

HINTS ON DOG SEMEN FREEZING, CRYOEXTENDERS, AND FROZEN SEMEN ARTIFICIAL INSEMINATION

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Having a practice directed towards breeders may generate many more clients each year from among their puppy buyers. A way to attract the breeders is to offer them expert help in breeding matters such as determining the proper time for mating of the bitch, checking the semen quality in stud dogs, preserving semen for shipping or long-time storage and performing artificial insemination (AI). There is a steadily increasing interest in canine frozen semen, as breeders are discovering that improved methods are leading to better results. The AKC registration figures for frozen semen litters, consequently, have doubled from 1997 to reach 658 in 2000.

Starting up a canine frozen semen service.

What are the alternatives if you want to extend your services to also encompass semen freezing? You will need to know quite a bit about sperm physiology and function, as well as about various pathologies in order to be able to do a good job in your semen laboratory. This means that unless you are already used to working with semen you will need to attend some courses. You will also need to get the necessary equipment, such as a proper cooler, some large liquid nitrogen (LN₂) tanks for storage of semen, and a number of smaller LN₂ dewars for shipment, a quality microscope, a centrifuge, a sperm counting chamber or a haemocytometer calibrated for dog spermatozoa, and you may also need special AI equipment.

You must then decide which semen freezing system you want to use. Canine semen is mainly frozen either as pellets or in straws. In the first successful studies on canine semen freezing (Seager 1969; Seager et al., 1975) a pellet system was used, and some of the commercial collection and storage facilities are still using this system, whereas Andersen in 1972 introduced the straw freezing method for dog semen. I think that it is correct to say that most people today prefer the straw freezing method. There is probably no difference in semen quality between semen frozen in pellets and straws, but the pellets are considered to carry a greater risk for contamination by infectious agents and spermatozoa from other dogs, and are sometimes not as easy to identify as the straws. The pellets are also by many considered more difficult both to store and thaw than the straws. To open the small screwcap of a cryotube with a forceps or to cut the edge of the cryovial with a pair of scissors at -197°C wearing protective gloves can be a delicate operation and unless the vial containing the pellets is opened immediately at removal from the LN₂-tank it may explode, scattering the pellets. Practice makes perfect, no doubt, but for many it is a matter for concern. The straws come in two sizes, mini straws containing 0.25 ml and medium straws containing 0.5 ml. Usually medium straws are used for dog semen.

Another important decision to make is whether to join one of the commercial companies, usually on a franchise basis, or to use non-commercial canine semen cryopreservation systems. There are advantages and disadvantages to both. Advantages from joining one of the companies is that they may provide training, extenders and other necessary equipment. They also often have a network of associates offering nation-wide and sometimes also international coverage. Some have a

central semen storage location, so that you do not have to invest in dewars and space for a semen bank. They may also help you organize the semen shipments. Some of the disadvantages are the costs involved in franchising, that some companies request that you sign a contract agreeing to exclusively use their system, and you are also dependent on them for timely delivery of the extenders etc. In general you will not receive any information about the results from the particular proprietary protocol you are using. Advantages of using the non-proprietary cryopreservation systems for dog semen are that you are free to use the method that you find works best for each particular dog, you can make your own extenders (if need be with the help of a pharmacist), and you know every step of the procedure. You can count on that any further developments to the protocols and AI-results will be published. Disadvantages are that you are on your own and must get your own training, buy your own equipment and organize your own semen bank and semen shipments etc (see Linde-Forsberg, 2001a, b).

You will, no doubt, want to make sure that you are choosing a good system. If you are joining a company which does not reveal its success rate, you will just have your own results to go by. So, what should we today expect in the way of AI-results from cryopreserved dog semen? Looking back there has been a slow but steady increase in the whelping rates from frozen-thawed dog semen. It was reported to be 30-40% in the late 1960-ies and early 1970-ies (Seager 1969; Seager et al., 1975), 40-50% during the 1980-ies (e.g. Linde-Forsberg and Forsberg, 1989) and 50-60% during the early 1990-ies (e.g. Linde-Forsberg and Forsberg 1993; Linde-Forsberg 2000) and are now reaching 70 to 85% (Nöthling et al., 1995; Linde-Forsberg et al., 1999; Thomassen et al., 2001) and are thus approaching those obtained from well planned natural matings. This improvement has come about because of our vastly increased understanding of the physiology of the estrous cycle of the bitch and which days of the cycle are the most fertile, that we now have regular access to progesterone and LH assays, the appreciation for that canine semen should be deposited in the bitch's uterus and methods to achieve this, and improved protocols for dog semen cryopreservation. Using several of the available freezing systems and intrauterine semen deposition it should, thus, be reasonable to expect an average whelping rate of between 60 and 70% on a regular basis.

What do we do to the spermatozoa when we freeze and thaw them?

During cryopreservation the spermatozoa will be first cooled from body temperature to the temperature of liquid nitrogen (LN₂, -197°C), and subsequently rewarmed immediately prior to AI. The challenge for the spermatozoa is not to withstand the very low temperature at which they are preserved, frozen spermatozoa can be stored practically indefinitely, for up to 2000 years or more, but to survive the changes in temperature, both above and below 0°C, to which they are exposed during freezing and thawing.

