CRYO-DAMAGE OF CANINE SEMEN AND ITS MINIMIZATION: PRESENT SCENARIO

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Introduction

Although dog was the first species to be used for Artificial Insemination (AI) and become pregnant by Spallanzani in 1780, 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. 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 (Foote, 1964[a]; Andersen, 1972[a]; Davies, 1982; Yubi, 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 (Froman *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.

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. Since the first birth of line 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 (Farstad, 1996). 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.

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.

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Early studies utilized skim-milk extenders for cryopreservation (Martin 1963[b]: 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 (1984) and Yubi (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.

Basic principle of semen preservation

During freezing of a cell suspension, ice forms as the temperature falls below the freezing point of the extra-cellular material. Later water crystallizes out or solution, leaving the remaining solutes at a higher concentration. As the cell membrane prevents the extension of ice crystals into the cell, the intracellular fluid becomes super-cooled; this results in the formation of an osmotic gradient across the plasma membrane and water tends to move out of the cell. If the cooling rate is sufficiently slow and the cell is permeable to water, the cell becomes progressively dehydrated; these cells are functional upon thawing. If, however, the cooling rate is too rapid or the cell is relatively impermeable to water, intracellular fluid can not move out of the cell sufficiently quickly, the degree of super cooling increases and intra-cellular ice formation may occur (Mazur, 1965); in this case cells are dead upon thawing.

The formation of intra-cellular ice does not necessarily result in cell death, because it is both the amount of ice and. the size of crystals that influences survival (Farrant *et al.,* 1977). Cells frozen rapidly may contain small ice crystals and will survive unless thawing is slow, in which case re-crystallization (ice crystal growth during thawing) occurs and reduces cell survival. At freezing rates less than optimal, the loss of viability i.s related to several events - decreasing intracellular water content and resultant increasing concentrations of intracellular solutes, precipitation of these solutes and subsequent disturbances in pH (Van den Berg & Soliman, 1969). Meryman (1910) considered that the major 'solution effect' was reduction in cell volume below a minimum compatible with survival; more recently, however, Mazur (1984) found that slow freezing injury was the result of decreasing size of unfrozen extracellular channels, rather than of intracellular solute concentration or a shrinkage.

The possibility of damage during slow thawing has been discussed previously; osmotic forces are important during this process. For example, when a permeating cryo protectant is used, too rapid thawing may not allow the agent to leave the cell fast enough to maintain osmotic equilibrium. Thus producing cell swelling following the entry of water (Griffiths *et al.,* 1979). Successful freezing and thawing of cell solutions should, therefore, allow for the maintenance of osmolarity, pH and ionic strength, in addition to providing a source of energy and preventing cryo injury.

Cryo- Damage

Damage to spermatozoa by cooling from physiological temperatures to the freezing point is called cold shock (Watson, 1981). The ability of spermatozoa to withstand chilling and freezing differs between species. Spermatozoa from horse, cat, dog and humans are relatively insensitive to cold shock, whereas spermatozoa from cattle, sheep and goat show medium sensitivity, and boar sperm are extremely sensitive (Watson and Plummer, 1985; Bwanga, 199l).

Physical and chemical alterations in the cell membrane caused by cooling may be irreversible and differ from the damage caused by freezing and thawing; old shock and freezing damage may therefore be considered separate phenomena (Paulenz, 1993). Irreversible alterations in the sperm membrane i.e. disturbances in the protein lipid bilayer structure, such as decreased membrane fluidity, increased membrane permeability, acrosomal damage, dehydration, enzyme and phospholipids liberation, reduced metabolic activity and diminished consumption of ATP, are all consequences of cooling and freezing which may partly or totally compromise fertility (for review, see Hammerstedt *et al.*, 1990). The most evident result of these changes is loss of sperm motility (Paulenz, 1993). However, studies on frozen-thawed silver fox and blue fox spermatozoa have

shown that reduced fertility is not revealed by reduced motility or even y the degree of acrosomal integrity (Farstad *et al.,* 1992[c]). Also, in the dog no clear relationship has been found between post-thaw motility and fertility (Kosiniak *et al.,* and Thomas *et al.,* 1993).

