COMPARATIVE EVALUATION OF CRYOPROTECTIVE **COMPOUNDS FOR DEEP FREEZING OF RAM SPERMATOZOA IN TRIS DILUENT**

BY

MOHAMMED SANI AHMED
DVM (A.B.U., 1978), M.P.V.M (U.C. Davis, 1982)

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Department of Veterinary Surgery and Medicine,
Faculty of Veterinary Medicine,
Ahmadu Bello University,
Zaria, Nigeria.

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DECLARATION

I hereby declare that the work in this thesis was carried out by me in the Reproductive Physiology Laboratory of the Department of Animal Reproduction and Artificial Insemination, National Animal Production Research Institute, Ahmadu Bello University, Zaria, under the Supervision of Dr. O.A. Osinowo, Prof. E.C.I. Molokwu and Prof. E.O. Gyang.

No part of this thesis has been presented in any previous application for a higher degree. All sources of information are specifically acknowledged by means of references.

Dr. Mohammed Sani Ahmed

CERTIFICATION

This thesis entitled *Comparative evaluation of cryoprotective compounds for deep freezing of ram spermatozoa in Tris diluent* by Mohammed Sani Ahmed meets the regulations governing the award of Doctor of Philosophy of Ahmadu Bello University and is approved for its contribution to scientific knowledge and literary presentation.

Internal Examiners

Dr. A.D. Osinowo
B.Sc., M.Sc., Ph.D
Small Ruminants Programme
N.A.P.R.I.,
Ahmadu Bello University
Zaria

Prof. E.O. Gyang
DVM, M.Sc., Ph.D
Dept. of Vet. Surgery and Medicine
Ahmadu Bello University
Zaria

Prof. E.C.I. Nolokwu
DVM, M.Sc., Ph.D
Dept. of Vet. Physiology and Pharm.
Ahmadu Bello University
Zaria

External Examiner

Dr. B.I. Orji
Reader in Reproductive Physiology
Dept. of Animal Science
University of Nigeria
Nsukka

20 - 9 - 89
Date

DEDICATION

TO MY WIFE AND CHILDREN

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ABSTRACT

A comparative evaluation of cryoprotective compounds for deep freezing of ram spermatozoa in tris diluent was carried out. The experimental work is presented in four sections. The first section contains the experiments which screened the effect of some cryoprotective compounds on post-thawing survival of ram spermatozoa deep frozen in tris diluent. The viability of spermatozoa was influenced by cryoprotective compound concentration, dilution rate of the cryoprotective compounds concentration, dilution rate and post-thawing incubation. The optimum concentration for most of the cryoprotective compounds used in this study was found to be 0.5M while 1:4 (semen:diluent, v/v) dilution rate was best. There was a general decline in post-thawing motility with increase in incubation time. Compounds such as DMSO, polyethylene glycol and acetamide gave no appreciable protective effect to ram spermatozoa during freezing. But glycerol, erythritol and ethylene glycol gave protection to the freezing ram spermatozoa. The experiments revealed interactions between some of the factors examined.

In section 2 a comparative evaluation of the cryoprotective compounds (used in section 1) was carried out. These was no significant difference in post-thawing survival of spermatozoa when thawed at 37°C, but there was a poor post-thawing survival after thawing at 27°C. Ram spermatozoa can be frozen in highly concentrated form without greatly affecting the post-thawing recovery rate. Freezing at concentrations of up to 2 x 10^9 cells per ml gave satisfactory post-thawing recovery rate following two-step dilution technique. Glycerol, erythritol and ethylene glycol showed the best post-thawing motility. There were interactions between some of the factors examined.

The third section dealt with the effects of combination and substitution of some cryoprotective compounds on post-thawing survival of ram spermatozoa deep-frozen in tris diluent. The combination of glycerol and erythritol offered

better protection to frozen spermatozoa than glycerol. ethylene glycol or erythritol as single compounds or combination of all three. In the substitution technique which involved a two-step dilution, glycerol as an initial compound gave the highest post-thawing motility compared to ethylene glycol, acetamide, DMSO and erythritol. There was a highly significant interaction ($P < 0.001$) between initial and freezing cryoprotective compounds which was mainly due to the presence of glycerol as the initial cryoprotective compound. The combination and substitution techniques demonstrated the tolerance of ram spermatozoa to high osmotic pressure of more than 1000 mosm. The post-thawing motility was substantially improved following substitution and combination techniques as there was no significant decline in motility, during the 6 hr post-thawing incubation. This findings is of practical importance when it comes to artificial insemination since the semen could be thawed and kept at 37°C without much decline in motility.

In these experiments the acrosomal membrane integrity and live-dead ratio were examined after freezing. The integrity of acrosomal membrane was influenced by some cryoprotective compounds and post-thawing incubation. It was observed that with frozen-thawed ram spermatozoa it was not possible to categorize the acrosomal damages. The damage appear uniform irrespective of cryoprotective compounds used. The live and dead ratio was generally influenced by cryoprotective compound treatments and incubation time.

In section 4 fertility trials were reported. Though cryoprotective compounds treatment was the only factor considered, there were some variations in the four fertility trials. Use of PMSG and insemination dose and insemination technique were varied. Generally the overall lambing rate following insemination with deep-frozen semen after estrus synchronization was poor. However, important in fertility was observed after insemination at the second estrus after synchronization.

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INTRODUCTION

The greatest advances artificial insemination have come from development in techniques for the dilution and storage of semen. Simple isotonic media can be used for dilution of semen that is to be inseminated within a short time after collection. For storage however, the medium should provide some buffering capacity and perhaps some substrates for metabolism, but in addition, it should also provide protection against what has been termed "dilution effect" on spermatozoa (Polge, 1973). Good storage medium usually contains protein, and the addition of egg yolk or milk to a simple phosphate or citrate buffer has proved to be very satisfactory for most species. Egg yolk and milk provide protection against deleterious effects of chilling, and semen diluted in these media can then be cooled to temperatures a little above 0°C without damage. Motility and metabolism of the spermatozoa are thereby reduced and their survival *in vitro* is considerably extended.

In all the available techniques before 1949, the period of storage was very strictly limited, but a great step forward was made when methods were discovered that permitted the long-term preservation of spermatozoa at very low temperatures. Normally spermatozoa are killed if the semen is frozen. Most cryobiologists date the beginning of the modern era of cryobiology from the discovery of the cryoprotective properties of glycerol against lethal effects of freezing and thawing on spermatozoa by Polge *et al.* (1949). This served as the key that opened the door to future developments in the preservation of semen by deep freezing. With this discovery it was possible to store semen in the frozen state almost indefinitely. Since this landmark discovery, cryoprotective

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agents have been used to secure the survival of many living systems after storage at low temperatures. Despite this impressive degree of success, few cryopreservation techniques today permit 100 per cent survival after freeezing and thawing. The preservation of many important living systems has proven unsatisfactory or unsuccessful. In other words, cryoprotectants are generally not panaceas, even when used at optimized cooling and warming rates (Fahy, 1986).

Most of the research work on semen preservation and artificial insemination in sheep carried out elsewhere were specific on exotic breeds such as the merino. Despite the presence of four main breeds of sheep in Nigeria, there is no concerted effort on artificial insemination in sheep. The breeds of sheep include Yankasa, Balami, Uda and West African Dwarf. Each of these breeds has its own merit over the others, ranging from fast growth of Balami to disease resistance of Yankasa. These breeds could be improved through selection and cross breeding. Where natural mating is not possible, artificial insemination could be an alternative. Also, with the breeds located in various ecological zones, it would be much cheaper to transport semen from one part of the country to the other rather than transporting the live animals. Even if transported, the animals might die before adjusting to the new environment. At present, a crop of Nigerian elite livestock farmers are coming up who might appreciate artificial insemination in sheep.

The need for artificial insemination for sheep is becoming increasingly apparent. In an intensive system utilising controlled reproduction, artificial insemination is required to simplify management and to increase the number of ewes that can be mated to an individual ram at a synchronized oestrus under field conditions. In addition, artificial

insemination is important because of its potential for exploiting the genetic merit of superior rams. The application of artificial insemination to sheep is presently not practised in Nigeria. The country being in the tropics, the keeping quality of fresh semen and even extended semen at ambient temperature would be limited. Deep frozen storage of ram semen could be the answer since such semen keeps indefinitely only to be used when needed. However, if the economics, conveniences and advantages that can be realised through artificial insemination are to be made available to the sheep industry, artificial insemination procedures suited to Nigerian conditions must be developed.

The survival and fertilizing capacity of frozen spermatozoa are good for some species but poor in the majority of species. Not all factors involved in the frozen storage of semen have been fully investigated. Many specific effects of cryoprotectants are not fully understood. For example, some known cryoprotectants become damaging when concentrated by freezing (Lee, 1971; Show *et al.*, 1975 and Fahy, 1986).

The present study was designed to investigate the use of various cryoprotective compounds on deep frozen storage of ram spermatozoa. The main objective of the study was to develop improved crypreservation techniques for ram spermatozoa which could be used in the sheep industry. The specific objectives of this study were to evaluate the efficacy of various cryoprotective compounds, singly or in combination, on the post-thawing recovery, survival and fertilizing capacity of deep frozen ram spermatozoa. The experimental work was conducted as follows:

1. Screening of several cryoprotective compounds while determining optimum semen dilution rate and cryprotectant concentration.

2. Undertaking a comparative evaluation of cryoprotectants selected for their cryoprotective action on ram spermatozoa.

3. Evaluating the effects of combination and substitution of selected cryoprotective compounds on ram spermatozoa, and

4. Fertility tests with ram semen frozen using single and combined cryoprotective compounds, following oestrus synchronization.

1. REVIEW OF THE LITERATURE

1.1 Cryoprotective Compounds

To deep freeze cells and tissues, it is necessary to add cryoprotective compounds to the diluting medium. The type and concentration of the cryoprotectant is very important. Basically there are two kinds of cryoprotective compounds (Meryman, 1971; McGan, 1978):

1. Penetrating cryoprotective agents: These include glycerol, dimethylsulphoxide (DMSO), propanediol, erythritol, acetamide, ethanol and other alcohols. Even though Sherman (1963) observed that intracellular glycerol was not essential for maximum survival of certain cells, it was found that these compounds must penetrate into the cell in order to produce their maximum protective effect. The effects of these substances mostly occur in multimolar concentrations (1-4 M). Rapid and thorough penetration is necessary to produce the best protective effect. However, the danger of toxicity is quite considerable.

2. Non-penetrating cryoprotective agents: Examples are polyvinyl pyrollidone (PVP), polyethylene glycol-600 (PEG), sucrose, glucose, mannitol and other sugars. Their protective effect is produced outside the cell. The effect is generally greater at low molar concentrations (0.01-0.2M). In general, these substances are considerably less toxic than the penetrating (intracellular) cryoprotective agents.

Basically there are three possible ways in which the protective effect of cryoprotective agents are believed to work:

a. They reduce damage from solution effect and intracellular freezing (Farrant, 1965; Lepock et al., 1978). Glycerol, for example, reduces the sodium ion concentration in the extracellular medium quite considerably (Rall *et al.*, 1978).

b. They lower the temperature at which intracellular freezing occurs between −35 and −45°C when a cryoprotective agent is present (Leibo *et al.*, 1978). In its absence freezing will occur at about −10°C.

c. They stabilize the cell membrane (Maure, 1978), though the exact mechanism of the protective effect is yet to be understood.

Cryoprotective agents can reduce or prevent freezing damage but they themselves can damage the cells as a result of osmotic changes (Rall *et al.*, 1978; Fahy, 1986). The osmotic conditions in the medium alter dramatically with the addition of the cryoprotective agents. Addition of 1.0-1.4 M of glycerol can raise the osmotic pressure of the diluent from about 300 mOsm to about 1500 mOsm (Niemann, 1982). It is recognised that cryoprotective agents should be added in a stepwise manner to give the sensitive cells sufficient time for equilibration. The spermatozoon has the same basic requirements of protection as other mammalian cells against thermal and osmotic damage. Water is removed from solution during freezing with a resultant ice crystal formation and concentration of solutes (Foote, 1978). Certain unprotected cells such as red blood cells and a few nucleated mammalian cells can survive freezing in water or in simple salt solution if they are cooled at optimum rate. These cells will survive better if a protective additive such as glycerol is present. Various phospholipids, lipoproteins and proteins, provide some protection to the spermatozoa during cooling and

freezing. However, buffered egg yolk or milk are generally superior to various organic extracts in their protective activity, particularly against cold shock.

The cryoprotective effect of glycerol was discovered by Polge *et al.*, (1949). It remained the most effective cryoprotectant until Lovelock and Bishop (1959) demonstrated protection by dimethyl sulphoxide (DMSO). However, other solutes, chiefly sugars and macromolecules such as lactose and polyvinyl pyrrolidone (PVP) can protect some cells under certain conditions (Doebbler, 1966). Several cryoprotective compounds have been used for the preservation of spermatozoa of various animal species (Graham and Crabo, 1972; Wilmut and Polge, 1972; Salamon *et al.*, 1973; Hanada and Nagase, 1980; Jeyendran and Graham, 1980). A variety of agents such as erythritol, adonitol, acetamide, dimethyl sulphoxide, polyvinyl pyrrylidone have been recognised for their ability to protect red blood cells, ova, cells in tissue culture, and spermatozoa of some species other than farm animals during deep freezing. The degree of cryoprotection offered by these compounds has been found to vary considerably from one type of cell to another, as well as between similar types of cells from different species of animals. Some compounds have been examined for deep-freezing of ram spermatozoa, but relatively few of them offered sufficient cryoprotective action.

Platov (1965) reported that 1.75% and 2% ethylene glycol gave satisfactory recovery rates similar to 3.5% glycerol, after deep-freezing ram spermatozoa. Jones (1965a; 1965b) observed the toxicity of DMSO to ram spermatozoa. Salamon (1968) investigated glycerol, ethylene glycol and DMSO separately and in combination as cryoprotective compounds for freezing of ram spermatozoa. It was observed that glycerol (5%) gave better protection to spermatozoa during freezing

than ethylene glycol (5%) or DMSO (5%) or their combination.

Despite the considerable investigations on cryoprotective compounds for mammalian spermatozoa, glycerol remains the most widely used agent for deep-freezing spermatozoa (Salamon, 1968; Foote, 1978; Jeyendran and Graham, 1980). The optimum level of glycerol in the diluted ram semen was observed to be about 4% (Fraser, 1968; Jones, 1969; Johnson *et al.*, 1974; Colas, 1975). However, other investigators used as high as 11.5% glycerol (Fiser *et al.*, 1981). Still other workers used values below the reported optimum and obtained satisfactory spermatozoa recovery rates. Platov (1965) used 3.5% glycerol while Salamon (1970) observed that glycerol in 2% concentration was sufficient to obtain satisfactory revival of frozen ram spermatozoa. The latter author speculated on the possibility of further decrease in or even exclusion of) glycerol when freezing. Colas (1975) observed that the tendency towards a decrease in glycerol level might probably be used to improve results obtained when semen was diluted to a constant concentration of 900 x 10^6 spermatozoa/ml. By this the quantity of glycerol per cell remained more constant than at a constant dilution ratio of 1:4 (semen:diluent, v/v) when the quantity of glycerol per cell would be variable, because of the variation in number of spermatozoa between semen samples. This seems to suggest that the optimum level of glycerol in diluted semen is perhaps related to its final concentration, relative to the spermatozoa. This relationship does not appear to have been considered by other workers and may be one reason for the diversity of published findings on freezing of ram spermatozoa.

Salamon (1968) observed that optimum glycerol level seems

to be dependent on type of sugar used in the diluent. Lactose gave acceptable survival with only 1% glycerol or even without glycerol. The type of sugar used also appeared to determine whether glycerol should be added at 30°C (one-step) or after cooling to 5°C (two-steps). In either case an equilibration time of 2.5 h appeared beneficial especially after the two-step dilution. In a recent evaluation of cryoprotective compounds for bull spermatozoa, addition of the cryoprotective compounds appeared to be better at 35°C than at 5°C (Jeyendran and Graham, 1980).

It has been observed that apart from cryoprotectants added to semen diluent, egg yolk also provides some cryo-protection to the spermatozoa. Pace and Graham (1974) reported that glycerol provided little protection during freezing in the absence of egg yolk. Infact it has been observed that some cryoprotective compounds do not protect spermatozoa in the absence of egg yolk (Graham and Crabo, 1972). This suggests that egg yolk and glycerol interact with each other or with the sperm membrane to yield a synergistic effect during freezing. Jeyendran *et al.* (1979) observed a macromolecular interaction between egg yolk and seminal plasma proteins. The use of fluorescent probes has demonstrated a coating of egg yolk proteins on the spermatozoa membrane (Smith and Polge, 1950). It therefore appears that this interaction between egg yolk and seminal plasma proteins may be responsible for coating the spermato-zoa surface which then aids in cryoprotection. A low molecular weight compound such as glycerol may influence occurrence of the above interaction.

Most cryoprotectants appear to protect against solution effects rather than against intracellular freezing as demon-strated by Mazur (1970; 1977). He observed that increases in

the concentration of glycerol raised the survival of slowly cooled stem cells and cow and human red blood cells more than that of rapidly cooled cells: The recovery rate of rapidly cooled cow and human red blood cells were effectively decreased (Mazur, 1968; Rapatz and Luyet, 1968). It has been demonstrated that glycerol and DMSO offer protection on a molar basis, and that the two compounds act by reducing the electrolyte concentration in the residual unfrozen solution in, and around a cell at any given temperature (Lovelock, 1953). Lovelock (1954) concluded that only low molecular weight hydrophilic solutes with low eutectic points could protect cells, because only such solutes could yield solutions of high molar concentration at sufficiently low temperature. He also stated that to be protective, a solute must permeate a cell, or else it would not prevent a rise in intracellular electrolyte concentration during freezing. Meryman (1966) reached a similar conclusion about additives on the bsis of this hypothesis, ascribing a solution effect damage to osmotic forces.

Although protection by glycerol, DMSO and most other low molecular weight solutes is explicable on the basis of molarity and solubility (Lovelock, 1954; Nash, 1962), protection by macromolecules is not. Doebbler and Rinfret (1965) found that when red blood cells are suspended in PVP, polyethylene glycol, serum albumin, or in dextran solution, they survived freezing at a cooling rate of 300°C/minute. Protection at that cooling rate was correlated, not with the molarity of the additive, but with the molarity of the potential hydrogen bonding groups on the solute. An alternative explanation of the anomalously high freezing point depression (FPD) by PVP is that the preferential binding reduced the amount of free water available for the solution of electrolytes and actually

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raised their concent-ration in the residual unbound water.

The protection by macromolecules raises questions about Lovelock's and Meryman's conclusion that protection requires permeation. Penetration of more than trace quantities of molecules with molecular weights greater than 10,000 into cells seems unlikely. There is some doubt whether glycerol has to permeate in order to protect. Lovelock (1953) observed that the recovery of frozen human red blood cell was poorer (63% recovery) when glycerol permeation was blocked by copper ions than when it was not (97% recovery). However, the results obtained when glycerol permeation was blocked was far better than the 2% recovery rate in the absence of glycerol. It was suggested that the ability of a cell to survive freezing may depend more on protection of the cell surface than on protection of the cell interior. Perhaps the cell interior is protected by the high concentrations of macromolecules normally within it (Sherman, 1963; Pickett *et al.*, 1984).

