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Cryopreservation of ram semen for artificial insemination.

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CRYOPRESERVATION OF RAM SEMEN FOR ARTIFICIAL INSEMINATION

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BY

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SUMMARY

A theoretical study showed that AI can greatly affect the efficiency of sheep breeding schemes provided fertility is maintained at the highest levels.

Factors that affect the survival of ram spermatozoa during preservation were studied. A pH range between 6 and 7 was well tolerated. The addition of 4% (v/v) glycerol to the diluted ram semen in Tris buffer lowered the motility and survival of spermatozoa during 5 hours of storage at 30°C. Following insemination of chilled ram semen, with and without glycerol in the diluent, lambing percentages of 59% and 73% respectively were obtained.

Ram semen was frozen in 0.25 ml straws using various cooling combinations. The optimal procedure was found to be to cool rapidly from 5°C to -120°C at -20°C/min. When semen so treated was compared in a fertility trial with semen frozen by the pellet method of Evans and Maxwell (1987), lambing percentages of 14% and 18% respectively were obtained.

Attempts were made to formulate a vitrifying diluent for ram semen. A method was developed for the assessment of semen in highly concentrated cryoprotective solutions. Semen tolerated 10% concentrations of each of glycerol, acetamide and propylene glycol applied together, but when concentrations were raised above this level sperm mortality was very high.

A simple spectrophotometric procedure for the objective assessment of vigour of ram semen was developed and tested.

Raffinose 66 mM in the freezing diluent improved the post-thawing revival rate of spermatozoa from 46% to 71%, and increased the post-thawing recovery of the swimming-up vigour ($P \leq 0.01$). Raffinose treatment reduced the ATP content of semen but did not reduce the rate of glucose oxidation by diluted spermatozoa at either the pre-freezing or post-thawing stages.

Frozen storage of ram spermatozoa as pellets was best achieved using two volumes of Tris buffer diluent containing 18% (v/v) egg-yolk, 6% (v/v) glycerol and 66 mM raffinose to one volume of semen. The diluted semen was chilled to 5°C and frozen as 0.10 ml pellets on dry ice. For frozen storage of ram semen in 0.25 ml straws, best results were obtained when the Tris buffer diluent contained 18% (v/v) egg-yolk, 9% (v/v) glycerol and 66 mM raffinose, and cooling was at a rate of -30°C/min from 5°C to -120°C.

Non-return rates were 21%, 20% and 31% for ewes inseminated with semen samples frozen as standard 0.20 ml pellets, as raffinose containing 0.10 ml pellets, and as raffinose containing 0.25 ml straws respectively. Of the *in vitro* tests, only the swim-up test was correlated with non-return rates ($r=0.904$, $P \leq 0.1$). Post-thawing survival of the spermatozoa was improved by the addition of raffinose which had no deleterious effect on fertility.

CONTENTS

CHAPTER TWO

GENERAL MATERIALS AND METHODS

SECTION TWO

CHAPTER SIX

VITRIFICATION AS A METHOD FOR THE CRYOPRESERVATION OF RAM SPERMATOZOA

CHAPTER ONE

REVIEW OF LITERATURE

PART ONE

SHEEP BREEDING AND ARTIFICIAL INSEMINATION

Artificial insemination of sheep has the potential for a significant impact on sheep production (Evans, 1988). To fulfil this, the methods for semen preservation and delivery need improvement.

1. The features of artificial insemination which make it advantageous:

1.1. In sheep production:

A selected ram can produce many more lambs if its semen is distributed by artificial insemination rather than if it is used for natural service only. Maxwell (1984) illustrated the potential capacity of one ram for producing progeny. At the average lambing percentage in South Western Australia of 65%, a single ram serving 33 ewes would produce an average of 22 lambs per year by natural mating. By AI using fresh semen, the ram could service 1000 ewes which, with lambing rate of 50%, would produce about 500 lambs. However, semen collected and frozen from the same ram over a 9 month season at 9 ejaculates per week could be used to inseminate 25000 ewes producing up to 12,000 lambs per year (Table 1). An alternative estimate (Agriculture Training Board, Technical Note, 130.5) suggests an annual production of up to 8000 doses of chilled semen or 5000 doses of frozen semen per ram.

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growth rate, efficiency of feed utilisation, body weight or wool production, is usually called a 'performance test' (Dalton, 1985).

In countries, like Australia and New Zealand, where wool or milk are the primary products from the sheep industry, selection programmes based on individual selection have been successfully followed for many years to identify superior animals. Harvey et al. (1988) estimated that an additional increase of 25% in clean fleece weight would be possible if artificial insemination were employed to disseminate superior genes of high performance rams, in comparison with conventional natural mating.

1.3. In breed improvement schemes:

a) Group breeding schemes:

Group breeding schemes or cooperative breeding schemes were developed mainly through the efforts of progressive breeders in New Zealand. In the UK, the first group breeding schemes were founded in Wales in 1976 (Meat and Livestock Commission, sheep yearbook, 1989). In a Group Breeding scheme for sheep a few (10-15) interested breeders contribute their top performing ewes to a central unit, normally called the nucleus. If breeders continue to contribute animals to the nucleus after its initial formation then the scheme has an 'open nucleus'. If no further animals are received from the members after the initial formation of a central flock, the scheme has a 'closed nucleus'. Screening and selection is continued in both contributor's flocks and in the nucleus.

Clark et al. (1986) visualised a pyramidical structure of gene dissemination in cooperative breeding schemes, in which ram breeders at the top distribute genetic improvements to commercial producers at the base. There is an intermediate layer of multiplying ram breeders who buy in rams from the nucleus. The same authors anticipated that the commercial flocks would lag behind the genetic merit of the nucleus flock because of the time it takes to transfer genetic improvement. The advantage of AI is that this genetic lag can be reduced by more intensive and widespread use of better quality nucleus rams.

b) Sire referencing schemes:

In a sire referencing scheme a few selected rams are used for mating with high quality ewes from contributing flocks in the scheme either simultaneously or within a short period of time. From the progeny of this mating, all rams tested are ranked for different traits and outstanding lamb rams are identified for the following mating season. The main obstacle to the widespread use of sire referencing schemes is the difficulty of using sires simultaneously over several flocks (MLC sheep yearbook, 1989). The distribution and widespread use of rams in such schemes is facilitated by the application of artificial insemination, preferably using frozen ram semen. AI can thus greatly facilitate genetic progress in the sheep industry through progeny testing of reference sires.

2. Disadvantages of artificial insemination:

2.1. Inbreeding:

A potential disadvantage of AI is that the intensive selection and exploitation of a limited number of sires could lead to inbreeding, particularly when breeding units are small. The answer is to avoid inbreeding by using large flocks or cooperative schemes (as explained in 1.3.a). In such schemes semen would be collected at a central farm and stored for distribution among the members of the scheme (Clarke et al., 1986).

2.2 Extra costs and professional labour:

Before using AI in a system of sheep production, the extra costs of AI and the problems of management must be allowed for. In animal breeding economic gain is the main justification for genetic improvement.

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quoted by Salamon et al., 1979)). Lopyrin and Robcev (1968) reported an increase in embryonic mortality after insemination with ram semen stored only for 1, 2 and 3 days, but Salamon et al. (1979) rejected their findings on the basis of insufficient numbers of experimental animals. He concluded that It was unlikely that AI using chilled stored ram semen would affects the level of early embryonic losses.

El-Gaafary (1987) obtained mean embryonic losses of 8.0 and 32.6% after using chilled semen stored for 3 and 24 hours respectively, though the difference between treatments was not significant at the 5% level.

1.1. Diluents for the chilled storage of ram semen:

In normal freshly ejaculated ram semen, the average concentration of spermatozoa is 3600 million per ml during the breeding season (Agricultural Training Board, Technical Note, 130.5). During natural mating several thousand million spermatozoa will be shed, although only about 100-140 million spermatozoa, are necessary to fertilise egg(s) when administered by cervical insemination (Evans and Maxwell, 1987). Therefore, using freshly diluted semen a sheep breeder can inseminate many more ewes with a single ejaculate than can a ram.

An extender of ram semen has to contain certain components:

a) Buffer to prevent harmful changes in pH due to the production of acid, particularly lactic acid.

b) A source of energy.

c) Electrolyte to maintain the proper osmotic environment for the spermatozoa.

d) Antibiotics to inhibit bacterial growth.

e) Agents to protect against the harmful effects of cooling.

f) Sufficient volume to dilute the semen so that multiple insemination can be performed (Foote, 1974; Graham et al., 1978a).

1.1.1 Buffers:

Different groups of organic and inorganic buffers have been used to prevent

changes of pH in diluted semen. Usually, one of three types of buffer has been employed:

Phosphate buffer

Citrate buffer

Tris buffer

Phosphate and citrate buffer were commonly used to extend bull and ram semen. Salamon and Robinson (1962b) reported that citrate buffered egg-yolk glycine diluent gave a longer half-life and total survival time than did phosphate based egg-yolk fructose or cow milk. An optimum pH of 7.0-7.2 for phosphate or citrate-buffered was suggested by Mann (1964) whilst Watson (1979) suggested an optimum pH of 6.9. Watson (1979) modified the pH of buffer solutions based on phosphate or citrate by adding citric acid in the presence of egg-yolk. Generally, yolk-phosphate or yolk-citrate have been regarded as standard diluents for ram semen (Emmens and Robinson, 1962).

Lapwood and Martin (1972) studied the storage of ram spermatozoa at 37°C and 5°C in diluents containing various buffers. They found the optimum composition to be 185 mM sugar, 31 mM sodium chloride, and 20 mM phosphate buffer. Of the buffers used to store semen at 37°C, phosphate was the most satisfactory, but caused a decline in motility at higher concentrations. Lapwood and Martin (1972) reported that citrate, at a level of 10 and 20 mM, reduced the viability of spermatozoa stored in a fructose containing synthetic diluent at 5°C. This loss of viability was particularly marked in diluents containing the higher citrate level together with 0.8% (w/v) milk solids. Dauzier et al. (1954) as quoted by Lapwood and Martin (1972) obtained satisfactory fertility with an egg-yolk citrate diluted semen when used within 2 hours of collection.

However, Salamon and Robinson (1962b) concluded citrate based buffer to be superior to phosphate based buffer, when semen was frozen at -79°C , while Lapwood and Martin (1972) found phosphate buffers gave better performance when semen was stored at 37°C or 5°C.

Tris buffer:

Tris (Tris [hydroxymethyl] aminomethane) is an organic buffer. The superiority of

an egg-yolk glycerol diluent, containing 0.2 or 0.25 M Tris, to egg-yolk citrate glycerol was demonstrated, when bull spermatozoa was preserved better by its inclusion at both 5°C or -85°C (Davies et al., 1963). Foote (1970) confirmed that a 0.20 M Tris egg-yolk extender buffer at pH 6.50 or 6.75 was superior to the other buffers tested for preserving the motility of sperm.

The first report on the use of a Tris-based diluent for pellet freezing of ram spermatozoa came from Samouilidis (1970 as quoted by Salamon and Visser, 1972). He examined the effects of Tris concentration and type of sugar in the freezing diluent, and of composition of the thawing solution, on the survival of ram spermatozoa following the freeze-thaw procedure. The pH of the Tris-based diluent was adjusted to 7.0 using citric acid. The best survival rates of motile spermatozoa were observed when the semen was frozen with 250 and 350 mM Tris diluent, and subsequently thawed in 250 mM Tris solution. Thus, ram spermatozoa tolerated a considerable range of Tris concentration (250 to 400 mM) in the diluent. However, cell survival varied depending on the type of sugar included in the Tris diluent and on the composition of the thawing solution. Amongst the sugars glucose, fructose, lactose and raffinose, glucose was the best to be included with the Tris buffer. Salamon and Visser (1972) reported that their best diluent for extending ram semen for freezing was as follows: Tris (300 mM), glucose (27.75 mM), egg-yolk (15% v/v), and glycerol (5% v/v), used at a semen dilution of 1:4. Tris (300 mM), fructose (55 mM), thawing solution (dilution ratio 1:3, pellet: thawing solution, v/v).

1.1.2. Sugars and osmolarity:

Generally, spermatozoa can glycolyse hexoses such as fructose, glucose, and mannose (Mann, 1964). Lapwood and Martin (1966) found that the benefit of a sugar for spermatozoa was related to the temperature of semen storage. In their trial, ram spermatozoa survived well when stored at 37°C with the inclusion of 185 mM of the readily metabolisable hexoses glucose, mannose, and fructose, while pentoses and galactose were superior when semen was stored at 5°C, glucose gave very poor results at

the lower temperature. The same authors showed that there was no difference in the survival of spermatozoa in diluents containing 185 mM of fructose, glucose, lactose or sucrose for storage at 5°C degree centigrade for 4 days. When fructose and lactose were compared in diluents at this temperature for storage for 6 days, fructose was superior.

Whilst the main purpose of adding a sugar is to provide a source of energy in diluted semen, it serves several other functions. It may maintain the osmotic pressure of the diluent or it may protect spermatozoa during freezing. In particular, non-penetrating sugars such as lactose and raffinose have a protective effect (Watson, 1979). Broadly speaking, during freezing procedures, spermatozoa retain their motility better in hypertonic solution rather than in a hypotonic environment (Watson, 1979; Griffith, 1978; Fiser et al., 1981).

Fiser et al. (1981) investigated the importance of tonicity on the viability of frozen ram spermatozoa. They prepared a range of osmolarities, using reconstituted skimmed milk, which had been heated for 15 minutes in a boiling water bath, cooled, enriched with 500,000 IU penicillin G and 625 mg dihydrostreptomycin/litre diluent and at pH 6.65-6.70 Osmotic pressure was adjusted with a concentrated solution of 800 mM trisodium citrate. The following osmolarities of diluents (Table 4) were used by Fiser et al. (1981) to test the effect of osmolarity on semen motility and the pregnancy rate of inseminated ewes.

Table 4:

Diluents	Percentages of skimmed milk	Osmolarity (mOsm/kg)	Tonicity
A	7	200	Hypotonic
B	11	320	Isotonic
C	15	450	Hypertonic
D	19	600	Hypertonic
E	23	750	Hypertonic

The authors demonstrated that spermatozoa motility improved with increasing osmolarity of freezing solution irrespective of whether thawing was at room temperature (20°C) or in a water bath at 39°C. Higher percentages of motilities of 49% and 53% respectively were obtained for diluents D and E, while diluents A, B, and C resulted in only few cells survived. There was a substantially lower percentage of motility when isotonic diluent B was used. The hypotonic diluent A yielded extremely poor cryosurvival results. When ewes were inseminated at an induced oestrus with semen frozen in hypertonic diluent (600 mOsm) and thawed at 39°C, a pregnancy rate of 67.5% (n=40) was obtained, compared with 78.9% (n=19) using fresh semen.

1.1.3. Antibiotics:

A combination of 500 IU penicillin/ml and 500 µg streptomycin/ml is most commonly used for the preservation of ram semen in the UK (MLC Veterinary Services Technical Bulletin, 1982). This is added to diluted ram semen in heated milk for short term storage.

1.1.4.Egg-yolk:

Kampschmidt et al. (1953) identified a resistance factor in egg-yolk which protected sperm against cold shock during freezing. Watson (1981) considered lecithin, which is present in both phospholipid and lipoprotein preparations, as a resistance factor for protecting spermatozoa in a variety of cold stresses. The 'storage' factor has been related to the protein alone or a lipoprotein complex which consists of the phospholipids, lecithin, and cephalin (Blackshaw, 1954 & 1958; Quinn et al., 1980; Watson, 1981).

Sudden decreases in the temperature of ram semen from body temperature to a storage temperature of either 15 or 5°C, irreversibly reduce spermatozoa motility. This is called cold shock which results in a disruption of cell membranes and the loss of intracellular constituents (Quinn et al., 1969). Quinn et al. (1980) examined the mechanism whereby phospholipid dispersions protect spermatozoa against cold shock damage. They washed spermatozoa after adding lecithin dispersions and observed the

effect of rapid cooling on the motility of cells. This suggested that the exogenous phospholipid provided immediate protection against cold shock. This protective effect of phospholipid against cold shock was probably due to a 'loose' interaction of the lipid structures with the plasma membrane of the cells. If the exogenous phospholipids of spermatozoa are washed and removed, loss of protection against cold shock occurs. However, it was shown by Watson (1981) that diluents containing egg-yolk protected spermatozoa more effectively during freezing and thawing than did the exogenous phospholipids.

Release of intracellular enzymes is another manifestation of cold shock which can be prevented by using the phospholipid lecithin of egg-yolk at a minimum concentration of 0.12 percent (Blackshaw, 1954). The concentrations of egg-yolk that have been most widely used, are up to 12% in final concentration of diluted ram semen (Evans and Maxwell, 1987). This level of egg-yolk has a beneficial effect on the post-thaw motility of spermatozoa (Salamon and Visser, 1972; Watson and Martin 1972).

Blackshaw (1958) demonstrated that the metabolic activity of ram spermatozoa was drastically reduced by declining temperature. Over a temperature shock range from 30°C to 0°C the decline in metabolic activity was linear for both control semen and that diluted with yolk medium. At 7.5°C semen in a lecithin diluent showed an abrupt decline in lactic acid accumulation which was not seen in semen diluted with egg-yolk. This result showed that, although egg-yolk protected ram sperm to some extent, it did not prevent the occurrence of cold shock as fully as it does for bull semen (Blackshaw and Salisbury, 1957). This was because ram spermatozoa are very susceptible to rapid temperature changes particularly to 10°C, 5°C, or 0°C (Fiser and Fairfull, 1986). So egg-yolk and lecithin did not give sufficient protection against severe cold shock to enable the spermatozoa to maintain their full metabolic activity during a subsequent period of incubation at 37°C. The failure to regain full metabolic activity and motility when rewarmed was ascribed to the disruption of cell membranes and the loss of intracellular

constituents (Quinn et al., 1969).

Watson (1981), compared the protective effect of pure phospholipid, lecithin (phosphatidylcholine), and low-density lipoprotein fraction of egg-yolk (LDF) under conditions of both cold shock and cold storage, on ram spermatozoa cooled to below 5°C slowly or subjected to cold shock by transference to ice-water at 0°C for 2 minutes. His results were in agreement with those of Kampschmidt et al. (1953) who showed that while lecithin and egg-yolk lipoprotein provided equal protection to spermatozoa during the brief but severe stress of cold shock, the lipoprotein fraction was superior to lecithin during cooling and cold storage. Furthermore, it was shown that a significant increase in sperm survival occurred in diluents containing a range of lipid:protein ratios between 0.2 and 1.67. Survival of the spermatozoa decreased as this ratio fell below 1.67 compared with a ratio of 4.76 for LDF of egg-yolk. Higher ratios of lipid:protein only provided little further benefit.

1.1.5. Milk diluent:

Lapwood et al. (1972) used a reconstituted milk preparation as a diluent and found about 60% of spermatozoa to be highly motile after incubation for 5 hours at 37°C. The same authors reported that the fertility of ewes inseminated with diluted semen in skim milk was higher than that of ewes inseminated with diluted semen in glucose diluent, 40.2% and 35.3% respectively.

A fertility trial conducted by the Meat and Livestock Commission showed reconstituted milk diluent containing 10 g skim milk powder, 0.3 g sulphanilamide, and 90 ml distilled water, antibiotics (500 IU penicillin/ml and 500 μg streptomycin/ml) added after cooling to be a satisfactory diluent for storing ram semen at 15°C. The storage time could be prolonged provided that the semen was used for the second of two insemination within 14 hours. So the first insemination must be done within 5 hours of semen collection (MLC Veterinary Services Technical Bulletin, 1982).