When cells are frozen they are subjected to stresses resulting from the water-solute interaction that arise through ice crystallization. Exposure of cells to the hyperosmotic, yet unfrozen, solution causes withdrawal of intracellular water, consequent cell shrinkage and possible influx of ions (Mazur, 1984). Thawing involves a reversal of these effects, and the consequent inward water flux may cause cell membrane disruption. The detrimental effects of slow freezing and therefore prolonged exposure of cells to hyperosmotic condition "solution effect"(Mazur *et al.,* 1970) have been viewed as balancing the deleterious consequence of rapid freezing which Cytoplasmic disruption through intracellular ice formation may be further compounded by the growth of ice crystals during thawing (re-crystallization). It has been suggested that a compromise-freezing rate exists where the damaging effects of these two different sources of cryo-injury can be minimized (Watson, 1990).

The incorporation of micro-architectural considerations into hypotheses of cryo-injury may help to explain why some spermatozoa survive the cryopreservation process intact, while others suffer acrosomal disruption, plasma membrane damage and loss of motility. If cell survival were dependent upon spatial orientation in relation to regions of heterogeneously distributed solute concentration, then different types of microstructure would alter the random likelihood of achieving optimal orientations.

Hypotheses of sperm cryo-injury must account for the known thermodynamic and structural properties of the sperm plasma membrane. It is well known that the sperm plasma membrane contains an unusual array of lipids (Lin *et al.,* 1993; Parks *et al.,* 1987) and that the plasma membrane is organized into different domains (Friend, 1984; Holt, 1984). The phospholipids typically adopt unusual configurations, with a high proportion of plasmalogens that contain ether-linked fatty acids instead of the more usual ester linkages. Phospholipids account for 65-70% of the total, and a large proportion of these contain a docosahexaenoic acid side chain, which may confer membrane fluidity and instability. Possibly to counteract these destabilizing effects, sperm plasma membranes contain variable amounts of sterols. The sperm plasma membrane lipids respond to temperature changes by alterations in their physical phase state. Although regions of fluid and gel phase lipids coexist at physiological temperatures, reductions of temperature favour fluid to gel transitions; the presence of sterols is thought to inhibit these phase changes.

As spermatozoa are not adapted to undergo the temperature changes involved in cryopreservation, they cannot modify their lipid content to suit the environmental conditions. This useful strategy is widely used in nature to compensate for the changes in ionic permeability and enzyme activity, which result from phase transitions. Spermatozoa undergo these lipid phase transitions (Crowe *et al.,* 1989; Drobnis *et al.,* 1993; Holt and North, 1984; 1986; Parks and Lynch, 1992) typically within the temperature range l7-36^{0}C. Their occurrence shows species dependence, which could go some way towards explaining the variations in cryopreservation sensitivity seen in spermatozoa from different species. It is also likely that during a typical freeze-thaw cycle, the sperm membranes must undergo phase transitions during both cooling and rewarming.

Evidence, that cold shock (i.e. damage due to rapid cooling above 0^{0}C) is caused by lipid phase transition effects was presented by Drobnis *et al.* (1993). Holt *et al.* (1992) obtained some evidence that phase transition might be involved in the manifestation of cryo-injury during the re-warming of cells after thawing. Ram spermatozoa were stained with fluorescein diacetate (FDA), a fluorescent probe of cell membrane integrity, cooled to a series of minimum temperatures (5^{0}C, -10^{0}C and -20^{0}C) and then rewarmed to 30^{0}C. Plasma membrane integrity was retained throughout cooling, but fluorescein leakage, indicating membrane disruption, occurred during the re-warming process. Conversely, performing similar experiments in the presence of external adenosine triphosphate (ATP) and a sperm reactivation medium showed that spermatozoa rendered immotile by cooling could be restored to motility by an influx of ATP when the plasma membrane

was breached. The threshold temperatures causing loss of membrane integrity were correlated with the minimum temperature reached during cooing. One interpretation of this data is that as the post-thaw temperature increases, the plasma membrane is subjected to structural rearrangements involving lipids and proteins, the extent and nature of which are governed by interactions of temperature and solute effects during the freezing process.

