog. J Reprod Fertil, 39 (Suppl), 3-25.
Cornett LE and Meizel S (1978) Stimulation of in vitro activation and the acrosome reaction of hamster spermatozoa. Biol Reprod, 20, 925-929.
Cross NL, Morales P, Overstreet JW and Hanson FW (1986) Two simple methods for detecting acrosome-reacted human sperm. Gamete Res, 15, 213-226.
Curry MR and Watson PF (1994) Osmotic effects on ram and human sperm membranes in relation to thawing injury. Cryobiology, 31, 39-46.
DasGupta S, Mills CL and Fraser LR (1993) Ca-related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. J Reprod Fertil, 99, 135-143.
Davies PR (1982) A study of spermatogenesis, rates of sperm production, and methods of preserving the semen of dogs. PhD Thesis, University of Sydney, Australia.
Davies RO, Drobnis EZ and Overstreet JW (1995) Application of multivariate cluster, discriminate function, and stepwise regression analyses to variable selection and predictive modeling of sperm cryosurvival. Fert Steril, 63, 1051-1057.
Dobrinski I, Lulai C, Barth AD and Post K (1993) Effects of four different extenders and three different freezing rates on post-thaw viability of dog semen. J Reprod Fertil, 47 (Suppl), 243-255.
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 Fert, 29, 443-445.
Dresser DW, Atkins CJ, Pinder A and Morrell JM (1993) Analyses of DNA content of living spermatozoa using flow cytometry techniques. J Reprod Fertil, 98, 357-365.
Ellington J, Scarlett J, Meyers-Wallen V, Mohammed HO and Surman V (1993) Computer-assissted sperm analysis of canine spermatozoa motility measurements. Theriogenology, 40, 725-733.
England GCW (1992) The cryopreservation of canine semen. PhD Thesis, London.
England CGW and Allen WE (1992) Factors affecting the viability of canine spermatozoa II Effects of seminal plasma and blood. Theriogenology, 37, 373-381.
Ericsson SA, Garner DL, Johnson LA, Redelman D and Ahmad K (1989) Assessment of the viability and fertilizing potential of cryopreserved bovine spermatozoa using dual fluorescent staining and two flow cytometric systems. Gamete Res, 22, 355-368.
Evenson DP, Darzynkiewic Z and Melamed MR (1982) Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J Histochem Cytochem, 30, 279-280
Evenson DP (1989) Flow cytometry evaluation of male germ cells. In: Yen A (ed): Flow cytometry: Advanced research and clinical applications, Vol 1, CRC Press, Florida, 217-246.
Evenson DP, Jost LK, Baer RK, Turner TW and Schrader SM (1991) Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. Reprod Toxicol, 5, 115-125.
Farlin ME, Jasko DL, Graham JK and Squires EL (1992) Assessment of Pisum sativum agglutinin in identifying acrosomal damage in stallion spermatozoa. Mol Reprod Dev, 32, 23-27.
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.
Ferrara F, Daverio R, Mazzini G, Bonini P and Banfi G (1997) Automation of human sperm cell analysis by flow cytometry. Clin Chem, 43, 801-807.
Foote RH (1964) Extenders for freezing dog semen. Am J Vet Res 25, 37-39.

Gao DY, Asworth E, Watson PF, Kleinhans FW, Mazur P and Critser JK (1993) Hyperosmotic tolerance of human spermatozoa: separate effects of glycerol, sodium chloride, and sucrose on spermolysis. Biol Reprod, 49, 112-123.

Garner DL, Gledhill BL, Pinkel D, Lake S, Stephenson D, Van Dilla MA and Johnson LA (1983) Quantification of the X- and Y-chromosome-bearing spermatozoa of domestic animals by flow cytometry. Biol Reprod, 28, 312-321.

Garner DL, Pinkel D, Johnson LA and Pace MM (1986) Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analysis. Biol Reprod, 34, 127-138.

Garner DL, Johnson LA and Allen CH (1988) Fluorometric evaluation of cryopreserved bovine spermatozoa extended in egg yolk and milk. Theriogenology, 30, 369-378.

Garner DL, Johnson LA, Yue ST, Roth BL, Haughland RP (1994) Dual DNA staining assessment of bovine sperm viability using SYBR-14 and Propidium iodide. J Androl, 16, 620-629.

Garner DL and Johnson LA (1995) Viability assessment of mammalian sperm using SYBR-14 and Propidium iodide. Biol Reprod, 53, 276-284.