Salamon and Robinson (1962b) concluded that the maximum duration of storage of ram semen in cow milk diluent at 10-15°C was 24 hours, and that even this storage period

resulted in a loss of fertilising capacity of about 15%. Lapwood et al. (1972) found that ram semen survived in skim milk medium for 5 hours, with a high motility (60%), when incubated at 37°C. The same authors reported a fertility (percentage of ewes lambing) of 40.2% for ewes inseminated with semen diluted in skim milk and 35.3% for ewes inseminated with semen diluted in glucose diluent.

Thatcker and Almquist (1953) obtained an optimum spermatozoan survival when the milk (homogenised or skim milk) had been heated to 95°C for 1 to 10 minutes. Motility and survival were extremely poor in unheated portions (2 and 28%) compared to portions of the same milk which had heat treatment (66 and 62%). They emphasised that gentle heating yielded better motility results than did vigorous heating. Reaching the appropriate temperature (92°C) was more crucial than the length of the heating period to destroy the toxic factor (lactenin) in unheated milk. Overheating of milk cause browning and the subsequent loss of Thiol group (SH) or the accumulation of new heat-generated products, such as volatile acids, furfuryl alcohol, ammonia, or maltol which can damage spermatozoa (Saacke et al., 1956). Another effect of overheating is to destroy catalase, an enzyme which exists in milk and breaks down peroxide, may have harmful effects on cell membranes (Colas et al., 1968).

Salamon and Robinson (1962a) observed that dilution of semen to 1:4 with heat treated cows milk, either skim or whole, did not depressed fertility, while ewe-milk diluent depresses fertility following a single insemination (68.6 v 47.3%). This superiority of cows milk to ewes milk, was confirmed by Schindler and Amir (1961).

Reconstituted skim milk plus antibiotics are routinely used in the UK, France and Ireland for ovine artificial insemination with liquid semen. Ram semen remained effective for as long as 14-16 hours at 15°C with a subsequent lambing rate of 65-75% following AI at oestrus (Colas and Courot, 1977; Gordon and Crosby, 1980).

Milk diluent alone is not a suitable storage medium for ram semen at 5°C, particularly if the storage time is extended beyond 8 hours. The rapid accumulation of

lactic acid results in a significant drop in the pH and acidity leads to a high mortality rate of spermatozoa (Tiwari et al., 1977. Generally, cows and ewes milk were found to be less effective than egg-yolk citrate diluent (Schindler & Amir, 1961); and egg-yolk glucose citrate was much more effective than heated cows milk as a storage diluent (Salamon and Robinson, 1962a).

There is almost general agreement that, the choice of diluent (egg yolk or skim milk) depends on the storage temperature to be used. At 15°C, skim milk preserves the fertilising capacity of ram spermatozoa better than does egg-yolk, while for storing ram semen at 4°C egg yolk is preferred (Salamon and Robinson, 1962b; Barlow et al., 1974; Colas and Courot, 1977; and Colas, 1983b). However, Colas and Courot (1977) stored ram semen at 5°C in milk diluent and in egg-yolk diluent. Their fertility results demonstrated that the egg-yolk extender was not as effective as milk extender for maintaining the fertilising ability of liquid ram semen. The lambing percent for egg-yolk extender and milk extender was 66.4% and 75.4% for natural oestrus or 38.3% and 54.6% for synchronised oestrus respectively.

Lapwood et al. (1972) showed that although the addition of egg yolk had little effect on the survival of spermatozoa at 37°C, it was very potent in prolonging the survival of spermatozoa stored at 5°C. El-Gaafary (1987) suggested that skim milk diluent was a good medium for preserving the fertilising ability of ram spermatozoa during storage for three hours at 5°C, but when the storage time was extended to 6 hours, Tris-based diluent gave better results.

2. LONG TERM STORAGE OF RAM SEMEN.

2.1. The basic concept of cryopreservation of cells:

When a cell suspension is cooled below 0°C extracellular ice crystals form, resulting in the concentration of the solutes in the remaining liquid water. The cell membrane acts as a barrier to prevent the spread of ice crystals into the intracellular compartments. Because of this, a differential vapour pressure for water is set-up across the plasma membrane and water tends to be transported out of the cell (Watson, 1979).

The transport of water depends on the permeability of the membrane to water which may vary with temperature, the surface to volume ratio of the cell, and the freezing rate. If the cell is sufficiently permeable to water or the freezing rate is slow enough not to allow the intracellular ice to form, the pressure around the cell remains low and dehydration results as water moves out of the cell to freeze extracellularly. Although extracellular ice crystals deform the cell, they do not rupture the plasma membrane or cause irreversible damage (Hafez, 1987). If, on the other hand, the cell is relatively impermeable to water or the freezing rate is rapid, intracellular water can not leave fast enough and is supercooled. Intracellular ice is formed which subsequently damages the cell compartments.

Karow et al. (1965) proposed that intracellular bound water is essential to cell integrity in term of the structure and function of its protein. Removal of this water by freezing would result in protein dehydration and denaturation and cell injury. The amount of water remaining in the cell would be proportional to the water activity of the surrounding solution. Injury is associated with solute concentration and freezing injury would be proportional to the rate of freezing because rapid freezing can disturb the function of membranes. In support of this idea Lovelock (1955) as quoted by Meryman (1971a) found that damage to red blood cells when they were frozen was related to the concentration of salt resulting from the freezing-out of water which produced haemolysis. He showed that whenever the extracellular concentration exceeded 0.8 M NaCl,

haemolysis began. Lovelock and Polge (1954) also attributed the immobilisation of spermatozoa by freezing in a concentrated salt environment, to the injury which resulted from the concentration of intracellular electrolytes produced by freezing.

Meryman (1968) claimed that the salt concentration could not be the cause of freezing injury, because marine organisms could survive and thrive in saline environments of osmolarity 0.5 M and in some cases even 0.8 M. He suggested that the membrane damage of frozen-thawed red blood cells resulted from the osmotic pressure produced by increasing the concentration of nonpenetrating extracellular solutes. Human red blood cells shrunk due to water loss until a volume at which the cell developed a resistance to further shrinkage. Cryoinjury would happen if cells were free to lose more water. Then a further increase in osmotic pressure gradient across the cell membrane would cause rupturing (Meryman, 1971a). He proposed that a cryoprotective agent must penetrate the cell without creating osmotic gradients, otherwise it will itself dehydrate the cell osmotically and exacerbate injury on freezing. It has to be nontoxic in the multimolar concentrations required for cryoprotection. The toxic effect was described by Meryman (1971b) as an irreversible effect such as solubilizing of membrane lipids or the denaturation of protein.

Mazur (1965) elaborated a two-factor hypothesis for freezing injury particularly in yeast and red blood cells. He claimed it was due to the combined effects of exposure to concentrated solutes (solution effect) and the formation of large intracellular ice crystals. A progressive fall in temperature causes more and more water to be withdrawn from the solution and deposited as ice until the solution becomes saturated with respect to solute. A further fall in temperature causes the solute to precipitate and all of the water to freeze.

Mazur (1970) developed equations from which he calculated the amounts of water remaining inside cells cooled to low temperatures at varying rates. He stated that the critical cooling rate which allows a cell to freeze internally depends on the ratio of cell volume to its surface area and on the permeability of the cell to water. However, below

-10°C to -15°C cell membranes can no longer block the passage of ice crystals and if the rate of cooling is rapid enough intracellular ice crystals will form. The same author suggested that 'solution effect' is responsible for injury when the cooling rate is slower than optimal, and intracellular freezing is responsible for injury when cooling is faster than optimal. The optimal rate, then, is a rate that is slow enough to prevent formation of intracellular ice but fast enough to minimise the length of time cells are exposed to solution effects. Although this hypothesis was developed to explain the survival curves of yeast and red cells, it is now a reliable base for explaining the survival of nucleated mammalian cells in freezing procedures. According to this theory, the main cause of cryoinjury are cooling rate and solution component effects, which should be carefully planned to obtain the best rate of survival of cells.

Recently, Holt and North (1991) investigated the involvement of cytoskeletal proteins in the maintenance of sperm plasma membrane integrity, and the effect of cooling stress in relation of the role of actin in ram spermatozoa. Their laboratory results showed the possibility of destabilising the actin membrane protein during cooling. They were unable to suggest a similar pattern for susceptibility of cryoinjury, due to variations in the cytoskeletal proteins among species. The complexity of sperm plasma membrane and the steps in a cryopreservation procedure where membrane perturbations can occur have been reviewed by Parks and Graham (1992).

2.2. Freezing of ram semen:

Cryoconservation of semen, was first reported by Polge et al. (1949) who, in their studies on the low temperature storage of fowl spermatozoa, recorded the cryoprotective properties of glycerol. Following the discovery of the protective effect of glycerol, successful freezing of bull spermatozoa (Smith and Polge, 1950) and of ram spermatozoa (Emmens and Blackshaw, 1950) were reported. Smith and Polge (1950) obtained the best survival rates by dilution of the semen in a 3.9% sodium citrate buffer containing 15% glycerol and by slowly cooling the diluted semen in ampoules from 0°C to -79°C in an alcohol bath.

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Visser (1974) obtained lambing results of 52.9% (37/70) and 54.4% (37/68) respectively for semen frozen stored for 5 years and for control semen frozen stored for 2 weeks. Examination of semen smears revealed no difference in the proportion of spermatozoa with undamaged acrosomes for 5 years old semen and 2 weeks (41% and 40%).

To explain the low fertility of frozen ram semen there are two schools of thoughts:

a: Ram semen is not particularly impaired by the freezing procedure, and the cause of low fertility is the tight cervix path as the main barrier of sperm transport. To overcome this obstacle, intrauterine insemination with injection of semen inside the uterus by means of laparoscopy, was suggested by Killeen and Caffery (1982); Maxwell et al. (1984a;b) and Haresign et al. (1986).

b: Ram semen is impaired during freezing by the formation of intracellular ice crystals. The major factors responsible are the 'solution effect' and the 'rate of cooling', which should be optimised to reduce freezing damage. If this could be achieved, frozen semen would be as effective as fresh semen and acceptable fertility of the ewe as in natural mating would be obtained (Evans, 1988).

2.3. The effect of diluents on the freezing process:

Diluents for freezing ram semen have the same components as described for short term storage of ram semen but include cryoprotective agent(s).

2.3.1. Cryoprotective agents:

Different cryoprotective agents have been tested by Salamon (1968) for pellet frozen ram semen. He found that varying glycerol concentration significantly altered the mean percentage of post-thaw motility obtaining 6.6%, 19.0%, 27.4%, 32.6%, and 26.7% for glycerol concentrations of 0%, 1%, 3%, 5%, and 7%, respectively. The highest rate of motility was obtained using 5% glycerol. When glycerol was added in either one or two steps, the post-thaw motilities were 26.2% and 27.5% respectively. An equilibration time of 2.5 hours increased percentage of post-thaw motility from 23.7% to 29.9% when compared with diluted semen that was frozen immediately after cooling to 5°C.

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indicated that the optimal level of glycerol in the diluted semen for freezing ram spermatozoa by the pellet method depended on the composition of the diluent, and in particular on the solution tonicity. Optimal glycerol concentration were from 2.8% to 5.6% (v/v) for freezing in sugar-yolk diluent or for the more hypertonic sugar-yolk citrate diluent respectively. Salamon and Visser (1972) obtained the highest percentage of spermatozoa post-thaw motility (39.5%) using 4% (v/v) glycerol, compared with 36.1% and 15.3% for 2% and 0% glycerol respectively. An optimal level of glycerol of 4% was recommended by Colas (1975) and Colas and Courot (1977). This is in agreement with the observation of Jones (1969), but does not conform with the results of First et al. (1961) who found that a final concentration of glycerol about 7% was optimal for ram semen frozen in ampoules.

2.3.2. Methods of glycerol addition and equilibration:

The addition of glycerol in one step at 30°C and in two steps at 30°C and 4°C was examined by Salamon (1968) who found that the sugar determined whether the glycerol should be added at 30°C or in two steps. An equilibration period of 2.5 hours was beneficial following glycerol addition especially after the two steps dilution methods. Since the cryoprotective activity of glycerol is needed during the crystallisation phase (Morris and Farant, 1972) it is more logical to add glycerol at 5°C (Colas and Courot, 1977). Lightfoot and Salamon (1969a) after laboratory tests claimed a single addition of glycerol to 5.6% followed by 2.5 hours equilibration before pellet freezing was the optimal situation. The same authors found that the optimal period of equilibration at 5°C prior to pellet freezing varied according to the composition of the diluent and the level of glycerol in the diluted semen. However, no advantage was obtained by equilibration longer than 4 hours for semen diluted in raffinose-yolk-citrate diluent with 4% glycerol. Colas (1975) investigated the effects of glycerol concentration and time allowed for equilibration. He demonstrated that increasing the exposure time of semen to glycerol before freezing had no significant effect on the viability of spermatozoa after thawing and incubation. Jones and

Martin (1965) found that a period of 5 hours equilibration at 5°C was better than 30 minutes. Fiser and Batra (1984) assessed the effect of equilibration time at 5°C on survival of ram spermatozoa frozen in straws. He observed that the percentage and the rate of forward progression of motile spermatozoa increased significantly from 0 to 2 hours equilibration with 4.5% glycerol, remained constant from 2 to 6 hours and decreased significantly at 18 hours.

2.4. The effect of the rates of cooling on chilled and frozen semen.

2.4.1. Chilling rates:

Ram and bull semen are relatively insensitive to rapid temperature change from 37°C to 15°C while they are susceptible to such changes from 15°C to 5°C (Wales and White, 1959 as quoted by Jones, 1969; Quinn et al., 1968). Chilling diluted ram semen to 5°C in 2.5 hours resulted a higher rate of recovery of motile frozen thawed ram spermatozoa than cooling to 5°C in 45 minutes (Patt and Nath, 1969). Fiser and Fairfull (1986) measured the survival of spermatozoa and demonstrated that rapid cooling of extended semen from 30°C to 15°C had little or no effect on spermatozoa survival before or after freezing. Rapid cooling of extended semen from 30°C to 10°C, 5°C, or 0°C was accompanied by a progressive decrease in the percentage of motile cells and of intact acrosomes before freezing, and a decrease in percentage of motile spermatozoa after freezing. In a fertility test, Fiser et al. (1987) obtained pregnancy rates of 73%, 67% and 80% respectively, following AI with the same semen processed by three methods of cooling and freezing:

1. Slow cooling from 30°C to 5°C before freezing in straws at a cooling rate of -20°C per minute to -100°C followed plunging to liquid nitrogen.

2. Slow cooling, but freezing on dry ice.

3. Rapid cooling from 30 to 16 and then further chilling and freezing as was explained for the first method.

2.4.2. Freezing rates:

Ram spermatozoa did not survive well when frozen at a rate of 1°C per minute and

recovery of ram spermatozoa was better for semen frozen at faster rate (Patt and Nath, 1969). Fiser and Fairfull (1984) assessed the effect of varying the concentration of glycerol from 0 to 16% on the survival of ram spermatozoa at increasing rates of cooling (1°C to 100°C) per minute or by directly plunging of spermatozoa in 0.5 ml straws into liquid nitrogen. After thawing at 39°C for 30 seconds, they obtained the best survival rates when the glycerol concentration was 4% to 6% and the rates of freezing ranged from 10°C to 100°C per minute. However, following direct plunging of straw to liquid nitrogen no spermatozoa survived. Colas and Courot (1977) reported that freezing rates of -10°C to -15°C per minute provided similar lambing results (33.3% and 35.6% respectively). These reports were in contrast with the earlier report of Hill et al. (1959) who found that slow rates of freezing gave better results than faster rates of freezing for ram semen frozen in ampoules.

2.5. The importance of thawing conditions on the revival of frozen thawed ram spermatozoa:

The importance of thawing temperature and thawing solution arises because injury to the spermatozoa occurs at both freezing and thawing stages (Salamon, 1968). He thawed pelleted frozen ram semen in solutions containing 2.6% or 3.1% sodium citrate at temperatures of 40°C, 30°C, and 15°C with percentage of motile spermatozoa 45.8, 44.1, and 20.8 respectively. He concluded that thawing frozen semen at 40°C was better than 30°C and that poor motility was obtained when semen was thawed at 15°C.

Salamon and Brandon (1971) examined the effect of the composition of the thawing solution on the survival of ram spermatozoa frozen by the pellet method. They froze the ram semen with a diluent (1:4) consisting of 166.5 mM raffinose, 68 mM sodium citrate, 15% egg yolk, and 5% (v/v) glycerol. They found that thawing solutions improved the survival rate of the ram spermatozoa frozen by pellet. The best survival rate was obtained with thawing solution containing 388.5 mM fructose, and inositol or glucose both at 210 mM concentration, each combined with 40 mM sodium citrate. Salamon and

Visser (1972) found that pellet frozen ram spermatozoa revived poorly when thawed without a thawing solution. They introduced a thawing solution which contains 300 mM Tris and 55.5 mM fructose when glucose had been used as the sugar for pre-freezing dilution. However, Evans and Maxwell (1987) for semen pellet-frozen in Tris-glucose egg-yolk diluent proposed a thawing solution containing 3.63 g tris (hydroxymethyl) aminomethane, 1.00 g fructose, and 1.99 g citric acid (monohydrate) to 100 ml distilled water.

Colas and Guerin (1981) introduced a new method for thawing frozen ram semen in the laboratory. They centrifuged semen after thawing and extended it in skim milk for storage at 15°C until utilised on the farm 2 to 6 hours later. They found that the fertility and prolificacy of synchronised ewes were slightly higher following AI with treated semen than with untreated semen (52.4% vs 44.2% and 155.0% vs 148.0% respectively). Thus the redilution and packaging of spermatozoa in the laboratory facilitated the use of frozen semen in practice.

2.6. Methods of packaging ram semen for freezing:

Three methods of packaging ram semen for freezing storage were described by Memon and Ott (1981).

2.6.1. In Glass ampoules:

Glass ampoules containing 0.5 to 1 ml, and aluminium packets, have been described for bull semen storage. Optimal recovery of bull sperm frozen in 1 centimetre cube ampoules followed an average freezing rate of 1°C to 2°C per minute (Smith and Polge, 1950). Salamon (1968) froze diluted ram semen in ampoules by slow freezing (placing them into an alcohol bath at -20°C) or rapid freezing (plunging directly into a dry ice-alcohol mixture at -79°C. He concluded that rapid freezing in ampoules (-79), gave poorer recovery of motile spermatozoa (16.2%) than slow freezing in ampoules (32.9%).

2.6.2. In Pellets:

Pellets are made by dropping extended semen onto the surface of dry ice. This method was used to freeze ram semen by Salamon (1968) who froze pellets either in liquid

nitrogen or on dry ice obtaining motile spermatozoa 8.8% and 34.5% respectively. His results showed that pelleting directly into liquid nitrogen was unsatisfactory while freezing pellets on dry ice was superior. Salamon (1971) examined the fertility of ram spermatozoa following pellet freezing on dry ice at -79°C and in liquid nitrogen vapour at -140°C. He obtained lambing percentages of 48.2% and 44.9% respectively. The author recommended pellet freezing of ram semen at -79°C. A pellet volume of 0.1 to 0.3 ml which is equal to 3 to 8 drops was suggested as optimal (Evans and Salamon, 1987).