Capacitation-like changes

A significant breakthrough in understanding has come with the recognition that cooled and re-warmed spermatozoa behave as if they were capacitated (Watson, 1995). However, we are unsure at present whether spermatozoa manifest the changes of capacitation or simply are enabled to by-pass capacitation and proceed directly to acrosomal exocytosis. We have begun to study this phenomenon and have shown that cooled spermatozoa display chlortetracycline staining and an increase in intracellular free Ca_2+, typical of capacitated spermatozoa, but that the tyrosine phosphorylation patterns are somewhat different in cooled and re-warmed spermatozoa (Watson and Green, in press). We are continuing to investigate these events to determine the nature of the membrane changes resembling capacitation.

Motility impairment

One obvious characteristic of cryopreserved spermatozoa is the decline in motility of the cells. While a minority seems to exhibit vigorous forward progression, the majority show a variable degree of impairment. This would seem to be an important contribution to their relatively poor fertilizing potential when introduced into the reproductive tract at artificial insemination. A study of cryopreserved human spermatozoa under IVF conditions found that both motility and forward progression were important factors (Kelly *et al.,* 1997). However, one recent observation reached a different conclusion; in a study of in vitro fertility of asthenoteratozoospermic dogs, the quality of motility though poor did not impair fertility (Hewitt and England, unpublished). These studies relate only to events associated with the final contact with the oocytes; transport to the site of fertilization may still require a minimum capability of forward motility, which may be compromised in the majority of cryopreserved spermatozoa.

Oxidative damage

More important is the observation that oxidative damage could impair sperm function.This subject is difficult to tackle because of the recognition that a degree of superoxide radical formation is necessary for the events preceding fertilization (de Lamirande *et al.,* 1997). However, it is now generally accepted that cryopreservation induces the formation of reactive oxygen species that are detrimental to subsequent performance (Alvarez and Storey, 1992; Bell *et al.,* 1993; O'Flaherty et al., 1997). Much of the published work on lipid peroxidation and cryopreservation relates to work on human spermatozoa.

The use of antioxidants in cryopreservation diluents is not commonplace, partly because there are a number of possible agents from which to select, and partly because different pathways (and hence, different antioxidants) are utilized in different species (Askari *et al.,* 1994; O'Flaherty *et al.,* 1997). Moreover, it is clear that oxidative damage is only one of a number of stresses the cryopreserved sperm cell experiences (Alvarez and Storey, 1993). This is clearly an area for further investigation.

Surface changes affecting recognition of receptors

An issue that has been recently examined is the extent to which the surviving population is impaired in its ability to interact with the oviductal epithelium (Ellington *et al.,* 1990). These interactions are now recognized as receptor-ligand interactions, often involving intracellular signaling mechanisms, which are actively being explored. A similar interaction involves spermatozoa and the oocyte and its vestments (Bwanga *et al.,* 1991; Ochninger *et al.,* 1993). The freeze-fracture observations, suggesting that the clustering of membrane proteins during lipid phase separations induced by cooling are not entirely reversible, may well have implications for receptor-lignad interactions. Although the evidence from IVF studies generally indicates that cryopreserved spermatozoa are capable of effecting fertilization

(e.g., Donaghue *et al.,* 1992), this is an artificial situation when hundreds of spermatozoa are present. In vivo, the reality may be quite different when only a few spermatozoa reach the site of fertilization.

Ability of sperm to sustain embryonic development

Finally, another area that has not been adequately studied is the ability of cryopreserved spermatozoa to sustain embryonic development. Technically, this is difficult to investigate since the evidence of early embryonic death often passes without overt signs. Nevertheless, a persistent suggestion is that frozen-thawed spermatozoa are associated with an increased incidence of early embryonic mortality (Salamon and Maxwell, 1995).

DNA Damage

The potential mechanisms can now be studied more effectively. DNA damage can be investigated with the COMET assay (Hughes *et al.,* 1996) or with flow cytometry (Karabinus *et al.,* 1990; Royere *et al.,* 1991) and may indicate functional damage to the nuclear structures (Ellington *et al.,* 1998). Moreover, the suggestion that the spermatozoon' s contribution to the zygote is more than the haploid male genome (Navara *et al.,* 1995) implies that there are more subtle ways in which the spermatozoon's function could be disrupted during cooling and cryopreservation. The post-syngamy fate of other sperm structures is now known better than ever before (Sutovsky *et al.,* 1996; Sathananthan *et al.,* 1997), and the possible importance of sperm RNA (Rohwedder *et al.,* 1996; Miller, 1997) to the events before the embryonic genome is activated cannot be disregarded. Any of these structures may suffer alteration during cryopreservation and can all now be investigated.