Damage inflicted on spermatozoa when they are cooled to 0°C is called cold shock. The magnitude of the damage depends on the species, the cooling rate, the temperature interval and temperature changes (Watson, 1990). Cold shock results in a lower proportion of motile spermatozoa, circular movements, diminished energy production, leakage of ions and molecules from a more permeable plasma membrane, and cell death (Amann and Pickett 1987). Such changes are related to phase transitions in lipids of the plasma membrane bilayer and other membranous structures (e.g. acrosome, mitochondria), resulting also in altered structure and

function of membrane proteins such as ion channel proteins. In spermatozoa, calcium regulation is affected by cooling, and the uptake of calcium results in destabilising membrane changes resembling capacitation (Watson 2000).

The main stresses to spermatozoa during freezing and thawing are induced by osmotic changes, dehydration and ice crystal formation, events occurring in a temperature zone from -15 to -60°C (Mazur 1985). The spermatozoa can be damaged both when they are frozen too slowly and too rapidly. During very slow freezing, as water leaves the cell and freezes outside it, spermatozoa can be damaged by exposure to a too high concentration of solutes (the so-called “solution effects”) and by excessive cell shrinkage. When freezing rates are too fast, on the other hand, water freezes inside the cells, being a potential source of damage. Small intracellular ice crystals are not necessarily harmful for spermatozoa. However, if the rewarming is not fast enough, the crystals may become larger and may mechanically damage the cell. A too fast thawing when the freezing rate has been very slow, in contrast, might create a sudden osmotic stress to the dehydrated cell, resulting in swelling of the spermatozoa (Mazur 1985, Griffiths et al 1979).

In order to minimize injuries derived from cold shock and the freezing-thawing process, spermatozoa are diluted in special extenders, cryoprotectants are added, and the rates of cooling and rewarming are controlled.

Cryopreservation extenders for dog semen

Freezing extenders should have a composition that protects the spermatozoa from cold shock and injuries during freezing and thawing, have an osmolarity similar to that of seminal fluid, maintain pH and provide substrate for sperm metabolism. They should also always contain some antibiotics to prevent growth of bacteria

Buffers. In the dog, the first pregnancies resulting from frozen-thawed sperm derived from semen diluted in lactose-based (Seager 1969) and Tris-hydroxymethyl aminomethane (Tris)-based (Andersen 1972) extenders. Since then, a number of diluents have been tested for dog semen cryopreservation (e.g. Davies 1982, Smith 1984, Battista et al., 1988, England 1992). Extenders containing the zwitterionic buffer Tris are today the most commonly used (e.g. Farstad 1984, Wilson 1993, Nöthling et al., 1995, Koutsarova et al., 1997, Rota et al., 1997, Peña et al., 1998a, Peña and Linde-Forsberg, 2000a, b). In diluents for canine semen, Tris is normally titrated against citric acid.

Cryoprotectant. The addition of a cryoprotectant is essential for sperm survival during freezing and thawing. Cryoprotectants can have either an extracellular (e.g. sugars, such as lactose) or an intracellular (e.g. glycerol or DMSO) action. Glycerol is the cryoprotectant most frequently used for dog semen cryopreservation, and its main effect is to reduce the salt concentration and improve the amount of unfrozen water both inside and outside the cell at any given temperature. Freezing and thawing rates and extender composition interacts with the concentration of the cryoprotectant, and therefore the percentage of glycerol in the extender varies between freezing methods. For canine semen, a wide range of glycerol concentrations (2 to 8%), have been tested (Davies 1982, Olar et al., 1989, Terhaer 1993, Peña et al., 1998b, Rota et al., 1998). Most protocols today use 5-8% glycerol.

Egg yolk. Egg yolk is routinely included in semen extenders for protection against cold shock, and although its action is not fully understood, it is thought to function at the plasma membrane site. Egg yolk contains phospholipids and low density lipoproteins. Most diluents for canine semen contain 10-20% egg yolk. Another source of proteins sometimes included in freezing extenders is milk (Rota et al., 2000).

Sugars. In semen extenders, sugars are included as energy supply to spermatozoa, for their action as cryoprotectants, and for their contribution to osmotic pressure. For dog semen, the sugars most commonly used are the monosaccharides glucose and fructose and the disaccharide lactose. Recently, different sugars were tested in freezing extenders for canine spermatozoa and trehalose, xylose and fructose yielded the best post-thaw survival (Yildiz et al 2000). Rigau et al., (2001a,b) showed that fructose induced a faster and more linear motility pattern than did glucose, whereas the motility obtained with glucose had a similarity to that described for hyperactivated canine spermatozoa. They also found that at low concentrations fructose was less efficient than glucose in maintaining elevated energy levels (Rigau et al., 2001 b). The sugars may be of greater importance for the chilled semen extenders, to maintain sperm longevity for longer periods without freezing.

