

RECENT ADVANCES IN THE AREA OF CANINE SEMEN PRESERVATION

A.K. Srivastava and A.K. Mathur

Secretary General, Indian Society for the Advancement of Canine Practice, Lucknow; Principal Scientist Project Directorate on Cattle, I.C.A.R., P.O. Box No. 17, G.F. Road, Meerut Cantt. (U.P.); India.

Although dog was the first species to be used for Artificial Insemination (AI) and become pregnant by Spallanzani in 1776, very little work has been done on preservation of dog semen to exploit the full genetic potential of pedigree males. Frozen semen is now inseminated routinely in cattle, sheep and goats and much research has been carried out with regard to the optimum conditions for its use in these species. In many countries there has been limited use of frozen dog semen, owing mainly to the poorer fertility when compared with fresh or chilled semen and also, to some extent, owing to the regulations controlling the use of artificial insemination. The use of frozen semen has, therefore, been limited to inter-continental transfer of genetic material and long-term storage of spermatozoa. The Authors review these turn of events necessitated a detailed investigation on canine semen preservation for A.I. in Bitches. However, the technique of AI gained momentum among dog breeders as a result of increased interaction and exchange of dog semen nationally and internationally. Increased emphasis on scientific dog breeding in recent time has encouraged many researchers to explore the possibilities of artificial breeding in dogs. Apart from exploitation of genetic potential of superior males, AI in dogs help psychological problems like shyness, inexperienced male, habitual premature erection and mating difficulties like female dominance, unequal size of dog and bitch and low libido in male dogs and long distance between stud dog and bitch, semen can be transported and bitch can be inseminated using artificial insemination. Although, excellent extenders for preservation of bovine semen in both chilled and frozen state have been developed, only few attempts have been made on the environmental conditions required to prolong the viability of canine sperm. The presentation deals with more recent advances about the protocols of Canine semen preservation. New perspectives, as the demand and use of Canine semen are also discussed.

Key Words: Dog, Canine Semen, Preservation, Cryo Preservation, Subzero temperature, Semen Extenders, A.I., Fertility, Breeding.

Introduction

Artificial Insemination (AI) was performed for the first time in dog but very

little work has been done on preservation of semen of this species as compared to others. Frozen semen is inseminated routinely in cattle, sheep and goats owing to much research carried out in these species. Although there have been many reports of the successful freezing and thawing of dog semen, the majority of workers have adopted methodology designed for other species, and investigations have merely comprised comparisons between diluents (Andersen, 1975; Davies, 1982; Yuhi, 1984). Thorough investigations have been attempted in only a limited number of studies (Olar, 1984; Smith, 1984) and these have limitations in that the end point has rarely been fertility in any suitable number of bitches.

Conception rates with frozen-thawed dog semen have generally been poor when compared with those of other species. It has been reported that frozen-thawed spermatozoa did not exhibit the vigorous motility of freshly ejaculated spermatozoa and that consequently their survival within the female tract and their fertilizing ability are likely to be reduced. This may be related to the poor penetration of oocytes by frozen-thawed (compared with fresh) dog spermatozoa (Fromian *et al.*, 1984). The problem is not entirely the result of poor semen quality after thawing, but also of the difficulty of identifying the optimal time for insemination of the bitch.

Spallanzani (1776) was the first to observe that a reduction in temperature reversibly reduced the metabolic activity of spermatozoa. It was the discovery of the cryoprotective action of glycerol (Polge *et al.*, 1949) that made a significant impact upon the methodology of spermatozoal cryopreservation. The first success in freezing dog spermatozoa was reported by Rowson (1954). Though, in 1969 Seager obtained the first canine pregnancy using frozen thawed semen, the potential development of cryopreservation and AI in dog is not equal to the industry of cryopreserved semen of other farm animals. Since last three decades interest for artificial

insemination (AI) in dogs has increased many folds. There can be two possibilities when semen is transferred for insemination. It may be diluted, cooled and stored at 5°C for several days (Pinto et al., 1999; Iguer-ouda and Verstegen, 2001) or semen may be extended in suitable extender and cryopreserved at -196°C (6-8).