2.6.3. In Straws:

Polyvinyl chloride straws containing 0.25 to 0.50 ml of extended semen have been recommended by Colas (1975). Reports suggest that ram semen can be frozen successfully in straws with generally similar fertility to semen frozen in pellets (Visser and Salamon, 1974; Graham et al., 1978a). However, Maxwell et al. (1980) reported that spermatozoa survived freeze-thawing procedure better when frozen as pellets than in straws.

Watson and Martin (1975b) found several advantages in the use of plastic straws for freezing. At a straw volume of 0.25 ml no semen is more than 1 mm from the surface. When crystallisation is initiated it spreads rapidly from the nucleation point in both direction along the straw. Because of the large surface area in relation to the volume of the straw, the latent heat of fusion can be removed rapidly, and a freezing rate approaching linearity can be achieved. It is therefore possible to produce an exactly specified freezing rate within the sample. Evans (1988) predicted that attention would turn more to the use of straws when selling semen from breeding centres became more common, with the consumer need for ready identification which can be met by the straw. A report by Hunton et al. (1987) indicated that semen frozen in straws was more motile than pelleted semen, but that, following intrauterine insemination, the difference in fertility was not significant (58.4% and 50.3% for pellet and straw respectively).

PART THREE

Methods for administering artificial insemination.

1. Methods for artificial insemination of ewes:

1.1. Vaginal insemination:

This method was originally developed in Russia as a simple method of insemination (Lopyrin, 1971 as quoted by Evans, 1988). Tervit et al. (1984), using freshly diluted semen obtained lambing rates of 64% for vaginal (blind) insemination and 69% for cervical insemination. Maxwell and Hewitt (1986) obtained similar pregnancy rates using cervical and vaginal insemination techniques (39/99 v 41/101). The Vaginal insemination required little skill, involved less handling and stress to ewes, and the speed of insemination was greater. However, the same authors obtained lower conception rates using frozen ram semen, with pregnancy rates of 8%, 14%, and 28% for vaginal, cervical, and intrauterine insemination respectively. Rival et al. (1984) reported vaginal insemination to be inferior to the other methods when insemination was done at 14 hours after the detection of oestrus, with a non return rate 15% lower than other methods.

1.2. Cervical insemination:

This method of insemination is currently used for both freshly diluted and frozen ram semen. However, generally low fertility has been reported following cervical insemination with frozen-thawed ram semen. Salamon (1967) obtained a 6% lambing rate. Salamon and Lightfoot (1970) found that the concentration of motile spermatozoa in the inseminate affected lambing percentage. Of the ewes inseminated, 43.8% and 23.7% lambed following insemination with concentrated ($1.5*10^9$/ml) and diluted ($0.5*10^9$) semen respectively. Deep cervical insemination, involving cervical traction, resulted in significantly lower fertility than normal cervical insemination (23.9% versus 37.1%). Two insemination gave a higher lambing rate than a single insemination (38.8% versus 22.6%). The best treatment was two cervical inseminations with 0.1 ml of concentrated ($1.5*10^9$ motile spermatozoa/ml) semen, with 61.9% of inseminated ewes lambing.

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commercial artificial insemination programme. In this, the ewes were positioned in dorsal recumbency with their hindquarters elevated. The vagina was dilated using a duck-billed speculum; the cervix was retracted using forceps; and an inseminating instrument was manipulated through the cervical canal. Uterine penetration was achieved in 82% of the ewes. This technique depends heavily on the expertise of the inseminator.

1.3. Intrauterine insemination:

Killeen and Gaffery (1982) developed a technique for intrauterine insemination with the aid of a laparoscope and Killeen et al. (1982) achieved a non-return rate of 69% and a fertility rate of 50% in a field test using $60*10^6$ frozen thawed spermatozoa.

Maxwell et al. (1984a;b) showed that intrauterine insemination of frozen semen by laparoscopy at 60 hours after progestagen sponge removal and PMSG injection was as effective as cervical insemination of fresh semen 55 hours after synchronisation treatment. The percentage of ewes pregnant when slaughtered after 100 days were 33/46 (71%) and 19/35 (54%) for cervical and intrauterine insemination respectively. The intrauterine insemination technique was more efficient than cervical insemination because of the much smaller dose of semen required (0.02 ml, containing $20*10^6$ spermatozoa).

Kerton et al. (1984) found no advantage using intrauterine insemination for fresh semen, obtaining conception rates of 56%, 53%, and 31% after cervical, intrauterine, and vaginal insemination respectively.

Haresign et al. (1986) examined the possible application of intrauterine insemination in field conditions in UK using frozen thawed semen. They inseminated ewes cervically using chilled and frozen semen and by the intrauterine route using frozen semen, and achieved lambing rate of 94.7%, 48.3%, and 36.7% respectively. Although the authors were in favour of intrauterine insemination, the lambing rate, using cervical insemination, was greater. In addition, the embryonic losses were higher using intrauterine insemination than with cervical insemination. The ewes lambing as percentage of ewes pregnant on day 19 was 100% for cervical but only 73.4% for uterine insemination. Findlater et al. (1991) investigated the potential application of the technique in commercial

sheep flocks. They obtained a conception rate of 90% following intrauterine insemination using fresh semen. The optimum time for intrauterine insemination was found to be between 54 and 60 hours after sponge removal and PMSG injection. However, the conception rate using frozen semen was between 51 to 69% which showed the freezing damages could not entirely be compensated for with intrauterine insemination. In the UK, the intrauterine technique was used on some 10,000 ewes in 1990 (McKelvey and Dingwall, 1991), it is an invasive procedure, requiring a veterinary applicator and expensive equipment which restricts its use. Recent figures from Texel Breed Society Record (1992) showed an average lambing rate of 45.1% with a range of 17% to 76% was obtained following intrauterine insemination in members flocks by laparoscopy using frozen-thawed ram semen. As the result of low fertility, they have reverted to the use of chilled ram semen up to 6 hours after collection (Alwyn Phillips, personal communication). For welfare consideration too, alternative are required. Evans (1988) and McKelvey and Dingwall (1991) regard laparoscopic insemination as a stop-gap technique until better techniques for cervical insemination are developed.

2. Transport of spermatozoa in the reproductive tracts of ewes:

The reproductive tract of the ewe is comprised of its vagina, cervix, uterus and oviducts.

a) The vagina is not an appropriate biological environment for spermatozoa. In the human, the immediate escape of spermatozoa to the cervix from the vagina has a biological advantage (Overstreet and Katz, 1981 as quoted by Overstreet, 1983).

b) Cervix, the second part of the reproduction tract, acts as a important reservoir from which migration of spermatozoa to the uterus and fallopian tubes continues for a prolonged period after mating (Mattner, 1966). Quinlivian and Robinson (1969) estimated that one hour after cervical insemination a considerable cervical population of sperm was established. Spermatozoa number in the cervix declined gradually by 12 hours, while it increased in the uterus and in the fallopian tubes by 24 hours. This indicated an important

role of cervix as a sperm reservoir supplying the uterus and fallopian tubes. The authors envisaged that the isthmus acts as a reservoir for the ampulla in much the same way but they did not determine the proportion of the time needed for establishment of a cervical or uterine reservoir or the time required for sperm to swim to the oviduct of the ovulated ewes.

The ampullary-isthmic junction of the oviduct is the fertilisation site of the ovine egg (Hunter, 1986). He stated that eggs are transferred by means of ciliary and myosalpinageal activity to this place within 15 to 30 minutes of ovulation. At this stage their fertilisable life is relatively short, and sperm penetration must occur within 6 to 8 hours of ovulation (Hunter, 1986). Other reports suggest that sheep and cow eggs have effective lifespans of 12 to 18 hours (Thibault and Wintenberger-torres, 1967; Killeen and Moore, 1970).

The crucial role of spermatozoa transport from the site of deposition in the female reproductive tract to the site of fertilisation becomes apparent when AI is used. Poor adjustment of the time of service causes a great reduction in conception rate.

As far as sperm passage through the female reproductive tract is concerned, there are two interpretations:

1. It has been claimed in cows that spermatozoa reach the site of fertilisation within a few minutes of mating, through the release of oxytocin and increase in the motility of the uterus (VanDemark and Hay, 1954). The entry of spermatozoa into the oviducts 15 minutes after insemination (Overstreet and Tom, 1982) and into the isthmus within 90 minutes (Overstreet et al., 1978 as quoted by Overstreet, 1983) was confirmed following a series of experiments on sperm transport and egg fertilisation in rabbits. It is perhaps relevant that rabbits ovulate shortly after coupling. Experiments with rabbits by Overstreet and Tom (1982) proposed that rapid transport might be initiated by the seminal plasma, since artificial insemination of nonmotile spermatozoa in seminal plasma was followed by rapid transport after 15 minutes of insemination. Rapid transport never occurred when nonmotile sperm were inseminated in saline. Therefore rapid sperm transport does not rely

on the motility of sperm cells, but must be due to passive transfer.

2. Dziuk (1973) as quoted by Hunter (1985) and Hunter et al. (1980) rejected the idea of rapid transport of spermatozoa through the reproductive tract of sheep and cattle which ovulate spontaneously some 20 and 30 hours, respectively, after the onset of receptivity to the male. Hunter et al. (1982) showed by surgical experiments on sheep that 8-10 hours were required after mating for sufficient spermatozoa to enter the oviducts to ensure fertilisation. He suggested that spermatozoa were transported to the site of fertilisation more rapidly at the end of oestrus than at the onset. This was presumably due to a slow initial build-up of sperm numbers by increasing contractile activity of the female tract close to the time of ovulation. Hunter et al. (1980) reported that, for ewes mated at the onset of oestrus the eggs were not fertilised if transection of the oviduct was carried out 4 or 6 hours after mating, whereas 30% and 100% of the eggs were fertilised after transection at 8 and 10 hours, respectively. The transport of viable spermatozoa in relation to the time of ovulation was examined by Hunter et al. (1982). They divided experimental ewes into two groups. The first group of ewes was mated at the onset of oestrus and the second group was mated 24 hours later. 8 hours after the second mating fertilised egg(s) were counted. 20% of egg(s) in the first group and 94% of egg(s) in the second group were fertilised.

Hunter et al., (1983a;b) measured the distribution of viable spermatozoa in the caudal oviduct, before and at the time of ovulation by tying off the caudal 1:5 to 2.0 cm along the oviduct. All ewes were mated at the beginning of oestrus. 10 to 27 hours later, the oviduct was transected. The majority of spermatozoa were found in the caudal 1.5-2.0 cm of oviduct until one hour before ovulation. From then until ovulation time they were released to move towards the fertilisation site. The conclusion from this investigation was that, the spermatozoa were held in the caudal section of the oviduct before ovulation. At ovulation the sperm was activated and released under the influence of ovarian hormones.

The more recent work of Hunter (1985) indicated that a minimum period of six to

eight hours was required for a functional population of spermatozoa to be established in the oviducts after natural mating in sheep and cattle. He transected the oviduct from the uteri of ewes at specific intervals after mating having placed double ligatures of braided silk at the uterotubal junction to prevent any further passage of spermatozoa. The ewes were slaughtered after ovulation and their eggs were flushed from the oviducts at autopsy and examined for fertilisation by phase contrast microscopy. His report suggested that the fertilising population of spermatozoa spent most of the pre-ovulatory interval in the oviduct, and that the caudal portion of the oviduct (the isthmus) rather than the cervix, acted as the functional sperm reservoir.

To attempt to improve the transport of frozen thawed ram spermatozoa through the ewe genital tract Lightfoot and Salamon (1970a); Salamon and Lightfoot (1970) used oxytocin to stimulate cervical contractile activity. They found that oxytocin treatment lowered the lambing percentage of ewes (14.9% for control and 4.5% for oxytocin injected ewes respectively). However, the concentration of spermatozoa in the inseminate was the major factor influencing the proportion of ewes lambing.

El-Gaafary (1987) supplemented diluted semen with prostaglandin to improve the fertility of ram semen. His results indicated that supplementation of ram semen with PGF2α during chilled storage at 5°C for three days at levels ranging from 150 and up to 600 µg/ml improved spermatozoa motility and did not have any adverse effect on acrosome morphology. But insemination of ewes with chilled ram semen supplemented with prostaglandin F2α did not improve lambing percent (33.3% and 35.0% respectively for 0 µg/ml and 600 µg/ml prostaglandin PGF2α). Insemination with frozen semen supplemented with a combination of prostaglandins (F2α and E2) improved the mean fertility by about 14.5%, but the differences were not significant. The lambing percent was 16.3% and 32.3% respectively for control and treated semen with prostaglandin.

3. Methods of assessment of semen.

3.1. *In vivo* assessment:

3.1.1. Fertility:

An ideal method of assessment of semen would be simple, rapid and objective with a high correlation with the ability of semen sample to cause conception in the ewes. But fertility itself depends on both male and female components, spermatozoa are only partially responsible for the outcome of the fertility. As an alternative, to waiting for ewes to lamb, the non-return rate (NRR) which is the failure of ewes to return to oestrus within 17 to 35 days, following insemination was employed by Salamon and Lightfoot (1970). In this respect Martin and Watson (1976) found that non-return rates were positively correlated with birth rates and provided a more rapid means of comparing the fertilising ability of semen samples.

3.2. *In vitro* assessments:

3.2.1. Motility:

Motility is important for spermatozoa transport in female genital tract (Lightfoot and Restall, 1971). They showed that motility was the main requirement for spermatozoa to penetrate between the deeply divided mucosal folds of the cervix, immotile spermatozoa did not penetrate.

In vitro, the proportion of motile spermatozoa is usually determined by visual microscopic appraisal. Although, this method of assessment is rapid and easy, it Is subjective. However, post-thaw motility assessment of bull semen was shown to be positively correlated with the non-return rate (Linford et al., 1976). On the contrary, Watson and Martin (1976) observed that ram semen diluted, cooled, and stored for 24 or 48 hours had a significant reduction in fertility while motility remained indistinguishable from the diluted controls.

Computer techniques have been employed by Dott (1975) to measure the proportion of motile spermatozoa in bull semen by the application of the image-analysis computer scans. The computer can count either the total number of spermatozoa or the number of immotile spermatozoa. Dott and Foster (1979) described a method for obtaining an objective assessment of motility which can be applied to semen or diluted

semen of bull, ram, and stallion. They used a specially constructed slide in which the base was a permeable membrane, so that a suspension of spermatozoa could be examined under controlled conditions with a microscope and the area change frequency measured with the image-analysis computer. Recently, the results of motility assessment by five individual persons were compared with a computer-assisted (HTM-200 motility analyser) by Tuli et al. (1992). They found that the motility analyser provided greater consistency than the individual persons.

A spectrophotometric procedure for measuring equine spermatozoa motility and ram spermatozoa motility objectively, was described by Jasko et al. (1989) and Suttiyotin et al. (1992). This method is reviewed in the literature to Chapter seven.

3.2.2. Morphological assessments:

Laboratory assessment of spermatozoa routinely includes live-dead staining which differentiates between live and dead sperm cells in semen smears (Mann and Mann, 1981). The dead spermatozoan is eosinophilic and appears red coloured. It is distinguishable from non-eosinophilic (live) as explained by Dott and Foster (1972). Acrosome morphological assessments of spermatozoa by giemsa staining of semen smears which revealed disruption of the acrosome in frozen ram spermatozoa was described by Watson (1975) and Tasseron et al. (1977).

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ATP is both carrier and donor of 'high-energy bonds', the basic source of energy for any living cell including the spermatozoa (Zanariev, 1985 as quoted by Grudova, et al., 1988). Wishart and Palmer (1986) inseminated poultry with the semen from 24 cockerels, and shown that the fertility was correlated positively with sperm motility (r=0.82) and with ATP content (r=0.76). The morphological integrity of the spermatozoa, measured subjectively by light microscopy, was also found to be correlated with fertility (r=0.67). Grudova et al. (1988) found a positive correlation between the motility and the ATP content of frozen-thawed ram semen. The ATP content of a cell is therefore likely to indicate its potential for activity unless its accumulation is a result of inability to utilise the stored energy. In motile sperm cells, the latter assumption is highly unlikely.

Sephadex G-15 filtration method has been suggested by Graham et al. (1978b) as an objective method of assessing semen quality, by determining the exact number of dead and live spermatozoa of bull semen. The technique involves the movement of spermatozoa through a short column of sephadex. Only motile spermatozoa pass through the column, even after repeated washing. Further investigation of sephadex G-15 separation of motile and nonmotile spermatozoa by Land et al. (1980) showed a G-15 sephadex column (40 to 120 μm) to be less effective when bovine sperm were extended in skim milk or homogenised whole milk rather than in egg-yolk citrate. The latest application of the G-15 sephadex filtration method was to remove abnormal and nonmotile bull sperm from an entire ejaculate (Graham and Graham, 1990).

3.2.4. *In vitro* fertilisation test:

Fertilisation of zona-free hamster or rabbit eggs has been used as a test for capacitation of spermatozoa from other species: for rat and mouse, (Handa and Chang, 1976); guinea-pig (Barros and Herrera, 1977); stallion (Padilla et al., 1991); and ram (Slavik, 1987).

CHAPTER TWO

MATERIALS AND METHODS

General materials and methods:

Introduction:

Semen collection, assessment and immediate dilution took place in the Pen-y-Ffridd Field Station where facilities for these have been established. The semen was transported in a vacuum flask at 30°C to the Animal Biochemistry laboratory 2 miles away for further experiments.

1. Sheep and handling method:

1.1. Rams:

Four shearling Cambridge rams, two experienced Cambridge rams and two Llyn shearling rams were provided from the U.C.N.W. College Aber farm flock, together with two draft Cambridge ewes to act as teasers. The rams and ewes were maintained at pasture except when housed for the collection of semen.

1.2. Training of rams:

The Rams was trained to serve into an artificial vagina of the type described by Emmens and Robinson (1962). The Rams were trained over several weeks in the presence of a teaser ewe confined in stocks. Rams were encouraged to mount the teaser ewe in front of a worker as described by Duff (1984). Care was taken to ensure the friendly contact between the rams and personnel working with them. Once training was completed, the rams readily mounted the teaser, allowing semen to be collected into a prepared artificial vagina (I.M.V., Rue Clemen Ceau, L,AIGIE, France).

2. Collection, handling and dilution of semen:

Before collections were made the A.V. was washed thoroughly, dried and assembled. All equipment used to collect and handle semen, including slides,coverslips, syringes, collection glasses, needles and test tubes were washed thoroughly and stored for use in an oven at 37°C. Semen collected by artificial vagina was transferred immediately into the field laboratory which was maintained at 25°C and placed in a water bath at 30°C. Semen was usually diluted immediately after collection 1:2 with Tris-glucose egg-yolk

diluent which was prepared fresh on the same day. The constituents of extender solution used for chilled storage (1.3.1.) and for frozen storage (1.3.2.) were usually similar to those described by Evans and Maxwell (1987). Alternative diluents are described as appropriate in the protocol of experiments.

2.1. Composition of extender for chilled storage:

Tris egg-yolk diluent:

Tris (Tris[hydroxymethyl] aminomethane)	3.634 g
Citric acid (Monohydrate)	1.99 g
Fructose	0.50 g
Egg-yolk	14.0 ml (v/v)

Distilled water was added to make 100 ml of solution which stirred by a magnetic stirred (Gallenhamp Magnetic Stirrer Hotplate 400). pH was adjusted using a pH meter (Electronic instruments limited pH Meter 7020) to 6.8 by the addition of a few drops of diluted hydrochloric acid.