Meryman *et al.*, (1977) observed that glycerol offered cryoprotection partly on the colligative basis by reducing further the amount of intracellular ice. It was further observed that the protection could be kinetically mediated through increase in the time taken for water to leave the cell. This was found to be in response to a decreased vapour pressure of adjacent ice, by increasing viscosity of extra - and intracellular solutions. It is the surface membrane that prevents ice from nucleating in cells at temperatures above -10 to -20°C. The permeability of the membrane to water is also the chief determinant of whether cells will equilibrate by dehydration or by intracellular freezing at lower temperatures. Furthermore, cells that are injured either by solution effects or by intracellular freezing undergo damage to

surface and internal membranes. The damage from the "solution effects" can be reduced by the use of certain additives that appear to act at the cell surface such as dextran and polyethylene glycol.

1.2. Diluent and Dilution

The requirements which should be met in providing a suitable environment for spermatozoa have been described by Foote (1972). Among those factors important for diluents used in deep freezing are osmotic pressure, pH, energy source, bacterial control, exclusion of toxic products, dilution effect, gaseous environment, protection of sperm from cold shock and freezing injury. The post-thawing recovery rate has been used as the main assessment criterion in judging the suitability of the diluting media.

1.2.1 Diluent Composition, pH and Osmotic Pressure

Many diluents previously used for liquid and frozen storage of ram semen were adapted from bull semen extenders (Salamon and Robinson, 1962; Sorensen, 1979). One extender was made utilising egg yolk-glucose citrate and heated cow milk by Salamon and Robinson (1962). However, Nagase and Niwa (1963) developed the most common extender for ram semen which consisted of egg yolk (25%), lactose (71.5%), and glycerol (3.5%). Diluents containing tris (hydroxy-methyl aminomethane) as a main component were initially used for frozen storage of bull (Davis *et al.*, 1963; Yassen and Foote, 1966; Hahn *et al.*, 1969) and boar semen (Rohloff, 1967; Groove *et al.*, 1968; Salamon, 1973; Osinowo,1975). The use of the tris-based diluent was later adopted for

pellet freezing of ram spermatozoa by Samouilidis (1970) and Salamon and Visser (1972).

According to Salamon and Visser (1972) ram spermatozoa can tolerate a relatively wide range of tris concentrations in the freezing diluent. They discovered that the type of sugar included in the tris diluent was important and that the performance of this diluent was influenced by the composition used for thawing the frozen semen. Steinbach (1967) examined tris concentrations ranging from 150-350 mM for freezing bull spermatozoa. It was observed that 200 mM was the optimum concentration in the tris-fructose egg yolk diluent, adjusted to pH 6.5. Tris at 350 mM concentration in the extender was unsuitable for freezing bull spermatozoa. Salamon and Visser (1972) reported that ram spermatozoa tolerated a relatively wider range of tris molarities. The most acceptable rate of cell survival following freeze-thaw procedure were observed in the range of 250 to 400 mM tris concentrations in diluent adjusted to pH 7.0.

The effects of diluent pH and osmotic pressure on survival and metabolism of spermatozoa during liquid and frozen storage have been studied most extensively in the bull than in other species (Johnson *et al.*, 1956; Cragle and Salisbury, 1959; Steinbach and Foote, 1967). Most of these investigators indicated that pH and osmotic pressure interacted, and that this relationship was further complicated by interactions with other factors such as glycerol and cation concentration of the diluent. Johnson *et al.* (1956) did not find any significant difference in the survival of bull spermatozoa when diluent with freezing point depression (FPD) of -0.50°C or -0.70°C and pH of 6.2, 6.6 or 7.0 were used for liquid storage of bovine semen. On the other hand, Cragle and Salisbury (1959) reported that a diluent with low pH and

high potassium concentration was inhibitory to the metabolism of bull spermotozoa during liquid storage. In a systematic investigation be Steinbach and Foote (1967) bull semen was extended and deep frozen in egg yolk-fructose extender buffered with five concentrations of tris (0.15, 0.20, 0.30 and 0.35 M) adjusted to four pH levels (6.0, 6.5, 7.0, 7.5) and containing two levels of glycerol (6.4 and 8.8%). The FPDs of the non-glycerol fraction of the extenders ranged from -0.58°C to -0.955°C. It was observed that the post-thawing motility of the spermatozoa was significantly affected by the tris molarity, pH and glycerol levels within the ranges examined. The investigators found an interaction between pH and osmotic pressure, with the higher pH levels tending to be detrimental at the low tris concentrations, and the lower pH levels showing a similar tendency at the higher tris concentrations. This relationship was further complicated by a significant second order interaction involving the tris molarity, pH and glycerol levels. The best overall result was obtained with an extender adjusted to pH 6.5 and containing 0.20 M tris, 6.4% glycerol, with a FPD of -0.62°C in the non-glycerol portion.

A similar study was carried out by Fiser *et al.* (1984) on ram spermatozoa frozen in skim-milk diluent. It was observed that spermatozoa motility improved with increasing osmolarity of the freezing solution. Diluents of 600 and 750 mOsm resulted in the highest motility, immediately after thawing. Inclusion of sugars in tris diluent was found to be beneficial to spermatozoa survival. However, glucose proved to be more suitable than fructose or raffinose. In earlier works, only fructose (55.5 mM) was included in tris extender in bull (Steinbach and Foote, 1967) and ram (Samouilidis, 1970) semen. It became evident that the amount of glucose

required in hypertonic tris extenders was relatively small (27.75 mM). Higher sugar concentrations had some depressing effect on the survival of spermatozoa following the freeze-thaw process (Salamon and Visser, 1972). However, in hypotonic tris media (150 mM), increasing sugar concentrations improved sperm cell recovery.

Recent reports have emphasized the importance of hypertonic conditions on the viability of several mammalian somatic cell types (Bolognani *et al.*, 1976; Dal Pra *et al.*, 1978; Griffith, 1978; and Tenchini *et al.*, 1980). The tolerance of spermatozoa to hypertonic conditions is not unique. Human red blood cells in culture, and Chinese hamster ovary cells (Griffith, 1978) exhibit relative tolerance to hypertonic solutions. Human embryonic epithelial cells exhibited improved survival rate, after freezing in hypertonic medium (Tenchini *et al.*, 1980). The cells were found to be well adapted to the medium and could infact be conditioned to survive higher hypertonicity after initial exposure to moderate/lower hypertonic medium (Bolognani *et al.*, 1976; Dal Pra *et al.*, 1978). The best post-thawing motility of ram spermatozoa was observed in hypertonic diluents which ranged in osmolarity from 600 mOsm/kg to 750 mOsm/kg (Fiser *et al.*, 1984).

Induced resistance to hypertonicity may give added protection against "solution effects" as the suspension of spermatozoa begins to freeze. Hyperosmotic diluents will also result in partial removal of freezable water from sperm even before freezing and so reduce the volume of potential intracellular ice formed during freezing.

1.2.2 Dilution of semen

Factors which influence semen dilution include holding time from collection until dilution, dilution tempe-rature, dilution method and rate of dilution. Considerable variation exists between the practices adopted by different investigators for the dilution of ram semen. No theoretical basis has yet been established regarding the holding of freshly collected semen for a period of time before dilution, though it has been used by a number of investigators (Graham *et al.*, 1971; Wilmut and Polge, 1972; Pursel and Johnson, 1974).

Jones and Holt (1974) observed that epididymal plasma had a slight initial stimulatory effect on motility of ram and bull sperm, but it had no effect on sperm survival. Seminal plasma on the other hand had marked initial stimula-tory effect on motility of ram spermatozoa, but was found to be detrimental to survival during storage. Even a 15-minute exposure to the fluid reduced sperm survival rate.

Holding time before dilution could adversely affect the survival of ram spermatozoa. There was a claim by Graham and Crabo (1972) that holding boar spermatozoa in its own seminal plasma might result in a protein coat that was protective. The holding time that has been used during processing of boar semen for deep freezing ranges from 45 minutes (Wilmut and Polge, 1972) to 2 hours (Johnson *et al.*, 1974; Pursel and Johnson, 1974). Beyond 2 h holding time, survival was signi-ficantly depressed. According to Osinowo (1975), holding boar semen for a length of time after collection before dilution had no beneficial effect on post-thawing survival following deep freezing. The same could apply to ram spermatozoa.

Dilution temperature varies between workers but three approaches seem predominant. These are, dilution at near body temperature (30-37°C), at room temperature (14 - 24°C) or at 5°C. Dilution at 30-37°C is most commonly used. Dilution at 5°C in most cases involves a 2-step dilution, the first being at a higher temperature (30-37°C). The dilution temperature which have been most commonly used in various reports is 0°C (Salamon, 1968; Salamon, 1970; Salamon and Visser, 1972; Visser and Salamon, 1973; Colas, 1975; Fiser *et al.*, 1981). Dilution methods for ram semen are either one-step or two-step. The one-step dilution involves slow addition of the glycerolated diluent to the semen, followed by gentle mixing (Salamon, 1968). In the case of two-step dilution, the semen is initially diluted containing no cryoprotective compound. The other fraction with cryoprotective agent is added after cooling the first fraction to 5°C (Salamon, 1968; Colas, 1975). Multi-step dilution is another method carried out by adding three or more equal portions of glycerolated diluents at certain time intervals (Colas, 1975). Dilution is carried out either on a volume per volume basis of semen and diluent, or alternatively to a fixed cell concentration. In a systematic study of the effect of dilution rate on the survival of ram spermatozoa, Salamon (1968) found a highly significant interaction between type of diluent and dilution rate.

Fresh semen may be diluted to a fixed cell concentration (Hess *et al.*, 1960) or the seminal plasma may be removed by centrifugation and the spermatozoa resuspended with diluent to a fixed cell concentration or to the original volume (Visser and Salamon, 1972). In the two-step dilution of semen, volume to volume dilution may be carried out at the first step, while the concentration of spermatozoa is taken into account at the second stage of the dilution (Salamon, 1968).

The two-step dilution is most applicable for fertility trials, because the spermatozoa could be concentrated in a small volume to reflect insemination dose.

The removal of seminal plasma from ram semen and subsequent resuspension of the spermatozoa with diluent to a fixed cell concentration could partly eliminate the variability between rams and ejaculates. This method may also facilitate freezing of semen in a more concentrated form which offers a saving in storage space and in time required for freezing and thawing procedures. However, it has been claimed that centrifugation has an adverse effect on the survival of spermatozoa. Mattos (1982) reported a better post-thawing motility in ram semen centrifuged at 5°C but percentages of intact acrosomes were higher in uncentrifuged semen.

1.3 Cooling and Equilibration of Semen

Extended semen should be cooled from 30°C to about 5°C before freezing, otherwise survival of spermatozoa is poor. The optimum cooling rate is not precisely known. Good post-thawing recovery rates were obtained after cooling from 30 to 5°C in 2 h (Salamon and Visser, 1972; Colas, 1975), 1.5 h (Salamon, 1970; Fiser *et al.*, 1981), 1 h (Visser and Salamon, 1973). There is not much variation between investigators in the cooling times used.

The equilibration period has generally been regarded as necessary to facilitate the penetration of spermatozoa by glycerol, so as to ensure better cryoprotection during freezing. However, this view has been challenged by Sherman (1963) and Meryman (1977) who observed that the protective action of glycerol is mainly extracellular. Equilibration of ram semen at 5°C was based on the practice adopted for bull semen. Blackshaw (1960) observed a better recovery

rate when spermatozoa were equilibrated for more than 6 h. But First *et al.* (1959) and Hill *et al.* (1969) observed no differences after equilibration for 0.5, 1 or 18 h. Salamon (1968) equilibrated ram spermatozoa at 5°C for 2.5 h before freezing and reported an increase both in motility and percentage of motile spermatozoa. The effect was more beneficial when glycerol was added after cooling to 5°C. However, in another observation by Salamon (1970), equilibration for 1-4 h prior to freezing gave similar survival rates. Colas (1975) obtained slightly better result after equilibration for 2.5 h which suggests that glycerol penetration into the ram spermatozoa is not as rapid as has been shown in the bull (Berndtson and Foote, 1972), or boar (Wilmut *et al.*, 1973). By contrast, Lightfoot and Salamon (1969b) found that there was no significant advantage in equilibration longer than 1 h when pelleting semen on dry ice.

1.4 Methods of Deep Freezing Semen

Spermatozoa are generally deep-frozen in straws, ampoules or in pellets. The methods used for pellet freezing could be classified into two:

1. Pellet freezing on dry-ice (Nagase and Niwa, 1964; Salamon and Lightfoot, 1969; Salamon, 1970).
2. Pellet freezing in liquid nitrogen vapour or directly in liquid nitrogen (Salamon, 1970).

Generally the frozen semen is stored in liquid nitrogen irrespective of method of freezing. The successful development of pellet freezing on dry-ice, and its relative simplicity, probably have rendered it more suitable than the other

methods. However, for international trade in frozen semen, straws are preferred. Pellet freezing which is the most commonly used method, eliminates the need for a packaging container and liquid nitrogen vapour during freezing. The technique allows freezing of concentrated semen sample in small volumes. Survival and fertility of ram spermatozoa following freezing by the pellet method on dry ice (Nagase and Niwa, 1964) have been examined in a number of studies (reviewed by Salamon and Lightfoot, 1969, 1970). Salamon (1970) reported on the survival of ram spermatozoa after freezing in pellet form at various temperatures below -79°C. Infact the result was very encouraging after freezing on stainless steel plate cooled to between -12 and -14°C. The survival of ram spermatozoa following pellet freezing on stainless steel plate at -12 and -100 to -160°C were not significantly different from freezing on dry ice at -79°C. Notwithstanding the possibility of very rapid freezing of ram semen at the indicated temperature, pelleting on dry ice (-79°C) at the present stage seems more advantageous due to its simplicity and resultant consistent survival rates. Although various freezing temperatures on the metal plate and on dry ice were not directly compared, it is noteworthy that spermatozoa tolerated the low temperatures at freezing range of -79°C to -180°C on dry ice better than on the metal plate. Freezing in synthetic straws which has been reported to give good results with bull semen (Cassou, 1964; Macpherson and King, 1966) was less effective with ram semen (Salamon, 1968). However, freezing of straws in liquid nitrogen vapour was superior to freezing of straws on dry ice. Freezing in ampoules at a slow rate offers good results for ram spermatozoa. However, recovery was poor when ampoules were plunged into a dry ice-alcohol bath at -79°C and only a few

spermatozoa were motile after dropping extended semen directly into liquid nitrogen (Salamon, 1970).

Lightfoot and Salamon (1969b) observed that the rate of cooling within the semen droplet placed into the cavity of dry ice is not uniform, but the difference in cooling rate throughout the pellet became less apparent with decrease in freezing temperature. It is possible that variation in cooling rate within the pellet could have some biological effect on the cell. Nevertheless, droplet volumes of 0.03 and 0.3 ml, with different surface-to-volume ratios gave similar results when frozen. There was no interaction between volume and temperature of pelleting.

Cooling velocity affects the physical-chemical events in cells during freezing. It also affects cell survival rate. The relationship between cooling velocity and survival is that maximum survival occurs at an optimum freezing rate. However, the specific value of the optimum freezing rate for different cell types varies over a wide range, from 1.6°C/minute for marrow stem cells to about 3000°C./minute for the human erythrocyte (Mazur, 1970).

There has been a tendency among investigators to consider freezing as a single stress. But the existence of an optimum temperature refutes this view, for it indicates that survival is affected by at least two opposing factors dependent on cooling velocity. It is a widely accepted practice to cool cells at 1°C/minute. Smith (1961) determined this cooling rate to be the optimum for bull spermatozoa. It also yields high survival rate for other cells (Polge and Lovelock, 1952; Smith, 1961; Meryman, 1966).

The suggestion has been made that an optimum cooling rate arises because of the interaction of the two classes of freezing events - dehydration and intracellular ice formation

(Mazur, 1980; Pickett *et al.*, 1984). According to this suggestion, solution effects are responsible for injury when cooling is slower than optimal and intracellular freezing is responsible for injury when cooling is faster than optimal. The optimum rate, then, is a rate that is slow enough to prevent production of intracellular ice and yet is rapid enough to minimize the length of time cells are exposed to solution effects.

There is evidently a connection between intracellular ice formation and the freezing rate on one hand and cell death on the other (Farrant and Woolger, 1972; Leibo *et al* 1978). The extent to which intracellular freezing is lethal to cells depends, first on the amount of ice formed and secondly, on the size of the ice crystals (Whittingham *et al.*, 1979; Mazur, 1980; Picket *et al.*, 1984). Rapid freezing rates produce small intracellular ice crystals which are not damaging to the cell.

1.5 Thawing and Thawing Solutions

An important factor associated with the thawing process is the thawing rate. The thawing rate depends on physical factors such as the thawing temperature, thermal conductivity of the container, use of thawing solution, size or shape of the frozen samples and the concentration of spermatozoa. In addition, the properties and composition of the freezing diluent and of the thawing solution may also be important. it should be mentioned, however, that the physico-chemical mechanisms involved during thawing of deep frozen mammalian spermatozoa are not fully understood. Both the recovery rate after thawing and the survival of spermatozoa during post-thawing incubation are important measures of cell viability

(Saacke, 1982).

There are numerous reports on the effect of thawing temperature on revival of spermatozoa after freezing, but there is no agreement among investigators (Pickett et al., 1965). Salamon (1968) indicated that rapid thawing is required in order to obtain maximum recovery of spermatozoa after fast-freezing. Many studies, especially with bull semen, have clearly shown tnat injury to the sperm is less when semen is thawed rapidly (Aamdal and Anderson, 1968; Rajamanan and Graham, 1968; Robbins *et al.*, 1972; Almquist and Wiggins, 1973; Meryman, 1974; Ennen *et al.*, 1976; Fiser *et al.*, 1986).

Salamon (1969) reported that temperature of thawing had a marked effect on recovery of frozen ram spermatozoa, the best result being obtained with thawing at 30 and 40°C and the poorest at 15°C. In the ram, Colas (1979) observed that recovery rate was lower following thawing at 25°C than at 38°C or 50°C. Generally, using higher temperatures and short thawing periods result in better sperm recovery.

Slow thawing rate has depressing effect on post-thawing recovery rate for samples frozen in liquid nitrogen. Salamon *et al.*, (1973) reported that thawing pellets of frozen boar semen at 37°C was superior to thawing at a lower temperature, and further increase of the thawing temperature to 50°C or 60°C was only slightly advantageous. Salamon (1970) observed that thawing frozen ram semen at 37 and 45°C yielded similar results. However, significant interaction was reported between thawing temperature and dilution rate at thawing.

Salamon (1970) observed that pellets prepared under a wide range of freezing condition showed similar recorvery after thawing at a rate of 1:3 (pellet:thawing solution, v/v) at either 37 or 45°C. However, the same author observed that

thawing frozen pellets in thawing solution at 37°C at the rate of 1:2 (pellet:thawing solution, v/v) gave good results

Increasing the pre-freezing dilution of semen which increases the volume of thawed semen compensated only partly for the benefit gained by using a thawing medium (Lightfoot and Salamon, 1969a). Concomitant examination of both freezing and thawing media revealed that a relationship exists between their compositions (Lightfoot and Salamon,1969). Further evidence concerning such a relationship indicated that variation in the constituents of the freezing diluent could require a change in the composition of the thawing solution in order to obtain maximum cell recovery. An appropriate thawing solution may offer some kind of "repairing" action, especially to spermatozoa which are not lethally injured during freezing in the absence of cryoprotectant. Such effect could be additive to the protection given by a cryoprotectant.

The choice of thawing solution was less critical in tris-based diluent when glucose was present than when fructose or raffinose was combined with the tris (Salamon and Visser, 1972). However, when optimum tris concentration of 300 mM was combined with glucose, fructose or lactose in the freezing diluent, spermatozoa recovery rate was improved by thawing in solutions consisting of inositol citrate or of 250 to 300 mM tris combined with citrate, glucose or fructose (Lightfoot and Salamon, 1969b; Salamon and Brandon, 1971). This indicates that a solution which acts beneficially at thawing is not necessary the optimal medium for maintaining the viability of sperm cells.