Cryopreservation of canine semen like that of other animal species is used for artificial insemination and for storage of semen samples from valuable dog breeds (Ivanova et al., 1992). The cryopreservation of dog semen is becoming increasingly popular since it allows for the transporting of genetic materials both within and between countries. In dogs the first successful AI using frozen thawed semen was reported by Seager, 1969. Since then different studies have been reported for testing various techniques, extenders etc. Since the first birth of live offspring from frozen dog semen (Seager, 1969), dog breeders world-wide have put increasing pressure on the scientific world to be part of the progress in reproduction biotechnology. And when developing new techniques for cryopreservation of spermatozoa, the goal is to minimize the damage caused to spermatozoa by the process in order to recover a maximum number of viable spermatozoa.

The cryopreservation process results in reduced fertility compared with fresh semen. It has been shown that this arises from a combination of both loss of sperm viability and

an impairment of function in the population of survivors. This situation needs to be borne in mind when strategies to improve the results are contemplated. We need to consider not only the cryopreservation protocol to optimize the number of survivors, but also the functional ability of the surviving population. Considering all these aspects the aim of this paper is to have an over view of the current status and advances made in the area of semen preservation in dogs.

Dog semen preservation in India

However, very little experimental work has done on semen preservation and AI in canine. Deshpande et al., 1970; collected dog semen in three different fractions viz., first, second and third and evaluated in Alsatian dog. Srivastava and Singh, 1986; developed a new semen extender for preservation of Canine semen at subzero temperature. Detailed evaluation of Canine semen was performed by Srivastava and Singh, 1988; and Spermatozoal Bent Tail Abnormality was detected by them causing infertility in male Golden Retriever Dog. An Observation on Survival of Stored Dog Sperm in Buffered- Yolk Extenders was made by Srivastava and Singh, 1989. Similarly, semen was collected by digital manipulation in mongrel dogs and physical and morphological characters were evaluated by Daiwadnya and Hukeri, 1993. Dabas et al., 1991; reported that whelping of 3-6 pups after AI with semen diluted in buffered-yolk extender.



COLLECTION OF SEMEN

Semen Evaluation

Color Healthy canine semen should be pearly white or translucent in color. Yellow semen indicates urine contamination, and

urine is toxic to sperm. Red discoloration indicates that blood is present either from trauma, prostate problems or infection.

Motility: Since sperm have to be able to

travel up the bitch's reproductive tract toward her eggs, the measure of how well sperm are moving and in what direction is important. A motility of 70% or greater is rated very good; 30% - 50% motility is fair, and 10% - 30 % is poor. Sperm should be moving rapidly forward, not in circles.

Concentration: The average sperm concentration of sperm rich fraction is reported to vary from 150-840 million. The wide variation in the sperm concentration of different fraction depends on method of semen collection, breed and size of dog, and presence of teaser bitch. It is more precise to calculate total number of sperm per ejaculate.

Morphology: Morphology is the evaluation of the structure of the individual sperm. Abnormal structure in any part may affect sperm movement or function. To be considered normal, a minimum of 80% of the sperm should have normal morphology.

Other cells or bacteria The final consideration is to look for cells and bacteria in the semen sample. Normally there are few cells seen in an ejaculate;

Fraction	Range in (ml)
1 st Fraction	0.5 – 2.0 ml
2 nd Fraction	0.5 – 1 ml
3rd Fraction	15 - 20 ml

Extenders dilute the semen and remain of prostatic fluid and provide favorable osmotic conditions, as well as provide energy to spermatozoa during the storage period between cooling/equilibration and freezing. Empirical adaptation of extenders used for cooling and freezing of bull spermatozoa initiated the use of skimmed milk (Martin, 1963), citrate (Harrop, 1962), chloride-phosphate; lactose (Seager, 1969), Tris (trimethylhydroxyaminomethane) and Tris-fructose-citrate (Andersen, 1975) buffers for dilution in early experiments with short-term (liquid) or long-term (frozen) preservation of dog spermatozoa. Srivastava and Singh, 1986; developed a new extender containing both di and tri saccharide sugars for Indian Journal of Canine Practice

however, certain prostatic diseases may cause higher number of cells to be present. Excessive bacteria may indicate the possibility of infection, either in the testicles or the prostate.