2.2. Composition of extender for frozen storage:

Tris (Tris[hydroxymethyl] aminomethane)	4.36 g
Citric acid (Monohydrate)	2.38 g
Glucose	0.6 g
Egg-yolk	18 ml (v/v)
Glycerol	6 ml (v/v)
Penicillin	100,000 (IU)
Streptomycin	100 mg

Distilled water was added to make 100 ml of solution. pH was adjusted as described in section 2.1.

3.Cooling and freezing of diluted semen:

The test tubes containing diluted semen were transferred to a 300 ml glass beaker containing water at 30°C up to the level of semen. The beaker was placed in a temperature

controlled refrigerator to cool down to 5°C at a cooling rate of 0.20°C per minutes over 1.5-2.0 hours (El-Gaafary, 1987).

Two methods of freezing ram semen were generally used:

3.1. Freezing semen by the pellet method:

A block of dry ice (solid carbon dioxide, -79°C) with a smooth surface was prepared. Appropriate holes to contain 0.1-0.3 ml or 2-6 drops of semen, were engraved in the surface of dry ice using a sterilised plastic pipette. For the freezing operation, the beaker in which the semen had been cooled was transferred from the refrigerator to a separate beaker containing ice-water to prevent any increase in the temperature of the semen. Immediately, 3 drops of cooled semen were pipetted into each hole on the dry ice surface using cooled pipettes. Pellets were allowed to freeze for 4-5 minutes. Three pellets were placed together inside cooled plastic tubes which were transferred into liquid nitrogen. Semen resulting from different experimental treatments were stored separately in clearly marked goblets in the liquid nitrogen refrigerator (Evans and Maxwell, 1987).

3.2. Freezing semen in straws:

Cold semen at 5°C was loaded into 0.25 ml French straws (IMV, Rue Clemen Ceau, L,AIGIE, France) by applying suction to the plugged end of the straw. This end of the straw sealed after contacting with semen. At the other end of the straw a small air gap was produced using a plastic comb, and this end was dipped into polyvinyl alcohol powder. Finally, the straw was dropped into water at 5°C to form the second seal. Immediately after filling and sealing, the straws were frozen in the cell freezer (Cell Freezer R204 Planer Products Ltd) using a freezing rate of -20°C per minute to -120°C and then plunged into liquid nitrogen (Evans and Maxwell, 1987).

4. Thawing of frozen semen:

4.1. Thawing of pellets:

2-3 pellets were removed from liquid nitrogen with forceps previously cooled in liquid nitrogen, and placed in a clean dry glass test tube. This was transferred into a water bath at 37°C and shaken for a minimum of 20 seconds until the pellet melted. In some

experiments a thawing solution with a ratio of 1:1 (volume of pellets:volume of thawing solution) was used.

The composition of the thawing solution was that described by Evans and Maxwell (1987), which is as follows:

Tris (Tris[hydroxymethyl] aminomethane)	3.634 g
Fructose	1.000 g
Citric acid (Monohydrate)	1.990 g

Glass-distilled water was added to 100 ml.

The thawed semen was used for further examination or artificial insemination of ewes.

4.2. Thawing of semen frozen in straws:

The straws were removed from the liquid nitrogen and immediately dipped in a water bath at 37°C. The straws were shaken in the water for a minimum of 20 seconds to ensure the semen was thawed and then wiped dry with a clean towel. The polyvinyl alcohol powder plug was cut off using scissors and the straw either placed into an inseminating gun designed for artificial insemination in sheep or used for laboratory examination (El-Gaafary, 1987).

5. Assessment of semen:

5.1. Volume and appearance:

The volume of each ejaculate was measured by aspiration into a graduated 5 ml warm plastic syringe. The semen was judged for normality by colour and smell by the method of Gordon (1984).

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Table 1: Scoring system for wave motion.

Score	Class	Description
5	Very good	Dense, very rapidly moving waves. Individual sperm cells cannot be observed. 90% or more of the spermatozoa are active.
4	Good	Vigorous movement, but the waves and eddies are not so rapid as for score 5. About 70-85% of sperm are active.
3	Fair	Only small, slow moving waves, individual spermatozoa can be observed. 45-65% of sperm cell are active.
2	Poor	No waves are formed, but some movement of spermatozoa is visible. Only 20-40% of sperm cells are alive, and their motility is poor.
1	Very poor	Very few spermatozoa (about 10%) show any signs of life, with weak movement only.
0	Dead	All spermatozoa are motionless.

5.3. Progressive linear motility:

Two ml 0.9% saline was warmed in a 37°C water bath. One drop of semen was added to the test tube using a warm Pasteur pipette and mixed by gentle agitation. After two minutes a drop of mixed semen was taken and placed on a warm (37°C) slide. The drop was covered by a warm coverslip and observed under the X40 objective of microscope. The percentage of individual spermatozoa which moved in straight lines with a normal vigorous swimming motion across the field of vision was estimated.

5.4. Concentration:

The number of spermatozoa per ml of semen sample was estimated visually as described by Gordon (1984), or by using a haemocytometer cell count in conjunction with measuring the optical density of a 1:200 dilution of semen (Evans and Maxwell, 1987).

5.4.1. Haemocytometer procedure:

The semen was diluted 1:200 in a red cell counting pipette with 3% saline. After

shaking the pipette for 3-5 minutes between the thumb and forefinger, 4-5 drops was discarded from the stem of the pipette. Diluted semen was applied to the haemocytometer chamber and allowed to settle for 5 minutes. The number of spermatozoa were then counted in 5 large squares (each containing 16 small squares). The convention of recording those in contact with the top and right side line and omitting those touching the bottom plus left side line was observed. Spermatozoa per ml of sample was calculated as follows:

Spermatozoa count/ml = Total count in 5 large squares * 10^7.

5.4.2. Optical density procedure:

0.05 ml of undiluted semen was added to 10 ml 0.9% saline or 2.9% sodium citrate to make a 1:200 dilution (Evans and Maxwell, 1987). The optical density of diluted semen was read at 439 nm in a spectrophotometer (Cecil instruments Ltd) using a saline or citrate blank and the sperm concentration estimated by reference to a standard curve.

5.5. Abnormality and Live/Dead ratio:

The percentage of abnormal spermatozoa and the live/dead ratio were assessed from a stained semen smear (MLC Veterinary Service Technical Bulletin, 1982).

Material:

Nigrosin/Eosin stain was prepared from the following constituents:

1. Nigrosin	10.0 g
2. Eosin	1.68 g
3. Sodium citrate	2.90 g
4. Distilled water to	100 ml

The components were dissolved in a boiling water bath for 20 minutes, cooled, and then filtered.

Method:

One drop of semen was mixed with seven drops of warm stain and incubated at 32°C for five minutes in a water bath. After incubation a smear was prepared by placing a

drop of the mixture on a warm microscope slide and gently drawing out a film of the mixture across the slide using the edge of another slide as a spreader. The smear was allowed to air dry and was examined under the high power magnification (X 1000) of a microscope. At least 100 spermatozoa were examined, and the percentage of abnormal cells was recorded. The live/dead ratio was obtained by recording those cells which were alive in the stain (white) and those which were dead (pink).

5.6. Acrosome staining techniques:

Acrosomal damage of spermatozoa was assessed using giemsa stain and light microscopy by the method of Watson (1975).

Materials:

Three solutions were needed for this technique.

1. Buffered formol saline was made by mixing 2.9 g tri-sodium citrate dehydrate with 0.1 ml of a 40% solution of formaldehyde in 100 ml deionised water (Dott and Foster, 1975).

2. Buffered giemsa solution was prepared by grinding 3.8 g solid giemsa stain (Allied Chemicals National Aniline Division) with 375 ml absolute methanol (Argrade). The stain was stored at 37°C for one week. During this period it was shaken for a few minutes each day.

3. Sorensen,s M/15 phosphate buffer, pH 7.0. To make this solution 3.05 g anhydrous di-sodium hydrogen phosphate (Na2H2 Po4) was added to 1.95 g sodium dihydrogen orthophosphate (NaH2 Po4) and diluted fifteen times with distilled water (Dawson et al., 1986).

For staining fixed smears of spermatozoa, 3.0 ml of this solution was diluted with Sorenson,s M/15 phosphate buffer (2.0 ml), and distilled water (35 ml).

Method:

A drop of diluted semen was smeared on a pre-warmed slide and dried in a current of warm air. The smear was fixed by immersion in buffered formol saline for 30 minutes and then washed in running tap-water for 15 minutes. After being dried, the slide was

immersed in the buffered giemsa solution for 90 minutes and then rinsed in distilled water and dried. Acrosomes were examined with a microscope equipped with an oil-immersion lens at a magnification of 1350X using a green light filter coded VG4 (Carl Zeiss, Jena).

5.7. Osmotic resistance test:

The integrity and functional activity of the sperm membrane was assessed by the method of Jeyendran et al. (1984).

Materials:

A hypo-osmotic (150 mOsmo) of equi-molar proportions of fructose and tri-sodium citrate was prepared by dissolving 7.35 g tri-sodium citrate.2H2O and 13.51 g fructose in 1000 ml distilled water.

Method:

1 ml of hypo-osmotic solution was added to 0.1 ml of diluted or undiluted semen and incubated at 37°C. After 30 minutes, the spermatozoa were observed with phase contrast microscopy and the proportion of sperm cells exhibiting tail swelling was determined.

5.8: Swim-up test:

A swim-up procedure to measure sperm vigour is described in Chapter seven.

5.9: ATP test:

Determination of ATP content and measurement of rate of oxidation of glucose were carried out as described in Chapter eight.

6. Artificial insemination of ewes:

Ewes were synchronised for artificial insemination using progestagen impregnated intra-vaginal sponges followed by an injection of pregnant mare serum gonadotrophin (PMSG) (MLC sheep yearbook, 1987). 12 days after insertion, the sponges were withdrawn from the vagina and then each ewe was injected with 400 IU PMSG. Single insemination was carried out 56 hours after sponges withdrawal. For inseminations a built-in light speculum was inserted into the vagina to locate the cervix of the ewe which

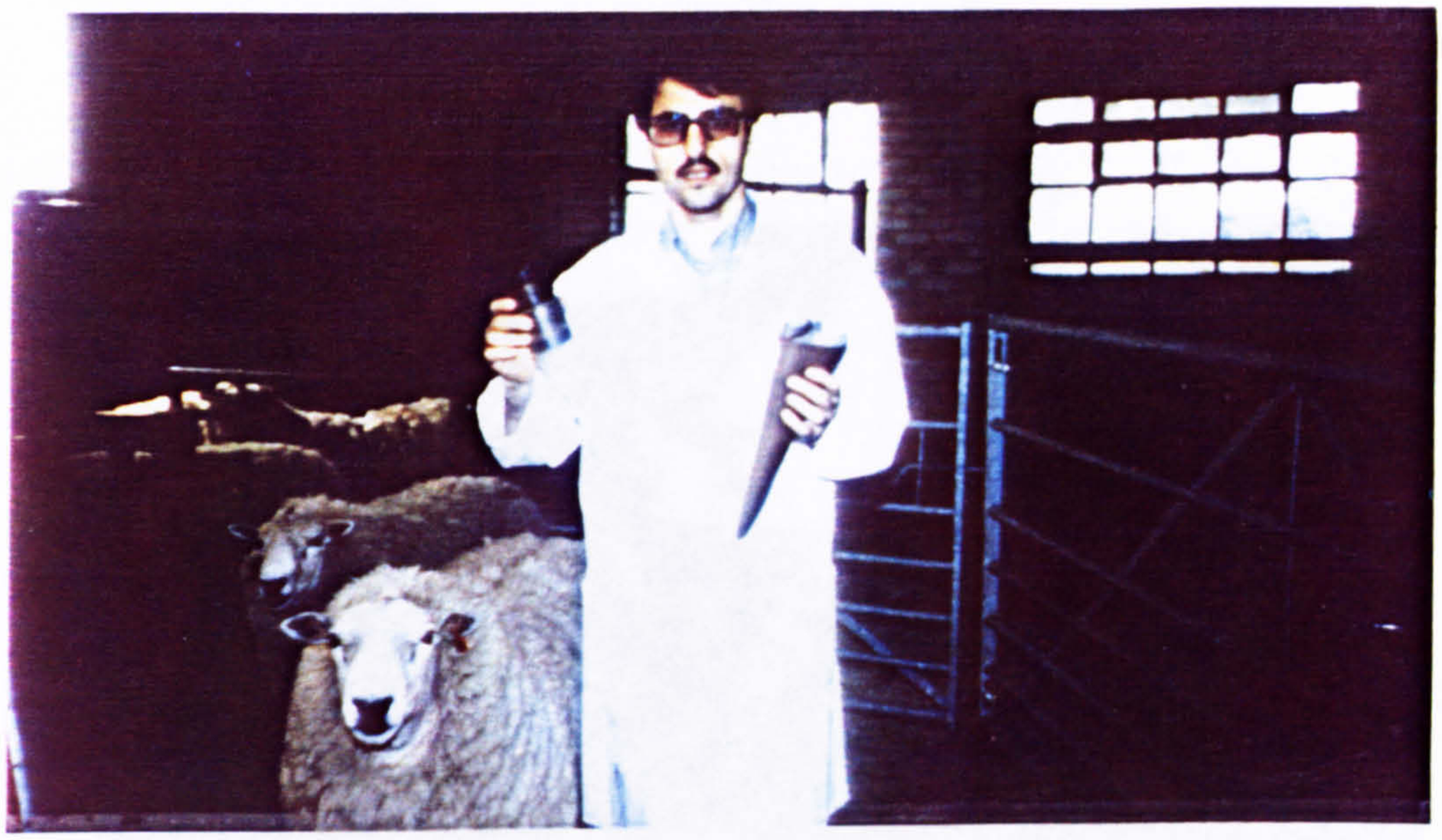
was restrained over a bale of straw. For semen packed in straws, liquid or frozen-thawed semen was discharged into the first fold of the cervix using the inseminating gun. For pellet frozen semen, the thawed semen was sucked into straws and inseminated as described for straws. Artificial insemination was carried out on synchronised ewes under farm condition inside a trailer parked adjacent to the sheep pens.

7. Data analysis:

For Chapter three the spreadsheet Quattro Pro was used. Other data were analysed by analysis of variance (General linear model), regression, correlation and Chi-square test, as indicated, using the Minitab statistical package.

A: The collection of rams for artificial insemination.

B: Ram serving into an Artificial vagina.

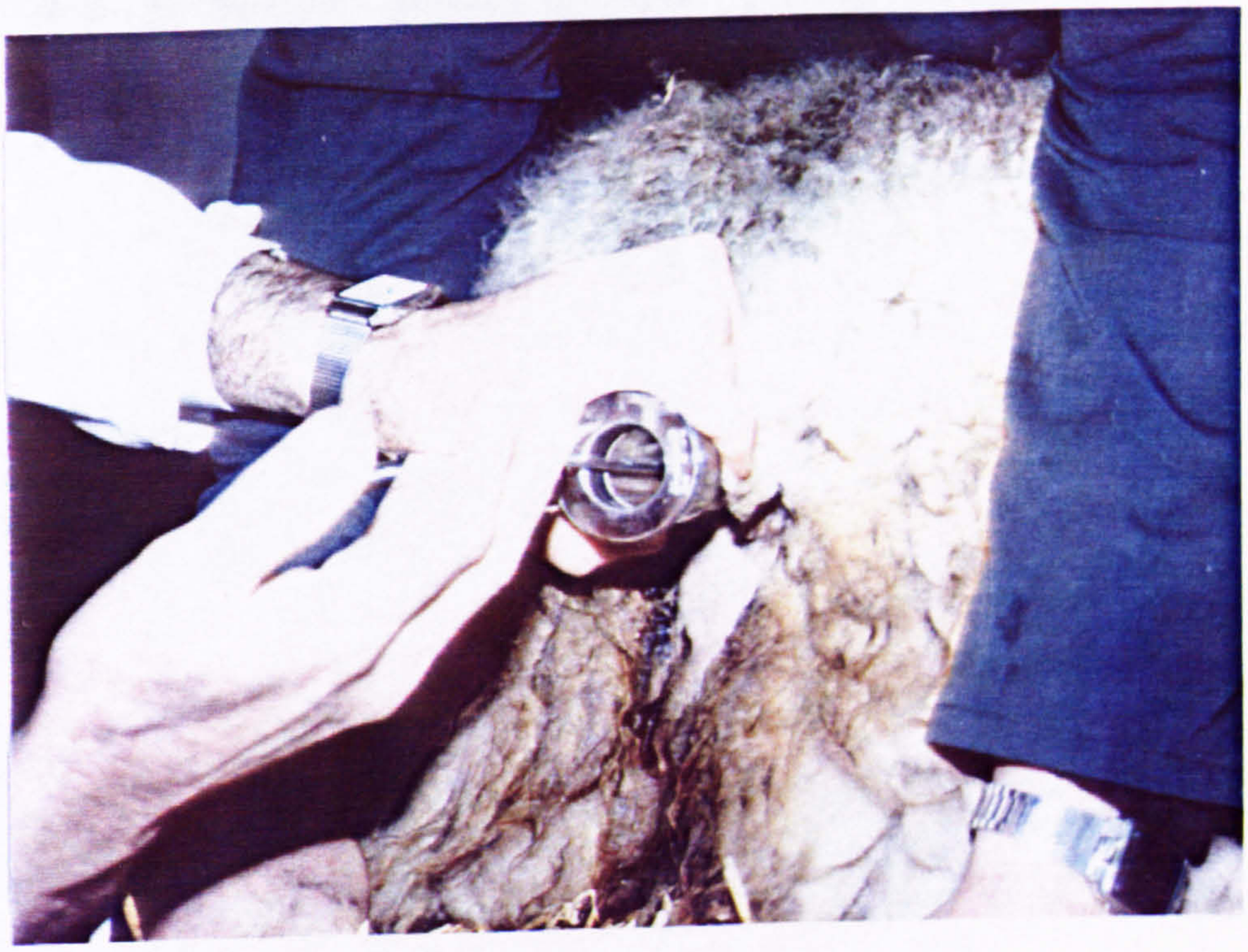


C: Artificial vagina, rams and operator.

D: Dilution of semen in a water bath.

E: Storage of frozen semen in a Liquid Nitrogen Refrigerator.

F: Cervical insemination of ewes.



CHAPTER THREE

AN EVALUATION OF THE POTENTIAL IMPACT OF ARTIFICIAL INSEMINATION WITHIN SHEEP GROUP BREEDING SCHEMES.

Introduction:

Group breeding schemes were introduced as a strategy to exploit sheep genotypes for improved flock performance (Owen, 1988). In sheep breeding programmes, small average flock size is a problem and group mating schemes have been advocated as a means of increasing effective population size and selection intensity (Notter and Hohenboken, 1990). They allow several small-scale breeders to combine into an effective breeding structure, involving the selection of superior males and females for use in a nucleus flock to promote within-breed improvement (Owen, 1987). They can also be used as a vehicle for the efficient incorporation of foreign genetic material (rams, semen or embryos) into adapted sheep breeds (Owen, 1987).