1.6 Post-thawing Survival of Spermatozoa

Sperm viability measured before, during and after storage has been the basis for development of methods of semen preservation in the research laboratory. For artificial insemination, sperm viability becomes a measure of man's interaction with the semen. Man's effeciency in developing successful methods of semen preservation is therefore contingent upon the relationship of tests of semen quality to fertility (Saacke, 1982b). In the case of fresh ram semen, an estimation of motility or the percentage of motile cells after dilution gives a good idea of its fertility. After freezing, the relationship between the fertilizing ability and *in vitro* semen characteristics is more difficult to establish.

Different *in vitro* tests to predict the fertility of frozen ram semen have been proposed. The most common is the recovery rate immediately after thawing. But this has been found to be imprecise, at least for certain diluents, as it does not take into account the survival ability of the cells which is known to be reduced after freezing. When only the recovery rate is used, the assessment can be misleading, as the survival of the spermatozoa during subsequent *in vitro* incubation may be poor, despite good initial post-thawing motility (Martin, 1965). Survival rate during post-thawing incubation therefore seems to be a better criterion for judging the functional integrity of spermatozoa after freezing. Though subjective, post-thawing motility in conjunction with determination of [illegible]al integrity is widely used.

Results have been contradictory on the value of incubation of frozen semen at 35 to 37°C, post-thawing, prior to evaluation. One report revealed that semen evaluation after

incubation for 2 to 4 h post-thaw is more closely related to fertility than viability measured immediately after thawing (Saacke and White, 1982). Other workers observed that viability measured immediately post-thaw is equally or more closely correlated with fertility than that measured after incubation (Pace, 1980; Saacke *et al.*, 1980; Pace *et al.*, 1981). Salamon (1972) incubated thawed semen at 37°C for 6 h and assessed the post-thawing survival by motility after every 2 h of incubation. Colas and Courot (1979) observed a significant correlation ($r = 0.66$) between survival and post-thawing motility of spermatozoa at 0 and 1 hour incubation at 38°C. Basically, evaluation of semen after incubation at or near body temperature following preservation by freezing appears rational, because spermatozoa must maintain viability at that temperature for some time in the female genitalia. However, *in vitro* incubation in preservation medium is probably different from that encountered by sperm *in vitro* where they become surrounded by the fluids and conditions of the female genital tract. Wright and Bondioli (1981) showed that spermatozoa, when incubated *in vitro*, achieved higher *in vitro* oocyte penetration than did spermatozoa incubated *in vitro*. The use of more than one measure of viability to evaluate methods of semen preservation appears to be desirable. But, since motility is highly regarded as an important characteristic of fertile spermatozoa it is most often used. However, sperm transport in the female genitalia and penetration of the ovum are only partially dependent upon motility (Overstreet and Katz, 1977; Overstreet *et al.*, 1979; Katz and Overstreet, 1980).

1.7 Morphological Changes

The morphology of mammalian spermatozoa has been described by Austin and Bishop (1958) and Saacke and Marshall (1968). Spermatozoa can be damaged during all the stages of dilution, cooling, freezing and thawing, resulting in morphological changes. These result in a cummulative acrosomal damage of about 40%, detected by light microscopy and about 70%, detected by electron microscopy (Tasseron *et al.*, 1977). The higher percentage of damage detected by electron microscopy after freezing and thawing consisted mainly of moderate bubbling and swelling of the acrosome. The acrosome receives more attention in morphological studies of spermatozoa, probably because it exhibits the most visible changes. Freezing causes profound changes in the acrosome morphology of ram (Quinn *et al.*, 1969; Tasseron *et al.*, 1977), bull (Somade *et al.*, 1974) and boar spermatozoa (Kunze *et al.*, 1973). Changes in the structure of the middle-piece and tail of spermatozoa have been studied to a lesser extent. Electron microscopy has revealed that cold-shock and freezing cause changes in the appearance of the acrosomes of ram spermatozoa. In addition, structural changes in the middle piece, particularly in the matrix of the mitochondrial sheath, were observed (Quinn *et al.*, 1969).

Comparative studies have revealed that the spermatozoa of the bull, ram and boar differ in their resistance to freezing injury. Watson and Martin (1972) reported that the acrosome appeared to be more severely damaged in ram than in bull spermatozoa. Healey (1969) observed that both boar and ram spermatozoa were more severely damaged by freezing than bull and stallion spermatozoa. An investigation by Jones (1973a) showed that the plasma membrane of bull spermatozoa

was more resistant to breakage than that of the boar or ram, when stressed by dilution and fixation. The author suggested that this could be the reason why bull spermatozoa are more resistant to damage. According to Jones and Martin (1979) there is a relationship between structure and function of spermatozoa which may be of use in evaluating semen quality. However, factors at the molecular level must also be considered since ultrastructural changes alone do not explain the reduced fertility due to processing and storage of ram semen. More information is required on the structure of a spermatozoon which is capable of fertilizing an ovum. It has been suggested that acrosome swelling may not prevent the acrosome reaction and fertilization, and that much of the plasma membrane breakage observed occur during fixation. It is also noteworthy that the susceptibility of spermatozoa to membrane breakage during fixation varies between males, and within and between species (Jones, 1975).

The proportion of spermatozoa with normal acrosome following deep freezing could be influenced by a variety of factors associated with semen processing. Such factors include diluent type, dilution, cooling and equilibration, freezing and thawing (Tasseron *et al.*, 1977). Visser and Salamon (1974) observed that the mean percentage of boar spermatozoa with normal acrosome after freezing, thawing and incubation were higher for this than for glucose diluent. It was generally accepted that post-thawing incubation at 30°C for 6 h caused marked increase in the proportion of spermatozoa with severe damage. Wiggins and Almquist (1974) investigated the effects of equilibration time and thawing rate on the acrosomal damage of bull spermatozoa. The mean percentage of spermatozoa with normal acrosome increased for each increase in thawing rate. There was an interaction between

thawing rate and equilibration time. The shorter the equilibration time in association with rapid thawing the higher the percentage of spermatozoa with normal acrosomes. Tasseron *et al.* (1977) investigated the acrosome damage of ram spermatozoa during dilution, cooling nd freezing, and reported that about 50% of the damaged spermatozoa were due to dilution and cooling alone. They suggested that by improving the diluent (raffinose-sodium citrate-egg yolk -5% glycerol) the damage could be reduced.

Swelling of the acrosome was mainly caused by cooling spermatozoa to 5°C (Jones and Martin, 1973). It was further observed that acrosomes and mid-pieces were affected by cooling to 5°C and storage at this temperature for up to 72 h. The acrosomal changes involved swelling and vacuolation of outer acrosomal membrane with condensation of mid-piece in a small proportion of spermatozoa. Freezing and thawing of spermatozoa caused disruption of the acrosome and damage to the middle piece. Consequently, any further loss in viability attributed to the processing of semen probably occurs during the freezing and thawing process (Jones and Stewart, 1979). Healey (1969) showed that freezing and thawing caused qualitatively similar acrosomal damage to boar, chinchila and ram spermatozoa. A detailed discussion of the literature on freezing injury to cells and methods for its prevention have been made by Lindemann *et al.*, (1982). It is not clear however, whether the changes in the cell membrane are the results of a selective effect of the freezing process on the cell membrane alone or are, infact, a single manifestation of generalised damage to the entire cell structure. Although it is possible to demonstrate that specific structures are damaged when a given cell type is subjected to freezing, it is usually not determined which cell organelles are left

unaffected after freezing and thawing (Lindermann *et al.*, 1982).

1.8 Biochemical Changes

The biochemical changes in spermatozoa induced by deep freezing have been investigated by several workers, with the aim of establishing a relationship with the loss in fertilising capacity. In these studies, greater emphasis has been placed on the changes in enzyme activity and deoxyribonucleic acid (DNA) content of spermatozoa. In addition, the phospholipid and fatty acid components have been examined to some extent. The effects of low temperatures and freezing on enzymes and enzyme systems in biological organisms have been reviewed by Tappel (1966).

Extracellular enzyme activities are sometimes used to assess cell membrane damage during *in vitro* storage of spermatozoa (Crabo *et al.*, 1970; Bower *et al.*, 1973; Foulkes and Watson, 1975). Some attempts have been made to relate acrosomal enzymes to capacitation (Austin, 1968; Allison and Hartree, 1970; Rogers and Martin, 1973). Generally, the pattern of enzyme release by mammalian cells is complex (Schmidt and Schmidt, 1974) and differences between pathological and physiological release are not clear.

The loss of intracellular enzymes into extracellular medium during freezing of spermatozoa was examined by Graham *et al.* (1970). The activities of glutamic oxaloacetic transaminase (GOT), lactic acid dehydrogenase (LDH), hyaluronidase, acid and alkaline phosphatases in the extracellular fluid of bull, boar, stallion and turkey semen increased in proportion to the extent of cellular damage after deep freezing (Graham *et al.*, 1970; Brown *et al.*, 1971).

According to Kurilov (1972) the extracellular activities of glucose phosphate isomerase (GPI) and alkaline and acid phosphatases in boar semen did not increase following deep freezing, although aldolase increased more than 2-fold. However, Osinowo (1981) observed that significant amounts of LDH and GPI in ram and bull semen were lost by spermatozoa as a result of washing. The release of certain enzymes may therefore be poorly correlated with cellular damage. White (1972a, b) observed that cytoplasmic droplets in spermatozoa contained enzymes which leaked into the extracellular medium during dilution, cooling or freezing. Osinowo (1981) speculated that leakage of intracellular material during dilution could be a mechanism to eliminate osmotic differences between intracellular and extracellular fluids, in order to control cell volume. It has also been observed that enzymes leaked from washed spermatozoa under conditions which seemed to preclude cellular damage. Crabo *et al.* (1970) reported that the dilution of semen caused a release of GOT from spermatozoa, although the amount released varied with the types of buffer used. It was observed that higher glycerol levels could cause increase in the loss of GOT (Bower *et al.*, 1973). Graham *et al.* (1970) proposed that the amount of enzymes left in the cell, after all procedural manipulations, could be important to the potential fertilizing capacity of the spermatozoa. In a subsequent study however, Crabo and Graham (1972) observed that the amount of GOT left in boar spermatozoa after deep freezing, and the amount of hyaluronidase released were poorly correlated with motility or acrosome damage. This indicates that enzyme leakage assay mightnot be a good criterion for assessing frozen spermatozoa viability.

The DNA content of spermatozoa has been intensively

studied in liquid and frozen semen in relation to the loss of fertilizing capacity. Sharobaiko *et al.* (1974) found that *in vitro* storage of semen did not affect the nucleotide composition of the DNA of live spermatozoa. An investigation on the effect of freezing and thawing on the stability of the double helix of DNA was carried out with no structural changes detected following freezing at temperatures as low as -192°C (Shikama, 1965; Hanada *et al.*, 1968). It was reported that storage of bull spermatozoa for up to 15 days at 4°C, or for 100 days at -79°C, or X-irradiation, followed by storage for up to 8 days, did not significantly alter the denaturation temperature of the DNA (Berchtold *et al.*, 1971). The stability of the DNA component component of spermatozoa during *in vitro* storage has been further confirmed by Blackshaw and Salisbury (1972).

Although there was no loss of DNA from cold-shocked or frozen ram spermatozoa during 30 minutes period, loss of DNA occurred when the spermatozoa were incubated for 6 h at 37°C (Quinn *et al.*, 1969). The longer incubation required to demonstrate the DNA loss from ram spermatozoa may be due to the action of deoxyribonucleases. A highly active DNase-1 is present in ram seminal plasma. This enzyme passes into the spermatozoa on freezing (Quinn, 1968). Degradation of DNA into dialysable fragments occurs slowly in senescent ram semen. But the process is accelerated if the semen has been cold-shocked or frozen (Quinn and White, 1969a).

The different claims by investigators concerning the stability of DNA content of spermatozoa during storage can partly be explained by the various methods and techniques of DNA determination and analysis carried out (Bouters *et al.*, 1967; Hanada and Nagase, 1968; Blackshaw and Salisbury, 1972)

Relationship between the polyunsaturated and saturated

fatty acid ratio of the phospholipids of spermatozoa and susceptibility to cold-shock has been established (Poulos *et al.*, 1973; Darin-Bennet *et al.*, 1974). The role of cholesterol in low temperature tolerance of other cells has been observed (de Gier *et al.*, 1968; Rottem *et al.*, 1973) and the ability of this lipid to promote close packing of phospholipids, particularly those lipids with unsaturated fatty acid chains (Benedetti and Emmelot, 1968; Phillips, 1972) is well recognized. The mean amount of cholesterol found per 10^9 spermatozoa varied between 280 to 560 µg depending on the species. Ram spermatozoa had 279.0 ± 23.3 µg of cholesterol/10^9 spermatozoa (Darlin-Bennet and White, 1977) while bull, rabbit and human spermatozoa had higher values. Variation in the amount present in the different species is not unusual, as significant quantitative differences have been noted when comparing the steroid content of membranous structures (Van Deenen, 1965). Ram and bull sperm contain approximately half the amount of cholesterol present in human and rabit spermatozoa. Sensitivity of spermatozoa to cold-shock (Wales and White, 1959) have been linked to the polyunsaturated - saturated fatty acid ratio of their phospholipids (Poulos *et al.*, 1973). There appears to be a correlation between the structure of the spermatozoa membrane which is strongly influenced by the ratio of cholesterol to phospholipid, the ratio of polyunsaturated to saturated fatty acids, and the subsequent ability of the membrane to withstand stress such as cold-shock. The different interaction of cholesterol with saturated and unsaturated fatty acids is well documented in model membrane systems (Chapman and Walloch, 1968; Phillips, 1972; Hauser and Barratt, 1973; Janson *et al.*, 1973). Relatively large amount of saturated fatty acids results in a more rigid structure than is the

case with unsaturated acids (Beneditti and Emmelot, 1968). The nature of the fatty acids also affects the ability of cholesterol to decrease membrane permeability (Daniel *et al.*, 1968). The transition temperature of the phospholipid is decreased as the level of unsaturation rises and chain length decreases (Phillips, 1972). This might explain why cholesterol, in combination with the more saturated fatty acids in the phophoslipids of the rabbit and human spermatozoa yield a membrane structure of intermediate fluidity that can withstand greater environmental stress such as freezing.

1.9 Fertility of Deep Frozen Ram Spermatozoa

Insemination methods involving cervical bypass resulted in considerably higher fertilization rate than did cervical insemination (Gustafsson, 1978). Loginova and Zeltobryjuh (1968) showed that insemination of frozen-thawed semen in the oviducts gave 88% fertilization compared to only 12% obtained at cervical insemination. A study conducted by Mattner *et al.* (1969) very clearly demonstrated the same phenomenon. Frozen-thawed semen yielded the same number of fertilized ova as did fresh semen by intra-uterine insemination via a surgical incision. Furthermore, the results obtained by Mattner *et al.* (1969) indicated that with intra-uterine insemination, the fertilization rate is comparable to natural mating even with an insemination dose containing only 30 x 10^6 motile sperm of frozen ram semen and 3 x 10^6 of fresh ram semen. A comprehensive study by Lightfoot and Salamon (1970) which compared cervical and intra-uterine inseminations demonstrated that inseminating frozen semen into the uterus via a surgical incision gave about 90% fertilization

rate compared to about 45% when inseminated into the posterior cervix. Similar results were obtained by Anderson *et al.*, 1973); Fukui and Roberts, 1976 and Graham *et al.*, 1978). However, Anderson *et al.* (1973) deposited the semen intra-uterine by passing the catheter through the cervix and obtained a conception rate of 89% compared to 48% by cervical deposition.

Killeen and Caffrey (1982) described a technique for intra-uterine insemination in ewes by laparoscopy. Preliminary results by the technique indicated that high levels of egg fertilization could be obtained in ewes following intra-uterine insemination with fresh or frozen-thawed semen. More recently Maxwell *et al.* (1984) reported an increase in fertilization and pregnancy rates with time of insemination, using intra-uterine insemination of frozen-thawed ram semen with the aid of laparoscopy. They observed that fertilization and pregnancy rates following intra-uterine insemination with frozen ram semen 60 h after sponge removal were not statistically different from the fresh semen when cervically inseminated as control. Furthermore, the intra-uterine insemination technique is more efficient than cervical insemination because of the relatively small dose of semen needed (0.02 ml, containing 20 x 10^6 spermatozoa). The intra-uterine insemination technique is technically a very difficult and time-consuming procedure. The earlier techniques (Lightfoot and Salamon, 1970a, b; Anderson *et al.*, 1973; and Fukui and Roberts, 1976) have not proved very successful as they have relied either on deposition of semen in the uterine horns after laparotomy or a forceful entry to the uterus through the cervix (Maxwell *et al.*, 1984). Another method to overcome the cervical barrier for easy sperm transport is the use of hormones. Substances such as oxytocin

(Bedford, 1972), relaxin (Salamon and Lightfoot, 1970), and prostaglandin (Bygdeman and Holmberg, 1966; Edqvist *et al.*, 1975; Gustafsson *et al.*, 1977) have been tried either on the ewe or incorporated in the semen diluent. Only prostaglandin showed some promise in improving the sperm transport in the female genital tract (Gustafsson, 1978).

Reports on embryo losses due to insemination with frozen ram semen are conflicting. According to Anderson *et al.*, (1973) the non-surgical intra-uterine technique did not result in high incidence of early embryonic loss. But Maxwell *et al.* (1984) observed that there was considerable embryonic loss between 2-3 and 100 days after insemination. The embryonic survival rate was higher for inseminations at 48 and 60 h than at 24 and 35h after sponge removal (36/43 v 9/31).

This indicates that the survival rates of embryos were lower when inseminations were performed too early. However, according to Edey (1979), embryonic loss in sheep is relatively high even after natural mating. The situation may be further aggravated after insemination with spermatozoa subjected to frozen storage and/or ageing (Salisbury *et al.*, 1976).

2. EVALUATION OF SOME CRYOPROTECTIVE COMPOUNDS FOR DEEP FREEZING RAM SPERMATOZOA IN TRIS DILUENT

ABSTRACT

Six experiments were conducted to investigate the optimum concentration of six cryoprotective compounds and the most appropriate dilution rate for deep frozen storage of ram spermatozoa in Tris diluent.

Glycerol, erythritol, DMSO, acetamide, ethylene glycol and polyethylene glycol-600 were found to have highly significant ($P < 0.001$) effect on post-thawing survival of ram spermatozoa. However, glycerol, erythritol and ethylene glycol offered more cryoprotection to the spermatozoa. The optimum concentration of these agent was 0.5 M, while the best dilution rate was 1:4 (semen:diluent, v/v).

Among the cryoprotective compounds used, glycerol was found to be superior to others which is in agreement with previous findings (Platov, 1965; Salamon, 1968). Presumably, the superiority of glycerol over the other compounds could be due to its nature of being a normal intermediary product of lipid metabolism in mammals. Erythritol though not routinely used as cryoprotective agents in freezing ram spermatozoa offered cryoprotection as good as glycerol. This observation was similar to the findings of Wilmut and Polge (1973) and Osinowo (1975) who worked on boar spermatozoa.

2.1 INTRODUCTION

The discovery by Polge *et al.* (1949) that glycerol minimized the harmful effects of deep freezing on spermatozoa opened the era of deep frozen storage of semen. Since then, several other compounds have been used as cryoprotective agents in deep frozen storage of spermatozoa and other cells and tissues of various species (Graham and Crabo, 1972; Salamon, 1980; Jeyendran and Graham, 1980; Hanada and Nagase, 1980; Harvey, 1983).