Semen Processing

Successful preservation of spermatozoa by cooling, freezing and thawing is dependent on a series of steps aimed at reducing damage to the cell and securing adequate longevity *in vitro* and *in vivo*, i.e. optimal dilution, addition of extenders, type of buffer, cooling protectant and cryoprotectant addition, cooling rate and equilibration time, seeding, freezing and thawing rate, and possibly, also the removal of cryoprotectant after thawing. The later may be dependent on *in vitro* post-thaw storage time prior to insemination and on the method of semen deposition (i.e. vaginal or intrauterine).



Semen Extenders

canine semen preservation at subzero temperature.

Most of the recent research groups have used the original Tris-fructose buffer without modifications, or have continued to develop the Tris buffer, for instance by substituting monosaccharide fructose with glucose, which is nutritional and osmotic, or by the nonpenetrating disaccharides sucrose and lactose, which act as extracellular cryoprotectants in addition to providing a favorable osmotic effect (England, 1996). The commercially available Triladyl (Minitub, Tiefenbach, Germany) also contains Tris as the major buffer. England (1996) preferred a mixture of Tris and TES (n-Tris

(hydroxymethyl) methyl-2-amino ethane-sulphonic acid, N-(2-(hydroxy-1, 1-bis(hydroxymethyl)ethyl)amino) ethane sulphonic acid), but found the regular Tris buffer to work better for some dogs (England, 1996). Addition of the detergent sodium dodecyl sulphate to Tris-citrate extender was found to improve the longevity of frozen-thawed spermatozoa.

Also a variety of commercial companies have developed their own buffers (Laichipos 478, L'Aigle, IMV, France, Silva *et al.*, 2002; C.L.O.N.E, Pennsylvania, USA; ICG, Pennsylvania, USA). The exact composition of many of the commercially available extenders is unknown. In recent publications, Tris-citrate buffer has been shown to be superior to other buffers for either short-term storage of chilled semen (Rota and Linde-Forsberg, 1995) or for freezing of semen in straws.

History of Frozen Semen in the Dog

Spallanzani (1776) was the first to observe that a reduction in temperature reversibly reduced the metabolic activity of spermatozoa, thus allowing their storage. However, it was the discovery by Polge *et al.* (1949), of the cryoprotective action of glycerol, that made a significant impact upon the methodology of spermatozoal cryopreservation. The first success in freezing dog spermatozoa was reported by Rowson (1954). In 1969 Seager obtained the first canine pregnancy using frozen-thawed semen. A new extender for preservation of Canine Semen at sub zero temperature was developed by Srivastava and Singh (1986).

Early studies utilized skim-milk extenders for cryopreservation (Takeishi *et al.*, 1976); however, pregnancy in most scientific reports has been achieved using a modification of only two diluents - the egg-yolk-lactose-glycerol diluent (Nagase & Graham, 1964) and the Tris-egg-yolk-glycerol diluent (Davis *et al.*, 1963). The most recent studies into the cryopreservation of dog semen include those of Davies (1982), Smith (1984), Olar (1983) and Yuhi (1984). These workers aimed to improve the diluent and freezing methods; however, only Smith (1984) attempted more than simple modifications of the basic bovine diluent. The use of frozen semen was first approved by the American Kennel Club (AKC) in 1981.

Short Term Preservation

The use of short term preserved chilled semen has already shown some good results (Goodman and Cain, 1993; England and Ponzio, 1996). However the quality of semen at 4°C preservation varies. The quality of chilled semen deteriorates with time. It has been reported that storage of dog semen at 5°C for 24 h did not significantly impair the physical and functional integrity of spermatozoa (Kimi-Daika and Badtram, 1994) further, it was observed that first two days after collection semen quality was always good and it was suggested on the basis of semen evaluation that chilling was the most suitable method of semen storage if semen samples are used within approximately 4-5 days of collection (England and Ponzio, 1996; Rota and Linde-Forsberg, 1995; Province *et al.*, 1984). After the study of Linde-Forsberg, 1995; no studies were reported on the-effect of different extenders on the semen preserved for longer periods since it was accepted that the fertility of chilled semen would only be preserved for 12 to 24h. Later Iguer-ouada and Versteegen, 2001; concluded that at 4°C egg yolk extender was superior in protecting sperm motility parameters and the optimal results were obtained using egg yolk supplemented Tris glucose extender, which protects motility and acrosome integrity in chilled dog semen for up to 10 days.