In the UK, commercial group breeding schemes have been established for the improvement of several sheep breeds including the Welsh Mountain (Ap Dewi, 1990), the improvement of prolificacy and milk yield in the Cambridge breed (Owen 1984), and for the improvement of prolificacy and ewe milking ability in the Lleyn breed (Owen, 1987). The Lleyn is a white faced lowland breed originating from the Lleyn peninsula in North Wales. The Lleyn breeding group has a total of 1200 breeding ewes distributed in flocks varying in size from 20 to 250. A nucleus flock, based at the University farm (University College of North Wales, Bangor) has 100 mature ewes, including twelve 3 years old ewes, one from each member, on loan annually to the nucleus. The members of the scheme benefit by being able to select rams from the nucleus. The nucleus is maintained partly by self replacement of ewe lambs (30%) at one year old. The selection of replacements for the nucleus is based on an index which is weighted on prolificacy and ewe milking ability (Owen, 1987).

The aim of the current study is to investigate the potential effect of AI on sheep breeding schemes with particular reference to the nucleus flock of the Lleyn Group Breeding Scheme.

Materials and methods:

This study is based on a simulated sheep flock, similar to the nucleus flock of the Lleyn Group Breeding Scheme, containing 100 mature ewes. A computer spreadsheet model was developed, as shown in appendix A, using the programme Quattro Pro. The method described by Falconer (1981) was used to evaluate genetic progress within the flock with regard to lamb weaning weight. The application of this methodology for the Lleyn group was described by Williams and Owen (1980). Response per annum was calculated as:

Response per annum (kg) = $((i1*h^2*Ps)+(i2*h^2*Ps))/(L1+L2)$

Where:

i1 = Selection intensity for males.

i2 = Selection intensity for females.

h^2 = Heritability of lamb weaning weight.

Ps = Phenotypic standard deviation of lamb weaning weight.

L = Generation length for rams (L1) or for ewes (L2) was calculated as:

$L = 0.5 ((N/Ns) -1 + 2A)$

Where N = The total number of ewes in the flock or the total number of rams used for mating annually for the calculation of L1 and L2 respectively.

Ns = The number of ewe lambs or rams selected per annum for the calculation of L1 and L2 respectively.

A = Age of ewes or rams when their first progeny are produced (i.e. age at first lambing for ewes) for the calculation of L1 and L2 respectively.

Values of 0.20 and 1.50 were used for h^2 and Ps respectively (Williams and Owen, 1980).

Within the model, the ewes to ram ratio and the percentage of ewes that lambed were altered to predict the annual genetic gain for lamb weaning weight (kg). The ewes to ram ratio was varied from 10 to 100 and the percentage of ewes lambed ranged from 40 to

100. Ewes to ram ratios of between 10 and 40 for natural mating, and between 50 and 100 for artificial insemination, were assumed.

Results:

The result of simulation are shown in Table 1, Figure 1, and Figure 2.

Table 1: The effect of the ewes to ram ratio and the percentage of ewes that lambed on the response to selection for lamb weaning weight (kg) in a self contained lowland flock.

Ewes to ram ratio	Percentage of mated or inseminated ewes that lambed							
	30	40	50	60	70	80	90	100
10	0.047	0.069	0.083	0.120	0.143	0.160	0.175	0.188
20	0.094	0.109	0.120	0.155	0.177	0.193	0.206	0.219
30	0.115	0.129	0.139	0.172	0.196	0.209	0.223	0.234
40	0.129	0.141	0.151	0.184	0.205	0.220	0.233	0.242
50	0.139	0.151	0.160	0.192	0.213	0.228	0.242	0.253
60	0.146	0.158	0.166	0.199	0.220	0.234	0.248	0.260
70	0.154	0.164	0.172	0.204	0.225	0.240	0.253	0.264
80	0.158	0.169	0.177	0.209	0.230	0.242	0.257	0.269
90	0.163	0.173	0.182	0.214	0.234	0.248	0.261	0.273
100	0.166	0.177	0.185	0.217	0.237	0.252	0.265	0.276

Figure 1 shows that the genetic gain increased as the ratio of ewes to ram increased. Genetic gain also increased with increasing lambing rate (Figure 2). The results in Table 1 show that the rate of genetic improvement for lamb weaning weight can be increased by the use of AI compared with normal mating. Lamb weaning weight increased by 0.119

and 0.088 kg/annum from a ewes to ram ratio of 10 to 100 at 30% and 100% of ewes lambed respectively. It was also shown that the annual response in lamb weaning weight increased with an increasing proportion of ewes that lambed. However, the lambing rate had a greater effect on the annual response in lamb weaning weight than did altering the ewes to ram ratio. The increases in lamb weaning weight from the lowest response of 0.047 (at 30% lambing and a ewes to ram ratio of 10) were 0.141 kg and 0.119 kg when the lambing rate was altered from 30% to 100% and the ewes to ram ratio was altered from 10 to 100 respectively.

Discussion:

In natural mating systems, the ewes to ram ratio is normally 10:1 for synchronised ewes and 30:1 for unsynchronised ewes (Colas 1975; Smith et al., 1978; Boland et al., 1981 and Maxwell, 1984). The use of AI results in a higher ewes to ram ratio. In the present study a ewes:ram ratio of 100:1 increased genetic response for lamb weaning weight compared to a lower ewes:ram ratio (30) provided that between 60% and 70% of the inseminated ewes lambed (assuming 90% lambing from naturally mated ewes). Hackett et al. (1979) compared the effect of artificial insemination and natural mating on reproductive performance of sheep during anestrous season in an intensive system and found that fertility (the number of ewes lambing per 100 ewes mated or inseminated) was 47% and 33% for AI and naturally mated ewes respectively. McKelvy et al. (1991) estimated that on a daily basis a ram, giving several ejaculates, might cover up to 60 ewes using cervical insemination with fresh semen. In the fertility trial reported in Chapter four a lambing percentage of 73% was obtained. With a lambing rate of 70%, and ewes to ram ratio of 10, the annual response for lamb weaning weight would be 0.143 kg. This would increase to 0.237 kg by altering the ewes to ram ratio to 100. It has been reported that between 50 to 60 million ewes are inseminated worldwide annually (Agricultural Training Board Note 130.3). If the use of AI, by altering the ewes to ram ratio, increased the weaning weight of each lamb by 0.094 kg annually, this would yield an annual increase of 5640,000 kg more lamb at weaning to the sheep industry worldwide.

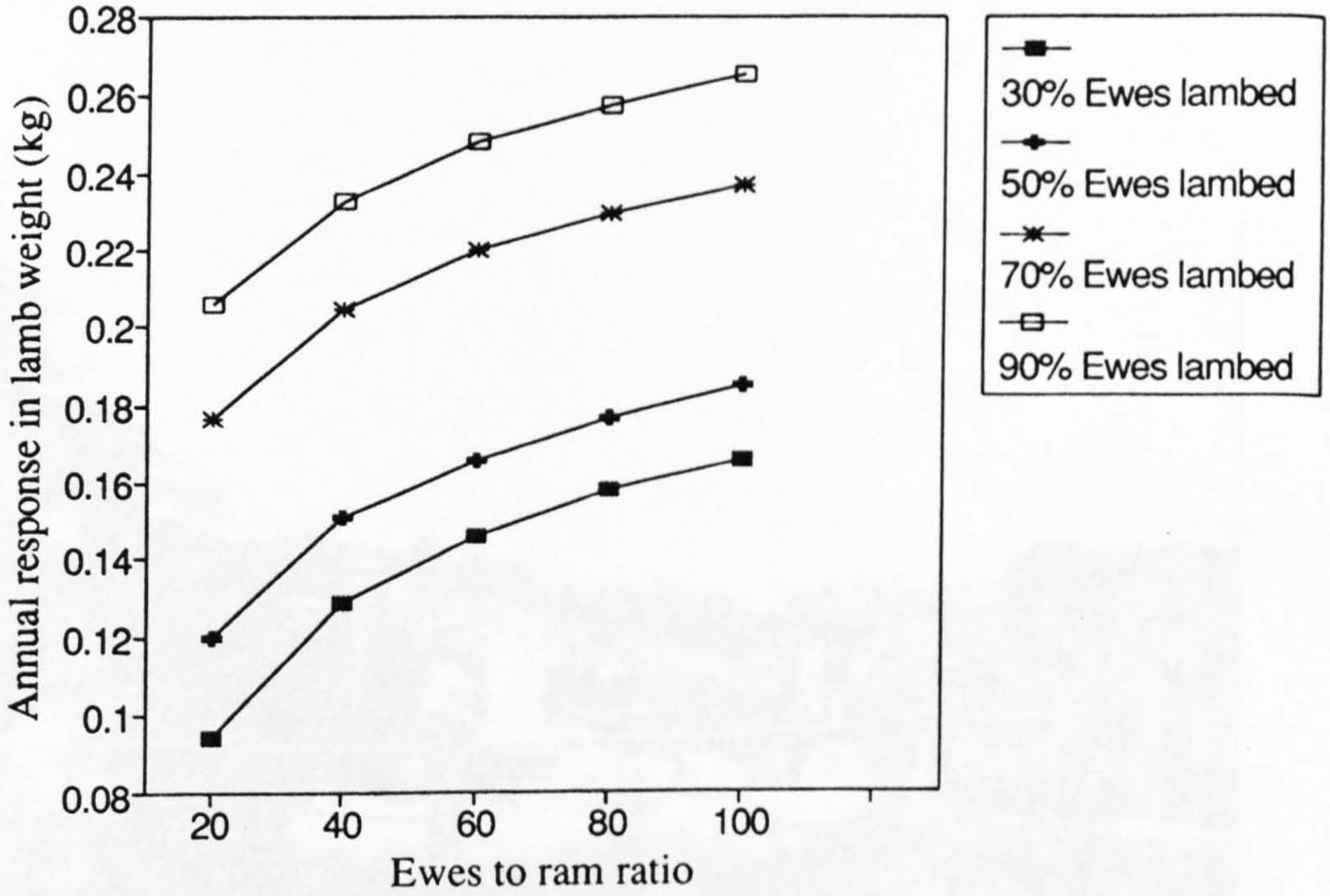


Figure 1: The effect of ewes to ram ratio and the proportion of ewes mated or inseminated lambing on the annual response in lamb weaning weight (kg).

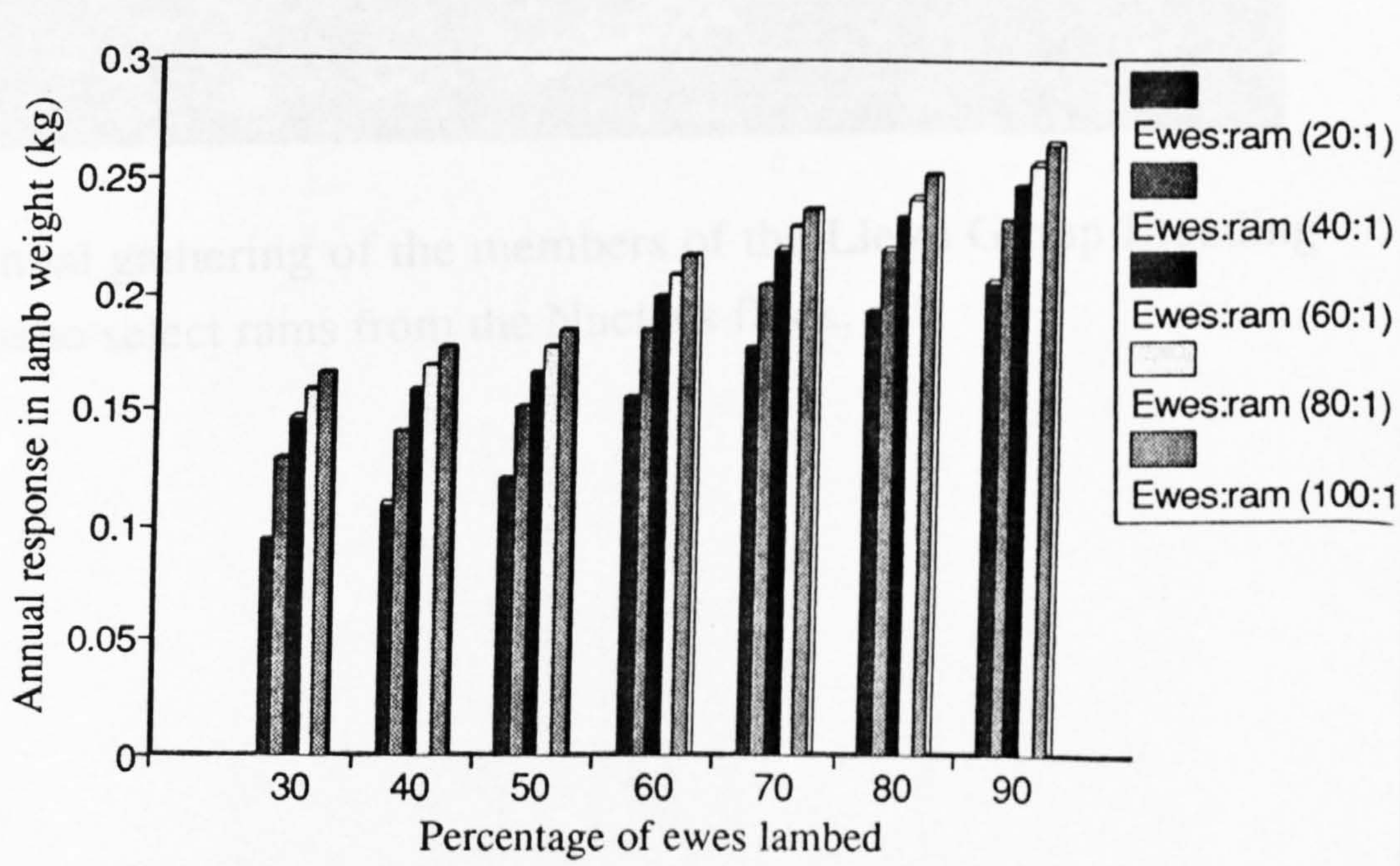


Figure 2: The effect of proportion of ewes mated or inseminated and ewes to ram ratio on the annual response in lamb weaning weight (kg).

A: Annual gathering of the members of the Lleyn Group Breeding Scheme to select rams from the Nucleus flock.

CHAPTER FOUR

THE EFFECT OF GLYCEROL IN THE SEMEN DILUENT ON RAM SPERMATOZOA

***In vitro* test:**

Introduction:

The purpose of including glycerol in the freezing medium is to preserve spermatozoa during ice crystal formation. The cryoprotective effect of glycerol during the crystallisation phase of freezing is well established (Mazur, 1965, 1966, 1970). However, glycerol has a toxic effect on ram spermatozoa even at a concentration of 4% (Colas, 1975).

Slavik (1987) investigated the effect of glycerol addition on the ability of fresh ram spermatozoa to penetrate zona-free hamster eggs. He concluded that glycerol at concentrations of 2% upwards negatively affected sperm motility even before freezing. Prolongation of semen storage in a medium with glycerol did not improve penetration ability. The author attributed the lower conception rate after insemination with deep-frozen ram semen to the induction of the acrosome reaction before the sperm reached the surface of zona pellucida in ewes (Crozet and Dumont, 1984 as quoted by Slavik, 1987). Alternatively, its harmful effect has been attributed to interference in the carbohydrate metabolism of spermatozoa by entering glycolysis through dehydrogenation to dihydroxyacetone phosphate (Mann and White, 1957). Mountford (1989) examined the effect of glycerol on ram spermatozoa at concentration of 4% v/v using an oxygen electrode, and 14-C labelled glucose. He showed that glucose oxidation, oxygen consumption, and lactate production all decreased in the presence of glycerol (Table 1).

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motility of glycerol treated semen declined more rapidly, while non glycerol treated semen had a constant decline of motility during incubation.

Motility was significantly reduced with increasing time of incubation from 0 to 5 hours for both treatment. The effect of glycerol was to increase the rate of decline at the 10% level of significance ($P \leq 0.066$). The interaction shows that the effect of glycerol increased with time. If the declines in spermatozoa motility are assumed to be linear with duration of incubation, the mean motilities could be expressed by the following equation:

$$\text{Motility\%} = 72.0 + 4.2\,G - 7.2\,T - 3.6(G*T)$$

Where G indicates the presence of glycerol in the buffer (%) and T is the time of incubation in hours.

The effect of glycerol addition was to increase the rate of decline in sperm motility by 50%. The motility data, together with the regression lines for sperm incubation with and without glycerol are shown in Figure 1. The significance of the parameters is shown in Table 3.

Table 2: The effect of the presence of glycerol 4%·(v/v) on the motility of semen over a five hours incubation period at 30°C.

Time of incubation (hours)	Concentrations of glycerol (%) 0	4	Means(SE)
0	73.2 (2.1)	72.0 (2.5)	72.6 (0.6)
1	65.0 (5.2)	69. 2 (1.2)	67.1 (0.6)
2	54.2 (3.8)	56.0 (4.0)	55.1 (0.6)
3	50.6 (3.8)	45.0 (6.3)	47.8 (0.6)
4	47.6 (2.2)	34.0 (5.3)	40.8 (0.6)
5	27.0 (0.0)	20.0 (5.4)	22.4 (1.0)
Means	52.7	49.2	50.9
(Lsd=6.5)*1	(Lsd=20.3)*2		(Lsd=10.1)*3

*The least significant differences (Fisher's method) for comparing means for time and glycerol are shown in the Table 2 were *1=Lsd value for time of incubation, *2=Lsd value for the glycerol effect and *3=Lsd value for interaction between glycerol treatment and time of incubation with the significant level ($P \leq 0.05$).

Table 3: Regression of motility on presence of glycerol (G) and time of incubation (T).

Predictor	t-ratio	probability
Constant	24.11	0.000
Glycerol	1.01	0.319
Incubation	-6.19	0.000
Glycerol*Time	-2.35	0.022

B. Spermatozoa survival:

The mean data for percentage of live spermatozoa in the five occasions are presented in Table 4.

The survival of spermatozoa declined more rapidly in the presence of glycerol with increased time of incubation ($P \leq 0.001$). Assuming the declines in sperm survival to be linear with duration of incubation, the mean percentage of live spermatozoa could be described by the following equation:

$$\text{Live\%} = 72.4 + 7.73\,(G) - 5.88\,(T) - 6.39\,(G*T)$$

Where G indicates the presence of glycerol in the buffer (%) and T is the time of incubation in hours.

The effect of glycerol addition was to increase the rate of at which the spermatozoa died (Table 4). The significance of the parameters is shown in Table 5.

Table 4: The effect of the presence of glycerol 4% v/v on the percentage of live spermatozoa over a five hours incubation period at 30°C.

Time of incubation	Concentrations of glycerol (%)		
(hours)	0	4	Means (SE)
0	75.7 (0.7)	76.0 (1.0)	75.8 (0.4)
1	65.2 (2.3)	69.3 (0.7)	67.0 (0.4)
2	57.2 (4.4)	64.3 (0.7)	60.3 (0.4)
3	52.0 (2.7)	38.0 (1.1)	46.0 (0.4)
4	53.0 (4.0)	30.3 (0.3)	43.3 (0.4)
5	----	----	----
Means	60.6 (0.2)	55.6 (0.2)	58.1
(Lsd=4.6)*1	(Lsd=13.3)*2		(Lsd=7.1)*3

*The least significant differences are shown in the above table as was described in Table 2.

Table 5: Regression of live sperm (%) in the presence of glycerol (G) and with time of incubation (T).