A variety of compounds including erythritol, adonitol, acetamide, dimethyl sulphoxide (DMSO) and polyvinyl pyrrolidone (PVP) have been recognized for their ability to protect red blood cells, cells in tissue culture, ova, and spermatozoa of species other than farm animals during deep freezing. The degree of cryoprotection by these compounds has been found to vary considerably from one type of cell to another, as well as between similar types of cells from different species. Some investigations have therefore been carried out in search of compounds which could give better protection than glycerol during deep freezing of ram spermatozoa (Platov, 1965; Salamon, 1968). Of the compounds tested, only ethylene glycol gave satisfactory recovery rates after deep freezing of ram spermatozoa, similar to that by glycerol. Other compounds such as DMSO which is routinely used as cryoprotectant agent for deep freezing of red blood cells proved to be toxic to ram spermatozoa (Platov, 1965; Salamon, 1968).

The experiments in this section are aimed at screening some compounds for possible cryoprotective action during deep freezing of ram spermatozoa in Tris diluent. The compound so screened were glycerol, DMSO, ethylene glycol, acetamide,

erythritol and polyethylene glycol-600. Their optimum concentrations for cryoprotection were also determined, as well as the optimum dilution rate for deep freezing ram spermatozoa Tris diluent.

2.2 MATERIALS AND METHODS

2.2.1 Semen Collection

Semen was collected by artificial vagina from six Yankasa rams. The ejaculates were pooled after checking for good initial motility. The sperm concentration of the pooled semen was than determined by photoelectric method using a spectrophotometer (Model C-400, Beckman) at 600 nm wave length.

2.2.2 Semen Processing

Diluent The Tris diluent used had the following composition: 300 mM Tris (hydroxymethyl aminomethane), 94.7 mM citric acid, 27.75 mM glucose, 15% (v/v) fresh egg yolk, 1000 IU per ml penicillin G and 1 mg per ml streptomycin sulphate. The diluent was prepared in double-glass distilled water without warming (Appendix 1). Diluents were either used soon after preparation or were stored at 5°C at double concentration for use within 24 h. Fresh egg yolk was added to the diluent on the day of each experiment.

Cryoprotective agents The cryoprotectants used were glycerol, erythritol, ethylene glycol, acetamide, dimethyl sulphoxide and polythylene glycol, at 0.0, 0.5, 1.0 and 1.5 M concentrations.

Dilution and cooling The semen samples were diluted with the appropriate diluent in test tubes placed in a water bath at 30°C. The dilution rates used in the experiments were 1:3, 1:5, and 1:7 (semen:diluent, v/v). After dilution at 30°C, the extended semen samples in the test tubes were placed in a beaker containing small quantity of water at 30°C to cover the level of the extended semen in the test tubes. The beaker was placed in the refrigerator to cool from 30°C to 5°C in about 2 h, covered with filter paper to prevent water from dropping into the samples.

Freezing and storage: On cooling to 5°C the extended semen was frozen on dry-ice in pellet form (Nagase and Niwa, 1964). Three drops of semen were placed by Pasteur pipette into small indentations made on blocks of dry ice allowed to freeze into pellets. The pellet volume was 0.09 ml. After the semen was completely frozen, the pellets were transferred into marked pre-cooled test tubes in liquid nitrogen for storage at -196°C for at least 48 h before evaluation.

Thawing, incubation and motility assessment: A minimum of three pellets per sample were thawed in coded dry test tubes in a water bath at 37°C. The test tubes were shaken gently until the semen was completely thawed. The samples were randomly presented for estimation of motility. Post-thawing incubation was at 37°C for 6 h.

The percentage of motile cells in each sample of thawed semen was assessed under coverslip using a phase-contrast microscope equipped with a warm stage kept at 37°C. The motility assessments were carried out immediately after thawing at 0 h, and at 2, 4 and 6 h of post-thawing incubation. In all experiments three replicates were used and

examination of the spermatozoa was carried out at 400x magnification.

2.2.3 Experimental Design

Six factorial experiments were conducted to investigate the optimum concentration of six cryoprotective compounds and the most appropriate dilution rate for deep frozen storage of ram spermatozoa (Appendix 3).

Experiment 1 examined the effects of glycerol concentration (0.0, 0.5, 1.0 and 1.5 M) and dilution rate (1:3, 1:5 and 1:7, semen:diluent, v/v) on post-thawing survival of deep frozen ram spermatozoa.

Experiment 2 examined the effects of dimethyl sulphoxide (DMSO) concentration (0.0, 0.5, 1.0 and 1.5 M) and dilution rate (1:3, 1:5 and 1:7, semen:diluent, v/v) on post-thawing survival of deep frozen ram spermatozoa.

In experiment 3, the effects of erythritol concentration (0.0, 0.5, 1.0 and 1.5 M) and dilution rate (1:3, 1:5, and 1:7, semen:diluent, v/v) on post-thawing survival of deep frozen ram spermatozoa in Tris diluent were investigated.

Experiment 4 involved investigation of the effects of ethylene glycol concentration (0.0, 0.5, 1.0 and 1.5 M) and dilution rate (1:3, 1:5 and 1:7, semen:diluent, v/v) on post-thawing motility of deep frozen ram spermatozoa in Tris diluent.

In experiment 5, the effects of acetamide concentration (0.0, 0.5, 1.0 and 1.5 M) of acetamide and dilution rate (1:3, 1:5 and 1:7, semen:diluent, v/v) on post-thawing survival of deep frozen ram spermatozoa in Tris diluent were investigated.

Experiment 6 examined the effects of polyethylene

glycol-600 concentration and dilution rates (1:3, 1:5 and 1:7, semen diluent, v/v) on post-thawing motility of deep frozen ram spermatozoa in Tris diluent. The concentrations of polyethylene glycol-600 used were 0.0, 3.0, 6.0 and 9.0 mM instead of 0.0, 0.5, 1.0 and 1.5 M used in experiments 1-5, because at higher concentrations the polyethylene glycol-600 coagulated the egg yolk.

2.2.4 Statistical Analysis

Motility data for each experiment were analysed, following angular transformation (Steel and Torrie, 1960) using the statistical package for the social sciences (SPSS) factorial analysis by regression approach (Kim and Kohout, 1975).

2.3 Results

2.3.1 Experiment 1

Results of the Experiment 1 on effects of glycerol concentration and dilution rate on post-thawing survival of deep frozen ram spermatozoa in Tris diluent are presented in Table 1.1. Glycerol concentration and incubation period showed highly significant ($P < 0.001$) effect on post-thawing motility of ram spermatozoa. The glycerol concentration which produced the best result was 0.5 M. Motility of spermatozoa during post-thawing incubation were 24.4, 20.8, 17.0 and 13.4% at 0, 2, 4 and 6 h respectively. There was a highly significant interaction ($P < 0.001$) between glycerol concentration and dilution rate (Table 1.2). The interaction was due to the response to glycerol concentration which changed with dilution rate. The optimum glycerol concentration at

1:3 dilution rate of 1:3 (semen:diluent, v/v) was 1 M, while for 1:5 and 1:7, it was 0.5 M.

2.3.2 Experiment 2

In Experiment 2, dimethyl sulphoxide concentration, dilution rate and incubation period showed highly significant effects ($P < 0.001$) on post-thawing motility of spermatozoa (Table 1.3). The dimethyl sulphoxide concentration which showed the best result was 0.5 M, while 1:7 (semen:diluent, v/v) dilution rate gave the best motility. Post-thawing motility declined as the incubation period increased. The mean motility was 13.3% at 0 h and 4.3% after 6 h of incubation. There was a significant interaction ($P < 0.05$) between dimethyl sulphoxide concentration and dilution rate (Table 1.4). The best motility was without DMSO at 1:5 and 1:7 dilution rates, while 0.5 M DMSO concentration could be considered the optimum level at 1:3 dilution rate.

2.3.3 Experiment 3

Results of Experiment 3 on the effects of erythritol concentration, dilution rate and incubation period on post-thawing survival of deep frozen ram spermatozoa in Tris diluent are presented in Table 1.5. Erythritol concentration and incubation period showed highly significant ($P < 0.001$) effects on post-thawing motility of spermatozoa. The mean percentage of motile spermatozoa for erythritol concentrations of 0.0, 0.5, 1.0 and 1.5 M were 17.7, 20.3, 20.6 and 21.4% respectively. The erythritol concentration which produced the best result was 1.5 M. The motility declined as with incubation time, with the best motility of 23.6% shown

at 0 h, compared to 15% at 6 h.

2.3.4 Experiment 4

In Experiment 4 ethylene glycol concentration ($P < 0.001$), incubation period ($P < 0.001$) and dilution rate ($P < 0.01$) had significant effects on post-thawing motility of spermatozoa (Table 1.6). The mean percentages of motile spermatozoa were 7.4, 13.9, 13.3, and 10.4% for 0.0, 0.5, 1.0 and 1.5 M concentrations of ethylene glycol. The best motility was shown by 0.5 M ethylene glycol. There was no significant difference in post-thawing motility at the three dilution rates, while there was a decline in motility with increase in incubation time. There was a significant interaction ($P < 0.05$) between dilution rate and ethylene glycol concentration. There was due to a decline in post-thawing motility at high dilution and high ethylene glycol concentrations. This was also a highly significant ($P < 0.001$) interaction between dilution rate, ethylene glycol concentration and incubation time (Table 1.7).

2.3.5 Experiment 5

As shown in Table 1.8, post-thawing motility of ram spermatozoa was significantly affected ($P < 0.001$) by acetamide concentration and incubation period in Experiment 5. The mean percentage of motile spermatozoa were 4.0, 8.8, 9.6, and 6.1% for 0.0, 0.5, 1.0 and 1.5 M concentrations of acetamide respectively. The acetamide concentration which produced the best result was between 0.5 and 1.0 M. There was a decline in motility with incubation time. There was also a significant interaction ($P < 0.01$) between dilution

rate and concentration (Table 1.9). This was mainly due to a decline in acetamide in motility with increase in acetamide concentration and dilution rate.

2.3.6 Experiment 6

In Experiment 6 polyethylene glycol-600 (PEG-600) concentration and incubation period had highly significant ($P < 0.001$) effects on motility of spermatozoa after freezing and thawing (Table 1.10). The PEG-600 which produced the best result was 3 mM. There was a decline in motility with an increase in incubation time. The best motility was at 2 h incubation. There was a highly significant interaction ($P < 0.001$) between PEG-600 concentration and dilution rate (Table 1.11). The interaction was due to an inconsistent motility, with the highest at 1:5 dilution when the PEG-600 concentration was 3 mM and at 1:7 dilution when the PEG-600 concentration was 6 mM.

2.4 DISCUSSION

In this study various compounds were screened to evaluate their cryoprotective activities on deep-frozen ram spermatozoa. Glycerol, erythritol, DMSO, acetamide, ethylene glycol and polyethylene glycol-600 were found to have highly significant ($P < 0.001$) effects on post-thawing survival of ram spermatozoa deep frozen in Tris diluent. Of these, glycerol, erythritol and ethylene glycol appeared to give the best post-thawing motility rates. Generally the optimum concentration of these cryoprotective compounds was found to be in the range of 0.5-1.0 M. The best dilution rate was 1:4 (semen: diluent, v/v) for most of the compounds screened. However, where dilution rate had no significant effect on post-thawing survival there was a significant interaction between dilution rate and cryoprotective agent concentration. Incubation period had highly significant ($P < 0.001$) effect on post-thawing survival of deep-frozen ram spermatozoa. There was a general decline in post-thawing motility as the incubation progressed.

Among cryoprotective compounds used, glycerol was found to be superior, which is in agreement with previous findings (Platov, 1965; Salamon, 1968). Presumably, the superiority of glycerol over the other cryoprotective compounds could be due to its nature of being a normal intermediary product of lipid metabolism in mammals.

Erythritol, though not routinely used as a cryoprotective compound for deep freezing ram spermatozoa, offered some cryoprotection. When included in the diluent at a concentration range of 0.6 to 1.5 M, erythritol offered cryoprotection as good as glycerol in respect of both post-thawing recovery and survival rates during post-thawing incubation.

This observation agrees with the findings of Wilmut and Polge (1973) and Osinowo (1975) who worked on boar spermatozoa.

Ethylene glycol and dimethyl sulphoxide did not offer any appreciable cryoprotection to ram spermatozoa. The effect of ethylene glycol was in contrast to the findings of Platov (1965) where it was observed that 1.75 and 2% ethylene glycol gave protection to ram spermatozoa during pellet freezing similar to that of 3.5% glycerol. But it could be speculated that the ethylene glycol concentration used in this study was much higher than what Platov (1965) used in his studies. It could be observed that ethylene glycol at higher concentration on molar basis has given less protection to the freezing ram spermatozoa.

There was a general decline in post-thawing recovery rate with all concentration of dimethyl sulphoxide used in this study. Though dimethyl sulphoxide is routinely used in deep freezing embryos of various species, it proved toxic for deep freezing ram spermatozoa. The findings in this study confirmed earlier reports by Jones (1965), Meryman (1966) and Salamon (1968) of toxicity of dimethyl sulphoxide to ram spermatozoa and human red blood cells. There was an inverse correlation between sperm recovery and DMSO concentration (Kahn, 1978).

Even though acetamide and polyethylene glycol-600 have not been reported as cryoprotective compounds for deep freezing ram spermatozoa, they gave some cryoprotection. Acetamide could be said to have a mild cryoprotective effect on recovery rate of deep frozen ram spermatozoa compared to glycerol or erythritol. On the other hand, polyethylene glycol-600, the only high molecular weight compound used as a cryoprotective agent in this study coagulated the egg yolk in

the diluent at higher concentrations. It performed poorly even at lower concentrations, rarely giving any cryoprotection to the spermatozoa. This agrees with the findings of Jeyendran and Graham (1980) on the effect of polyethylene glycol on bull spermatozoa.

The effective concentration of most of the cryoprotective compounds used in this study was found to be about 0.5 M while 1:4 dilution rate was found to be optimal. There was a decline in post-thawing motility with an increase in incubation time. The decline was generally gradual from 0-2 h of incubation; after which there was a sharp decline in motility. The practical application of this finding could be that, the thawed semen should not be kept for long before insemination.

3. COMPARATIVE EVALUATION OF SOME CRYOPROTECTIVE AGENTS FOR DEEP FREEZING RAM SPERMATOZOA IN TRIS DILUENT

3.1 INTRODUCTION

The use of glycerol in the diluents for deep freezing of bull, ram and boar spermatozoa stemmed from the discovery of its cryoprotective action by Polge *et al.* (1949). Other compounds such as erythritol, acetamide, dimethyl sulphoxide and ethylene glycol were used at various concentrations as cryoprotective agents during deep freezing of various cell types. For deep freezing of ram spermatozoa a number of compounds have been examined, but relatively few have offered sufficient cryoprotective action. Despite the considerable amount of investigations on cryoprotective agents, glycerol remains the most widely used for deep freezing ram spermatozoa. A number of studies have been carried out to determine its optimum level in the diluent. Investigators differed in their report on optimum glycerol levels ranging from 1.0 to 11.5% (Salamon, 1970; Foote, 1978; Fiser *et al.*, 1981).

The effectiveness of some cryoprotective agents such as glycerol is influenced by a variety of factors including initial dilution rate and final spermatozoa concentration at freezing.

The experiments carried out here were to compare cryoprotective agents used in the previous experiments (Chapter 2), except DMSO and polyethylene glycol-600, at their optimal concentrations. The effects of initial dilution rate and final spermatozoa concentration at freezing on the survival of deep frozen ram spermatozoa in Tris diluent were also examined.

3.2 MATERIALS AND METHODS

3.2.1 Semen Collection

Semen was collected by artificial vagina from six Yankasa rams. The ejaculates were examined visually for any contaminants and were pooled after checking for good initial motility under a phase contrast microscope (x 200). The sperm concentration of the pooled semen was determined by photoelectric method using a spectrophotometer (Model C-400, Beckman) at 600 nm wave-length.

3.2.2 Semen Processing

Diluent: The diluent (see chapter 2) was used.

Cryoprotective compounds: The cryoprotective compounds examined were glycerol, erythritol, ethylene glycol and acetamide at 0.0, 0.2, 0.4 and 0.6 M in experiments 1, 2 and 3. In experiment 4, only glycerol was used at 0.5 M while in experiment 5, glycerol, erythritol, ethylene glycol and acetamide were used at either 0.5 or 1.0 M.

Dilution and cooling: The dilution and cooling of semen was carried out as in the previous experiments. Dilution rate of 1:4 (semen:diluent, v/v) was used in all the experiments except experiments 2 and 4 where 1:2 or 1:4 and 1:2 or 1:6 dilution rates were examined respectively. In experiments 4 and 5, two-step dilution was used.

Freezing and storage: The cooled, extended semen was deep frozen in triplicates on dry ice in pellet form (Nagase and Niwa, 1964) and stored in liquid nitrogen at -196°C for at

least 48 h as in the previous experiments.

Thawing, incubation and motility assessment: A minimum of three pellets per sample were thawed in coded dry test tubes at 37°C in a water bath, except in experiment 3 where thawing was at 27, 37 and 47°C. The test tubes were shaken gently until the semen was completely thawed. This procedure was common to all experiments except experiment 4 where thawing solution (Tris 300 mM, citric acid 94.7 mM and fructose 55.5 mM) was used for thawing the pellets at a uniform concentration of 1.0 x 10^9 cells/ml. The samples were randomly presented for estimation of post-thawing motility. Post-thawing incubation was at 37°C for 6 h.

The percentage of motile cells in each sample of thawed semen was assessed under coverslip using a phase contrast microscope equipped with a warm stage kept at 37°C. The motility assessments were carried out immediately after thawing at 0 h, and at 2,4 and 6 h of post-thawing incubation. In all the experiments, three replicates were used and examination of the spermatozoa was carried out at 400 x magnification.

3.2.3 Experimental Design

Five experiments were carried out to futher exmine the optimum concentration of four cryoprotective compounds. Experiment 1 was conducted to examine the effects of different concentrations (0.0, 0.2, 0.4 and 0.6 M) of glycerol and erythritol on the post-thawing survival of deep frozen ram spermatozoa in Tris diluent.

In experiment 2 four concentrations of glycerol and erythritol (0.0, 0.2, 0.4 and 0.6 M) were investigated at 1:2

and 1:4 (semen:diluent, v/v) dilution rates, for their effects on the post-thawing motility of spermatozoa.

Experiment 3 involved investigating the effects of concentrations (0.0, 0.2, 0.4 and 0.6 M) of glycerol, erythritol, acetamide and ethylene glycol on the post-thawing survival of spermatozoa thawed at 27, 37, and 47°C.

Experiment 4 examined the effects of three initial dilution rates (1:2, 1:4 and 1:6, semen: diluent,v/v) and three spermatozoa concentrations at freezing (0.5, 1.5 and 2 x 10^9 cells/ml) on post-thawing motility of spermatozoa using glycerol as cryoprotective compound. In this experiment, thawing solution (Tris 300 mM, citric acid 94.7 mM, fructose 55.5 mM) was used for thawing the pellets at uniform concentration of 1.0 x 10^9 cells/ml.

In experiment 5 the temperature of cryoprotective compound addition was investigated, using glycerol, ethylene glycol, acetamide and erythritol as the cryoprotective compounds. The experiment involved two-stage dilution at 30°C and 5°C. An overall dilution rate of 1:4 (semen:diluent, v/v) was used, with the diluent fraction containing the cryoprotective compound added either at 30°C or 5°C, or at 30 and 5°C. The concentration of each cryoprotective compound varied in the diluent depending on whether it was added once (at 30 or 5°C) or twice (at 30 and 5°C). These concentrations were 1.0 M and 0.5 M for one and two additions.