Detergents. In addition, a number of other substances have been tested and can be included in freezing extenders for dog semen. Especially Sodium Dodecyl Sulphate (SDS) or compounds containing this detergent (Equex STM paste, Orvus ES paste), added to a Tris-based extender increased post-thaw sperm survival and longevity in dogs (Thomas et al., 1992, Rota et al., 1997, Peña et al., 1998a, Peña and Linde-Forsberg, 2000a,b). Addition of Equex STM paste (Nova Chemical Sales Inc. Scituate, MA, USA) resulted also in an increased number of frozen-thawed dog spermatozoa able to bind in vitro to the dog oocyte zona pellucida (Ström-Holst et al., 2000) and in pregnancy rates of 70% or higher, both after vaginal and intrauterine insemination (Nöthling et al., 1995, Rota et al., 1999a, Kong et al., 2000). (Please note that Equex from Minitüb has a different composition compared to the Equex STM paste from Nova Chemical Sales and has by Peña et al [2002] been found to lack the protective effects of the Equex STM Paste from Nova).

Antibiotics. Semen extenders should also contain antibiotics, usually penicillin and streptomycin, to prevent the growth of bacteria in the extended semen.

There is a large number of agencies world-wide which freeze dog semen using proprietary systems. The American Kennel Club, for instance, lists around 100 U.S. collection and storage facilities for frozen canine semen. Many of those are franchises of for instance Canine Cryobank, Cryogenetic Laboratories of New England (CLONE), International Canine Semen Bank (ICSB), or Symbiotics and utilize their proprietary extenders. Other agencies use commercial extenders with proprietary composition developed for other species such as for instance EZ-MIXIB CST with amikacin (Animal Reproduction Systems, Chino, CA) or Triladyl (Minitüb, Tiefenbach, Germany). In many countries in Europe and elsewhere non-proprietary cryopreservation systems such as the Norwegian method described by Andersen (1972), or the Swedish "Uppsala method" (Rota et al.,

1997; Peña and Linde-Forsberg 2000a,b) are used. The Norwegian and Uppsala protocols and extenders are described below.

Freezing methods

A number of freezing methods have been described in the literature in the last 30 years (for review e.g. Concannon and Battista 1989, England 1993, Farstad 1996; Linde-Forsberg et al., 1999, Peña and Linde-Forsberg 2000a, b).

Centrifugation of the ejaculate. In most protocols, the second, sperm-rich fraction of the ejaculate is collected, evaluated and centrifuged at 600-700g for 5-10 min, in order to eliminate the excess prostatic fluid which has been demonstrated to have a detrimental effect on chilled dog spermatozoa (Rota et al., 1995) as well as on motility and viability after freezing and thawing (Sirivaidyapong et al., 2000), and to standardize the extension of the semen to a controlled final sperm concentration and glycerol concentration etc. It also reduces the number of straws from each collection. If the ejaculate is collected well fractionated, however, it can be diluted with an extender and frozen without prior centrifugation (Andersen 1972).

To calculate the g-force of a centrifuge the following formula can be used:

$$\text{g-force} = R \times 11.18 \times \frac{[\text{RPM}]^2}{[1000]}$$

where R = radius in cm from centrifuge spindle to the end of the tube and RPM the number of rotations by the spindle per minute.

Final sperm concentration. After centrifugation the sperm pellet is mixed with extender to the desired final sperm concentration. In most systems dog semen is frozen at a final concentration of 50-100 million spermatozoa per 0.5 ml straw, and 2-4 straws are used per AI. When different spermatozoal concentrations were tested using a Tris-based extender, the best post-thaw results were obtained at a final sperm concentration of 200×10^6 spermatozoa/ml (Peña and Linde-Forsberg 2000a).

Dilution. The dilution can be done in one (Andersen 1972) or two steps. When two-step dilution is used the first is done at room temperature and the second after chilling/equilibration and just before the freezing (Rota et al., 1997, Linde-Forsberg et al., 1999, Peña and Linde-Forsberg 2000a,b). No significant difference was found in post thaw viability or longevity of canine spermatozoa whether glycerol was added all at once or in two steps with increasing concentrations (Smith 1984, Fontbonne and Badinand 1993, Peña and Linde-Forsberg 2000b). However, recent studies indicate that when Equex STM paste (Nova Chemical sales Inc.) is used it is better to add it immediately before freezing, therefore, when this compound is used, dilution should always be done in two steps (Peña and Linde-Forsberg 2000b).

Equilibration. Prior to freezing, the diluted semen needs to be “equilibrated”. This procedure consists of cooling the semen to 4°C and to hold it at this temperature for a period of time during which the spermatozoa develop a higher resistance to the effects of freezing. For dog semen this

time is usually 1-2 hours but may range from 1 to 4 hrs in different protocols. Although canine spermatozoa are considered relatively resistant to cold shock, it has been shown that also in this species a comparatively slow cooling is preferable (White 1993, England 1992). When a controlled cooler is not available, the tube containing diluted semen can be placed in a refrigerator and, if a slower cooling rate is desired, the tube can be surrounded by a room temperature water jacket (Linde-Forsberg et al., 1999).

Packaging of semen. Dog semen is generally frozen in 0.5 or 0.25 ml straws, or in pellets. Other packaging systems such as ampoules, maxi-straws and aluminum tubes have been tested in the canine. Although both straws and pellets have been found equally good for dog semen cryopreservation (Seager and Fletcher 1973, Davies 1982, Nizanski et al., 2001), straws are more hygienic, are easier to identify, store and thaw, and are therefore usually preferred. Semen doses should be easily identifiable: breed, name and registration number of the dog, together with the date and place of freezing, should be clearly marked on the package.