Predictor	t-ratio	probability
Constant	30.41	0.000
Glycerol	2.13	0.042
Incubation	-6.04	0.000
Interaction (G*I)	-4.31	0.000

Fertility test of chilled ram semen:

The aim of this experiment was to assess the effect of glycerol on the fertility of chilled stored semen expressed as lambing percentage.

Materials and methods:

50 Welsh mountain ewes, 3-4 years old were synchronised in oestrus using progesterone impregnated sponges, as described by Evans and Maxwell (1987). Twelve days after insertion the sponges were pulled out from 44 ewes. Two teaser rams were introduced to stimulate oestrus. Cervical insemination, was carried out as described by Evans and Maxwell (1987).

Semen was collected using A.V. from 5 Cambridge rams in November 1989. samples with good wave motion > 4 were pooled together to be used for AI The concentration of spermatozoa in the pooled semen, was $1.7*10^9$ per ml. The semen was divided into two equal parts. One sample of semen was extended 1:1 with Tris egg-yolk diluent as described in materials and methods. The second sample of semen was extended with the same diluent but including 8% glycerol. Both samples of semen were allowed to cool to 14°C over 1.5 hours. The semen was sucked into plastic straws. These were sealed and stored at 16°C until used for insemination 2 hours later, 56 hours after sponges withdrawal.

Results:

The overall lambing percentage was 66 (Table 4).

Table 4: The fertility of chilled ram semen, with and without 4% (v/v) glycerol.

Type of semen diluent used	No. of ewes inseminated	No. of ewes lambing	Lambing %
With glycerol	22	13	59
Without glycerol	22	16	73
Total and means	44	14.5	66

Table 5: Chi-square analysis of fertility results.

Comparison	d.f.	Chi-square values for fertility data
With glycerol vs Without glycerol	1	0.910*

*This is significant at the 66% level.

*For $P \leq 0.05$ Chi-square is 3.84.

The lambing percentage was lower for the glycerol containing semen (59% cf 73%) than when the glycerol free diluent was used (Table 4). However, the effect of glycerol (4%) treatment on the percentage of lambing was not significant at the 5% level (Table 5).

Discussion:

In this series of tests glycerol had a consistently deleterious effect on the survival and activity of ram spermatozoa. The effect was reflected in the lambing data of the ewes, although the difference in fertility obtained did not reach an acceptable level of significance.

The semen used for insemination was 3-5 hours old at the time of use. But it is likely that any metabolic effect of glycerol would be checked at the temperature of chilling, and that following insemination the active sperm would soon become separated from the fluid in the inseminate.

The lambing rate following insemination using chilled ram semen was 73% which was in harmony with the result of Maxwell et al. (1980) who, in a trial, using chilled semen previously diluted in reconstituted skim milk without glycerol obtained 82% lambing rate.

The small increase of spermatozoa motility immediately after the addition of glycerol was possibly due to entry of glycerol into the metabolic pathway of glycolysis producing more energy for the cells (Mann and White, 1957).

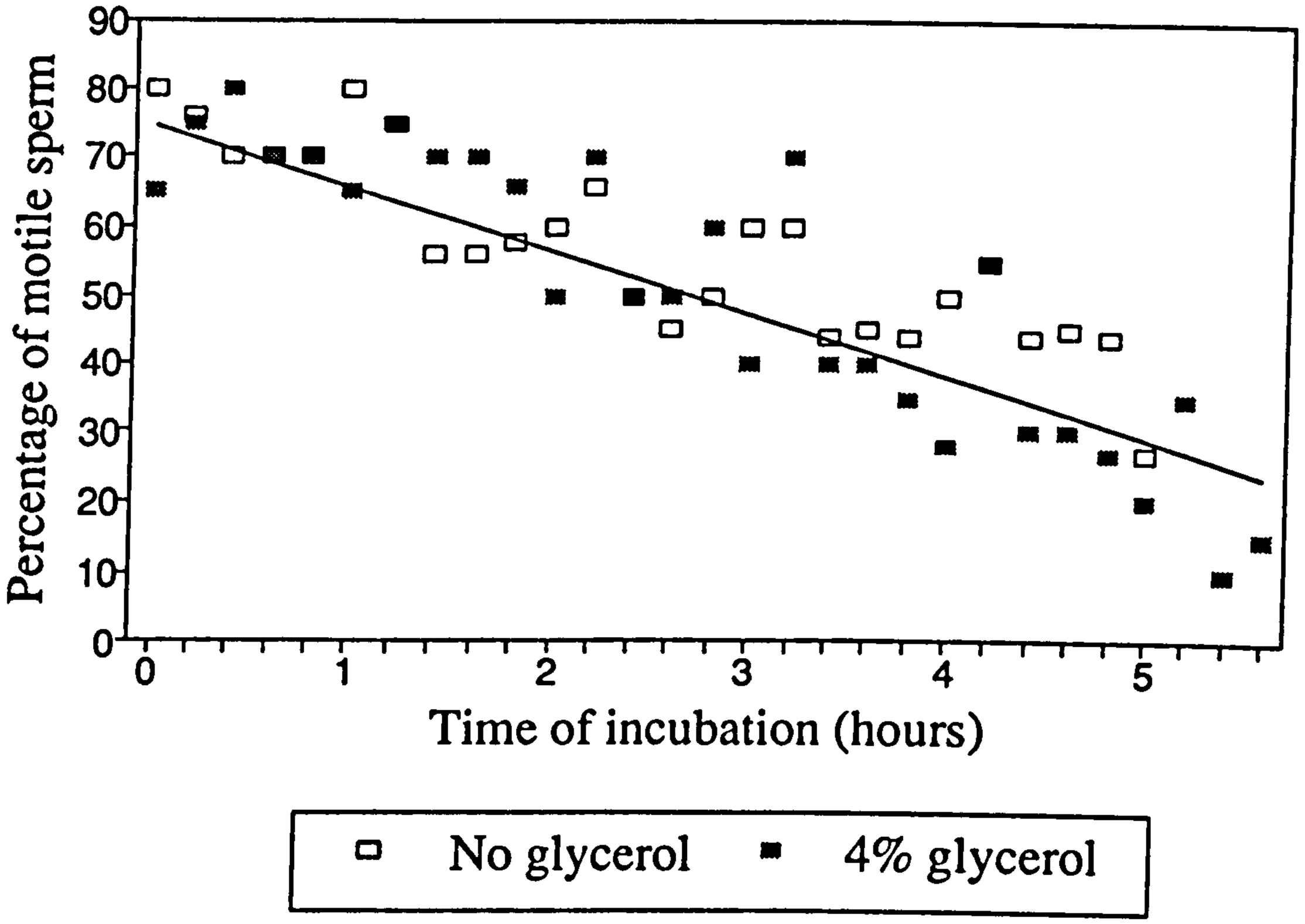

Figure 1: The effect of glycerol (4%) in diluent on the motility of ram spermatozoa.

CHAPTER FIVE

STUDIES ON FREEZING METHODS FOR RAM SEMEN USING CONVENTIONAL PROCEDURES

Section one:

Studies on the temperature of freezing ram semen

In this section a series of experiments to establish the optimum cooling routine for freezing ram semen using 0.25 ml French straws, is reported.

Introduction:

Ram semen is usually chilled slowly to 5° before it is frozen. It is then cooled rapidly to an intermediate temperature before being plunged into liquid nitrogen (Quinn et al., 1968; Evans and Maxwell, 1987).

The effect of intermediate temperature of freezing on ram semen quality has been studied mainly by Salamon (1970, 1971). His results showed that temperatures within the range -79°C to -140°C, were equally effective in permitting the recovery of active spermatozoa following pellet freezing. Colas (1979) reported that the optimum intermediate point of freezing was -79°C for freezing ram semen in 0.25 ml French straws.

The use of either slow rates of cooling or rapid rates of cooling for freezing ram semen was discussed by Colas and Courot (1977); Colas (1983b).

Materials and methods:

Collection, assessment of semen characteristics and dilution

Semen was collected by means of an artificial vagina from 4 Cambridge and 2 Welsh Mountain rams during the non-breeding season 'Spring'. The semen samples were pooled and sperm concentration was estimated by spectrophotometer to predict the number of sperm/ml of diluted semen. The dilution was carried out (1:2) semen:diluent as described in Chapter two.

Table 1: The composition of diluent used.

Tris (hydroxymethyl aminomethane)	4.361 g
Citric acid (monohydrate)	2.400 g
Glucose	0.600 g
Egg yolk	18.0 ml
Glycerol	6.0 ml
Distilled water to	100.0 ml

The Tris, citric acid and glucose were dissolved in distilled water; the glycerol and egg yolk were then added and the final volume made up to 100 ml with distilled water. The pH of the diluent was then checked and adjusted to 6.8 using 1 M HCl if necessary.

The final concentration of spermatozoa/ml of the diluted semen samples was usually about $0.7*10^9$. The motility was checked before freezing.

Chilling and freezing:

The diluted semen samples in three replications were transported to the laboratory within ten minutes, keeping a constant temperature of 30°C, and then chilled to 5°C over 2 hours at a rate of approximately-0.17°C per minute. The chilled semen was loaded into three 0.25 ml plastic straws for each replication and frozen in a programmable freezer (Cell Freezer R204, Planer Products Ltd), which operated with liquid nitrogen at a selected rate of freezing to a chosen intermediate temperature, and then stored in liquid nitrogen (-196°C) for at least 24 hours before being thawed and assessed.

Thawing and assessment:

The semen was thawed by dipping the straws at 37°C for 20 seconds and immediately assessed for wave motion, progressive linear motility, live and dead ratio, osmotic resistance test and acrosome damage as described in Chapter two.

Experiment one:

The aim of this experiment was to find the best starting point for freezing of chilled ram semen.

Procedure:

Collection, dilution, transport, assessing, cooling, and loading into the straws were carried out as described in materials and methods. Chilled semen samples (5°C) were loaded into the programmable freezer and programmed to freeze rapidly (-20°C/minutes) to -120°C (control), or to cool slowly (-1°C/minute) to -1°C and then to freeze rapidly (-20°C/minute) to -120°C, or to cool slowly (-1°C/minute) to -5°C and then to freeze rapidly to -120°C.

Results:

The result of *in vitro* assessment of the ram semen sample after thawing is summarised for each treatment in Table 2.

Table 2: Mean and standard errors of post-thaw sperm characteristics for each treatment.

Characteristic	Temperature of starting point of fast freezing (°C)			
	+5	-1	-5	F ratio
W.Motion	2.67 (0.44)	2.33 (0.33)	2.82 (0.17)	0.70
Motility (%)	20.33 (2.96)a	12.33 (0.33)b	19.33 (3.67)a	4.44$
Live (%)	32.00 (2.00)a	26.67 (2.33)b	28.33 (1.67)b	7.05*
Osmotic Resis.	7.67 (1.20)	8.67 (1.20)	7.67 (1.20)	1.26
Acrosome Dam.	47.67 (1.45)	48.00 (2.31)	46.00 (4.62)	0.42

$ $P \leq 0.10$

* $P \leq 0.05$

The percentage of motile and live spermatozoa after freezing was greater when chilled to 5°C rather than to -1°C or -5°C. The probabilities were $P \leq 0.10$ and $P \leq 0.05$ for percentages of motile and live sperm respectively. There were no significant difference between treatments on osmotic resistance or acrosome damage.

Experiment two:

The aim of this experiment was to find an optimum rate of cooling for freezing ram semen.

Procedure:

Collection, dilution, transport, assessment, cooling, and loading into the straws were carried out as described in general materials and methods. Chilled semen samples (5°C) were loaded into the programmable freezer which was programmed to cool dawn to -120°C at rates of -10°C, -20°C and -30°C/minute.

Results:

The result of *in vitro* assessment of the ram semen after thawing was summarised for each treatment in Table 3.

Table 3: Mean and standard errors of post-thaw sperm characteristics for each treatment.

Charcteristic	Rate of freezing (°C/minute)			
	-10	-20	-30	F ratio
W.Motion	2.83 (0.17)	3.17 (0.17)	3.33 (0.41)	0.76
Motility (%)	21.67 (1.20)	19.67 (2.89)	25.33 (5.51)	0.44
Live (%)	36.33 (1.76)	32.67 (2.67)	32.00 (5.03)	0.42
Osmotic Resis.	12.67 (1.20)	13.00 (0.57)	20.00 (4.51)	1.87
Acrosome Dam.	54.33 (5.36)a	45.33 (4.37)b	41.67 (1.67)b	10.82*

* $P \leq 0.05$

The means of post-thaw revival of ram spermatozoa were greater using faster rate of freezing (-30°C). But this was not significant at the 5% level. Acrosome integrity was significantly greater at the faster freezing rate ($P \leq 0.05$).

Experiment three:

The aim of this experiment was to find the critical temperature at which semen is frozen on the survival of spermatozoa.

Procedure:

Collection, dilution, transport, assessing, cooling, and loading into the straws were carried out as described in materials and methods. Semen samples chilled slowly to 5°C were frozen rapidly at -20°C/minute, to -15, -25°C, -35°C, -45°C, -55°C, -65°C, -75°C or

-120°C and held at that temperature for 2 minutes before cooling was resumed to -120°C. The straws were then transferred to liquid nitrogen. This test was done twice and the data was pooled as replications.

Results:

The result of *in vitro* assessment of the ram semen after thawing is summarised for each treatment in Table 4 and illustrated in Figure 1.

Table 4: Mean and standard errors of post-thaw sperm characteristics for each treatment.

Characteristic	Intermediate temperature held for two minutes during freezing of straws (°C)								
	-15	-25	-35	-45	-55	-65	-75	-120	F
W.Motion	1.5b	2.0b	2.5a	2.6a	2.6a	3.0a	2.5a	3.0a	9.1***
S.E.	0.29	0.00	0.29	0.17	0.17	0.00	0.29	0.00	
Motility%	8.0a	8.7a	15.3b	21.3b	16.0b	20.7b	20.0b	21.7b	13.7***
S.E.	0.00	0.88	0.67	0.88	2.08	0.67	0.57	3.18	
LIVE%	22.6a	15.0b	26.3a	20.6a	26.3a	31.3c	27.0a	29.0c	10.3***
S.E.	1.45	2.89	0.33	0.88	2.03	0.67	0.57	2.08	
Osmo.Res.	6.6a	7.3a	10.3a	14.6b	11.3b	13.6b	13.6b	12.0b	8.6***
S.E.	1.45	0.33	0.88	0.33	0.88	0.88	1.20	1.53	
Acros.D.	54.3	51.6	48.0	54.0	51.3	54.3	51.0	41.7	2.2
S.E.	2.03	0.88	1.15	1.15	1.33	2.03	0.57	6.69	

*** $P \leq 0.005$

These show highly significant differences ($P \leq 0.005$) between semen samples staged at -15°C, -25°C and -35°C, and those staged at -45°C or cooler. For motility, survival and osmotic resistance, with semen cooled rapidly to the lower temperature

displaying the better characteristics. There were no significant ($P \leq 0.05$) differences in post-thaw characteristics between those semen samples that were staged between -45°C and -120°C. Cooling only to -25°C or less produced the greater loss of motilities.

Experiment four:

The aim of this experiment was to identify the optimum intermediate temperature to which ram semen should be cooled in straws before storage in liquid nitrogen.

Procedure:

Collection, dilution, transport, assessment, cooling, and loading into the straws were carried out as described in materials and methods. Chilled semen samples (5°C) were cooled at a fixed freezing rate of -20°C/minute to -60°C, -80°C, -100°C and to -120°C, before being plunged into liquid nitrogen (-196°C). This test was done twice and the data was pooled as replications.

Results:

The result of *in vitro* assessment of ram semen after thawing is summarised in Table 5 and illustrated in Figure 2.

Table 5: Mean and standard errors of post-thaw sperm characteristics for each treatment.

	Intermediate temperature of diluted ram semen (°C)				
Characteristic	-60	-80	-100	-120	F ratio
W.Motion.	1.33a	1.66a	3.0b	3.0b	30.18***
S.E.	0.33	0.17	0.00	0.00	
Motility (%)	9.67a	11.33b	14.00c	21.00d	117.22***
S.E.	0.33	0.33	0.57	0.57	
Live (%)	18.33a	22.67a	28.00b	38.00c	46.45***
S.E.	1.67	0.33	0.00	1.67	
Osmotic Resis.	4.67a	5.67a	10.67b	13.67c	28.17***
S.E.	0.33	1.20	0.33	0.88	
Acrosome Dam.	58.33a	55.66a	35.67b	32.33b	31.91***
S.E.	0.88	4.70	0.33	1.20	

*$P \leq 0.05$

***$P \leq 0.005$

There were highly significant ($P \leq 0.005$) differences in the motility and survival of diluted semen samples cooled to intermediate temperature before transfer to liquid nitrogen. The semen in straws plunged into liquid nitrogen after being cooled to -100°C or -120°C was significantly ($P \leq 0.005$) superior to that cooled to -60°C or to -80°C in respect to all the parameters measured. Cooling to -120°C before plunging produced semen with the best characteristics after thawing (Table 5).

Discussion:

The first experiment showed that freezing chilled semen from 5°C produced the best mean characteristics, although the difference in semen quality between temperatures was not significant. In field conditions, it is convenient to chill ram semen by ordinary refrigerator to 5°C. This experiment confirmed that this choice of temperture is soundly based as well as being easy to achieve.

Faster cooling rates to induce freezing (-20°C/minute and -30°C/minute) were less harmful to the acrosome integrity of spermatozoa cells than the slower rates tested (-10°C) and generally also lead to a higher rate of post-thaw revival of spermatozoa. These results agree with the work of Patt and Nath (1969) who showed ram semen did survive better when cooling was speeded up around the freezing temperature (-1°C/min from 5 to -10, -2°C/min from -10 to -18°C, -4°C/min from -18°C to -35°C, and -5°C/min from -35 to -70°C) than compared with semen frozen at the slow and steady rate of -1°C/min. They obtained mean motilities of 71.3% and 51.0% for ram semen frozen at the faster rate of cooling and the slower rate of cooling respectively. Fiser et al. (1987) obtained pregnancy rates of 73% and 67%, after freezing semen in 0.50 ml straws, using a freezing rate of -20°C per minute to -100°C and freezing on dry ice (-79) respectively.

Once freezing had commenced, the sperm survived better if cooled rpidly to below -45°C. Cooling rapidly to the intermediate temperature of -15°C, -25°C and -35°C produced a lower rate of revival of spermatozoa after freezing. This is in agreement with the finding of Watson and Martin (1975b).

Freezing the ram semen to -120°C before it was plunged into liquid nitrogen was beneficial for post-thawing revival of spermatozoa.

Section two:

In this section a series of experiments were carried out using diluents of varying composition for freezing ram semen in 0.25 ml French straws. Results were compared with that obtained using semen frozen as pellets by the method of Salamon (Evans and Maxwell, 1987).

Introduction:

The role of pre-freezing dilution of semen is to protect spermatozoa during cooling and freezing, and to result in the required number of sperm in an insemination volume (Colas, 1975). Various rates of dilution of ram semen were studied by Lightfoot and Salamon (1969a) and Jones and Martin (1965).

Based on the buffer used as a pre-freezing diluent, various pH levels have been recommended as follows: For citrate based buffer a pH of 7.0-7.2 (Mann, 1964), for phosphate based buffer a pH of 6.9 (Watson, 1979); for Tris based buffer a pH of 6.65-6.7 (Fiser et al., 1981) or a pH of 7.0 (Salamon and Visser, 1972).