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.

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 cryoprotectants addition, cooling rate and equilibration time, seeding, freezing and thawing rate, and possibly, also the removal of cryoprotectants after thawing. The latter 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).

Extenders in semen

Extenders dilute the semen and remains 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 (Wales and White, 1963), lactose (Seager, 1969), Tris (trimeti'fylhydroxyaminomethane) (Gill *et al.,* 1970) and Tris-fructose-citrate (Foote, 1964; Andersen, 1972; Andersen, 1975) buffers for dilution in early experiments with short-term (liquid) or long-term (frozen) preservation of dog spermatozoa.

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 non--penetrating disaccharides sucrose and lactose, which act as extracellular cryoprotectants in addition to providing a favorable osmotic effect (Ivanova-Kicheva *et al.,* 1992; England, 1992; Thomas *et al.,* 1993; Wilson, .1993; Ivanova-Kicheva *et al.,* 1995; Nothling *et al.,* 1995). The commercially available Triladyl (Minitub, Tiefenbach, Germany) also contains Tris as the major buffer (Nothling *et al.,* 1995). England (1992) preferred a mixture of Tris and TES (n-Tris(hydroxymethyl) methyl-2-amino ethane-

sulphonic acid, N-(2-<<hydroxy-l, 1-bis(hydroxymethyl)ethylamino ethane sulphonic acid, but found the regular Tris buffer to work better for some dogs (G. W. England, personal-communication, 1993). Addition of the detergent sodium dodecyl sulphate to Tris-citrate extender was found to improve the longevity of frozen-thawed spermatozoa (Thomas *et al.,* 1992).

Also a variety of commercial companies have developed their own buffers (Laichipos 478, L'Aigle, IMV, France, Silva et al., 1995; 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 et al., 1995) or for freezing of semen in straws (Thomas et al., 1993).

Cooling protectants and cryoprotectants

The addition of cooling protectants such as egg -yolk and cryoprotectants such as glycerol or DMSO (dimethyl-sulphoxide), the sugars lactose or sucrose, macro-molecular polymers or detergents such as polyvinyl pyrrolidone, Orvus ES-Paste (Olar, 1984) -70°C has been successful both for dog semen (England, 1992). Also, rapid freezing by placing straws on a rack already placed above liquid N2 ,with a constant flow of vaporized nitrogen has yielded good results for canine semen (Andersen, 1972; Farstad, 1984; Alar, 1984; Wilson, 1993; Fontbonne and Badinand, 1993). However, Dobrinski *et al.* (1993) claimed. That a slower overall cooling and freezing rate of -5°C min- from + 3 to - 157°C gave better motility values than an intermediate rate of -8°C min -I from + .3 to - 164°C 01: fast rate of -18°C min.; -I from +. 3 to 191°C for all extenders tested when thawed at 37°C for, 2 min. The different freezing rates were achieved by placing straws different distances from the liquid nitrogen and for different periods of time. Freezing by lowering straws onto a rack placed above the surface of liquid N2 is a static method which has little control over the freezing procedure (different containers, metal or plastic Jacks), and it is advised that controlled dynamic freezing (variable flow of Nitrogen vapor) in freezing machines be used for commercial purposes.

Thawing rate

It is well established that optimum cell survival requires that the rate chosen for freezing be paired with the appropriate rate of thawing (Mazur, 1984; Hammerstedt *et al.*, 1990). Generally it is believed that fast freezing requires fast thawing to reverse the osmotic balance, rehydrate and restore the lipid protein configuration of the membrane in a fashion similar to the events induced during freezing. A variety of freezing regimes and thawing protocol. It has been reported for dog semen, even when the same packaging method and extender is used. For 0.5 ml PVC straws, thawing regimes in a water bath at 70°C for 6.5 s (Andersen, 1972), 70°C for 8 s (Farstad, 1984; Hofmo, 1988 (fox), 50°C for 30 s (Silva *et al.,* 1995), 30°Cfor30 s (Oettle, 1982), 37°C for 2 min and 55°C for 5 s (*Dobrinski et al.,* 1993) have been reported. These are moderate to fast rates of thawing compared with thawing at 4°C which Rofmo (1988) found was inferior to fast thawing of silver fox semen. Usually pelleted semen is thawed in a thawing solution (usually saline or sodium citrate) at 37°C (Linde-Forsberg, 1995), but thawing in plastic bags has also been reported (Thomas *et al*.. 1993). It is advisable to use the thawing rate recommended by the person or laboratory providing the frozen semen, since thawing rates are closely connected with the freezing protocol.