All the post-thawing motility data were analysed by factorial analysis of variance with a regression approach (Kim and Kohout, 1975), after angular transformation.

3.3 RESULTS

3.3.1 Experiment 1

Results of Experiment 1 are summrised in Tables 3.1.1 and Table 3.1.2, Post-thawing motility was significantly affected ($P < 0.001$) by the type and concentration of cryoprotective compound and incubation time. The mean percentages of motile spermatozoa for glycerol and erythritol were 36.7 and 30.1% respectively while the cryoprotective compound concentration which produced the best result was 0.6 M. There was a decline in post-thawing motility with increasing incubation time.

The mean percentages of motile spermatozoa for the incubation periods of 0, 2 and 6 h were 37.7, 34.4 and 28.1% respectively. There was a significant interaction ($P < 0.05$) between type and concentration of cryoprotective compounds (Table 3.1.2). The interaction was mainly due to an increase in post-thawing motility with increase in glycerol concentration, while erythritol increased at 0.2 M, declined slightly at 0.4 M and increased appreciably at 0.6 M.

3.3.2 Experiment 2

In Experiment 2, type and concentration of cryoprotective compound, dilution rate and incubation period had highly significant effects ($P < 0.001$) on motility of spermatozoa (Table 3.2.1). The mean percentages of motile spermatozoa for glycerol and erythritol were 23.0 and 16.8% respectively. There was a highly significant interaction ($P < 0.001$) between type and concentration of cryoprotective agent (Table 2.4). The interaction was mainly due to an increase in

motility with increase in concentration of glycerol with optimum at 0.4 M, while the response of erythritol changed with its concentration, rising at 0.2 M, and declining at 0.4 M and 0.6 M.

3.3.3 Experiment 3

The result of this experiment are summarized in Tables 3.3.1, 3.3.2 and 3.3.3. Type of cryoprotective compound, thawing temperature and incubation period showed highly significant effects ($P < 0.001$) on Post-thawing motility of ram spermatozoa. Glycerol produced the best result followed by ethylene glycol. Thawing at 37 or 47°C had equal effects on post-thawing motility. Percentge of motile spermatozoa averaged 26.2, 28.6, 21.8 and 15.4% at 0, 2, 4 and 6 h respectively. There were significant interactions between type of cryoprotective agent and thawing temperature ($P < 0.001$), and between type of cryoprotective agent and incubation time ($P < 0.001$). There was a general increase in motility for all types of cryoprotective agents as the thawing temperature was raised from 27 to 47°C (Table 3.3.2). There was a general decline in motility across all cryoprotective agents with increase in incubation time from 0 to 6 h (Table 3.3.3).

3.3.4 Experiment 4

Results of this experiment on the effects of initial dilution rate, freezing concentration and incubation period on post-thawing motility of ram spermatozoa in Tris diluent are presented in Table 3.4.1. Sperm freezing concentration and incubation period showed highly significant effects

(P < 0.001) on post-thawing motility. Freezing concentrations of 0.5 x 10^9 and 2 x 10^9 cells/ml produced post-thawing motility of 26.7 and 20.8% respectively. The post-thawing motility declined with increase in incubation period: 23.2, 28.2, 24.0 and 20.7% for 0, 2, 4 and 6 h incubation respectively.

3.3.5 Experiment 5

Results of this experiment on the effects of type and temperature of addition of cryoprotective agents on post-thawing survival of deep frozen ram spermatozoa in Tris diluent are presented in Table 3.5.1 and 3.5.2. Type and temperature of addition of cryoprotective agent and incubation period had highly significant (P < 0.001) effects on post-thawing motility of ram spermatozoa. Glycerol produced the best result (24.3%) while for ethylene glycol, acetamide and erythritol the motility percentages were 20.0, 7.5 and 16.5% respectively. Addition of cryoprotective agent at 30 and 5°C (two-stage dilution) gave slightly better post-thawing motility (23.9%) compared to addition at 30 or 5°C (one-stage dilution (21.4 and 20.9% respectively). There was a slight decline in motility from 0 to 4 h (19.7% to 18.4%) of incubation with a sharp decline between 4 and 6 h (18.4 - 11.6%). There was a highly significant (P < 0.001) 1st order interaction between type of cryoprotective agent and temperature of its addition (Table 3.5.2).

DISCUSSION

The present study shows that glycerol, erythritol and ethylene glycol offered some cryoprotection to ram spermatozoa deep frozen in Tris diluent. The post-thawing recovery rate and survival of spermatozoa were significantly improved. Glycerol gave the best cryoprotection to the spermatozoa compared to ethylene glycol and erythritol. The optimum concentrations of the compounds were 0.5 M for glycerol and ethylene glycol and 0.6 M for erythritol. Ethylene glycol offered better cryoprotection to ram spermatozoa at lower concentrations compared to higher concentrations in the previous experiments. The effect of ethylene glycol in this study was similar to the findings of Platov (1965) who observed that 1.75-2.0% of ethylene glycol gave protection to ram spermatozoa during pellet freezing, similar to that of 3.5% glycerol. However, it was observed in this experiment that the concentration of ethylene glycol could not be reduced below 0.5 M without losing its cryoprotection.

One-step dilution has a critical effect in deep freezing ram spermatozoa since tha same spermatozoa concentration is maintained throughout cooling and freezing. Dilution at 1:4 (semen:diluent, v/v) resulted in a better recovery rate than 1:2 or 1:7 dilution ratios. Lower dilution probably lead to fast accumulation of metabolic wastes especially during the cooling period which could result in cell death before freezing. On the other hand, at higher dilution rate, the metabolic wastes do not accumulate as fast, as such no significant cell death may occur prior to freezing. The findings in this study showed poor post-thawing recovery rate at lower dilution rate compared to a better post-thawing motility at higher dilution rate. This observation was in agreement with

the findings of Mizuho *et al.* (1963).

Thawing at temperatures higher than 27°C was more beneficial to the frozen spermatozoa as evidenced by higher post-thawing recovery and survival rates. The frozen-thawed semen showed similar recovery rates after thawing at either 37 or 47°C. This was similar to findings of previous investigators (Salamon, 1968; Colas, 1979; Fiser *et al.* 1981). According to Fiser *et al.* (1981) the decreased post-thawing recovery rate after thawing at 27°C was due to cryo-injury caused by re-vitrification of minute ice crystals during the slow thawing.

Contrary to the observations of Graham and Crabo (1972) with boar semen, ram spermatozoa can be frozen in highly concentrated form without greatly affecting the post-thawing recovery or survival rates. Freezing at concentrations of up to 2 x 10^9 cells per ml gave satisfactory post-thawing recovery and survival rates. Graham and Crabo (1972) maintained same spermatozoa concentration throughout cooling, freezing and the post-thawing period. In the present study, the concentration of spermatozoa in the diluted semen was kept low during cooling to 5°C and after thawing. This restricted the stage of high cell concentration to the period during freezing only, when metabolic activities were minimal, as such improving the post-thawing motility. A similar conclusion was reached by Osinowo (1975) after using the same processing technique with boar semen.

The findings of this study are of practical importance to the deep-frozen storage of ram spermatozoa for artificial insemination. Freezing at high cell concentrations allows for considerably reduction in processing time and storage space. There was no difference between freezing at 0.5 or 2.0 x 10^9 cells per ml. When two-step dilution was followed.

There was a difference in recovery rates of frozen-thawed ram spermatozoa when cryoprotective agents were added either once (at 37 or 5°C), or twice (at 37 and 5°C), the latter giving a better recovery rate. This finding is in contrast to an earlier observation with frozen bull semen by Jeyendran and Graham (1980).

4. EFFECTS OF COMBINATION AND SUBSTITUTION OF SOME CRYOPROTECTIVE COMPOUNDS ON POST-THAWING SURVIVAL OF RAM SPERMATOZOA DEEP-FROZEN IN TRIS DILUENT

ABSTRACT

Six experiments were conducted to examine various factors such as effects of combination and substitution on some selected cryoprotectants on the survival of deep frozen ram spermatozoa.

Following combination of cryoprotective agents the survival rate of frozen spermatozoa significantly increased (46.3 v 8.4%) compared to non inclusion of cryoprotective agent in the diluent. Combination of glycerol and erythritol (46.3) had better post-thawing motility than glycerol (44.6) and a combination of glycerol and ethylene glycol (40.0). In the substitution experiments glycerol was found to be superior to erythritol, ethylene glycol, DMSO and acetamide as initial cryoprotective agent. The choice of initial cryoprotective agent was observed to be critical ($P < 0.001$) to the survival of the deep frozen ram spermatozoa. Even though the choice of freezing cryoprotective agent was not critical to the survival, there was a significant interaction ($P < 0.001$) between initial and freezing cryoprotective agents. This indicated that the order at which the sequence of cryoprotective agents use could be critical to the survival rate of deep frozen ram spermatozoa (DMSO substituted by glycerol 14.2% v glycerol substituted by DMSO 40.4%).

In experiment 6, combination of glycerol and erythritol (34.6%) had the best post-thawing motility compared to any sequential use of cryoprotective agents.

The finding in this study was in contrast to reports by Salamon (1968, 1973) who observed thaat there was a depressing effect when any low molecular weight compound was combined with or used after glycerol.

4.1 INTRODUCTION

The inability of most known cryoprotective agents to confer more than partial protection on ram spermatozoa during deep freezing has led to a number of attempts aimed at combining the protective action of glycerol with that of other compounds (Platov, 1965; Salamon, 1968). In these studies, however, glycerol gave equal or better protection when used alone than in combination with other cryoprotective compounds. Salamon (1973) reported that the inclusion of low molecular weight polyols in diluent containing glycerol had no additional effect on survival of deep frozen boar spermatozoa. However, Osinowo (1975) reported an improvement in post-thawing survival of boar spermatozoa after replacing glycerol by erythritol in the diluent prior to freezing. No such attempts have been made with ram spermatozoa.

The experiments described here were carried out to examine the effects of combination and substitution of some selected cryoprotectants on the survival of deep frozen ram spermatozoa.

4.2 MATERIALS AND METHODS

4.2.1 Semen Collection

Semen was collected by artificial vagina from six Yankasa rams. Samples were checked visually for any contaminants and for good initial motility under a phase contrast microscope and pooled. Sperm concentration of the pooled semen was determined by spectrophotometer (Model C - 400, Beckman) at 600 nm wavelength.

4.2.2 Semen Processing

Diluent and cryoprotective compounds Tris diluent was used as in previous experiments. The cryoprotective compounds used were glycerol, erythritol, acetamide, ethylene glycol and DMSO, at various concentration ranging from 0.33 to 1.0 M

Dilution and cooling This procedure was carried out as in the previous experiments. A two-step dilution equivalent to overall dilution rate of 1:4 (semen: diluent, v/v) was used in all the experiments. After dilution at 30°C, the extended semen was cooled to 5°C in about 2 h. After cooling, the semen samples were centrifuged at 2500 rpm for 15 minutes in all experiments using a refrigerated preparative centrifuge at 5°C (model J-4, Beckman) to remove the initial diluent. The freezing diluent was then added at 5°C to resuspend the semen to 1 x 10^7 cells per ml concentration and frozen.

Freezing and storage The cooled semen was frozen on dry ice in pellet form. A 100 µl pipette was used in dropping the semen into small indentations made on dry-ice blocks. After freezing, the pellets were stored in liquid nitrogen at -196°C for at least 48 h.

Thawing, incubation and motility assessment A minimum of three pellets per sample were thawed in coded dry test tubes at 37°C in a water bath. The test tubes were shaken gently until the semen was completely thawed. The samples were randomly presented at 2 h intervals for estimation of post-thawing motility. Post-thawing incubation was at 37°C for 6h

Live/dead count This was carried out only in experiments 4, 5 and 6. Semen smears were stained with eosin-nigrosine stain prepared by the method of Dott and Foster (1972). The proportions of spermatozoa stained or with damaged acrosomal membrane were determined (100 counts/slide, 3 replicates/treatment) without clearing. A phase contrast microscope (model - Swift phase master) was used with oil immersion at 1,000 x magnification.

4.2.3 Experimental Design

Six experiments were conducted to examine various factors indicated below. Experiment 1 was conducted to examine the effects of combination of cryoprotective agents on post-thawing survival of deep frozen ram spermatozoa in Tris diluent. This involved a two-step dilution, at 30°C and 5°C. An overall dilution rate of 1:4 (semen: diluent, v/v) was used, with the diluent fraction containing the cryoprotective compound added after cooling to 5°C. Glycerol, ethylene glycol and erythritol were used as single cryoprotective compounds, while glycerol + ethylene glycol, glycerol + erythritol, ethylene glycol + erythritol and glycerol + ethylene glycol + erythritol were the combinations of 2 or 3 cryoprotective compounds examined. The concentration of each cryoprotective compound varied in the diluent depending on whether it was used singly, or in combination with one or with two other compounds. The concentrations for single, double or triple cryoprotective agents were 1.0, 0.5 and 0.33 M respectively.

The next four experiments (2-5) were conducted to investigate the effect of sequential substitution of cryoprotective compounds on post-thawing survival of deep-frozen ram spermatozoa in Tris diluent. Each of the cryoprotective

agents was used at 0.5 M concentration in the diluent. An initial dilution rate of 1:4 (semen: dilution, v/v) was used with diluent containing the initial cryoprotective compound, at 30°C. The extended semen was cooled to 5°C in about 2 h. The samples were transferred to precooled centrifuge tubes at 5°C and centrifuged at 2500 rpm for 15 minutes at 5°C to remove the initial diluent. The spermatozoa were resuspended to a concentration of 1 x 10^9 spermatozoa per ml with diluent containing the second cryoprotective compound. The resuspended samples were immediately frozen on dry ice and stored in liquid nitrogen for at least 48 h.

In experiment 2, four cryoprotective agents (glycerol, ethylene glycol, acetamide and DMSO) were investigated for their additive cryoprotection to deep frozen ram spermatozoa in Tris diluent.

Experiment 3 was similar to experiment 2, and involved five instead of four cryoprotective compounds (glycerol, ethylene glycol, acetamide, DMSO and erythritol in the sequential substitution.

Experiment 4 was a comparison of 8 selected substitutions from experiments 2 and 3. These were glycerol/DMSO, glycerol/glycerol, ethylene glycol/ethylene glycol, glycerol/erythritol, erythritol/glycerol, ethylene glycol/erythritol, acetamide/glycerol and erythritol/ethylene glycol.

Experiment 5 was also designed to further investigate the additive cryoprotection of the best four substitutions from experiment 4. This involved glycerol/glycerol, glycerol/erythritol, ethylene glycol/erythritol, acetamide/glycerol. An additional substitution (erythritol/erythritol) was also investigated. Four replicates were used in this

experiment.

Experiment 6 was designed to make the final comparison of the best combination and substitution treatments from the previous experiments. From the combination experiment (Expt. 1) two best combinations were used: These were glycerol + ethylene glycol and glycerol + erythritol. From the substitution experiments (Expt. 5) three best substitutions were used: These were glycerol/glycerol, glycerol/erythritol and ethylene glycol/erythritol.

The post-thawing motility data were analysed by a factorial analysis of variance with a regression approach (Kim and Kohout, 1975) after angular transformation.

4.3 RESULTS

4.3.1 Experiment 1

The results of experiment 1 on the effects of combination of cryoprotective compounds on post-thawing motility of deep frozen ram spermatozoa in Tris diluent are presented in Table 4.1.1. There was a highly significant effect ($P < 0.001$) of type of cryoprotectant combination and incubation period on post-thawing motility of ram spermatozoa. Combination of glycerol and erythritol produced the best results (Table 4.1.2). There was no significant difference in motility during incubation up to the 4th hour (37.6, 38.6 and 36.7% for 0, 2 and 4 h respectively) but there was a sharp decline in post-thawing motility between 4 and 6 h (36.7 and 25.6% respectively).

4.3.2 Experiment 2

Results of experiment 2 on the effects of initial and final cryoprotectants on post-thawing motility of deep frozen ram spermatozoa are presented in Table 4.2.1. The type of initial cryoprotectant and incubation period showed highly significant ($P < 0.001$) effects on post-thawing motility of ram spermatozoa. Glycerol, as an initial cryoprotectant, gave the best results. The mean motilities of spermatozoa during post-thawing incubation were 33.2, 32.5, 23.5, and 12.7% at 0, 2, 4 and 6 h respectively. There was a highly significant ($P < 0.001$) interaction between initial and final cryoprotective agents (Table 4.2.2).

4.3.3 Experiment 3

The results of experiment 3 on the sequential substitution of glycerol, ethylene glycol, acetamide, DMSO and erythritol in Tris diluent are presented in Table 4.3.1. The initial and final cryoprotective agents, and incubation period, had highly significant effects ($P < 0.001$) on post-thawing motility of ram spermatozoa. Glycerol followed by erythritol produced the best results both as initial and final cryoprotective agents. There was a decline in post-thawing motility with increase in incubation time with (28.7, 22.9, 19.1 and 11.6% motility at 0, 2, 4 and 6 h respectively). There was a significant ($P < 0.005$) interaction between initial and final cryoprotective agents (Table 4.3.2).

4.3.4 Experiment 4

Results of experiment 4 on effect of type of cryoprotective agent substitution on post-thawing survival of deep frozen ram spermatozoa in Tris diluent is presented in Table 4.4.1. Substitution of cryoprotectants and incubation period showed highly significant effects ($P < 0.001$) on post-thawing motility of ram spermatozoa. Glycerol substituted by erythritol and ethylene glycol substituted by erythritol produced the best results (Table 4.4.2). There was a decline in post-thawing motililty with increase in time of incubation (8.8, 24.6, 24.4 and 16.0% for 0, 2, 4 and 6 h respectively).

The type of cryoprotective agent substitution and post-thawing incubation period showed significant effects ($P < 0.001$) on % live sperm (Table 4.4.3). Glycerol substituted by glycerol or erythritol showed the highest percentage of live spermatozoa (Table 4.4.4). There was a decline in post-thawing percentage of live spermatozoa with increase in incubation time.

As regards the proportions of spermatozoa with damaged acrosomal membranes (Table 4.4.5) the type of cryoprotective agent replacement had a significant effect ($P < 0.005$). Ethylene glycol replaced by erythritol and the reverse order of cyoprotectants resulted in the highest proportion of damaged acrosomal membranes among the cryoprotectant substitution treatments (Table 4.4.6).

4.3.5 Experiment 5

The results of experiment 5 on the effects of cryoprotective agent substitution on post-thawing motility of deep-frozen ram spermatozoa, are present in Table 4.5.1.

Cryoprotectant substitution and incubation period showed significant effects ($P < 0.001$ and $P < 0.005$ respectively) on post-thawing motility.

Glycerol substituted by glycerol showed the best result followed by glycerol/erythritol, ethylene glycol/erythritol and acetamide/glycerol (Table 4.5.2). There was a gradual decline in post-thawing motility with increase in incubation time (32.3, 35.5, 33.8 and 29.0% at 0, 2,4 and 6 h respectively).

Type of cryoprotective agent substitution and post-thawing incubation time had highly significant effects ($P < 0.001$) on percentage of live spermatozoa (Table 4.5.3). Glycerol replaced by glycerol or erythritol showed the highest post-thawing percentage of live spermatozoa (39.1 and 30.3% respectively) compared to the rest of the substitutions. However, there was a gradual decline of live spermatozoa with increase in incubation time (30.3, 31.1, 27.6 and 24.1% at 0, 2, 4 and 6 h respectively). As regards the morphology, only post-thawing incubation time had a highly significant effect ($P < 0.001$) on percentage of spermatozoa with damaged acrosomes (Table 4.5.4). There was an increase in the percentage of damaged acrosomes with increase in the post-thawing incubation time (10.8, 18.3, 24.2 and 29.6% at 0, 2, 4 and 6 h respectively).