Glycerol (4%) is commonly used as a cryoprotective agent for freezing ram semen (Evans and Maxwell, 1987; Colas, 1975). The use of various concentrations of glycerol have been reported as optimal. There are shown in Table 6.

Table 6: Recommended concentrations of glycerol (v/v) for methods of freezing used for freezing ram semen.

Author	Glycerol concentration (v/v)	Method of freezing
Fiser et al. (1961)	7%	Ampoules
Salamon (1968)	5%	Pellets
Lightfoot & Salamon (1969a)	2.8-5.6%	Pellets
Salamon & Visser (1972)	4%	Pellets
Colas (1975)	4%	Straws
Fiser & Fairfull (1984)	4-6%	Straws

Colas and Courot (1977) suggested that it would be more logical to add glycerol at 5°C than 30°C, but this was not confirmed by the results of First et al. (1961), and Salamon (1968) found that the best time for the addition of glycerol depended upon the type of sugar in the diluent. The spermatozoa need time to equilibrate with the diluent before undergoing freezing. Optimum equilibration times were suggested to be 5 hours and 2-6 hours by Jones & Martin (1965) and Fiser and Batra (1984) respectively.

Egg-yolk has been mostly considered as a protective agent during chilling (Watson and martin, 1975a), but Lightfoot and Salamon (1969a) and Watson and Martin (1975a) have discussed a possible role for egg-yolk during the freezing procedure. Their results showed that the presence of egg-yolk during chilling gave a marked improvement in the survival of spermatozoa after freezing, ($P \leq 0.005$) and reduced acrosome damage.

Experiment one:

Four rates of dilution of semen, 1:1, 1:2, 1:3 and 1:4 (semen:diluent) were

examined to find the effect of rates of dilution of semen on post-thaw characteristics.

Procedure:

Semen samples were collected from 5 Cambridge rams on Autumn. The dilution of pooled semen samples with Tris yolk glycerol buffer as described in Chapter two was carried out but at different rates of dilution (semen:diluent) from 1:1, 1:2, 1:3, and 1:4. Transporting, chilling, freezing,thawing and assessing were carried out as described in materials and methods of this Chapter.

Results:

The result of *in vitro* assessment of ram semen after thawing is summarised in Table 7 for the different semen dilutions prepared.

Table 7: Mean and standard errors of post-thaw sperm characteristics for each rate of dilution.

	Rate of dilution (semen:diluent)				
Characteristic	1:1	1:2	1:3	1:4	F ratio
W.Motion	2.00	1.83	1.83	2.33	0.62
S.E.	0.00	0.17	0.60	0.17	----
Motility (%)	23.00	22.67	16.67	23.00	1.82
S.E.	1.53	1.45	4.41	1.53	----
Live (%)	34.67	36.67	32.33	34.67	1.19
S.E.	0.90	0.90	3.30	0.90	----
Osmotic Resis.	5.33	3.67	5.67	6.67	1.75
S.E	1.40	0.90	1.2	0.3	----
Acrosome Dam.	59.00	56.67	56.67	57.67	0.62
S.E.	2.1	1.7	0.7	1.2	----

There was no significant ($P \geq 0.05$) effect of dilution rate on post-thaw viability, motility and integrity of ram spermatozoa.

Experiment two:

The aim of this experiment was to find the most appropriate level of pH of diluent for freezing ram semen.

Procedure:

Tris egg-yolk glycerol diluent for freezing was prepared as described in materials and methods of this Chapter. The pH of samples of the diluent were adjusted to 6.00, 6.50, 6.60, 6.70, 6.80, 6.90, and 7.00 by adding few drops of hydrochloric acid (0.1 M), and 7.20, 7.50 by adding few drops of sodium hydroxide (0.1 M). Collection, dilution, transport, chilling, freezing, thawing and assessment were carried out as described in materials and methods of this Chapter. This test was carried out twice in Spring and in Autumn. The data was pooled for analysis.

Result:

The result of *in vitro* assessment of ram semen after thawing are summarised in Table 8 and illustrated in Figure 3 for the effect of different pH of diluent.

Table 8: Mean and standard errors of post-thaw spermatozoa characteristics for pH of diluent.

Characteristic	pH of diluent									
	6.00	6.50	6.60	6.70	6.80	6.90	7.00	7.20	7.50	F ratio
Wave motion	1.50c	2.25b	3.04a	3.04a	3.04a	3.04a	3.16a	2.80a	2.33b	6.66***
S.E.	0.08	0.08	0.13	0.13	0.13	0.13	0.08	0.13	0.10	----
Motility (%)	17.17d	24.67c	32.33b	33.67ab	35.00ab	35.00ab	37.50a	32.00b	27.00c	18.89***
S.E.	0.54	0.67	0.82	0.84	0.85	0.85	0.54	0.85	0.54	----
Live (%)	27.67c	37.50b	39.54b	39.54b	42.54ab	42.21ab	46.50a	38.88b	34.80c	9.05***
S.E.	0.70	0.70	1.02	1.02	1.02	1.02	0.70	0.70	0.70	----
Osmotic Res.	6.83c	10.33b	11.75a	11.75a	11.75a	11.75a	11.75a	10.75b	7.67c	14.05***
S.E.	0.20	0.20	0.27	0.27	0.27	0.45	0.45	0.30	0.30	----
Acrosome D.	47.33a	44.17b	42.46c	42.46c	42.46c	42.46c	41.00c	44.13b	48.33a	5.05***
S.E.	0.45	0.41	0.61	0.61	0.61	0.61	0.41	0.60	0.45	----

***P≤0.005

The results, in Table 8, showed that pH significantly ($P \leq 0.05$) affected post-thaw motility, viability and integrity of ram spermatozoa, but had no significant effect ($P \geq 0.05$) on the response of the cells to the osmotic resistant test. Motility and survival of spermatozoa was maximal between pH=6 to pH=7 and declined at higher and lower pH levels (Figure 3).

6·6

Experiment three:

The aim of this experiment was to find an optimum level of glycerol and egg yolk in the freezing procedure.

Procedure:

Three different Tris yolk glycerol buffers were used as was shown in Table 4 and adjusted to pH 6.8. Collection, assessment, transport, chilling, freezing, thawing and assessment were carried out as described in Chapter two. Pooled semen samples were divided to three portions and sample of each portion was in turn diluted with each buffer at a ratio of one part semen to two parts buffer.

Table 9: The composition of buffers for 2:1 dilution with semen sample.

Components	Buffer one	Buffer two	Buffer three
Tris	4.361 g	4.361 g	4.361 g
Citric acid (Monohydrate)	2.40 g	2.40 g	2.40 g
Glucose	0.60 g	0.60 g	0.60 g
Egg-yolk	18.0 ml	18.0 ml	12.0 ml
Glycerol	9.0 ml	6.0 ml	9.0 ml
Distilled water to	100.0 ml	100.0 ml	100.0 ml

Result:

The results of *in vitro* assessment of the frozen-thawed semen samples are shown in Table 10.

Table 10: Mean and standard errors of post-thawing sperm characteristics for semen diluted with buffers varying in their content of glycerol and egg-yolk.

	Different concentrations of glycerol and egg-yolk (%)			
Characteristic	G6E12 (Buffer.1)	G4E12 (Buffer.2)	G6E8 (Buffer.3)	F ratio
W.Motion	2.50a	1.17b	1.33b	9.50*
S.E.	0.30	0.17	0.17	----
Motility (%)	15.67a	7.00b	10.33b	17.20**
S.E.	1.45	0.57	1.45	----
Live (%)	25.33a	14.67b	19.33b	8.92**
S.E.	2.70	2.60	1.33	----
Osmotic Resis.	6.00a	2.67b	3.00b	10.71**
S.E.	0.00	0.88	0.57	----
Acrosome Dam.	49.33a	64.00b	51.33a	8.99**
S.E.	0.67	3.79	1.33	----

**$P \leq 0.01$

G=Glycerol

E=Egg-yolk

There were significant ($P \leq 0.05$) effects of composition of buffer on post-thaw characteristics of ram spermatozoa. The use of the buffer containing the highest levels of glycerol (6%) and egg-yolk (12%) resulted in the highest percentages of motility, viability,

osmotic resistant and integrity of acrosome after thawing.

Experiment four:

The aim of this experiment was to find the optimum concentration of glycerol and the optimum time for the addition of glycerol on the post-thawing survival of ram spermatozoa.

Procedure:

Collection, assessment and cooling was the same as described for the previous section. The number of ejaculated spermatozoa per ml was $2.4*10^9$. Pooled semen were diluted with three Tris egg-yolk buffers in four replications, either at 30°C or after cooling to 5°C, to give final concentration of 2%, 4% or 6% glycerol. The chilled semen samples were loaded at 5°C into 0.25 ml French straws and frozen to -120°C at a rate of -20°C/minute in the programmable freezer. Thawing and assessment of the semen samples were carried out as already described.

Result:

The mean assessments of the frozen thawed semen for the various temperatures are shown in Table 11.

Table 11: Mean and standard errors of post-thaw sperm characteristics for the effect of various concentrations of glycerol and adding times.

	Concentration of glycerol (%)						
	added at 30°C			added at 5°C			
Charateristic	2	4	6	2	4	6	F ratio
W.Motion	1.87	2.50	3.25	2.37	3.0	3.50	0.24
S.E.	0.12	0.24	0.00	0.20	0.32	0.20	----
Motility%	9.25b	17.75a	22.5a	11.0b	22.50a	22.50a	7.04**
S.E.	1.89	1.49	4.77	1.78	2.10	2.63	----
Live%	22.50b	32.75a	39.50b	26.25b	36.75a	39.50a	7.79
S.E.	2.10	1.55	4.33	1.75	1.65	4.11	----
Osmo.Resis	2.75b	4.00b	7.75a	3.00b	9.00a	6.75a	9.36***
S.E.	0.75	0.40	1.44	0.70	0.70	1.80	----
Acros.Dam.	53.00a	55.00a	40.25b	57.25a	50.75a	37.75c	10.5***
S.E.	4.36	1.25	1.84	0.94	2.69	1.31	----

**P≤ 0.01

***P≤0.005

These show that the glycerol concentration affected semen characteristics significantly (P≤ 0.01). Generally, post-thaw motility and survival was greater at a glycerol concentration of 6%. In the osmotic resistance test 4% glycerol added at 5°C produced the greatest number of undamaged cells during incubation.

The analysis of variance relating to this data is summarised, for the effects of glycerol concentrations in Table 12.

Table 12: Mean and standard errors of post-thaw sperm characteristics for the effect of glycerol.

	Glycerol concentrations (%)			
Characteristic	2	4	6	F ratio
W.Motion	2.12(0.2)b	2.75(0.1)b	3.37(0.2)a	18.00***
Motility (%)	10.12(1.2)b	20.12(1.5)a	22.50(2.5)a	12.00***
Live (%)	24.38(1.4)b	34.75(1.3)a	39.50(2.8)a	14.87***
Osmotic Res.	2.87(0.48)b	6.5(1.0)a	7.25(1.1)a	9.36***
Acrosome Dam.	55.13(2.2)b	53.0(1.6)b	39.00(1.1)a	27.29***

*** $P \leq 0.005$

There was a significant ($P \leq 0.05$) effect of glycerol concentration on the post-thaw semen characteristics. These improved with glycerol increase. Except for progress linear motility in which the increase was not significant at the 5% level. There was a significant ($P \geq 0.05$) reduction in acrosome damage with 6% glycerol comparing with 4% and 2% glycerol (Table 12).

Table 13 shows the effect of time of addition of glycerol on the semen characteristics assessed.

Table 13: Mean and standard errors of post-thaw sperm characteristics for the effect of the time of adding glycerol.

	Temp. of glycerol addition (°C)		
Characteristic	5	30	F
W.Motion	2.54 (0.2)b	2.95 (0.2)a	6.00*
Motility (%)	16.5 (2.3)	18.67 (2.0)	0.98
Live (%)	31.58 (2.6)	34.17 (2.2)	1.24
Osmotic Resistance	4.83 (0.8)	6.25 (1.0)	2.57
Acrosome Damage	40.9 (2.5)	48.58 (2.6)	0.22

* $P \leq 0.005$

These show no significant ($P \geq 0.05$) effect of adding time of glycerol on post-thaw semen characteristics except for wave motion which was greater for glycerol added at 30°C ($P \leq 0.05$).

Experiment five:

The aim of this experiment was to compare the effects of different methods of glycerol addition (at once at 30°C or slowly from 30°C to 5°C), two concentrations of glycerol (4% or 6%), two methods of packaging and freezing in (straws or in pellets), and two lengths of time of equilibration of semen samples with diluent before freezing.

Procedure:

Three Tris egg-yolk buffers containing 0%, 6% or 9% glycerol were prepared. Semen samples were collected from 5 Cambridge rams during autumn. The dilution was

carried out at 30°C by dividing the pooled semen samples into two aliquots. The first aliquot was divided into two portions and diluted 1:2 with the Tris buffers containing glycerol, giving final glycerol concentrations of 4% and 6% in the diluted semen portions. The second aliquot was diluted with the same Tris buffer but containing no glycerol. Glycerol was added slowly to portions of the second aliquot over 2.5 hours of chilling from 30°C to 5°C in three steps at 30°C, at 15°C and at 5°C to make final concentration of 0%, 4% or 6%. The chilled semen samples were further divided to two aliquots. Samples from the first aliquot were frozen immediately either in straws or as pellets as described in Chapter two. The second aliquot was allowed to equilibrate for a further hour at 5°C and then also frozen by the two methods. After one week, semen samples were thawed and motility of spermatozoa was assessed during a 6 hours incubation as described in Chapter two.

Results:

The mean motilities of the frozen thawed semen samples are shown in Table 14, the Anova results in Table 15-16, and the interactions in Appendix B.

Table 14: Mean of motility of ram spermatozoa during incubation up to 6 hours and the effect of equilibration time, methods of packaging, levels and methods of glycerol addition.

		Adding glycerol at once (30°C)				Adding glycerol slowly (2.5 hours)			
		4% glycerol		6% glycerol		4% glycerol		6% glycerol	
Equilibration (hours)	Incubation (hours)	Straw	Pellet	Straw	Pellet	Straw	Pellet	Straw	Pellet
2.5	0.00	34.6(2.4)	38.7(0.7)	40.0(0.0)	38.0(0.0)	26.0(0.6)	34.0(1.2)	30.0(0.0)	29.0(1.0)
	2.00	25.0(0.0)	30.0(0.0)	30.0(0.0)	16.7(0.9)	12.0(0.0)	28.0(1.2)	17.3(1.3)	16.0(0.6)
	4.00	17.0(0.5)	10.7(0.7)	13.0(0.7)	7.7(0.9)	4.0(0.6)	6.0(0.6)	7.3(1.2)	5.3(0.6)
	6.00	5.3(0.3)	4.0(0.0)	5.7(3.7)	3.7(0.3)	1.3(0.3)	3.0(0.0)	2.6(0.3)	1.7(0.3)
Means 3		20.5	20.8	22.2	16.5	10.8	17.7	14.3	13.0
3.5	0.00	33.0(1.0)	40.7(0.6)	35.7(2.3)	33.3(1.7)	33.3(4.2)	26.0(0.6)	31.7(4.4)	26.7(0.9)
	2.00	24.0(1.5)	26.3(0.7)	29.7(2.6)	23.0(0.0)	20.0(2.5)	13.0(1.0)	20.0(0.0)	15.0(0.0)
	4.00	11.0(1.5)	8.0(0.0)	14.7(2.7)	7.3(1.3)	7.7(0.9)	4.3(0.3)	7.7(2.7)	8.0(1.2)
	6.00	3.7(0.7)	3.3(0.3)	2.7(0.3)	1.7(0.3)	2.3(0.3)	1.0(0.0)	2.7(0.3)	2.0(0.0)
Means 3		17.9	19.6	20.7	16.3	15.8	11.1	15.5	12.9
Overall means		19.2	20.2	21.5	16.4	13.3	14.4	14.9	12.9

Table 15: Summary of analysis of variance of sperm post-thawing motility during 6 hours incubation at 3°C.

Source of variation	DF	Percentage of motility 0 hour MS	F	2 hours MS	F	4 hours MS	F	6 hours MS	F
Replications	2	18.08	1.29	0.56	0.04	0.44	0.08	1.27	2.37
A	1	0.75	0.05	18.75	1.31	77.52	14.70**	6.75	12.57**
B	1	616.3	43.8***	752.1	52.4**	221.0	41.90**	33.3	62.1***
C	1	0.75	0.05	21.33	1.49	9.20	1.74	0.33	0.62
D	1	18.75	1.33	3.00	0.21	1.02	0.19	12.00	22.35***
A*B	1	30.08	2.14	44.1	3.07	38.5	7.3*	2.08	3.88
A*C	1	96.33	6.85*	341.3	23.78**	13.02	2.47	2.08	3.88
A*D	1	48.00	3.41	96.33	6.71*	7.52	1.43	0.08	0.16
B*C	1	0.75	0.05	0.33	0.02	6.02	1.14	3.00	5.59*
B*D	1	10.08	0.75	8.33	0.58	11.02	2.09	8.33	15.5***
C*D	1	16.33	1.16	70.08	4.88*	7.52	1.43	0.33	0.62
Total	47								

A=Methods of packaging semen (pellet or straw).
B=Methods of addition of glycerol (at once or slowly).
C=Percentages of glycerol (4% or 6%).
D=Equilibration time (2.5 hours or 3.5 hours).

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

Table 16: Means and standard errors of the sperm motility of post-thawing incubation for the method of packaging semen.

Treatments	Percentage of motility			
	0 hour	2 hours	4 hours	6 hours
Replications				
1	34.25(0.30)	21.81(0.40)	8.50(0.2)	3.18(0.15)
2	32.12(0.30)	21.43(0.40)	8.25(0.2)	2.94(0.15)
3	33.12(0.30)	21.62(0.40)	8.56(0.2)	2.62(0.15)
F ratio	0.29 0.04	0.08 2.37		
Methods of packaging semen samples (A)				
Pellet (A1)	33.29 (1.14)	21.00 (1.31)	7.17 (0.45)b	2.54 (0.22)b
Straw (A2)	33.04 (1.09)	22.25 (1.28)	9.71 (0.84)a	3.29 (0.32)a
F ratio	0.05	1.31	14.70***	12.57***
Methods of glycerol addition (B)				
At once (B1)	36.75 (0.72)a	25.58 (0.95)a	10.58 (0.65)a	3.75 (0.28)a
Slowly (B2)	29.58 (0.91)b	17.67 (1.05)b	6.29 (0.46)b	2.08 (0.16)b
F ratio	43.82***	52.39***	41.91***	62.08***
Percentage of glycerol (C)				
4% (C1)	33.29 (1.16)	22.29 (1.36)	8.00 (0.65)	3.00 (0.29)
6% (C2)	33.04 (1.07)	20.96 (1.22)	8.87 (0.78)	2.83 (0.27)
F ratio	0.05	1.49	1.74	0.62
Equilibration time (D)				
2.5 hours (D1)	33.79 (1.04)	21.88 (1.41)	8.29 (0.69)	3.42 (0.32)
3.5 hours(D2)	32.54 (1.17)	21.37 (1.17)	8.58 (0.75)	2.42 (0.20)
F ratio	1.33	0.21	0.19	22.35***

A=Methods of packaging semen (pellet or straw).
B=Methods of addition of glycerol (at once or slowly).
C=Percentages of glycerol (4% or 6%).
D=Equilibration time (2.5 hours or 3.5 hours).