Freezing. It is generally considered that freezing rates in the range 10-100°C/min are suitable for spermatozoa (Watson 1990) although the higher values in this range could be too fast for dog spermatozoa. Several freezing rates have been tested for dog semen and although results are sometimes inconsistent, cooling at a rate between 10 and 50°C per minute in the critical range -15/-60°C seems to be adequate (Olar et al., 1989, England 1992, Rota et al., 1998, Peña and Linde-Forsberg 2000b). Hay et al. (1997) compared a very fast rate (-99°C/min), which was clearly detrimental, and a very slow rate (-0.5°C/min), which was also detrimental, whereas freezing rates of -12°C/min or -28°C/min gave good results. Freezing can be done by using a programmable freezer or by placing the straws at different distances over LN₂. Programmable freezers are expensive both to buy and to run, and most of the time only few straws of dog semen are frozen in each batch. Canine semen is, therefore, usually frozen by placing the 0.5 ml straws in liquid nitrogen (LN₂) vapour on a rack 4 cm above the LN₂ surface in a styrofoam box for 10 min (Andersen 1972), or by lowering the straws in 3 steps directly into a LN₂ tank (Linde-Forsberg et al., 1999; Peña and Linde-Forsberg 2000a,b). If the semen is frozen in pellets, drops of diluted semen are frozen directly in small depressions made in solid carbon dioxide blocks, and then the pellets are immersed in LN₂ (Seager 1969; Goodrowe et al., 1998). The frozen semen has to be kept in LN₂ tanks for storage, or for shipment.

Thawing. For thawing, straws are immersed in a waterbath at the temperature and for the time recommended by the person/agency who did the freezing. This is important because an interaction has been found between freezing and thawing rates. In the case of dog semen, however, thawing straws at higher rates has been shown to improve spermatozoal viability. Thawing 0.5 ml straws in a waterbath at 70°C for 8 sec resulted in a higher post-thaw survival than thawing at 37°C for 15 to 60 sec (Rota et al., 1998, Peña et al., 2000b).

Thaw medium. In some protocols the semen is diluted in a thaw medium during, or immediately after the thawing. Semen frozen in pellets is always thawed in physiological saline in a sterile plastic Whirl-Pak bag, but in several protocols also straws are emptied immediately post-thaw in some thaw medium. The reason for the beneficial effects of this post-thaw dilution is not clear, but it is thought to reduce toxic effects of substances such as glycerol, Equex or egg yolk, and it increases the volume of the insemination dose (Nöthling et al., 1995; Peña and Linde-Forsberg

2000a). Although a positive relation between post-thaw semen dilution and fertility has not been demonstrated, dilution 1:2-1:4 was beneficial for *in vitro* sperm survival, and high whelping rates were obtained when cryopreserved semen was diluted prior to AI in seminal plasma or artificial media (Linde-Forsberg et al., 1999, Rota et al., 1999a, Nöthling et al., 2000, Peña and Linde-Forsberg 2000a).

Freezing of cold stored semen.

Dog semen is frozen at the various canine semen banks, which are comparatively few and therefore often at a great distance from many dog owners. A few studies have been made on the possibility to freeze dog semen that has been collected and then chilled and stored for 2-3 days before being frozen (Hermansson & Linde-Forsberg, 2002; Verstegen et al., 2002) to find out if it would be feasible to collect a dog nearer to home and ship the chilled semen to the semen bank for freezing. Preliminary results indicate that this could be a workable solution, although there still remains to do some field studies using frozen-thawed semen treated in this way.

Damages inflicted on sperm by the freezing procedure

To be fertile and survive in the female genital tract, a spermatozoon has to be motile and have an intact and functional plasma and acrosomal membrane. To be able to reach the site of fertilisation and penetrate the oocyte it must be able to express, or suppress, a number of attributes in a correct temporal sequence (Amann and Hammerstedt 1993).

The freezing-thawing process results in a significant decrease in viable and motile spermatozoa and a large proportion show morphological modifications or loss of the acrosome (Oettlè 1986, Ström-Holst et al., 1998). The acrosome is an important structure containing enzymes that have to be released during the acrosome reaction at the site of fertilisation. Any damage to the acrosome that results in a premature loss of its content may therefore render the spermatozoon infertile.

A large proportion of dog spermatozoa surviving the cryopreservation process show a shortened life-span during *in vitro* incubation at body temperature (Olar et al., 1989, England 1992, Ström et al., 1997). This reduced longevity is considered to be another reason for the lowered fertility of cryopreserved spermatozoa and may reflect a latent damage caused by the cryopreservation process, rather than being related to ageing as such during the storage. During cryopreservation, capacitation like changes seem to be initiated also in canine spermatozoa, and this could be one reason for the short temporal window of fertility of frozen-thawed spermatozoa in this species (Rota et al., 1999b).

A close relation between spermatozoa and the luminal epithelium of specific areas of the female tract ("sperm reservoirs") appears to be important to maintain sperm viability and to prevent premature capacitation and the acrosome reaction (Dobrinski et al., 1996; Scott 2000). From the reservoirs, small numbers of spermatozoa are continuously released to reach the site of fertilisation. Freshly ejaculated dog spermatozoa have been shown to bind to the epithelium of the uterine tube *in vitro*, and bound spermatozoa remain motile for 48 to 144 hrs (Ellington et al., 1995, Pacey et al., 2000). The decreased ability of frozen thawed spermatozoa to attach to these

oviductal cells might impair the formation of an adequate sperm reservoir in the female, thereby reducing fertility and longevity of cryopreserved semen.