4.3.6 Experiment 6

Results of experiment 6 on the effects of cryoprotective agent treatment (combination or substitution) and incubation period on post-thawing survival of ram spermatozoa deep-frozen in Tris diluent are presented in Table 4.6.1. The cryoprotective agent treatment and incubation period had

significant effects (P < 0.005 and P < 0.001 respectively) on post-thawing motility. Combination of glycerol and erythritol ws superior to that of glycerol and ethylene glycol. In the substitution experiment, when glycerol was replaced by glycerol, glycerol substituted by erythritol and ethylene glycol replaced by erythritol had identical results (Table 4.6.2). The post-thawing motility declined as incubation time increased (37.0, 36.0, 31.3 and 20.0% for 0, 2, 4 and 6 h respectively).

Neither cryoprotectant treatment and combination nor incubation period had any significant effect on percentage of live spermatozoa (Table 4.6.3). However, cryoprotectant treatment and incubation period had highly significant effects (P < 0.001) on post-thawing morphology of frozen ram spermatozoa (Table 4.6.4). The proportion of spermatozoa with damaged acrosomes was slightly higher for cryoprotectant substitution than for cryoprotectant combination. There was an increase in damaged acrosomes with increase in post-thawing incubation time. There was a highly significant (P < 0.001) two-way interaction between cyoprotective agent treatment and incubation period (Table 4.6.5).

DISCUSSION

An attempt has been made in the present experiments to find the effect of combining cryoprotective agents or using them sequentially. Combination of glycerol and erythritol (46.3%) offered better cryoprotection to frozen ram spermatozoa than glycerol (44.6%), ethylene glycol (32.8) or erythritol (29.7%) as simple cryoprotectants (Experiment 1). This finding was in contrast to an earlier reports by Salamon (1968) who observed a depressing effect on post-thawing recovery rate when any low molecular weight compound was combined with glycerol.

The cryoprotective agent substitution technique involved a two-step dilution where an initial cryoprotectant was replaced by another prior to freezing. The viability of ram spermatozoa after freeze-thawing was better when glycerol was used as an initial cryoprotective agent compared to ethylene glycol, acetamide, DMSO or erythritol. However, there was an interaction ($P < 0.001$) between initial and freezing cryoprotective agents. This indicated that the order of cryoprotective agent used in sequential susbstitution could be critical to the post-thawing, survival of ram spermatozoa (Experiments 2 and 3). Example of such interaction is the replacement of glycerol by DMSO (40.4%) offering better cryoprotection than the reverse (14,2%). The depressing and toxic effect of some agents such as DMSO on ram spermatozoa could be ameliorated by first introducing glycerol as an initial cryoprotective agent. This finding was in contrast to report by Salamon (1973) on boar semen.

It has been demonstrated that ram spermatozoa tolerate solution of high osmotic pressure (more than 1000 mOsm) (Fiser *et al.*, 1981). The behaviour of the cryoprotective

agents in the substitution technique (Experiment 2-6) could be explained based on osmotic pressure. The 0.5 M concentration of glycerol, ethylene glycol, erythritol and acetamide have osmotic pressure of 1362.0, 1144.6, 935.5 and 919.7 mOsm respectively. A better tolerance for high osmotic pressure by ram spermatozoa was demonstrated whenever a high osmotic pressure compound such as glycerol or ethylene glycol was replaced by a compound of lower osmotic pressure such as erythritol or acetamide. However, when erythritol was used as an initial cryoprotectant it was as good as ethylene glycol unlike the observations of Osinowo (1975) with boar semen.

After examining eight selected substitution treatments (Experiments 4 and 5) of cryoprotective agents, glycerol replaced by glycerol, glycerol replaced by erythritol and ethylene glycol substituted by erythritol had the best post-thawing motility of 40.0, 33.1, and 32.5% respectively. (Table 4.5.2).

Even though there was a drop in diluent osmotic pressure when glycerol was replaced by any of the cryoprotective agents, there was an appreciable improvement on the post-thawing recovery and survival rates. Glycerol when used as an initial cryoprotective agent might have penetrated the cells during the 2 h looking period to 5°C and as such, the centrifugation might not remove it completely. Since osmotic equilibrium would have been attained by the glycerol, resuspension of the cells in diluents with cryoprotective aspects of lower osmotic pressure might not cause any detrimental effect on the spermatozoa.

In this study an attempt for using motility, acrosomal membrane integrity and live-dead ratio as criteria for assessing sperm viability after freezing in Experiments 4,

and 6. Cryoprotective agents improved the percentage of live sperm recovery following deep freezing. However, it was observed that, with frozen-thawed ram spermatozoa it was not possible to categorise the acrosomal damage. The damage appeared uniform irrespective of cryoprotective agents treatment. But, the extent of damage to acrosome varied with cryoprotective agents.

It was observed that at the end of the 6 h incubation at 37°C there was no significant decline in motility. This showed that post-thawing survival rate was improved by the combination and substitution of cryoprotective agents. But as with earlier findings (Lovelock, 1953; Morris, 1982; Takahashi *et al.*, 1985) the damaged acrosomes increased with the time of incubation.

The sequence in which cryoprotective agents are brought in contract with spermatozoa before freezing is very important. But glycerol proved to be superior when used as initial agent to other cryoprotective agents. Only a combination of glycerol and erythritol was better than glycerol as a single cryoprotective agent.

5. FERTILITY TEST OF RAM SPERMATOZOA DEEP FROZEN USING ONE OR MORE CRYOPROTECTIVE COMPOUNDS

5.1 INTRODUCTION

Intensive laboratory studies led to considerable progress regarding semen freezing and thawing procedures to give fairly good post-thawing survival rate (Salamon and Visser, 1972; Colas, 1975, Graham *et al.*, 1978). However fertility following insemination of frozen ram semen tends to be considerably lower than for fresh or chilled semen (Gustafsson, 1978; Maxwell *et al.*, 1984).

To improve fertility of deep frozen ram semen, attempts have been made to bypass the cervix during insemination, either by laparotomy, forceful entry into the uterus or by laparoscopy (Anderson *et al.*, 1973; Fukui and Roberts 1979; Maxwell *et al.*, 1980; Killeen *et al.*, 1982; Maxwell *et al.*, 1984). To date, these intrauterine insemination techniques have not been widely adopted due to their impractical nature under field conditions. However, satisfactory levels of fertility have been reported by many workers following intrauterine insemination with frozen ram semen (Killeen *et al.*, 1982; Maxwell, 1983; Maxwell *et al.*, 1984; Kerton *et al.*, 1984).

The present study examined the fertility of ram semen frozen with one or more cryoprotective agents, using cervical or intrauterine (surgical) inseminations.

5.2 MATERIALS AND METHODS

5.2.1 Semen Collection

Semen was collected by artificial vagina from Yankasa yearling rams. The ejaculates were checked for good initial motility. The sperm concentration was determined using a spectrophotometer (Model C-400, Beckman) at 600 nm wave-

length. Only ejaculates with minimum concentration of 3 x 10^9 cells per ml were used.

5.2.2 Semen Processing

Diluent The Tris diluent used had the following composition: 300 mM tris (hydroxymethyl amino methane), 94.7 mM citric acid, 27.75 mM glucose, 15% (v/v) fresh egg yolk, 1000 IU per ml penicillin G and 1 mg per ml streptomycin sulphate. The diluent was prepared in double-glass distilled water without warming. Diluents were either used soon after preparation or were stored at 5°C at double concentration for use within 24 h. Fresh egg yolk was added to the diluent on the day of use.

Cryoprotective agents The cryoprotective agents used were glycerol and erythritol as single compounds, a combination of glycerol and erythritol, and of ethylene glycol substituted by erythritol. All cryoprotective agents were used at 0.5 M concentration. In Trial 1, glycerol and erythritol were used as single compounds. For the other three subsequent trials glycerol and erythritol were used singly or in combination, while a substitution treatment, involving ethylene glycol substituted after cooling by erythritol, was also used.

Dilution and cooling The semen was diluted with appropriate diluent in test tubes placed in a water bath at 30°C. In Trial 1, the semen was diluted to 1.25 x 10^9 cells per ml in Tris diluent. In Trials 2 to 4, cryoprotective agent combination (glycerol and erythritol) and substitution (ethylene glycol replaced by erythritol) were used in addition to the single compounds. The semen was initially

diluted 1:4 (semen:diluent, v/v) at 30°C using diluent without cryoprotective agent in the case of combination. In the substitution technique the initial dilution was with diluent controlling the initial cryoprotective agent. After cooling to 5°C in about 2 h, the semen was centrifuged at 2500 rpm for 15 min at 5°C to remove the initial diluent. The centrifuged spermatozoa were resuspended to 2.0 x 10^9 cells per ml with the freezing diluent.

The semen was frozen on dry-ice in pellet form (Nagase and Niwa, 1964). The pellet volume was 0.1 ml. After freezing, the pellets were stored in liquid nitrogen or at least 48 h after which the post-thawing motility was assessed before pooling the pellets according to ram and treatment.

Thawing The pellets were thawed at 37°C in a water bath in dry test tubes. The test tubes were shaken gently until the semen was completely thawed. The post-thawing motility was assessed prior to insemination. Only semen batches with at least 30% motility were used for insemination.

5.2.6 Synchronization of oestrus

Trials 1, 2, 3 and 4 51, 50, 51 and 130 mature Yankasa ewes were used respectively. They were treated with intra-vaginal sponges containing 60 mg medroxyprogesterone acetate (Veramix, Upjohn) for 12 days after which they received an intra-muscular injection of 500 IU PMSG (Folligon, intervet) at sponge withdrawal in Trials 2 and 3 only. The ewes were joined with vasectomized rams and checked for signs of heat twice daily (at 0800 and 1500 h).

5.2.7 Cervical insemination

Ewes detected in heat in the morning were inseminated twice, first in the evening and then the following morning. When detected in the evening, they were inseminated the following morning and evening. Ewes were randomly allocated to the various treatments.

Frozen semen was thawed in dry test tubes at 37°C, three pellets per tube, and the post-thawing motility was assessed before insemination. For Trials 1 and 2 insemination dose was 312 x 10^6 cells, while in Trials 3 and 4 each dose contained 500 x 10^6 cells in 0.25 ml of thawed semen. In trial 4, the cervical insemination was carried out twice, at first and second oestrus after synchronization. A vagino-scope was used in depositing the frozen-thawed semen at the external orifice of the cervix with an inseminating pipette. Ewes serving as controls were naturally mated using the same intact rams from which semen was collected.

5.2.8 Surgical insemination (Trials 3 and 4)

Intra-uterine insemination with frozen semen was performed under local anaesthetic (Xylocaine, Astra). The uterine horns were located after exposing the reproductive tract by mid-ventral incision. The semen (0.01 ml) containing 20 x 10^6 total spermatozoa (Trial 3) and 0.01 - 0.02 ml) containing 20 x 10^6 motile cells (Trial 4) was introduced into each uterine horn half-way between the bifurcation and the uterotubal junction. The insemination procedure was carried out 48 to 55 h after sponge withdrawal using a 25 guage needle attached to a dispensing micropipette in Trial 3. For Trial 4 a 23 gauge needle attached to 1ml tuberculin syringe

was used.

5.3 RESULTS

5.3.1 Fertility trial 1

In the first fertility trial the 21-day non-return and lambing rates showed no difference between semen trated with glycerol or erythritol (Table 5.1.1). The control was superior to all the treatments lambing rate was generally lower than conception rate.

5.3.2 Fertility trial 2

In Trial 2 more treatment were introduced (Glycerol, erythritol, glycerol + erythritol and ethylene glycol substituted by erythritol). For cervical insemination, semen treated with ethylene glycol substituted by erythritol had three non-returns, while semen treated with glycerol and erythritol had two and one ewes not returning to heat after 21 days respectively (Table 5.2.1). Combination of glycerol and erythritol resulted in no conception.

All the ewes not returning to heat after 21 days in the treatment groups did not lamb. In the control four out of 7 ewes lambed giving a lambing rate of 57.1%. For intrauterine insemination (Table 5.2.2) the non-return rate for the control (fresh semen), semen treated with glycerol + erythritol were the same (50%) while semen treated glycerol and erythritol had 40 and 25 per cent conception rates respectively. Semen treated with ethylene glycol substituted by erythritol resulted in no conception. Semen treated with combination of glycerol and erythritol had higher lambing

rate of 40% compared to 50% of the control while other treatment groups had no lambing.

5.3.1 Fertility trial 3

In Trial 3 the conception and lambing rates were very poor following cervical insemination for all the treatment groups and the controls (Table 5.3.1). The few ewes not returning to heat after 21 days did not lamb. As regards the intrauterine insemination the non-return rate was uniform at 40%. Lambing rate was lower than the non-return rate (40 v 20%) except for first treatment (glycerol) which had no lambing (Table 5.3.2).

5.3.1 Fertility trial 4

The non-return rate for trial 4 (cervical insemination) for the treatment groups (27.3, 0, 20.0, 54.5%) were lower than the control (100%). Lambing rates were generally lower than the non-return rates (Table 5.4.1). The non-return rate for the intrauterine insemination (Table 5.4.1) of Trial 4 was generally good (40-60%). But the lambing rates were poor (0-20%).

Cervical insemination following natural estrus had the best non-return rate (20-70%). Glycerol treated semen was similar to the control (natural service). The lambing rate were lower than the non-return rates (Table 5.4.3).

DISCUSSION

The results obtained in the first fertility test showed that the conception rate based on 21-day non-return was higher than the lambing rate (35.5 v 11.5%). There was no difference in both conception and lambing rates between semen treated with glycerol and erythritol following cervical insemination. The observations in this study contrast with those reported by most investigators (Visser and Salamon, 1973; Salamon and Visser, 1974; Crosby *et al.*, 1983; Maxwell, 1983; Maxwell *et al.*, 1984), but compared favourably with the findings of Loginova and Zeltobryuh (1968) and Trajo (1983). Since the non-return rate was higher than the lambing rate, this indicated embryonic mortality or abortion. This finding compares with observations of Edey (1976) and Salamon *et al.*, (1979) that fertility levels could be disappointing as a result of high embryonic mortality in the ewes.

Fertility tests 2 and 3 have similar low non-return rates following cervical insemination. Despite the increase in insemination dose from 312 to 500 x 10^6 and the use of 500 IU PMSG to enhance the fertility of the ewes. In trial 3 there was no lambing from the ewes not returning to estrus. There was no doubt that the PMSG enhanced signs of estrus virtually 24 h after sponge withdrawal, but there was a general failure of most of the ewes to conceive based on low non-return rate. This finding contrasts with that of Maxwell *et al.*, (1984). Though Oyedipe *et al.*, (1987) reported 500 IU PMSG to be adequate for Yankasa ewes even resulting in mild superovulation in dry ewes, this observation might not apply to lactating ewes. According to Thimonier *et al.*, (1968) higher doses of PMSG might be required to get higher

percentage of lactating ewes to ovulate. In this experiment the use of PMSG was very effective as the animals started exhibiting signs of heat barely 24 h after sponge withdrawal but ovulation might not have taken place in time to effect fertilization after insemination.

In fertility trials 2 and 3 intrauterine insemination was performed by laparotomy. The results revealed that intrauterine insemination was better than cervical insemination. This compares with the findings of Takenaka *et al.*, (1987) Among the semen treatments only the substitution of ethylene glycol by erythritol resulted in no non-return in Trial 2. Despite the poor results in the intrauterine insemination no difference was observed in conception rate between semen treated with glycerol, glycerol + erythritol and the control in trial 3. But the lambing rate of semen treated with glycerol + erythritol was better than glycerol treatment and the control. In fact semen treated with glycerol or erythritol resulted in no lambing despite the non-return of 40 and 25% respectively.

In the fourth fertility test carried out, the non-return following cervical insemination was generally lower than the intrauterine insemination. For the cervical insemination, erythritol resulted in no conception while the ethylene glycol replaced by erythritol resulted in the highest non-return rate for all cervical inseminations (54.5%). The lambing rates were also lower than expected. There was no much difference based on non-return rate between the four semen treatments and the control following intrauterine insemination. This indicated that the treatments were equally effective. But lambing rates were still lower than the conception rates. This was in contrast to the findings of Maxwell (1983) and Maxwell *et al.*, (1984). Beside the

cervical and intrauterine insemination after estrus synchronization, another group of ewes were inseminated following natural estrus. This last group was used to shed more light whether the estrus synchronization and use of PMSG had adverse effects on fertility of the ewes, at the first oestrus.

From the results obtained in the last group of ewes the non-return and lambing rate were better than other fertility tests.

In the earlier fertility tests involving intrauterine insemination most of the ewes checked at the time of insemination (about 50 h after sponge withdrawal) had not ovulated. This was in contrast to the findings of Maxwell *et al.*, (1984) and Eppleston and Roberts (1987). This showed that what was obtained with other breeds of sheep might not necessarily apply to the Yankasa especially the time of ovulation following the first signs of estrus.

The ewes used in these fertility tests were from an intensive breeding programme for twice-yearly lambing. Estrus synchronization was carried out virtually 2-3 weeks post-partum. The ewe response to the synchronization compare to the findings of Van Niekerk (1979) and Osinowo and Ekpe (1985). But the ultimate fertility was below expectations though this compares with result obtained by Nel (1965) with Karakul ewes. Some investigators (Van Wyk, *et al.*, 1972; Gould yearly lambing programme indicating low conception following early rebreeding. This led to the conclusion that the ewes in these experiments were not given enough time for complete physiological normalcy in the reproductive tract to return before rebreeding. This might have accounted for the low fertility following artificial insemination with frozen/-thawed spermatozoa. Since the ewes inseminated following natural estrus had better lambing rates indicated that after estrus synchronization the ewes should be bred at the second estrus. Also the non-return inclusion of PMSG treat-ment improve on the fertility of the Yankasa ewes.

6.0 GENERAL DISCUSSION

A comparative evaluation of cryoprotective compounds for deep freezing of ram spermatozoa in Tris diluent has been carried out in this thesis. When cryoprotective agents were used singly, glycerol and erythritol were found to be superior to ethylene glycol, acetamide, DMSO and polyethylene glycol-600 with regard to post-thawing recovery rate of frozen ram spermatozoa. When more than one cryoprotective agent was used for deep freezing of ram spermatozoa, the post thawing survival rate was best improved by glycerol and erythritol combination. When cryoprotective agent substitution technique was tried, ethylene glycol replaced by erythritol was found to be superior to other substitutions.

In fertility trials, the results were generally poor. No significant difference was found in lambing rate among the cryoprotective agents examined. Erythritol, ethylene glycol and glycol gave equally poor results.

In the cryoprotective compounds screening (chapter 2), glycerol, erythritol glycol were found to give good post-thawing survival rate. However, glycerol showed the best survival rate. The effects of cryoprotective agent concentration, dilution rate and incubation time were examined. The factors examined in these studies could not work independently of each other. If one factor has no direct effect, it might have interactive effect with the other factor(s). As a result no single factor can be changed without possibly affecting other factors. Though the type and concentration of cryoprotective compounds had direct influence on survival rate, interaction with other factors such as dilution rate and incubation period were also obtained. However, 0.5 M was found to be the optimum concentration of cryoprotective agents while dilution rate of 1:4 (semen: diluent, v/v) was the best.