Garner DL, Thomas CA and Gravance CG (1999) The effect of glycerol on the viability, mitochondrial function and acrosomal integrity of bovine spermatozoa. Reprod Dom Anim, 34, 399-404.

Gill HP, Kaufman CF, Foote RH and Kirk RW (1970) Artificial insemination of beagle bitches with freshly collected, liquid-stored and frozen-stored semen. Am J Vet Res, 31, 1807-1813.

Goltz JS, Gardner TK, Kanous KS and Lindemann CB (1988) The interaction of pH and Cyclic Adenosine 3', 5'-monophosphate on activation of motility in Triton X-100 extracted bull sperm. Biol Reprod, 39, 1129-1136.

Govette G, Linde-Forsberg C and Ström B (1996) A successful concept for freezing of dog semen. 13th Int Cong Anim Reprod (ICAR), 2, 5-8.

Günzel-Apel AR, Günther C, Terhaer P and Bader H (1993) Computer-assisted analysis of motility, velocity and linearity of dog spermatozoa. J Reprod Fert, 47 (Suppl), 271-278.

Graham EF, Rajamannan AHJ, Schmehl MKL, Makilaurila M and Bower RE (1971) Fertility studies with frozen boar spermatozoa. A.I. Digest, 19 (6), 6-7.

Graham JK, Kunze E and Hammerstedt RH (1990) Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biol Reprod, 43, 55-64.

Hammerstedt RH, Graham JK and Nolan JP (1990) Cryopreservation of mammalian sperm: what we ask them to survive. J Androl, 11, 73-88.

Hammerstedt RH (1993) Maintenance of bionenergetic balance in sperm and prevention of lip peroxidation: a review of the effet on design of storage preservations systems. Reprod Fertil Dev, 5, 675-690.

Harrison RAP and Vickers SE (1990) Use of fluorescent probes to assess membrane integrity of mammalian spermatozoa. J Reprod Fertil, 88, 343-352.

Harrison RAP, Asworth PJC and Miller NGA (1996) Assessment of sperm function under fertilizing conditions. Reprod Dom Anim, 31(1), 25-30.

Harrison RAP and Miller NGA (1998) Applying flow cytometry to the investigation of live sperm suspensions. Proc BAS Advanced Topics in Andrology. Sperm Biology: New Techniques, New Inshights, 1-3.

Haugland RP (1996) Probes useful at near-neutral pH. In: Spence MTZ (ed): Handbook of fluorescent probes and research chemicals, Eugene, OR: Molecular Probes Inc, 23 (2), 555-556.

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

Holden CA, Hyne RV, Sathananthan AH and Trounson A (1990) Assessment of the human sperm acrosome reaction using Concanavallin A lectin. Mol Reprod Dev, 25, 247-257.

Holt WV (1996) Can we predict fertility rates? Making sense of sperm motility. Reprod Dom Anim, 31, 17-24.

Holt WV, Head MF and North RD (1992) Freeze-induced membrane damage in ram spermatozoa is manifested after thawing: observations with experimental cryomicroscopy. Biol Reprod, 46, 1086-1094.

Holt WV and North RD (1994) Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa. Biol Reprod 51, 414-424.

Hoskins DD, Stephens DT and Hall ML (1974) Cyclic adenosine 3',5'-monophosphate and protein kinase levels in developing bovine spermatozoa. J Reprod Fert, 37, 131-133.

Hyams JS and Borisy GG (1978) Isolated flagellar apparatus of Chlamydomonas: Characterization of forward swimming and alteration of waveform and reversal of motion by calcium ions in vitro. J Cell Sci, 33, 235-253.

Ivanova-Kicheva MG, Subev MS, Bobadov ND, Dacheva DP and Rouseva IA (1997a) Effect of thawing regimens of the morphofunctional state of canine spermatozoa. Theriogenology, 44, 563-569.

Ivanova-Kicheva MG, Bobadov ND and Somlev B (1997b) Cryopreservation of canine semen in pellets and in 5 mL aluminium tubes using three extenders. Theriogenology, 48, 1343-1349.

Januskauskas (1995) Studies on the assessment of post-thaw sperm viability in Swedish dairy AI bulls. Licenciate Thesis, Swedish University of Agricultural Sciences.

Jeffcoate IA and Lindsay FEF (1989) Ovulation detection and timing of insemination based on hormone concentrations, vaginal cytology and the endoscopic appearance of the vagina of domestic bitches. J Reprod Fertil, 39 (Suppl), 277-287.

Johnson LA, Flook JP and Hawk HW (1989) Sex preselection in rabbits: Live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod, 41, 199-203.