The capacity to bind to the zona pellucida is significantly reduced in cryopreserved dog spermatozoa (Ivanova et al., 1999, Ström-Holst et al., 2000). A reduced binding ability of sperm cells might reflect an ultrastructural damage to the receptors on their plasma membrane, responsible for the interaction with the oocyte. Other receptors thought to be important for fertilisation have recently been identified on the surface of canine spermatozoa such as the progesterone receptors. In the dog progesterone seems to be very important in the induction of the acrosome reaction (Sirivaidyapong et al., 1999). There are indications that in the canine the response of frozen-thawed spermatozoa to progesterone is reduced, and it was hypothesised that this might be due to alterations of progesterone receptors deriving from freezing injury (Cheng et al., 2000).

These damages inflicted on sperm cells result in that higher numbers of spermatozoa have to be used for each frozen semen insemination, and to that spermatozoa need to be deposited close to the site of fertilisation. It is generally recommended to use $150-200 \times 10^6$ spermatozoa/AI (Andersen 1975) for frozen-thawed semen, although there is some evidence that $25-50 \times 10^6$ /AI may be enough to obtain fertilisation (Wilson 1993, Kong et al., 2000). It seems reasonable that fewer spermatozoa would be needed in smaller breeds of dogs than in the larger breeds, but this has not been studied. Ten times as many spermatozoa are required when fresh (Tsutsui et al., 1988) as well as frozen-thawed (Linde-Forsberg et al., 1999) dog semen is deposited in the vagina to obtain the same results as by intrauterine AI.

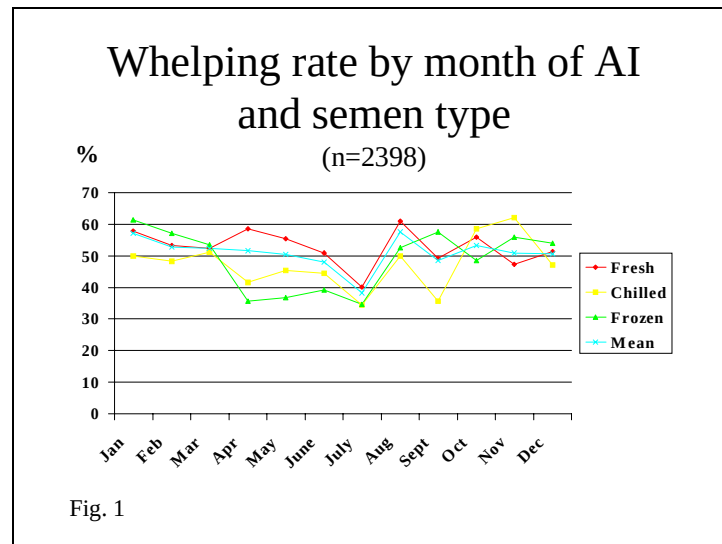
SUCCESSFUL EXTENDERS AND TECHNIQUES

Despite the steadily increasing interest for canine semen preservation and artificial insemination world-wide, comparatively few studies have been published within this field, and it is particularly difficult to find data on results from artificial inseminations. One reason for the scarcity of data is that it is expensive to do experimental studies in dogs, and few research centers can keep large enough dog colonies for this kind of studies. Another reason is that most of the large companies processing and distributing canine semen for dog breeders are working on a commercial basis, and neither the composition of the extenders or the methods for preservation of the semen, nor their results are disclosed. Any knowledge thus gained will, therefore, remain with the different companies, and will not be of benefit to the scientific world, and thus neither to the dog breeders in general.

The only studies on canine artificial insemination reporting on fertility results from a large number of inseminations are those by Seager *et al.* (1975) on 156 frozen-thawed semen AIs using vaginal deposition, Linde-Forsberg & Forsberg (1989; 1993) on 470 and 527 AIs respectively, using both fresh, chilled extended and frozen-thawed semen and vaginal as well as intrauterine AI, Linde-Forsberg *et al.* (1999) on 327 frozen-thawed semen AIs, comparing vaginal and intrauterine AI, Linde-Forsberg (2000) reporting on 2041 AIs with fresh, chilled extended, and frozen-thawed semen and using both vaginal and intrauterine AI, and Thomassen et al. (2001) on 312 frozen-thawed mainly intrauterine AIs.

Seasonal influence on results by AI in the dog?

Compilation of the Swedish AI-data from 1990 – 2001 (n=2398) indicates that there may be a seasonal influence on fertility in the dog (Fig. 1). It is interesting to note that the lowest success rate by AI in the dog is from the AI:s that are done during the summer month of July, and especially that this is seen not only in the AI:s that were performed with fresh and chilled semen, but also with frozen-thawed semen. The latter finding points to that there is a strong female factor involved, because the frozen semen had been collected during various times of the year. Whether this lower fertility during summer is a reflection of heat stress, which has been demonstrated to occur in several other species, or in the dog is dependent on the seasonal changes in day length is unclear. From the figures it would appear that for chilled and frozen-thawed semen the fertility is lowest during the period of increasing daylength in April – July, and increases abruptly during August when there is a marked decrease in daylength. In contrast, for fresh semen AI:s the second highest whelping rates are from the AI:s done during April. This may point to temporal differences in seasonal influence on male and female reproduction in this species, or to that the semen quality deteriorates during spring and is less resistant to the stresses of preservation.