Apart from the conventional freezing technique of using single cryoprotective compounds, ram spermatozoa was found to withstand the effect of combination and substitution of cryoprotective agents (Chapters 3 and 4). The post-thawing survival of deep frozen ram was improved when glycerol and erythritol were combined in the diluent. Also when ethylene glycol was used as initial cryoprotectant and later replaced

by erythritol as freezing cryoprotective agent the post-thawing survival was improved. These two techniques (combination and substitution) were carried out using two-step dilution.

Though erythritol has lower osmotic pressure compared to both ethylene glycol (appendix 2) at 0.5 M, the ram spermatozoa tolerated the change in osmotic pressure. Since glycerol's mode of action is by penetration and it was included in the initial dilution, the subsequent centrifugation after cooling might not have removed all the glycerol, as some would have penetrated the sperm cells, as such in the second suspension, any compound of lower osmotic pressure might not have detrimental effects on the sperm cells due to the residual effect of glycerol. In contrast to the findings of Dsinowo (1975) with boar spermatozoa, when erythritol was replaced by glycerol or ethylene glycol the post-thawing survival, though not as good as when glycerol or ethylene glycol were used as initial compounds, was better than the control or when glycerol was substituted by DMSO. This showed that the sequence in which cryoprotectants are brought in contact with spermatozoa before freezing is very important.

With the combination and substitution of compounds no significant decline in post-thawing was observed during the 6 h incubation period, compared to single compounds used in the initial experiments (chapter 2). This finding is of important in artificial insemination when frozen-thawed semen is used. With deep-frozen thawed ram spermatozoa it was not possible to categorize the acrosomal damages as they appeared uniform irrespective of cryoprotective used. However, the live percentage was similar to the post-thawing motility throughout the incubation period.

In this study it was established that ram spermatozoa

could be deep-frozen at high concentration of up 2×10^9 cells/ml without much detrimental effect on post-thawing survival. This gives an opportunity of using a smaller insemination dose in terms of volume at the same time having the desired number of spermatozoa.

From the fertility tests in chapter 4, the results were not as good as expected. The failure to achieve the desired goal could be attributed to various factors such as ovulation time, synchronization, PMSG treatment, heat detection and animal handling during insemination. Though perfection in deep freezing techniques of ram spermatozoa might not be far from being achieved but, systematic studies are needed on the factors enumerated above. Most of the earlier investigations were carried out on temperate breeds of sheep with seasonal breeding. What was obtained with them might not be true with the tropical breeds of sheep. The exhibition of signs of heat following estrus synchronization and PMSG treatment did not result in ovulation as expected (Maxwell, 1983; Maxwell *et al.*, 1984). However, ovulation might have taken place much later than what were reported. From the surgical intra-uterine insemination, it was observed that ewes did not ovulate up to 50 h after sponge withdrawal. Since cervical insemination was performed barely 9 h and 24 h after the onset of heat, this might explain the failure to achieve the expected conception rate. The use of PMSG at 500 IU on Yankasa sheep influenced the animals to show signs of heat at about 24 h after sponge withdrawal, but the ovulation rate was very poor. From the fertility tests carried out, the non-return and lambing rates were better when PMSG was not used at all.

Generally in all the fertility tests the non-return rates were higher than lambing rates. This difference

indicated that the animals probably conceived but the pregnancies were terminated. Return to heat following insemination with frozen ram semen were also reported by Edey (1976) and Salamon *et al.*, (1979). From these findings being the outcome of fertility tests on non-return rates may be misleading. A remarkable finding in this study was the identification of substitutes to glycerol for deep freezing ram spermatozoa. Either erythritol or ethylene glycol could be used since glycerol has been incriminated as a contraceptive in gilts (Neville, *et al.*, 1970). Recently, Slavik (1987) reported glycerol to accelerate acrosomal reaction in the ram spermatozoa leading to poor conception rate.

Table 2.1.1 Analysis of variance summary: The effects of glycerol level and dilution rate on post-thawing survival of deep frozen ram spermatozoa (Expt 1)

Source	df	MS
Glycerol concentration (GC)	3	417.5***
Dilution rate (DR)	2	44.0n.s
Incubation time (IT)	3	437.7***
2-way interaction GC x DR	6	59.3***
Remainder, 2-way interactions	15	6.2ns
3-way interaction GC x IT x DR	18	10.9n.s
Residual	96	15.3

*** P< 0.001
n.s Non-significant

Table 2.1.2 Effects of glycerol concentration and dilution rate on post-thawing motility of ram spermatozoa (Expt. 1)*

Glycerol concentration (M)	Dilution rate (v/v)			
	1:3	1:5	1:7	Mean
0.0	11.3**	16.3	12.1	13.2
0.5	16.7	25.0	26.7	22.8
1.0	20.8	21.0	22.1	21.3
1.5	20.6	17.1	17.1	18.3
Mean	17.4	19.9	19.5	18.9

* Glycerol concentration x dilution rate interaction (P < 0.001)
** % motile sperm

Table 2.1.3 Analysis of variance summary: Effects of DMSO concentration and dilution rate on post-thawing motility of deep frozen ram spermatozoa (Expt 2)

Source	df	MS
DMSO concentration (DC)	3	2276.3***
Dilution rate (DR)	2	210.7n.s
Incubation time (IT)	3	865.0***
2-way interaction GC xDR	6	53.3***
Remainder, 2-way interactions	15	19.6ns
2-way interaction GC x IT x DR	18	10.2n.s
Residual	96	19.1

* P < 0.05
*** P < 0.001
n.s Non-significant

Table 2.1.4 Effects of dimethyl sulphoxide concentration and dilution rate on post-thawing motility of ram spermatozoa (Expt. 2)*

DMSO concentration (M)	Dilution rate (semen:diluent, v/v)			
	1:3	1:5	1:7	Mean
0.0	9.7**	17.5	21.7	16.3
0.5	12.5	12.5	14.2	13.1
1.0	4.7	7.1	6.2	5.9
1.5	1.7	2.5	3.3	2.5
Mean	7.2	9.9	11.3	9.5

* DMSO concentration x dilution rate interaction (P < 0.05)
** Values are % motile sperm

Table 2.1.5 Analysis of variance summary: Effects of erythrito and dilution rate on post-thawing levels survival of deep frozen ram spermatozoa (Expt 3)

Source	df	MS
Erythritol concentration (EC)	3	46.7***
Dilution rate (DR)	2	1.0n.s
Incubation time (IT)	3	221.3***
2-way interaction GC xDR	21	7.1n.s
Pooled 3-way interaction	18	5.1n.s
Residual	96	6.4

*** P < 0.001
n.s Non-significant

Table 2.1.6 Analysis of variance summary: Effects of ethylene glycol and dilution rate on post-thawing motility of deep frozen ram spermatozoa (Expt 4)

Source	df	MS
Ethylene glycol concentration (EC)	3	402.1***
Dilution rate (DR)	2	33.9**
Incubation time (IT)	3	884.7***
2-way interaction DR x EC	6	14.8*
Remainder, 2-way interaction	15	8.0n.s
3-way interaction DR x EC x IP	18	15.4***
Residual	96	5.7

* P < 0.05
** P < 0.01
*** P < 0.001
n.s Non-significant

Table 2.1.7 Effects of dilution rate, ethylene glycol concentration and incubation period on post-thawing motility of ram spermatozoa (Expt. 4)*

Dilution rate	Incubation time (h)	Ethylene glycol concentration (M)				
		0	0.5	1.0	1.5	Mean
1.3	0	13.3	18.3	18.3	15.0	16.2
	2	10.0	18.3	18.3	11.3	15.0
	4	5.0	13.3	13.0	10.0	10.3
	6	0.0	8.3	10.0	5.0	5.8
Mean		7.1	14.6	15.0	10.8	10.9
1.5	0	10.0	18.3	16.7	11.7	14.2
	2	10.0	16.6	15.0	16.6	14.6
	4	6.7	15.0	13.3	11.7	11.7
	6	5.0	5.0	6.7	5.0	5.4
Mean		7.9	13.8	12.9	11.3	11.5
1.7	0	10.0	21.7	16.7	13.3	15.4
	2	10.0	15.0	15.0	11.7	12.9
	4	5.0	11.7	11.7	8.3	9.2
	6	2.5	5.0	5.0	3.3	4.0
Mean		7.0	13.3	12.1	9.2	10.4
Overall Mean		7.4	13.9	13.3	10.4	11.3

* Dilution rate x ethylene glycol concentration x incubation period interaction, $P < 0.001$.

Table 2.1.8 Analysis of variance summary: The effects of acetamide level and dilution rate on post-thawing survival of deep frozen ram spermatozoa (Expt 5)

Source	df	MS
Acetamide concentration (AC)	3	544.5***
Dilution rate (DR)	2	94.3n.s
Incubation time (IT)	3	1145.5***
2-way interaction DR x AC	6	106.2**
Remainder, 2-way interactions	15	35.6n.s
3-way interaction DR x AA x IT	18	18.8n.s
Residual	96	28.7

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$
n.s Non-significant

Table 2.1.9 Effects of dilution rate nd acetamide concentration on post-thawing motility of ram spermatozoa (Expt. 5)*

Acetamide concentration (M)	Dilution rate (v/v)			
	1:3	1:5	1:7	Mean
0.0	6.7	2.5	3.3	4.0
0.5	10.0	8.8	7.5	8.8
1.0	8.8	9.6	7.5	**8.6**
1.5	4.2	8.8	5.4	**6.1**
Mean	7.4	7.4	6.0	6.9

* Glycerol concentration x dilution rate interaction, ($P < 0.05$)

Table 2.1.10 Analysis of variance summary: Effects of PEG-600 concentration and dilution rate on post-thawing survival of ram spermatozoa deep frozen (Expt 6)

Source	df	MS
PEG-600 (PEGC) concentration	3	223.5***
Dilution rate (DR)	2	9.4n.s
Incubation time (IT)	3	21.3***
2-way interaction PEGC x AC	6	1375.9***
Remainder, 2-way interactions	15	22.5n.s
3-way interaction PEGC x DR x IT	18	14.9n.s
Residual	96	26.9

*** $P < 0.001$

n.s Non-significant

Table 2.1.11 Effects of polyethylene glycol-600 concentration and dilution rate on post-thawing motility of deep frozen ram spermatozoa (Expt. 5)*

Acetamide concentration (mM)	Dilution rate (v/v)			
	1:3	1:5	1:7	Mean
0.0	10.8**	4.6	1.3	5.5
3.0	1.3	17.9	6.7	8.6
6.0	1.3	1.3	15.4	6.0
9.0	7.9	1.3	1.6	3.6
Mean	5.3	6.3	6.3	6.0

* PEG-600 x dilution rate interaction, ($P < 0.05$)

** Percentge of motile sperm

Table 3.1.1 Analysis of variance summary: Effects of level of glycerol and erythritol on post-thawing survival of ram spermatozoa deep frozen in Tris diluent (Expt. 1)

Source	df	MS
Cryoprotective agent (CA)	1	272.2***
Cryoprotective agent concentration (CC)	3	486.3***
Incubation time (IT)	2	208.3***
2-way interaction, CA x CC	3	59.0*
Remainder, 2-way interactions	8	11.8ns
3-way interaction CA x CC x IT	6	9.8n.s
Residual	48	12.9

* P < 0.05
*** P < 0.001
n.s Non-significant

Table 3.1.2 Effects of type and concentration of cryoprotective agent on Post-thawing motility of ram spermatozoa (Expt. 1)*

Type of cryo-protective agent	Concentration of cryoprotectvie agent (M)				
	0:0	0:2	0:4	0:6	Mean
Glycerol	20.6**	39.4	42.8	43.9	36.7
Erythritol	21.7	31.7	29.4	37.8	30.2
Mean	21.2	35.6	36.1	40.9	33.4

* (P < 0.05)
** % motile sperm

Table 3.2.1 Analysis of variance summary: The effect of glycerol and erythritol levels and dilution rate on post-thawing motility of deep frozen ram spermatozoa (Expt. 2)

Source	df	MS
Type of cryoprotective agent (CA)	1	949.6***
Dilution rate (DR)	1	1276.2***
Cryoprotective agent concentration (CC)	3	1179.9***
Incubation time (IT)	3	595.0***
2-way interaction CA x CC	3	173.9***
Remainder, 2-way interactions	19	17.4n.s
Pooled 3-way interactions	24	13.6n.s
4-way interaction, CA x DR x CC x IT	9	11.0n.s
Residual	128	15.8

*** P < 0.001
n.s Non-significant

Table 3.2.2 Effects of type and concentration of cryoprotective agent on post-thawing motility of ram spermatozoa deep frozen in Tris diluent (Expt.2)*

Type of cryoprotective agent	Concentration of cryoprotectvie agent (M)				
	0:0	0:2	0:4	0:6	Mean
Glycerol	11.0**	22.9	31.3	26.8	23.0
Erythritol	10.6	18.3	16.5	16.0	15.4
Mean	10.8	20.6	23.9	21.4	19.2

* Type of cryoprotective agent x cryoprotective agent concentration interaction (P < 0.001)
** % motile sperm

Table 3.3.1 Analysis of variance summary: Effects of level of glycerol, erythritol, acetamide and ethylene glycol and thawing temperature on post-thawing survival of ram spermatozoa deep frozen in Tris diluent (Expt. 3)

Source	df	MS
Type of cryoprotective agent (CA)	1	1189.38***
Thawing temperature (TT)	2	384.77***
Incubation time (IT)	3	767.97***
2-way interactions, CA x TT	8	54.03***
CA x IT	12	56.78***
Remainder, 2-way interactions	6	15.05n.s
3-way interaction CA x TT x IT	24	15.6n.s
Residual	120	16.28

*** P < 0.001
n.s Non-significant

Table 3.3.2 Effects of cryoprotective agent and thawing temperature on post-thawing motility of ram spermatozoa (Expt. 3)*

Type of cryo-protective agent	Thawing temperature (°C)			
	27	37	47	Mean
Control	10.4**	12.9	19.2	14.2
Glycerol	31.3	33.8	36.2	33.8
Erythritol	20.4	22.5	22.1	21.7
Acetamide	10.4	19.2	21.3	17.0
Ethylene glycol	23.8	32.1	29.6	28.5
Mean	21.5	26.9	27.3	25.3

* Type of cryoprotective agent x thawing temperature interaction (P < 0.05)
** % motile sperm

Table 3.3.3 Effects of cryoprotective agent and incubation time on post-thawing motility of ram spermatozoa deep frozen in Tris diluent (Expt. 3)*

Type of cryo-protective agent	Thawing temperature (°C)				
	0	2	4	6	Mean
Control	16.1**	12.7	20.5	6.6	14.0
Glycerol	38.8	41.6	30.0	24.4	33.7
Erythritol	25.0	27.2	17.8	16.7	21.7
Acetamide	17.8	22.2	15.6	12.2	17.0
Ethylene glycol	33.3	37.8	25.0	17.8	28.5
Mean	28.7	32.2	22.1	17.8	25.3

* Type of cryoprotective agent x incubation time interaction ($P < 0.001$)

** % motile sperm

Table 3.4.1 Analysis of variance summary: Effects of initial dilution rate and freezing concentration on post-thawing survival of ram spermatozoa deep frozen in Tris diluent (Expt. 4)

Source	df	MS
Freezing concentration (FC)	3	96.38***
Incubation time (IT)	3	139.49***
Initial dilution (ID)	2	13.97n.s
Pooled, 2-way interactions	21	12.24n.s
3-way interaction, FC x IT x ID	88	6.02n.s
Residual	96	14.83

*** $P < 0.001$

n.s Non-significant

Table 3.5.1 Analysis of variance summary: The effects of type and temperature of addition of cryoprotective compound on post-thawing survival of ram spermatozoa in tris diluent (Expt. 5)*

Source	df	MS
Cryoprotective agent (CA)	3	1618.69***
Treatment (TR)	3	5590.42***
Incubation time (IT)	3	534.89***
2-way interaction CA x TR	9	279.29***
Remainder, 2-way interactions	18	29.99n.s
3-way interaction, CA x TR x IT	27	28.10n.s
Residual	128	25.47

*** P < 0.001
n.s Non-significant

Table 3.5.2 The effects of type and temperature of addition of cryoprotective compounds on post-thawing survival of deep frozen ram spermatozoa in Tris diluent (Expt. 5)*

Cryoprotective compound	Treatments¹				
	1	2	3	4	Mean
Glycerol	3.3**	27.1	28.8	37.9	24.3
Erythritol	4.7	29.2	27.9	22.5	20.0
Acetamide	7.6	10.8	6.3	10.8	7.6
Erythritol	2.5	18.3	20.8	24.2	16.5
Mean	4.5	21.4	21.0	23.9	17.1

* Type of cryoprotective compond x temperature of cryoprotective compound addition interaction (P < 0.001)
** % motile sperm
¹Treatments:
1. No cryoprotective compound
2. Cryoprotective compound at 30°C
3. Cryoprotective compound at 5°C
2. Cryoprotective compound at 30°C and 5°C

Table 4.1.1 Summary of analysis of variance: The effects of combination of cryoprotective compounds on post-thawing survival of ram spermatozoa deep frozen in Tris diluent (Expt. 1)

Source	df	ms
Type of cryoprotective agent combination (CAC)	7	1201.68***
Incubation time (IT)	3	513.88***
2-way interaction CAC x IT	21	12.72n.s
Residual	96	12.99

*** (P < 0.001)
n.s Non-significant

Table 4.1.2 Effect of cryoprotective agent combination on post-thawing survival of ram spermatozoa deep frozen in Tris diluent (Expt. 1)*

cryoprotective agent combination	% of motile spermatozoa
No cryoprotective agent	8.4
Glycerol	44.6
Ethylene glycol	32.8
Erythyritol	29.7
Glycerol + Ethylene glycol	40.0
Glycerol + Erythritol	46.3
Erythritol +Ethylene glycol	37.2
Glycerol + Ethylene glycol + Erythritol	38.8

* (P < 0.001)

Table 4.2.1 Summary of analysis of variance: Effects of sequential replacement of cryoprotective compounds on post-thawing survival of deep frozen ram spermatozoa in (Expt. 2)

Source	df	ms
Initial cryoprotective agent (ICA)	3	1527.74***
Final cryoprotective agent (FCA)	3	60.93n.s
Incubation time (IT)	3	2341.15***
2-way interaction ICA x FCA	9	259.55***
Remainder, 2-way interaction	18	18.00n.s
ICA x FCA x IF	27	8.56n.s
Residual	128	22.09

*** (P < 0.001)
n.s Non-significant

Table 4.2.2 Effects of cryoprotective agent combination: Interaction between initial and final cryoprotective agents (Expt. 2)*

Final cryoprotective agent	Intial cryoprotective agent				
	Glycerol	Ethylene Glycol	Acet-amide	DMSO	Mean
Glycerol	36.7**	36.3	12.1	14.2	24.8
Ethylene glycol	34.2	30.4	25.0	17.5	26.8
Acetamide	30.4	23.3	22.9	29.2	25.7
DMSO	40.4	25.8	16.3	15.3	24.0
Mean	35.4	29.0	19.1	18.5	25.3

* (P < 0.001)
** % motile sperm.