Post-thaw dilution

In bovine artificial intrauterine insemination with frozen semen, but intrauterine insemination of dogs carried out in Norway with domestically frozen semen, the removal of cryoprotectant from the semen occurred in the female genital tract. Some commercial companies have instructed inseminating veterinarians to dilute the semen after thawing prior to insemination with special, post-thaw extenders (C.L.O_N.E. and ICG, USA), although one recent instruction in the ICG manual is not to use post-thaw dilution when performing intrauterine insemination.

Vaginal insemination with post-thaw dilution of 3-5 ml homologous or heterologous Prostatic fluid has produced good results

(Nothling *et al.,* 1995). It is not known if the beneficial effect of redilution before insemination is only that of removal of cryo-protectant or if it has other effects as well. The addition of prostatic fluid points to some interesting possibilities because prostatic fluid does not maintain dog sperm motility as well as some extenders both at ambient (Nothling and Volkmalli, 1993) and refrigerator temperature (Rota *et al.,* 1995). It has been shown. That there are male to male variations in the composition of seminal plasma, such as in the content of sterol exchange proteins (Rammerstedt *et al.*, 1990). The possibility of the seminal plasma acting as a source of proteins which may interact with the sperm membrane by adsorption to the glycocalix region of the surface cannot be excluded. Such recoating of the membrane may be favorable when vaginal insemination is performed, since the vagina is not considered an appropriate environment for spermatozoa as opposed to the uterus. Research on protein additions to the thawed semen, with the goal of improving fertility results with vaginal inseminations is therefore recommended.

Evaluation of frozen-thawed semen

The ultimate test of success of semen processing, techniques for frozen-thawed semen is the evaluation of fertility, i.e. whelping rate and litter size. When the fertility of a semen sample in vivo is predicated using in vitro methods, a combination of different methods usually gives more reliable results than one test. The problems associated with fertility tests are, however, that they usually require a substantial number of animals and also depend on a variety of factors other than semen quality per se, such as insemination dose and volume, number of inseminations, site of semen deposition, timing of insemination and individual female factors. Using intrauterine deposition of semen (Andersen, 1975), one or two inseminations 24 h apart and doses of 50-200 million spermatozoa per insemination, our fertility results, pooled from 1994 until October 1995 for all inseminations with imported and domestic frozen-thawed dog semen, were: a 74% whelping rate (79/ 107) and a mean litter size of 5.5 pups (range 1-17). No selection of data with regard to number of inseminations (one or two), timing of insemination, insemination dose (Le. total number of spermatozoa in the inseminate), freezing protocol or semen quality, post-thaw was done.

Dog semen preservation in India

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. 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. Similarly, semen was collected by digital manipulation in mongrel dogs and physical and morphological characters were evaluated (Daiwadnya and Hukeri,1993). Dabas *et al.,*1991) reported that whelping of 3-6 pups after AI with semen diluted in buffered-yolk extender.

References

Alvarez, J.G., Storey, B.T., 1992. Evidence for increased lipid peroxidative damage and loss of superoxide dimutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J. Androl. 13, 232-241.

Alvarez, J.G., Storey, B.T.,1993. Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamaage in cryopreserved human sperm: glycerol and other polyols as sole cryoprotectant. J. Androl. 14, 199-209.
Animal ReproductionScience1996,42:251.

Askari, H.A., Check, J.H., Peymer, N., Bollendrof, A., 1994. Effect of antioxidants tocopherol and ascorbic acids in maintenancee of sperm activity during freeze-thaw process. Arch. Androl. 33, 11-15.

Bailey, J.L., Robertson, L., Buhr, M.M., 1994. Relationships among in vivo fertility, computeranalysed motility and in vitro Ca2+ flux in bovine spermatozoa. Can. J. Anim. Sci. 74,53-58.