There is also a seasonal variation in the number of bitches inseminated, with the majority occurring during late winter and early spring. This could be taken to indicate that there is a remaining tendency in this species to cycle like their wild ancestors, but the figures could also be biased by that breeders prefer to breed their bitches during this period of the year.

Results using frozen-thawed dog semen. Whelping rates of 84.4% from 167 frozen-thawed semen AIs (Linde-Forsberg et al., 1999) and 71% from 305 frozen-thawed semen AIs (Thomassen et al., 2001) have been reported when the semen was deposited in the uterus using the Scandinavian catheter (Linde-Forsberg, 2001a). In the same studies, when the semen was deposited in the vagina in 141 AIs and 7 AIs, respectively, whelping rates were 58.9% and 29%. In a 9-year field study including 286 frozen-thawed semen AIs with semen of variable quality that had been processed by a large number of different agencies world-

wide and AIs performed in bitches with varying fertility by many different veterinarians the whelping rate by vaginal AI was 34.6% compared to 52.0% by intrauterine AI (Linde-Forsberg, 2000). This was an average over the years, and results improved with time, being 64.7% during the 10th year (Linde-Forsberg and Andersson, unpublished; Tables 1 and 2).

The recent clinical studies (Linde-Forsberg et al., 1999; Linde-Forsberg 2000) are the first to demonstrate significantly better results when semen was deposited in the uterus rather than in the vagina in the dog, and not only for frozen-thawed semen but also for fresh, as well as chilled, extended semen. Also the mean litter size was larger, 5.4 ± 3.0 pups/litter by intrauterine AI, compared to 4.0 ± 2.7 by vaginal AI ($P < 0.001$).

Table 1. The influence of semen type used in 2210 AIs on the interval to whelping, whelping rate and litter size. (LSM and SEM)

Semen Type	Number of AIs	Interval to whelping*		Whelping* rate (%)	Litter size*
		from first AI	from last AI (days)		
Fresh	1333	61.7 $\pm$ 0.16ab	60.0 $\pm$ 0.16a	48.9a	6.5 $\pm$ 0.25a
Chilled	388	61.3 $\pm$ 0.26a	60.0 $\pm$ 0.25ab	49.0a	6.4 $\pm$ 0.40a
Frozen	320	62.2 $\pm$ 0.39ab	61.4 $\pm$ 0.38ab	53.8a	4.1 $\pm$ 0.60b
AI + mating	169	62.2 $\pm$ 0.32b	61.8 $\pm$ 0.31b	84.0b	7.0 $\pm$ 0.49a
Overall means (S.D.)		62.0 (2.4)	61.3 (2.3)	52.1	6.3 (3.6)

- Figures in a column with no letters in common are significantly different. In the model for litter size and interval to whelping month, breed (>10 litters), semen type, insemination type and interaction between the last two effects were included.

Table 2. Semen type, vaginal AI and intrauterine AI evaluated in correlation to the whelping rate and litter size. (LSM and SEM) (n=2210)

Semen type	Whelping rate (%) (n)*		Litter size (pups)*	
	Vaginal AI	Intrauterine AI	Vaginal AI	Intrauterine AI
Fresh	47.7a(1212)	62.0b (121)	6.5 $\pm$ 0.19a	6.4 $\pm$ 0.43a
Fresh + mating	82.9c (151)	88.9c (18)	6.5 $\pm$ 0.35a	7.5 $\pm$ 0.90a
Chilled	45.4a (348)	65.0b (40)	6.4 $\pm$ 0.31a	6.5 $\pm$ 0.71a
Frozen-thawed	36.7a (30)	55.5b (290)	2.9 $\pm$ 1.09c	5.2 $\pm$ 0.32b

*Figures with no letters in common are significantly different

The whelping rate by intrauterine AI, thus, increased with from 36% for fresh semen to 50% for frozen-thawed semen, and the mean litter size increased by 0.3 pups per litter for frozen-thawed semen and by 0.6 and 0.7 per litter for fresh, and chilled semen, compared to vaginal AI.

Methods to perform intrauterine AI in the dog.

Intrauterine AI in the dog can be done transcervically either by way of the Scandinavian catheter (Linde-Forsberg, 2001a), or by using a rigid fiberoptic vaginal endoscope to visualize the cervix and a dog urinary catheter to transverse it (Wilson, 1993; 2001). Intrauterine AI can also be accomplished by invasive methods such as laparoscopy, or full abdominal surgery. In many countries the latter methods are not considered ethically acceptable or are illegal.

Some non-proprietary freezing protocols:

The Norwegian Extender and Freezing Procedure:

(Andersen K: Fertility of frozen dog semen. Acta Vet Scand, 1972,13,128-130)

Tris	6.05 g
Citric acid	3.4 g
Fructose	2.5 g
Aqua dest	184 ml
Glycerol	16 ml
Penicillin	200.000 IU
DHS	0.2 g
Egg yolk	20% (v/v) is added immediately before dilution of the semen.