Johnson LA, Maxwell WMC, Dobrinsky JR and Welch GR (1996) Staining sperm for viability assessment. Reprod Dom Anim, 31, 37-47.

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

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.

Leipold SD, Graham JK, Squires PM, McCue PM, Brinsko SP and Venderwall DK (1998) Effect of spermatozoal concentration and number on fertility of frozen equine semen. Theriogenology, 49, 1537-1543.

Linde-Forsberg C and Forsberg M (1989) Fertility in dogs in relation to semen quality and the time and site of insemination with fresh and frozen semen. J Reprod Fertil, 39 (Suppl), 299-310.

Linde-Forsberg C and Forsberg M (1993) Results of 527 controlled artificial inseminations in dogs. J Reprod Fertil, 47 (Suppl), 313-323.

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.

Linford E, Glover FA, Bishop C and Stewart DL (1976) The relationship between semen evaluation methods and fertility in the bull. J Reprod Fertil, 47, 283-291.

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.
Maxwell WMC and Johnson LA (1997) Membrane status of boar spermatozoa after cooling or cryopreservation. Theriogenology, 48, 209-219.
Maxwell WMC, Welch GR and Johnson LA (1997) Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. Reprod Fert Devel, 8, 1165-1178.
Mazur P (1984) Freezing of living cells: mechanisms and implications. Am J Physiol, 247, 125-142.
Medeiros CMO and Parrish JJ (1996) Changes in lectin binding to bovine sperm during heparin-induced capacitation. Mol Reprod Dev, 44, 525-532.
Mendoza C, Carreras A, Moos J and Tsarik J (1992) Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using Pisum sativum agglutinin. J Reprod Fertil, 95, 755-763.
Morrell JM (1991) Applications of flow cytometry to artificial insemination: a review. Vet Rec, 129, 375-378.
Morton DB (1988) Artificial insemination with frozen semen in the dog: principles of 'DNA fingerprinting'. In: Joshua JO and Jones DE (eds): Reproductive clinical problems in the dog. 2nd ed. Wright, London, 169-186.
Nolan JP, Graham JK and Hammerstedt RH (1992) Artificial induction of exocytosis in bull sperm. Arch Biochem Biophys, 292, 311-322.
O'Connor MT, Amann RP and Saacke RG (1981) Comparisons of computer evaluations of spermatozoal motility with standard laboratory tests and their use for predicting fertility. J Anim Sci, 53, 1368-1376.
Oettlé EE and Soley JT (1988) Sperm abnormalities in the dog: A light and electron microscopic study. Vet Med Rev, 59, 28-70.
Olar TT (1984) Cryopreservation of dog spermatozoa. PhD Thesis, Colorado State University.
Olar TT, Bowen RA and Pickett BW (1989) Influence of extender, cryopreservative and seminal processing procedures on post-thaw motility of canine spermatozoa frozen in straws. Theriogenology, 31, 451-461.
Palacios A, Valencia J and Zarco L (1992) Efecto del sistema de envasado y la concentración espermática sobre el daño acrosomal y la motilidad postdescongelación del semen equino. Vet Mex, 4, 315-318.
Papaioannou KZ, Murphy RP, Monks RS, Hynes N, Ryan MP, Boland MP and Roche JF (1997) Assessment of viability and mitochondrial function of equine spermatozoa using double staining and flow cytometry. Theriogenology, 48, 299-312.
Parks JE and Graham JK (1992) Effects of cryopreservation procedures on sperm membranes. Theriogenology, 38, 209-222.
Parlevliet J, Malmgren L, Boyle M, Wöckener A, Bader H and Colebrander B. (1992) Influence of conservation method on the motility and morphology of stallion semen (an international projetc). Acta Vet Scand, 88, 153-162.
Parrish JJ, Susko-Parrish JL, Winer MA and First NL (1988) Capacitation of bovine sperm by heparin. Biol Reprod, 38, 1171-1180.
Penfold LM and Moore HDM (1993) A new method for cryopreservation of mouse spermatozoa. J Reprod Fertil 99, 131-134.
Peña AI, Barrio F, Quintela LA and Herradón PG (1998a) 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, Barrio F, Quintela LA and Herradón PG (1998b) Effect of different glycerol treatments on frozen-thawed dog sperm longevity and acrosomal integrity. Theriogenology, 50, 163-174.

Pinkel D, Garner DL, Gledhill BL, Lake S, Stephenson D and Johnson LA (1985) Flow cytometric determination of the proportions of X- and Y- chromosome bearing sperm in samples of purportedly separated bull sperm. J Anim Sci, 60, 1303-1307.