Bell, M., Wang, R., Hellstrom, W.J., Sikka, S.K., 1993. Effect of cryoprotective additives and cryopreservation protocol on sperm membrane lipid peroxidation and

recovery off motile human sperm. J. Androl. 144,472-478.

Bwanga, C.O., Hofmo, P.O., Ggrevlle, J.S., Einarsson, S., Rolriiguuez-Marrtinez, H., 1991. Invivro fertilizing capacity of deep-frozen boar semen packaged in plastic bags and maxi-straws. Zentralbl. Veterinaarmed., Reejhe A 38, 281-286.

Cochran, J.D., Amann, R.P., Squires, E.L and Pickett, B.W. (1983) Fertrility of frozen thawed stallion semen extended in lactose- EDTA egg yolk extender and packaged in 1.0 ml straws. Theriogenology, 20, 735-741.

Davies, P.R. 1982. A study of Spermatogenesis, rates of sperm prodction, and methods of pereserving the semen of dogs. Ph.D Thesis, University of Sydney, Australia.

Davis, I.S., Bratton, R.W. and Foote, R.H. 1963. Livability of bovine spermatozoa at 50, -25 and 850C in tris-buffered and citrate buffered yolk-glycerol extendors. Journal of Dairy Science, 46, 333-336.

Drobnis, E.Z., Crowe, L.M., Berger, T.,m Anchordoguy, Tol., Overstreet, J.W, Crowe, J.H. 1993. Cold shock damage is due to lipid phase-transitions in cell-membranes- a demonstration using sperm as a mopdel. J. Exp. Zool. 265, 432-437.

Friend, D.S., 1984. Membrane organization and differentiation in the guinea pig spermatozoa. In: Van Blerkom, J., Motta, P. M (Eds), Ultrastrucutre of reproduction, Martinus Nijhoff, Boston, pp. 75-85.

M (Eds), Ultrastrucutre of reproduction, Martinus Nijhoff, Boston, pp. 75-85.

J Fromian, D.P. Amann, R.P., Riek, P.M. and alar, T.T. 1984. Acrosin activity of canine spermatozoa as an index of cellular damageolournal of Reproduction and Fertility, 70, 301-308.

Holt, W.V., 1984. Membrane heterogeneity in the mammalian spermatozoa. Jntr. Rev. Cytol, 87, 159-194.

Holt, W.V., Head, M.F., North, R.D., 1992. Freeze-induced membrane damage in ram spermatozoa is manifested after thawing-observatioins with experimental cryomicroscopy. BioI. Reprod. 46, 1086-1094.

Hughes, C.M., Lewis, S.E.M., McElvey-Maartin, Vol., Thompson, W., 1996. A comparision of baseline and innducceed DNA damage in human spermatozoa from fertile and infertile men, using a mooddified comet assay. Mol. Hum. Reprod. 2, 66113-619.

Karabinuss, D.S., Evenson, D.P., Jost, L.K., Baer, R.K., Kaprrooth, M.T., 1990. Comparision of semen quality in young and mature Holstein bulls measured by light microscopy and flow cytomeetry. J. Dairy. Sci., 73,2364-2371.

Kelly, M.P., Corrson, S.L., Gocial, B., Batzer, F.R., Gutmann, J.N., 1997. Discontinoous percoll gradient preparation for donar insemination determinants for success. Hum. Reprod. 12,2682-2686.

Kelly, M.P., Corrson, S.L., Gocial, 8., Batzer, F.R., Gutmann, J.N., 1997. Discontinoous percoll gradient preparation for donar insemination determinants for success. Hum. Reprod. 12, 2682-2686.

Mazur, P. 1965. The role of cell membranes in the freesing of yeast ande other single cells. Annals of the New York Academy of Sciences, 125,658-676.

Mazur, P., 1984. Freezing of living cells: mechanisms and implications. Am, J. Physiol, 247, CI25-142.

Navara, C.S., Simerly, C., Zoran, S., Schatten, G., 1995. The sperm centrosome during fertilization in mammals: implications for fertility and reproduction. Reprod. Ferti!. Dev. 7, 745-754.

O'Flaherty, C., Beconi, M., Beorleugi, N., 1997. Effect of natural antioxidants superoxide dismutase and hydrogen peroxide on capacitation of froxen-thawed bull spermatozoa. Andrologia, 29, 269-275.