Long Term Preservation

Semen can be stored for long periods by deep freezing. One of the biggest merits of using frozen dog semen is to provide convenience for breeding dogs breeds from different countries. Another advantage of frozen semen is that it does not require a lot of male dogs for breeding. Valuable genetic lines can be saved by storing frozen dog semen and new offspring having some desired characteristics can be produced continuously. Cryo preservation leads to the availability of semen at any place and at any time.

Although cryopreserved dog spermatozoa, when used for AI, produces lower conception rates than fresh spermatozoa particularly when inseminated intra vaginally. (Linde-Forsberg and Forsberg, 1989; Linde-Forsberg *et al.*, 1999). However, it has been reported that on an average a post-thaw motility of 30-50% (Silva *et al.*, 2002; Yurdaydin and Kotzab, 1987) may be obtained after cryo survival of dog semen. It has been recommended that 40 to 50% sperm motility is necessary for success in AI with canine frozen

semen (Concannon and Battisa, 1989). However, pregnancies have been obtained with a- post-thaw motility between 20 to 30% (Linde-Forsberg and Forsberg, 1989). The post thaw motility of cryopreserved semen depend upon many factors which includes type of extender, dilution method and ratio cryoprotectants and semen additives used. The high individual variability in post-thaw semen quality and short life span of thawed spermatozoa in vitro (Pena *et al.*, 1998; Rota *et al.*, 2001) indicate that there is scope for the improvement for optimization of protocol of semen freezing in dogs, so that the post-thaw motility and longevity of sperm may be increased. This will improve the fertility rate obtained after insemination with frozen semen.

Number of extenders were used by different workers for freezing of dog semen. However, Tris along with fructose/glucose, egg yolk and the cryoprotectant glucerol was most commonly used (England and Ponzio, 1996; Silva *et al.*, 2002; Yurdaydin and Kotzab, 1987; Pena *et al.*, 1998). In Brazil coconut water extender with addition of 20% egg yolk and 4 to 6% glycerol was successfully used in cryo preservation of canine semen (Cardoso *et al.*, 2003). Recent studies have suggested that capacitation like changes in dog spermatozoa may be initiated by freezing methods (Rota *et al.*, 1998). These changes of spermatozoa results from membrane destabilizing events, hence mechanism, which prevents or minimize the occurrence of such event (by inducing membrane stabilizing mechanism, that modifies membrane fluidity and protect spermatozoa during cryo preservation) may be followed (Watson, 1975). Equex STM paste has shown better cryo survival when present in extender used for freezing (Pena and Linde-Forsberg, 2000). Addition of the detergent sodium dodecyl (SDS) to freezing has also been reported to improve the post-thaw survival (Pena *et al.*, 1998; Rota *et al.*, 2001).

Glycerol is an important constituent of extenders used in freezing for reducing freezing injury; however, it has been shown to damage the spermatozoa during its addition and removal and during freezing and thawing (Fahy, 1986; Hammerstedt and Graham, 1992). Hence the optimal concentration of glycerol is a compromise between its protective and toxic effect. After freezing and thawing the survival of dog spermatozoa was best when the glycerol concentration in extender ranged from 2-8% (Olar *et al.*, 1983).

It has been observed that the method of dilution of the semen before freezing also affects the post-thaw survival. Generally for freezing dog spermatozoa the semen is diluted in one step where as a two step dilution (Rota *et al.*, 1997; Strom and Linde-Forsberg, 1997) in which the extender containing higher glycerol concentration added just before the freezing operation so that its higher glycerol concentration does not exert a deterioration effect on the spermatozoa during equilibration period and results in better post-thaw survival. However, exposing the spermatozoa to 5% or 3% glycerol, respectively during equilibration had no significant effect on the post-thaw viability or longevity (Pena and Linde-Forsberg, 2000; Anderson, 1975; Fontbonne and Badinand, 1993; Seager and Fletcher, 1973). These studies indicates that glycerol at these levels is not toxic and it penetrates in spermatozoa which might be responsible for better cryo survival after thawing at a faster rate.