Table 4.3.1 Analysis of variance summary: The effects of sequential substitution of cryoprotective compound on post-thawing motility ram spermatozoa deep frozen in Tris diluent (Expt. 3)*

Source	df	ms
Initial cryoprotective agent (ICA)	4	484.50***
Final cryoprotective agent (FCA)	4	936.21***
Incubation time (IT)	3	2450.65***
2-way interaction ICA x FCA	16	73.52**
Remainder, 2-way interaction	24	21.89n.s
3-way interaction ICA x FCA x IT	48	15.35n.s
Residual	200	29.85n.s

** P < 0.005
*** P < 0.001
n.s Non-significant

Table 4.3.2 Effects of initial and final cryoprotective agents on post-thawing motility of ram spermatozoa (Expt. 3)*

Final Cryoprotective agent	Intial cryoprotective agent					
	Glycerol	Ethy. glycol	Acet-amide	DMSO	Ery.	Mean
Glycerol	32.7**	23.8	15.8	21.1	32.1	25.2
Ethylene glycol	23.8	22.9	16.7	15.8	26.7	21.2
Acetamide	25.8	23.3	2.9	13.3	24.2	19.2
DMSO	19.2	15.0	10.8	14.2	16.7	15.2
Erythritol	29.6	24.6	21.3	14.6	20.8	22.2
Mean	26.3	21.9	14.8	15.8	24.1	20.6

* P < 0.005
** % motile sperm.

Table 4.4.1 Summary of analysis of variance: Effect of substitution of cryoprotective compounds on post-thawing survival of ram spermatozoa deep frozen in Tris diluent (Expt. 4)

Source	df	ms
Cryoprotective agent substitution (CAS)	7	124.88***
Incubation period (IT)	3	345.57***
2-way interaction CAS x IT	21	9.93n.s
Residual	64	9.85

*** P < 0.001
n.s Non-significant

Table 4.4.2 Effect of cryoprotective agent substitution on post-thawing survival (Expt. 4)

Cryoprotective agent substitution	Motility %
Glycerol/DMSO	17.5
Glycerol/Glycerol	26.7
Ethyl glycol/Ethyl. glycol	21.3
Glycerol/Erythritol	30.0
Erythritol/Glycerol	22.1
Ethyl. glycol/Erythritol	28.8
Acetamide/glycerol	23.8
Erythritol/Ethyl. glycol	17.5
Mean	23.4

* P < 0.001

Table 4.4.3 Analysis of variance: The effect of cryoprotective agents substitution on post-thawing live/dead percentage of frozen ram spermatozoa (Expt. 4)*

Source	df	ms
Cryoprotective agent (CA)	7	138.50***
Incubation time (IT)	3	313.69***
2-way interaction CA x IT	21	11.08n.s
Residual	64	19.57

*** P < 0.001
n.s Non-significant

Table 4.4.4 Effect of cryoprotective agent substitution on post-thawing percentag of live spermatozoa (Expt. 4)

Cryoprotective agent (CA) substitution	% live sperm
Glycerol/DMSO	12.9
Glycerol/Glycerol	24.7
Ethyl. glycol/Ethyl. glycol	20.0
Glycerol/Erythritol	22.4
Erythritol/Glycerol	20.3
Ethyl. glycol/Erythritol	18.3
Acetamide/glycerol	18.7
Erythritol/Erythritol	11.9

* P < 0.001

Table 4.4.5 Summary of analysis of variance: The effect of of sequence of cryoprotective agents substitution on post-thawing acrosomal membrane damage of frozen ram spermatozoa (Expt. 4)

Source	df	ms
Cryoprotective agent substitution (CA)	7	50.90
Incubation time (IT)	3	24.68n.s
2-way interaction CA x IT	21	13.90n.s
Residual	64	17.62

* P < 0.005
n.s Non-significant

Table 4.4.6 Effect of cryoprotective agent substitution on acrosome damage of frozen ram spermatozoa (Expt. 4)*

Cryoprotective agent substitution	Damaged Acrosome, %
Glycerol/DMSO	22.3
Glycerol/Glycerol	25.4
Ethylene glycol/Ethylene glycol	28.3
Glycerol/Erythritol	22.9
Erythritol/Glycerol	25.2
Ethylene glycol/Glycerol	30.3
Acetamide/Glycerol	27.8
Erythritol/Erythritol	31.3
Mean	26.7

* P < 0.05

Table 4.5.1 Analysis of variance summary: The effect of cryoprotective compounds treatment on post-thawing survival of deep frozen spermatozoa in (Expt. 5)*

Source	df	ms
Cryoprotective agent substitution (CAS)	4	145.06***
Incubation period (IT)	3	54.91**
2-way interarction, CAS x IT	12	21.54n.s
Residual	60	13.94

** P < 0.005
*** P < 0.001
n.s Non-significant

Table 4.5.2 Effect of cryoprotective agent substitution on survival of deep frozen ram spermatozoa (Expt. 5)*

Cryoprotective agent substitution	Motility %
Glycerol/Glycerol	40.0
Glycerol/Erythritol	33.1
Ethylene glycol/Erythritol	32.5
Acetamide/glycerol	31.6
Erythritol/Erythritol	25.9
Mean	32.6

* $P < 0.001$

Table 4.5.3 Analysis of variance summary: The effect of sequential replacement of cryoprotective agents on post-thawing live/dead proportion of frozen ram spermatozoa (Expt. 5)*

Source	df	MS
CA substitution (CAS)	4	343.77***
Incubation time (IT)	3	88.15***
2-way interaction, CAS x IT	12	32.14n.s
Residual	60	28.29

*** $P < 0.001$
n.s Non-significant

Table 4.5.4 Summary analysis of variance: The Effect of substitution of cryoprotective agents on post-thawing acromosal membrane damage of frozen ram spermatozoa (Expt. 5)*

Source	df	ms
Cryoprotective agent (CA)	4	13.83n.s
Incubation time (IT)	3	746.65***
2-way interaction, CA x IT	12	6.07n.s
Residual	60	11.76

*** $P < 0.001$
n.s Non-significant

Table 4.6.1 Analysis of variance summary: The comparative effects of combination and substitution of cryoprotectants on the post-thawing motility of deep frozen ram spermatozoa (Expt. 6)*

Source	df	ms
Cryoprotective agent treatment (CAT)	4	33.26**
Incubation period (IT)	3	350.33***
2-way interaction, CAT x IT	12	12.50n.s
Residual	40	7.02

** P < 0.005
*** P < 0.001
n.s Non-significant

Table 4.6.2 Effects of cryoprotective agent treatment on post-thawing survival of spermatozoa (Expt. 6)*

Cryoprotective agent treatment	Motility, %
Combination	
Glycerol + ethylene glycol	27.1
Glycerol + erythritol	34.6
Substitution	
Glycerol/Glycerol	31.7
Glycerol/Erythritol	30.8
Ethylene glycol/Erythritol	31.3
Mean	31.1

* P < 0.005

Table 4.6.3 Analysis of variance summary: The effects of cryoprotectant treatment on post-thaw live/dead proportion of frozen ram spermatozoa (Expt. 6)*

Source	df	ms
Cryoprotective agent (CA)	5	45.96n.s
Incubation time (IT)	3	83.83n.s
2-way interaction, CA x IT	15	50.93n.s
Residual	48	28.51

n.s Non-significant

Table 4.6.4 Summary of analysis of variance: The effect of cryoprotective agents treatment on acromosal membrane damage of frozen spermatozoa (Expt. 6)*

Source	df	ms
Cryoprotective agent (CA)	5	52.37***
Incubation time (IT)	3	137.50***
2-way interaction, CA x IT	15	25.59***
Residual	48	4.90

*** P < 0.001

Table 4.6.5 Effects of cryoprotective agent and incubation time on acromosal membrane damage of frozen-thawed ram spermatozoa (Expt. 6)*

Cryoprotective agent combination and substitution	Incubation time (h)				
	0	2	4	6	Mean
Control	10.8**	11.3	21.3	19.3	16.4
Glycerol + Ethylene glycol	10.7	13.3	18.0	17.3	14.8
Glycerol + Erythritol	15.3	13.7	15.3	22.0	16.6
Glycerol/Glycerol	21.0	20.7	21.0	24.0	21.7
Glycerol/Erythritol	15.3	16.3	13.0	24.0	17.2
Ethylene glycol/Erythritol	11.3	23.7	21.7	27.0	20.9
Mean	14.1	16 5	18.9	22.3	17.9

* P < 0.001

** % motile sperm

Table 5.1.1 Effects of type of cryoprotectant on 21-day non-return rate and lambing rate following cervical insemination with frozen ram spermatozoa (Trial No. 1)

Cryoprotective agent	No. of ewes inseminated	No. of ewes not returning to heat after 21 days	No. of ewes lambing	Non-return rate, %	Lambing rate, %
Glycerol	17	6	2	35.3	11.8
Erythritol	17	6	2	35.3	11.8
Control	17	15	14	88.2	82.4

* Natural mating

Table 5.2.1 Effects of cryoprotective agent treatment on the 21-day non-return lambing rates following cervical insemination with frozen ram semen (Trial No. 2)

Cryoprotective agent treatment	No. of ewes inseminated	No. of ewes not returning to heat after 21 days	No. of ewes lambing	Non-return rate, %	Lambing rate, %
Glycerol	10	2	0	20.0	0
Erythritol	11	1	0	9.1	0
Glycerol + Erythritol	11	0	0	0.0	0
Ethylene glycol/ Erythritol	11	3	0	27.3	0
Control*	7	5	4	71.4	57.1

\+ Cryoprotective compound combination
/ Cryoprotective compound substitution
* Natural mating

Table 5.2.2 Effects of cryoprotective agent treatment on the 21-day non-return lambing rates after intrauterine insemination with fresh ram semen (Trial No. 2)

Semen treatment	No. of ewes inseminated	No. of ewes not returning at 21 days	No. of ewes lambing	Non-return rate, %	Lambing rate, %
Fresh semen	4	2	1	50	25
Glycerol	5	2	0	40	0
Erythritol	4	1	0	25	0
Glycerol + Erythritol	5	2	2	50	40
Ethylene glycol/ Erythritol	5	0	0	0	0

\+ = combination
/ = substitution

Table 5.3.1 Effects of cryoprotective agent treatment on the 21-day non-return and lambing rates following cervical insemination with frozen ram semen (Trial No. 2)

Semen treatment	No. of ewes inseminated	No. of ewes not returning at 21 days	No. of ewes lambing	conception rate %	lambing rate %
Glycerol	8	1	0	12.5	0
Erythritol	9	1	0	11.1	0
Glycerol + Erythritol	7	0	0	0	0
Ethylene glycol/ Erythritol	7	0	0	0	0
Control	7	3	0	42.9	0

+ = Combination
/ = Substitution
* = Natural mating

Table 5.5 Effects of cryoprotective agent treatment on the 21-day non-return and lambing rates following intrauterine insemination of ewes with frozen ram spermatozoa (Trial 3)

Cryoprotective agent	No. of ewes inseminated	No. of ewes not returning at 21 days	No. of ewes lambing	Non-return rate, %	Lambing rate, %
Glycerol	5	2	0	40	0
Erythritol	5	2	1	40	20
Glycerol + Erythritol	5	2	1	40	20
Ethylene glycol/ Erythritol	5	2	1	40	20
Fresh semen	5	2	1	40	20

+ = combination
/ = substitution

Table 5.4.1 Effects of cryoprotective agent treatment on the 21-day non-return and lambing rates following cervical insemination of ewes with frozen ram semen (Trial 4)

Semen treatment	No. of ewes inseminated	No. of ewes not returning to heat after 21 days	No. of ewes lambing	Non-return rate (%)	lambing rate (%)
Glycerol	11	3	1	27.3	9.1
Erythritol	11	0	0	0.0	0.0
Glycerol + Erythritol	10	2	0	20.0	0.0
Ethylene glycol/ Erythritol	11	6	2	54.5	18.2
Control*	11	11	4	100.0	36.0

+ = Combination
/ = Substitution
* = Natural mating

Table 5.4.2 Conception rate based on 21-day non-return and lambing rates following intrauterine insemination of ewes with frozen ram semen in single and combined cryoprotective agents (Trial 4)

Semen treatment	No. of ewes inseminated	No. of ewes not returning to heat after 21 days	No. of ewes lambing	conception rate (%)	lambing rate (%)
Glycerol	5	2	0	40	0
Erythritol	5	3	1	60	20
Glycerol + Erythritol	5	2	1	40	20
Ethylene glycol/ Erythritol	5	3	1	60	20
Fresh semen	5	3	0	60	0

+ = combination
/ = substitution

Table 5.4.3 Lambing rate following insemination with frozen raw spermatozoa after natural estrus in single and combined cryoprotective agents (Trial 4b)

Semen treatment	No. of ewes inseminated	Non-return	No. of ewes lambing	conception rate (%)	lambing rate (%)
Glycerol	10	7	5	70	50
Erythritol Glycerol +	10	5	2	50	20
Erythritol Ethylene glycol/	11	7	1	63	9
Erythritol	10	2	2	20	20
Control*	10	7	4	70	40

+ = Combination
/ = Substitution
* = Natural mating

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APPENDIX 1

Chemicals included in the diluents were analytical grade, except where otherwise stated in the list given below.

	Name of chemical	Mol. Wt	Manufacture
1.	Tris (hyrdoxymethyl aminomethane	121.14	Fisher
2.	Citric acid	210.14	Analar
3.	Glucose	180.16	Analar
4.	Glycerol	92.10	Analar
5.	Erythritol	122.12	BDH
6.	Acetamide	59.07	BDH
7.	Ethylene glycol	62.07	BDH
8.	Polyethylene glycol	600.00	Sigma
9.	Dimethyl sulphoxide (DMSO)	78.13	Analar
10.	Penicillin		Glaxo
11.	Streptomycin sulphate		Galenika

Diluent composition per 100 ml

Tris	300.00 mM
Citric acid	94.70 mM
Glucose	27.75 mM
Egg yolk	15.00 per cent
Penicillin	1000.00 IU/ml
Streptomycin	1.00 mg/ml

APPENDIX 2

Cryoprotective agent concentration, osmotic pressure, and pH in Tris diluent use for freezing ram spermatozoa

Cryoprotective agent (CA)	Concentration (M)	Osmotic Pressure (mOsm)	pH
Control no CA	–	310.0	6.69
Glycerol	0.5	1362.0	6.70
Glycerol	1.0	ND	6.65
Acetamide	0.5	919.7	6.66
Acetamide	1.0	1443.6	6.67
Erythritol	0.5	935.5	6.63
Erythritol	1.0	1529.4	6.62
Ethylene glycol	0.5	1144.6	6.66
Ethylene glycol	1.0	ND	6.64

ND = Not determined

APPENDIX 3a

The factorial design for experiments 2.1 to 2.6 was as follows:
Factors 4 x 3 4 : 3

1. Cryoprotective compound levels: 0.0 v 0.5 v 1.0 v 1.5 M

2. Dilution rates: 1:3 v 1:5 v 1:7 (semen:diluent, v/v)

3. Incubation time: 0 v 2 v 4 v 6 h

4. Replicates: Three replicates from pooled semen

In experiment 2.6 the factors were as above except the cryoprotective compund levels which were as follows:

Polyethylene glycol-600 levels: 0.0 v 3.0 v 6.0 v 9.0 mM

APPENDIX 3b

The factorial design for experiments 3.1 to 3.5

Expt. 3.1

Factors: 2 x 4 x 4 : 3

The following factors were considered:

1. Type of cryoprotective compound: Glycerol v Erythritol
2. Cryoprotective compound levels: 0.0 v 0.2 v 0.4 v 0.6 M
3. Incubation time: 0, 2, 4, 6 h
4. Replicates: Three from pooled semen

Expt. 3.2

Factors: 2 x 2 x 4 x 4 : 3

1. Type of cryoprotective compound: Glycerol v Erythritol
2. Concentration cryoprotective compounds: 0.0 v 0.2 v 0.4 v 0.6 M
3. Dilution rate: 1:2 v 1:4 (semen:diluent, v/v)
4. Incubation time: 0, 2, 4, 6 h
5. Replicates: Three from pooled semen

Expt. 3.3

Factors: 5 x 3 x 4 : 3

1. Type of cryoprotective compound: Control v Glycerol v Erythritol
2. Concentration cryoprotective agents: 0.0 v 0.2 v 0.4 v 0.6 M
3. Thawing temperature: 27 v 37 v 47°C
4. Incubation time: 0, 2, 4, 6 h
5. Replicates: Three from pooled semen

Expt. 3.4

Factors: 3 x 4 x 4 : 3

1. Initial dilution rate: 1:2 v 1:4 v 1:6 (semen:diluent, v/v)
2. Freezing concentration: 0.5 v 1.0 v 1.5 v 2 x 10^9 cells/ml
3. Incubation time: 0, 2, 4, 6 h
4. Replicates: Three from pooled semen

Expt. 3.5

Factors: 4 x 4 x 4 : 3

1. Type of cryoprotective compound: Glycerol v ethylene glycol v acetamide v erythritol
2. Cryoprotectant addition temperature: No CA[1] v 30 v 5°C v 30 v 5°C
3. Incubation time: 0 v 2 v 4 v 6
5. Replicates: Three from pooled semen

[1]CA = cryoprotective agent

\APPENDIX 3c

\ The following factors were considered in experiments 4.1 to 4.6

Expt. 4.1

Factors: 9 x 4 x 4 : 4

1. Cryoprotective agents: Control v Glycerol v ethylene glycol v erythritol v Glycerol + Ethylene glycol v Glycerol + erythritol v ethylene glycol x erythritol v Glycerol x ethylene glycol x erythritol

2. Cryoprotectant addition temperature: No CA v CA at 30 v CA at 5°C v CA 30 - 5°C

3. Incubation time: 0 v 2 v 4 v 6

5. Replicates: Four from pooled semen

Expt. 4.2

Factors: 17 x 4: 3

1. Sequence of CA substitution:

1st suspension	2nd suspension
Glycerol	Glycerol
v glycerol	Ethylene Glycol
v glycerol	Acetamide
v glycerol	Dimethyl sulphoxide
v ethylene glycol	Ethylene Glycol
v ethylene glycol	Glycerol

1st suspension	2nd suspension
v ethylene glycol	Acetamide
v ethylene glycol	Dimethyl sulphoxide
v acetamide	Acetamide
v acetamide	Glycerol
v acetamide	ethylene glycol
v acetamide	DMSO
v DMSO	DMSO
v DMSO	Glycerol
v DMSO	Ethylene glycol
v DMSO	Acetamide
v No CA	No CA

2. Incubation time: 0, 2, 4, 6, 6 h

3. Replicates: Three from pooled semen

Expt. 4.3

Factors: 5 x 5 x 4 : 3

1. Initial CA: Glycerol v ethylene glycol v acetamide v DMSO v erythritol

2. Freezing CA: Glycerol v ethylene glycol v acetamide v DMSO v Erytritol

2. Incubation time: 0, 2, 4, 6, 6 h

3. Replicates: Three from pooled semen

\Expt. 4.4

\ Factors: 8 x 4 : 3
Cryoprotective agent substitutions:

1st suspension	2nd suspension
Glycerol	DMSO
v glycerol	Glycerol
v ethylene glycol	Ethylene Glycol
v glycerol	erythritol
v erythritol	glycerol
v ethylene glycol	erythritol
v acetamide	Glycerol
v erythritol	ethylene glycol

2. Incubation time: 0, 2, 4, 6, 6 h

3. Replicates: Three from pooled semen

Expt. 4.5

Factors: 5 x 4 x 4 : 4

1. Cryoprotective agent substitution: Glycerol/glycerol v Glycerol/erythritol v ethylene glycol v erythritol v acetamide/Glycerol v erythritol/erythritol

2. Incubation time: 0, 2, 4, 6, 6 h

3. Replicates: Three from pooled semen

\Expt. 4.6

\ Factors: 6 x 4 : 3

Combination and substitution of cryoprotective agent:

Combinations

Glycerol + Ethylene Glycol1

v glycerol + erythritol

Substitutions

v glycerol/glycerol2

v glycerol/erythritol

v ethylene glycol/erythritol

v Control (No cryoprotective compound)

2. Incubation time: 0, 2, 4, 6, 6 h

3. Replicates: Three from pooled semen

1

\+ = Combination

2

/ = Substitution

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