Oehninger, S., Morshedi, M., Ertunc, H., Bocca, S.M., Acosta, A.A., Hoodgen, G.D., 1993, Validation of the hemizona assay (HZA) I monkey model: Kinetics of binding aanndd innffluueennce of cryopreesserved thawed sspeermatozoa. J. Assist. Reprod. Genet. 10,292-33011.

Olar, T.T. 1984. Cryopreservation of Dog

spermatozoa. PhD Thesis, Colorado State University.

Parks, J.E., Arion, J.W., Foote, R.H. 1987. Lipids of plasma-membrane and outer acrosomal membrane from bovine spermatozoa. BioI. Reprod., 1249-1258.

Parks, J.E., Lynch, D.V., 1992. Lipid composition and thermotrophic phase-behaviour of boar, bull, stallion and rooster sperm mem,branes. Cryobiology, 29, 225-266.

Polge, C., Smith, A.U. Parkes, A.S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperature. Nature, 164,666.

Robertson, L., Watson, P.F., 1986. Calcium transport in diluted or cooled ram semen. J. Reprod. Fertil, 77, 177-185.

Rohwedder, A., Liedigk, 0., Schaller, J., Glander, H.J., Werchau, H., 1996. Detection ofmRNA transcripts of beta 1 integrins in ejaculated human spermaqtozoa by nested reverse transcrfiptase-polymerase chain reaction. Mol. Hum. Reprod. 2,499-505.

Rowson, L.E.A. (1954) Infertility of cow, sow and bitch. Irish Veterinary journal, 8, 216-22l. Salamon, S., Maxwell, W.M.C., 1'»95. Frozen storage of ram semen: II. Causes of low fertility after cerivical insemination and methods of improvement. Anim. Reprod.Sci, 38, 136.

Sathannanthan, A.H., Tatham, B., Dharmawardena, V., Grills, B., Lewis, I., Trounson, A., 1997. Inheritance of centrioles and centrosomes in bovine embryos. Arch. Androl. 38, 3748.

Seager, S. W.J and Fletcher, W.S. 1973. Progress on the use of frozen semen in the dog. Veterinary Clinics of North America, 7, 757-764.

Seager, S.W.J. 1969. Successful pregnancies utilizing frozen dog semen. A.I. Digest, 17,6-7. Seager, S.W.J., Platz, C and Fletcher, W.S. 1975. Conception rates and related data using frozen dog semen. Journal of reproduction and fertility, 45, 189-192.

Smith, F.O. (1984). Cryopreservation of canine semen: Techniques and performance. PhD Thesis, University of Minnesota.

Smith, F.O. and Graham, E.F., 1984. Cryopreservation of canine semen: technique and performance. X the International congress on animal reproduction and artificial insemination. Urbana-champaign: 2, 216.

Spallanzani, L.1776. Opuscoli di fiscal animale e vegitabile. Opuscolo D. Obervatiozioni e sperienze intorno ai vermicelli sprmaici dell homo e degli animali. Modena 1776 (cited by Watson, 1979).

Sutovsky, P., Navara, C.S., Schatten, G., 1996. Fate of mitochondria, and the incorporation, conversion and disassembly of the sperm tail str4uctures duringv bovine fertilization. BioI. Reprod, 55, 1195-1205.

Takeishi, M., Mikami, T., Kodama, Y., Tsunekane, T. and Iwaki, T. 1976. Studies on reproduction in the dog. VIII. Artificial insemination duisng frozen semen. Japanese Journal of Animal Reproduction, 22, 28-33.

Waberski, D., Weitz, K.F., Gleumes, T., Schawarz, M., Wilmen, T., Petzoldt, R.,(1994. Effect of time of insemination relative to ovulation on ferti8lity with liquid and frozen boar semen. Theriogenology. 42, 831-840.

Watson, P.F. (1990). Artificial insemination and the preservation of semen. In: ing, G. Lamm (Ed.), Marshall's Physiology of reproduction vol. 2 Churchill Livingstone, Edinburgh, London, pp. 747-869.

Watson, P.F. (1995). Recent developments and concepts in the cryoperservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fertil. Dev. 7, 871-89l.

Yuhi, A.C. (1984). Investigations of dog semen with particular reference to freesing techniques. MVM Thesis. Faculty of veterinary medicine, University of Glasgow.

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