Attempts were made to de 'time the optimal freezing rates for dog spermatozoa (Dabrinski *et al.*, 1993; Hay *et al.*, 1979; Rota *et al.*, 1998). Although the results obtained are not conclusive, it seems that it is better to freeze dog spermatozoa at moderately fast freezing rates i.e. between -1 O°C/min and -50°C/min over the critical temperature range (-10 to -30°C). Workers have used as fast freezing rates as 99°C/min to as slow as -0.5°C/min (Dabrinski *et al.*, 1993) but both the conditions were proved to be detrimental, where as freezing rates of -12°C/min or -28°C/min gave good results.

Many of the workers have confirmed the hypothesis that cryopreserved spermatozoa are more damaged during thawing than freezing (Curry and Watson, 1994; GoaDY *et al.*, 1992; Holt and north, 1994) due to change from hypertonic conditions during freezing to isotonic conditions in thawing. Thawing rates had a significant effect on post-thaw survival. Workers have reported that post-thaw survival of dog spermatozoa was higher when thawed at a faster rate as compared to slow rates in variety of extenders, glycerol levels and freezing rates (Pena and Linde-Forsberg, 2000; Olar *et al.*, 1983). The reason of thermo resistance in dog spermatozoa is lowered at low temp thawing is not known but it may be attributed to temperature dependent lipid phase transitions.

Freezing and thawing not only causes reduction in the survival of dog spermatozoa in terms of motility and longevity but it also effects the acrosomal integrity of spermatozoa. The major ultra structural changes reported were extensive loosening of plasma membrane, vesiculation of outer acrosomal membrane and loosening of acrosomal contents. Almost all of these changes lower the fertility of frozen thawed semen. Hence, for further improvement of post-thaw semen quality in dogs efforts are required to optimize and standardize proper permutation and combination of various factors including selection of extenders based on previous experiences of various trial, type & concentration of cryoprotectant used equilibration period, semen additives, no of spermatozoa and proper cooling/freezing rates using programmable freezer.

Use of Chilled Extended Semen for Artificial Insemination

Like fresh semen insemination, chilled extended semen is usually successfully inseminated utilizing the anterior vaginal insemination technique, assuming proper procedures are followed for collection and processing, and accurate ovulation timing insures that inseminations are performed at the correct time. Chilled extended semen kits contain extenders that enhance sperm longevity, allowing the maintenance of good motility long enough to ship semen long distance. On occasion, samples of poorer quality necessitate intra-uterine insemination. Intra-uterine insemination may also be chosen to increase the chance of conception even when semen quality is high.

Use of Frozen Semen for Artificial Insemination

In India, because of commercialization of dog breeding and scientific kennels have been organized more in urban areas, the technique of Artificial Insemination has gained importance in recent years at dog breeding centers due to the increased demand of pedigree male dogs. Frozen semen may be inseminated either by anterior vaginal insemination or by intra-uterine insemination. However, many extenders used in semen freezing contain high levels of cryoprotectants that interfere with the sperm's ability to penetrate cervical mucus, and thus cannot be used with a vaginal technique. In addition, frozen semen is usually available in limited

numbers, usually has decreased vigor as compared to fresh or chilled extended semen, and has a severely limited lifespan once thawed. Thus, the most common type of insemination with frozen semen is surgical intrauterine implantation. Regardless of technique used, There is no doubt that regular examination of canine semen quality can improve the management of breeding stock, provide an early warning of potential problems, and give quantitative information on a particular individual dog. The longer duration of storage of chilled semen at 4°C is sufficiently to allow for national and internal shipments of semen.



Conclusion

The days are not far when the Kennel Club of India will start recognizing litters conceived from frozen semen A.I. and with subsequent acceptance of fresh chilled semen there will be a great demand on practitioners to assist clients using these breeding techniques. The gratification one feels when successful is one of the greatest rewards in veterinary medicine. The basis of fresh chilling and freezing semen is energy conservation within the sperm cell so that the semen can be shipped or used at a later date. The drawbacks to these methods are that even though some energy is conserved, enough energy is used to shorten the sperm cell's life. There is no doubt; the frozen semen has much potential advantage over chilled semen. However, pregnancy rate following A.I. with frozen-thawed semen in dogs remains unsatisfactory, particularly under clinical condition. This is mainly due to reproductive anatomy and physiology, very narrow period of fertility as well as to extreme sensitivity of dog semen freezing thawing and to practical difficulties of insemination especially when intra-uterine insemination are required. The future research should be focused to overcome these problems to get optimum pregnancy rate and litter size in dog after AI with frozen semen.

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