A well fractionated ejaculate is collected by digital manipulation. (If the ejaculate is not obtained well fractionated it should be diluted about 1 : 5 with the extender and then centrifuged at about 1000 g for 5 min and subsequently resuspended to the original ratio with the extender). The sperm rich fraction is diluted 1 : 5 at 35°C with the Tris extender (see above). Immediately before dilution 20% (v/v) egg yolk is added to the extender. The diluted semen is placed in a water bath at 35°C and cooled in the refrigerator for 2 hours. (The final temperature is then about 4°C). The material is filled in medium straws and frozen in N₂ vapour for about 10 min., 4 cm above the surface and then transferred to the LN₂ – container for storage. The final sperm concentration should be approximately 100 x 10⁶ spermatozoa per ml. The semen is thawed in a water bath at 70°C for 8 s.

Results using the Norwegian freezing procedure. Andersen (1980) reports on whelping rates of between 75 and 80% in two groups of 17 and 20 bitches. In the study by Thomassen et al. (2001) semen frozen according to the Norwegian procedure was used in 129 of the 312 bitches that were inseminated in Oslo between 1994-98. The overall whelping rate (312 bitches) was 70% and mean ($\pm$ SEM) litter size 5.3 $\pm$ 0.2 pups.

The Uppsala Extenders and Freezing Procedure:

(Rota A, Ström B, Linde-Forsberg C & Rodriguez-Martinez H: Effects of Equex STM Paste on viability of frozen-thawed dog spermatozoa during in vitro incubation at 38°C. Theriogenology, 1997, 47, 1093-1101; Peña and Linde-Forsberg, 2000a,b).

<u>Uppsala Equex I:</u>	<u>Extender 1</u>	<u>Extender 2</u>	<u>Thaw medium</u>
Tris	2.4 g	ditto	ditto
Citric acid	1.4 g	ditto	ditto
Glucose	0.8 g	ditto	ditto
Streptomycin	0.1 g	ditto	ditto
Aqua dest.	to 77 ml	to 72 ml	to 100 ml
Bensyl penicillin	0.06 g (in 0.3 ml dest H ₂ O)	ditto	ditto

Glycerol	3 ml	7 ml	none
Equex *	none	1 ml	none
Egg yolk	20 ml	ditto	none
pH =	6.53	6.48	6.60
Osmolarity =	740 mOsm	1370 mOsm	253 mOsm

*) Equex STM paste (Nova Chemical sales Inc., Scituate, MA, USA). **(Please note that the Equex from Minitüb has a different composition and effect, see Peña et al., 2002).**

Some batches for reasons unknown were seen to cause a degree of sperm agglutination in some dogs. Therefore the extender was modified:

<u>Uppsala Equex 2:</u>	<u>Extender 1</u>	<u>Extender 2</u>	<u>Thaw medium</u>
Tris	3.025 g	ditto	ditto
Citric acid	1.7 g	ditto	ditto
Fructose	1.25 g	ditto	ditto
Streptomycin	0.1 g	ditto	ditto
Aqua dest.	to 77 ml	to 72 ml	to 100 ml
Bensyl penicillin	0.06 g (in 0.3 ml dest H ₂ O)	ditto	ditto
Glycerol	3 ml	7 ml	none
Equex *	none	1 ml	none
Egg yolk	20 ml	ditto	none
pH =	6.72	6.74	6.76
Osmolarity =	865 mOsm	1495 mOsm	324 mOsm

*) Equex STM paste (Nova Chemical sales Inc., Scituate, MA, USA). **Please note that the Equex from Minitüb has a different composition, see Peña et al., 2002)**

The second sperm rich fraction of the ejaculate is collected. After assessing morphology and motility and counting the total number of spermatozoa the ejaculate is centrifuged at 700 x g for 6 min. The supernatant is removed (and if it still contains spermatozoa it can be centrifuged again) and the pellet diluted at room temperature in Uppsala Equex/Extender 1 (or Uppsala Equex 2 /Extender 1) to a concentration of 400×10^6 spermatozoa/ml and allowed to equilibrate for 60 – 75 min to +4°C. An equal volume of Uppsala Equex /Extender 2 (or Uppsala Equex 2 /Extender 2) is also cooled to +4°C and added after the equilibration period, immediately before filling 0.5-ml straws, resulting in a final concentration of 200×10^6 spermatozoa/ml. The straws are frozen in LN₂-vapor in an Apollo SX-18 or a TW-10 XT or similar LN₂-tank (Minnesota Valley Engineering, Inc., New Prague, MN, or Taylor-Wharton, Theodore, AL, USA) containing 15-18 ml of LN₂. The freezing is performed in three steps, with the goblets at the top of the canes and with the top of the canes 7, 13 and 20 cm (for 2, 2 and 1 min) below the opening of the tank, respectively, whereupon the canister is placed in the tank. Not more than 4 straws should be

placed in each goblet, and not more than 4 goblets (i.e. a total of 16 straws) be frozen in each batch.

The straws can also be frozen using a styrofoam box containing LN₂ with the straws lying horizontally on a rack placed 4 cm above the surface of the LN₂ for 10 min whereupon the straws are immersed into the LN₂.