Polge C, Smith AU and Parkes AS (1949) Revival of spermatozoa after vitrification and dehydration at low temperature. Nature 164, 666.

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

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, Ström B, Linde-Forsberg C and Rodriguez-Martínez 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.

Rota A, Linde-Forsberg C, Vannozzi J, Romagnoli S and Rodriguez-Martinez H (1998a) Cryosurvival of dog spermatozoa at different glycerol concentrations and freezing/thawing rates. Reprod Dom Anim, 33, 355-361.

Rota A (1998b) Studies on cryopreservation, capacitation and fertility of dog spermatozoa. PhD Thesis, Uppsala.

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-Martínez 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, 57, 199-215.

Rowson LEA (1954) Infertility of cow, sow and bitch. Irish Veterinary Journal 8, 216-221.

Saacke RG and White JM (1972) Semen quality tests and their relationship to fertility. Proc 4th Tech Conf Artif Insem Reprod, National Association of Animal Breeders, 2-7.

Saacke RG (1982) Components of semen quality. J Anim Sci, 55 (2), 1-13.

Saacke RG, Nadir S and Nebel RL (1994) Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. Theriogenology, 41, 45-50.

Seager SWJ (1969) Successful pregnancies utilizing frozen dog semen. A.I. Digest 17, 6-7.

Seager SWJ and Fletcher WS (1973) Progress on the use of frozen smen in the dog. Vet Rec, 92, 6-10.

Seager SWJ and Platz C (1977) Artificial insemination and frozen semen in the dog. Vet Clin North Am, 7, 757-764.

Silva LDM and Verstegen JP (1995) Comparisons between three different extenders for canine intrauterine insemination with frozen-thawed spermatozoa. Theriogenology, 44, 571-579.

Slaughter GR, MacDougall E and Means AR (1982) FSH activation and immunofluorescent localization of SCE testis glycogen phosphorylase. J Cell Biol, 95, 182.

Smith FO (1984) Cryopreservation of canine semen, technique and performance. PhD Thesis, University of Minnesota.

Spallanzani AL (1787) Expériences pour servir a l'histoire de la géneration des animaux et des plantes. Cited by Badinand F, Fontbonne A and Adoue C (1990) Préparation, conditionnement, conservation et utilisation de la semence du chien en insémination artificielle. Elevage et Insemination, 239, 3-15.

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

Ström-Holst B (1999) In vitro characterization of cryopreserved canine spermatozoa with special reference to post-thaw survival time and zona pellucida binding capacity. PhD Thesis, Uppsala.

Sukardi S, Curry MR and Watson PF (1997) Simultaneous detection of the acrosomal status and viability of incubated ram spermatozoa using fluorescent markers, Anim Reprod Sci, 46, 89-96.

Tao J, Critser ES and Critser JK (1993) Evaluation of mouse sperm acrosomal status and viability by flow cytometry. Mol Reprod Dev, 36, 183-194.

Tash JS and Means AR (1982) Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. Biol Reprod, 26, 745-763.

Tash JS and Means AR (1983) Cyclic adenosine 3', 5' monophosphate, calcium and protein phosphorylation in flagellar motility. Biol Reprod, 28, 75-104.

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. Proc 12th Int Cong Anim Reprod (ICAR), 4, 1823-1825.

Thomas PGA, Larsen RE and Hahn CN (1993) A comparison of three packaging techniques using two extenders for the cryopreservation of canine semen. Theriogenology, 40, 1199-1205.

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 (1989) Gamete physiology and timing of ovulation and fertilization in dogs. J Reprod Fertil, 39 (Suppl), 269-275.

Tuli RK, Schmidt-Baulain R and Holtz W (1992) Computer-assisted motility assessment of spermatozoa from fresh and frozen-thawed semen of the bull, boar and goat. Theriogenology, 38, 487-490.

Uhler ML, Leung A, Chan SYW, Schmid I and Wang C (1993) Assessment of human sperm acrosome reaction by flow cytometry: validation and evaluation of the method by fluorescence-activated cell sorting. Fertil Steril, 60, 1076-1081.

Wales RG and White IG (1963) Viability of diluted dog spermatozoa. J Reprod Fert, 5, 67-76.

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.

Williams WW (1920) Technique of collecting semen for laboratory examination with a review of several diseased bulls. Cornell Vet, 10, 87-94.

World Health Organization (1992) WHO Laboratory Manual for the examination of human semen and semen-cervical mucus interaction. Cambridge: The Press Syndicate of the University of Cambridge.

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