The semen is, thus, usually frozen so that the final number of spermatozoa per straw is between 100 and 200 x 10⁶. Depending on the semen quality usually 2-3 straws are used for each AI. In dogs of the smaller breeds which produce fewer spermatozoa per ejaculate it may be desirable to freeze the semen less concentrated to obtain more straws.

The straws are best thawed in a water bath at +70°C for 8 sec. (If this is impractical they can also be thawed at +37°C for 30-60 sec). Any water remaining on the outside of the straw is carefully wiped off before opening the straw. Each straw is emptied into 1 ml of the Uppsala Equex /Thaw medium (or Uppsala Equex 2 /Thaw medium) at +37°C and left at this temperature for approximately 5 min before assessing semen quality and performing the AI.

The Uppsala method is based on our experiences with the protocol of one of the commercial companies (Linde-Forsberg et al., 1999) which we found very practical and easy to use and with which we obtain excellent results. However, we wanted to increase post-thaw sperm longevity to improve results further, and so that the timing of the bitch's estrus period should not be so crucial and the method thereby be more useful also in the hands of those not so experienced. We continued using the freezing method but developed our own extenders. We found that the detergent Equex STM Paste (Nova Chemical Sales Inc., Scituate, MA, USA) was beneficial (Rota et al., 1998) and continued to test the various steps of the freezing protocol and the effects of final sperm concentration (Peña and Linde-Forsberg, 2000a, b):

We evaluated the effects of adding Equex STM Paste to an egg yolk-based Tris extender; compared the effects of a 1-step dilution with those of a 2-step dilution, in which the second extender was added after equilibration and immediately before freezing; compared the effects of two freezing procedures, i.e. placing the straws horizontally above the LN₂ surface in a styrofoam box (box method) versus gradually lowering them (3 steps) into a LN₂ tank (tank method); and compared the effects of two semen thawing rates, i.e. high (70°C for 8 s) versus low (37°C for 15 s).

The addition of Equex STM Paste to the egg yolk Tris-extender (Uppsala Equex extender) considerably increased the thermoresistance of canine spermatozoa and also appeared to prevent or reduce capacitation-like changes in thawed spermatozoa. However, *two step dilution* was better than one step dilution indicating that the exposure of spermatozoa to the detergent or to the detergent-treated egg yolk components during equilibration may have a negative effect. *Freezing in the box* was slightly better than freezing in the tank with regard to its effect on motility patterns and the percentage of motile sperm. Our results suggest that a rate of decrease in temperature of

about $-30^{\circ}\text{C}/\text{min}$ from the beginning of the freezing operation to around -50°C is better than lower rates, and that the freezing rate below -50°C may not be critical. Although the results are not conclusive, it seems that it is better to freeze dog spermatozoa at moderately fast freezing rates, i.e. between $-10^{\circ}\text{C}/\text{min}$ and $-50^{\circ}\text{C}/\text{min}$, over the critical temperature range (-10 to -30°C). In our study *thawing rate* had a significant effect on the sperm parameters studied. All of them were significantly better when the thawing was done at 70°C for 8 s as compared with 37°C for 15 s. Using a variety of extenders, glycerol levels and freezing rates, various workers have reported the post-thaw survival of dog spermatozoa to be higher when thawed at a fast rate than when thawed slowly (Davies, 1982; England 1992; Olar et al., 1989; Rota et al., 1998). Under the conditions used in our study, the fast thawing rate resulted in longer membrane stability at high temperature post-thaw. The reason why sperm thermoresistance in dog spermatozoa is shortened by thawing at low temperature is not known, but may be related to temperature-dependent lipid phase transitions. (Peña and Linde-Forsberg, 2000b).

We also evaluated freezing dog semen at concentrations of 50×10^6 , 100×10^6 , 200×10^6 and $400 \times 10^6/\text{ml}$ and diluting at a rate of 1:0, 1:1, 1:2 and 1:4 after thawing. Post-thaw motility was higher in samples frozen with 200×10^6 spermatozoa per ml, the integrity of sperm plasma membrane was higher after 18 h incubation at 38°C at sperm concentrations of 200 and $400 \times 10^6/\text{ml}$. The integrity of the sperm plasma membrane was higher at increasing post-thaw dilution rates, and motility was highest after 8 h of 38°C incubation at dilution rates 1:2 and 1:4. The best longevity from the 16 treatments was obtained when semen was frozen at $200 \times 10^6/\text{ml}$, and diluted post-thaw at 1:4 dilution rate. (Peña and Linde-Forsberg, 2000a)

Further clinical as well as experimental studies are ongoing to determine to what degree our in vitro results also apply in vivo, i.e. whether fertility rates can be enhanced by using the most advantageous combination of cryopreservation steps.

Conclusions

It is today possible to achieve high whelping rates using frozen-thawed dog semen. However, many factors may affect the rate of success. The quality of the semen and the methods used for freezing, storage and thawing are important for the post-thaw survival of spermatozoa. Accurate monitoring of the bitch's cycle in order to inseminate during the fertilisation period and the use of an appropriate technique for intrauterine semen deposition are equally important as the method used for cryopreservation. It is important to do a continuous follow-up on results. Although time-consuming, this follow-up obviously is necessary for a critical and objective evaluation of any semen processing system and insemination facility.

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