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

There was no significant effect of methods of packaging ram semen by pellet or straw until the 4th hour of post-thawing incubation. After 4 hours of incubation semen samples frozen in straws had a greater motility than those frozen as pellets ($P \leq 0.005$) (Tables 15 and 16). The method of addition of glycerol significantly ($P \leq 0.005$) effected post-thaw motility of ram spermatozoa throughout the incubation. The superiority of adding all the glycerol at 30°C was consistent throughout the 6 hours incubation (Table 16). There were no significant ($P \geq 0.05$) differences between using 4% glycerol or 6% glycerol, but the interaction of methods of packaging and the percentages of glycerol was significant ($P \leq 0.05$) for motility at 2nd hour of incubation and more significant ($P \leq 0.005$) for motility at 4th hour of incubation (Table 15). The means of interaction between methods of packaging and the percentages of glycerol in Table 16 showed that, freezing semen samples by pellet with 4% glycerol resulted in a higher percentage of motility than straws (34.83 to 31.75) respectively while semen samples frozen in straws with 6% glycerol had greater motility than pellets (34.33 to 31.75) respectively. The equilibration time either 2.5 hours or 3.5 hours, at 5°C, had no significant effect except for motility at 6th hour of incubation.

Experiment six:

Fertility test of frozen-thawed ram semen:

Introduction:

The aim of this test was to compare the fertility of semen frozen by the method of Salamon (Evans and Maxwell, 1987) in pellets, with semen frozen in 0.25 ml French straws using the best procedure identified in the above laboratory tests.

Materials and methods:

Semen samples were collected from 5 Cambridge rams and only those with the motility of greater than 70% were pooled. It was divided to two portions and each portion was diluted with Tris egg-yolk buffer contained, either 6% glycerol for pellets and 9% glycerol for straws, with a ratio of 1:2 (semen:diluent). The diluted semen samples were chilled to 5°C and frozen in pellets and straws as described in Chapter two. 44 Welsh mountain ewes were synchronised using progestagen sponges followed by the injection of PMSG as described in Chapter two. The ewes were divided randomly in two groups. Each group was inseminated cervically either by thawed pelleted semen or by thawed straw semen samples 56 hours after removal of sponges and PMSG injection. Lambing data was recorded for the ewes.

Results:

The lambing results are shown in Table 17 and the analysis in Table 18.

Table 17: The fertility of ram semen, frozen in pellets frozen semen or straws.

Method of freezing	Number of ewes inseminated	Number of ewes lambed	Lambing %
Pellet	22	4	18
Straw	22	3	14
Total and means	44	3.5	16

Table 18: Chi-square analysis of fertility results.

Comparison	DF	Chi-square value for fertility data
Pellet vs Straw	1	0.170*

*This is significant only at the 32% level.

*For $P \leq 0.05$ Chi-square is 3.84.

Cervical insemination of ram semen frozen by pellet yielded a higher of lambing percentage than semen frozen in French straws (18% and 14%) respectively, but this differences was not statistically significant ($P \geq 0.05$).

Discussion:

Although the rate of dilution entirely depends upon the required number of spermatozoa in the insemination volume (Colas, 1975), experiment one shows that from two to four fold dilution resulted a similar post-thaw survival. This is in agreement with the work of Lightfoot and Salamon (1969a).

A range of pH from 6.6 to 7.0 in pre-freezing diluent was found to be well tolerable by ram sperm. This is in agreement with the report of Fiser et al. (1981), El-Gaafary (1987) and Salamon and Visser (1972) who used pH of 6.65, 6.8 and 7.0. Zlatarev et al. (1988) found that after storage of ram semen at 0-4°C up to 48 hours, a pH of 6.3 resulted a greater motility and less acrosin disruption than pH 7.3. But they did not examine the effects of the pH between these limits which would have included those currently used.

In the attempt to identify the optimum concentration of egg-yolk and glycerol in pre-freezing diluent for ram semen, it was found that although 12% egg-yolk should be available, it was not as crucial as glycerol (Watson and Martin, 1975b). Various concentration of glycerol for freezing ram semen are reported in the literature (Table 6). However, glycerol as a cryoprotective component must be present in the freezing solution. Glycerol levels of 4% or 6% were more beneficial than 2%, no matter whether the addition happened at 30°C or 5°C. This could be due to the problem of adding glycerol slowly. An optimum concentration of glycerol for freezing ram semen was found to be 4% for pellets and 6% for straws. The adding time either at 30°C or 5°C was not important factor on post-thawing survival characters of spermatozoa (First et al., 1961), but Colas and Courot (1977) and Morris and Farant (1972) recommended the glycerol to be added at 5°C, since the crystallisation phase occur during the freezing procedure.

In this work an equilibration time of 2.5 hours and 3.5 hours at 5°C were equally effective in term of post-thawing survival of sperm. While, Jones and Martin (1965) and Fiser and Batra (1984) suggested up to 5 hours and 2-6 hours of equilibration at 5°C.

The fertility following insemination with ram semen deep-frozen in straws was higher than has been reported by Salamon (1967) and lower than has been reported by

Maxwell et al. (1980). Salamon (1967) obtained a lambing rate of 6.1% (3/49) and 4.2% (3/72) after single cervical insemination of ewes using pellets frozen semen and semen frozen in straws. This difference in lambing rates was also not significant ($P \leq 0.05$). However, the ranking in the present study of the two methods of freezing agree with that Maxwell et al. (1980) who inseminated synchronised ewes twice after 50 and 60 hours of sponge removal and PMSG injection, using semen frozen either in pellet form or in straws. They obtained lambing rates of 52% (16/31) for semen frozen in pellets and 29% (9/31) for semen frozen in straws but their difference also was not statistically significant ($P \geq 0.05$).

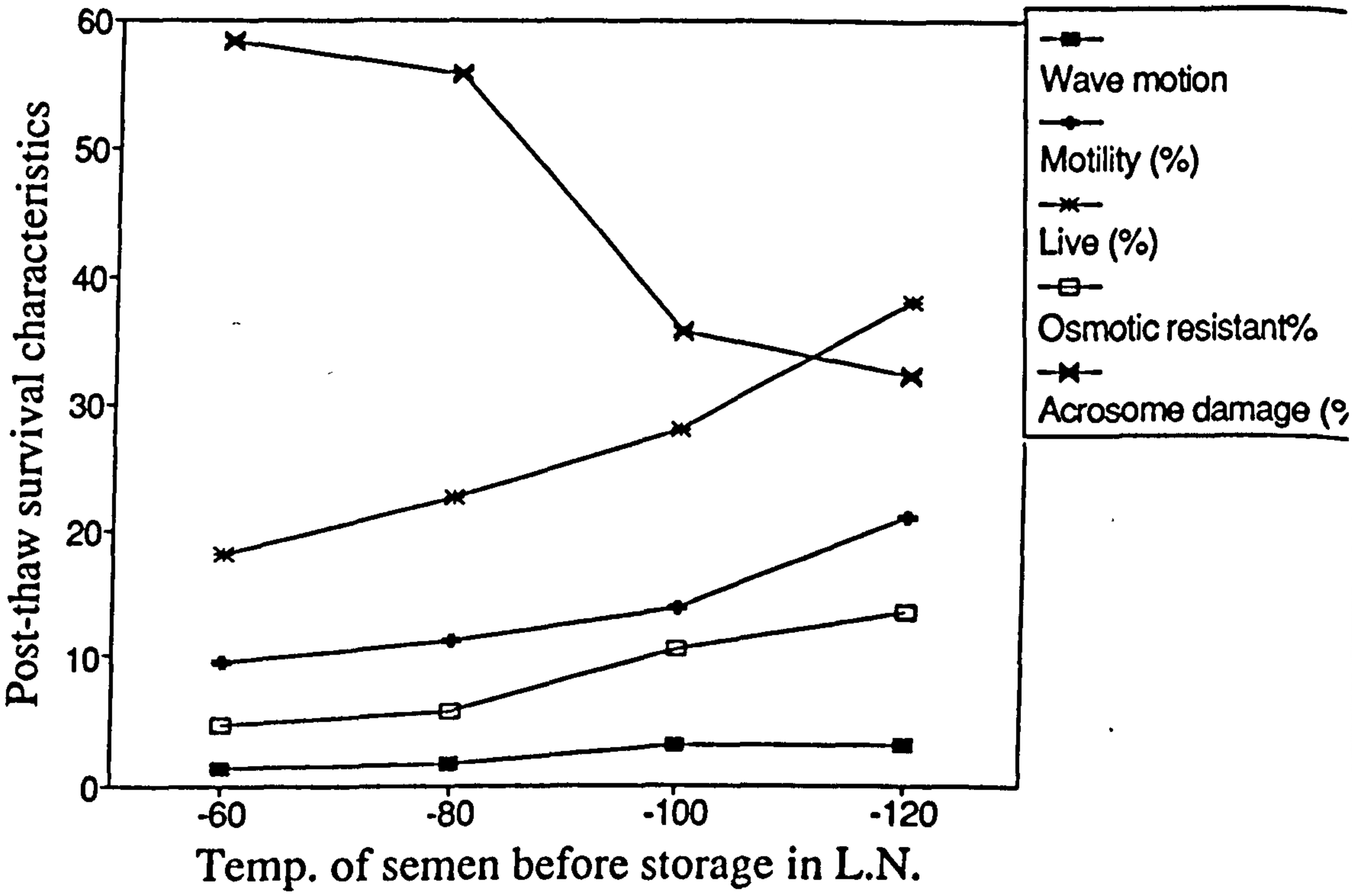

Figure 1: The effect of intermediate temperature during freezing on the post-thawing survival characteristics of ram semen.

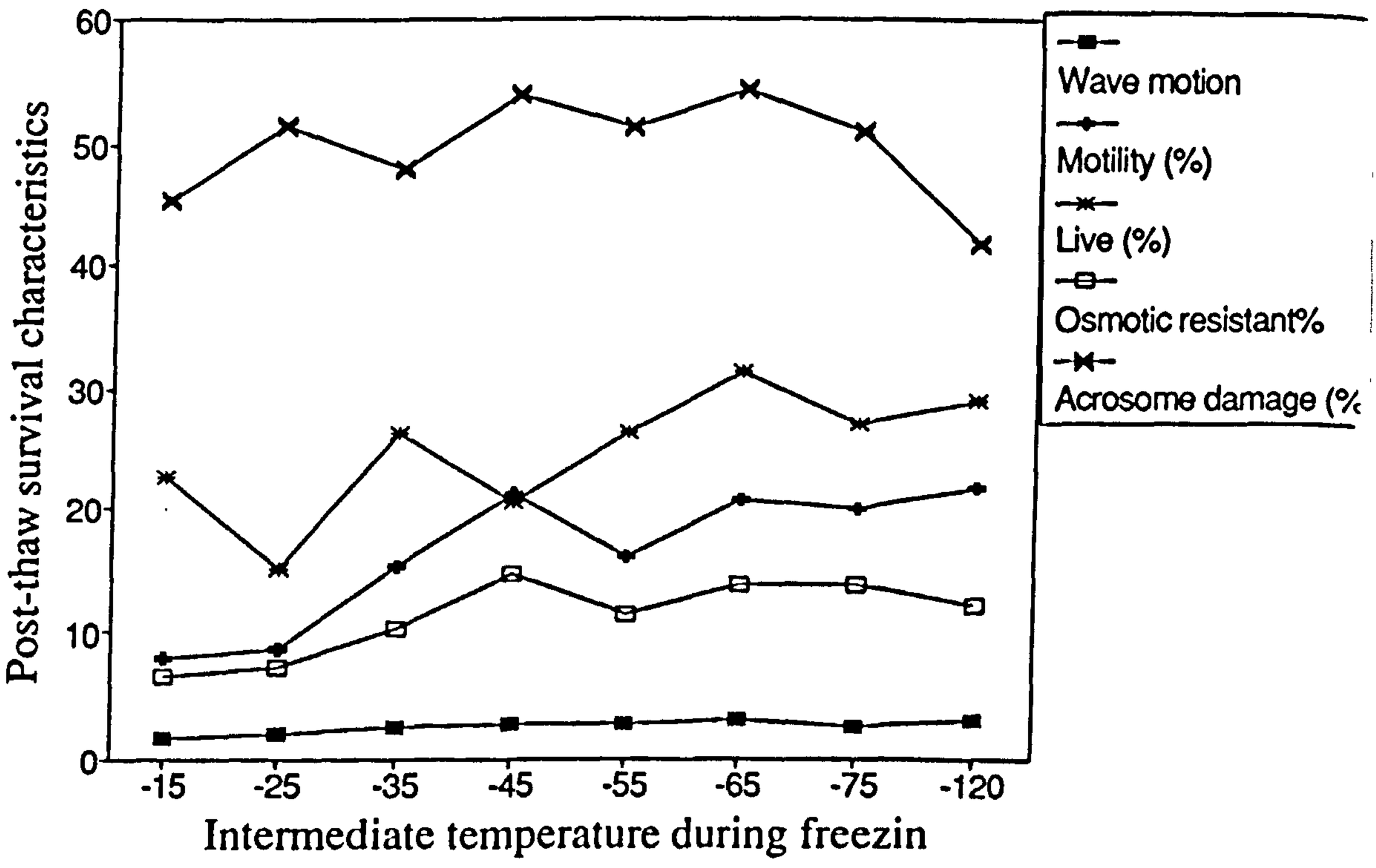

Figure 2: The effect of plunging temperature of frozen semen into liquid nitrogen on its post-thawing survival characteristics.

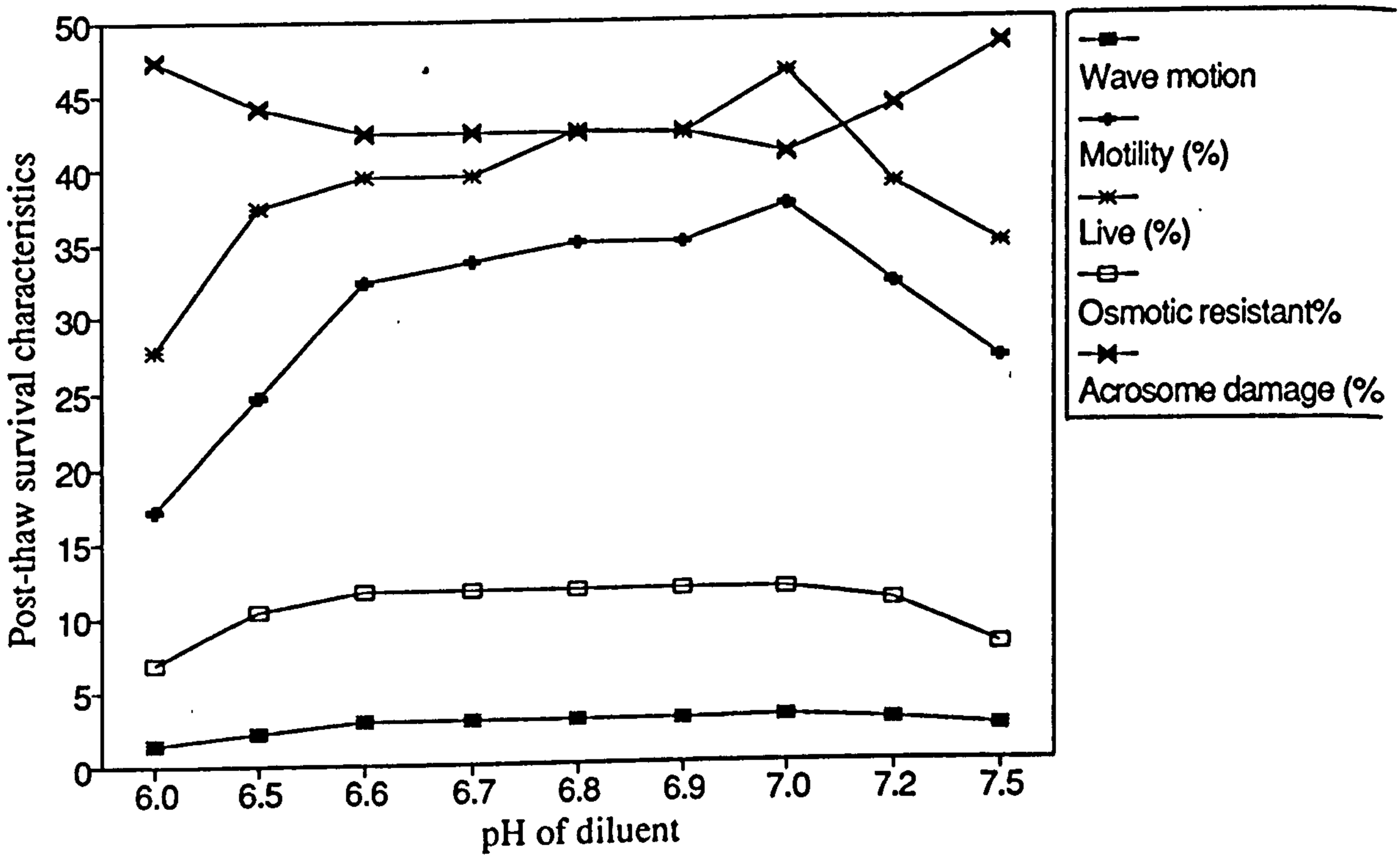

Figure 3: The effect of pH of diluent on the post-thawing characteristics of ram semen.

CHAPTER SIX

VITRIFICATION AS A METHOD FOR THE CRYOPRESERVATION OF RAM SPERMATOZOA

Introduction:

Vitrification has been reported for the cryopreservation of mouse embryos by Rall et al. (1987); Rall & Fahy (1985); and for cryopreserving sheep embryos by Schiewe et al. (1991) who preserved sheep embryos successfully using a vitrifying solution containing highly concentrated glycerol (6.5 M) and 6% bovine serum albumin. However, there is no record of ram semen being preserved in this way.

Rall et al. (1987) described vitrification as a process of solidification whereby an aqueous solution does not crystallise during cooling; instead, the viscosity of the solution increases with decreasing temperature until an amorphous glass-like solid forms. They used this approach on 8-cell mouse embryos by exposing them to a concentrated solution containing a mixture of three permeating solutes (20.5% (w/v) dimethyl sulphoxide, 15.5% acetamide, 10% propylene glycol) and a non-penetrating polymer (6% (w/v) polyethylene glycol 8000 Mr) in a modified Dulbecco's saline. The embryos were suspended in the mixture and then vitrified by plunging them into liquid nitrogen at -196°C. After rapid warming and thawing at a rate of about 2500°C/minute, and multiple steps dilution, nearly all of the embryos were apparently normal at the time of recovery. However the *in vitro* development of the recovered embryos was reduced significantly. The authors attributed this deleterious effect to the susceptibility of embryos to chemical toxicity and osmotic injury resulting from a highly concentrated cryoprotectant solution.

The advantages of vitrification are, namely, the prevention of mechanical damage from ice during the freezing procedure and the elimination of the need for special freezing equipment. It requires only the cryoprotectant solution and liquid nitrogen. Therefore, it has a great potential as a method for preserving ram semen under field conditions, which would be extremely useful for less costly operations.

Fahy et al. (1984) reviewed the principles of vitrification and found the concentration of cryoprotectants needed to vitrify (Table 1).

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was not possible. To assess the motility of semen preserved in this kind of solution, it had first to be rediluted to reduce the viscosity to that approaching 'normal' semen diluent, so that the spermatozoa could regain their proper speed and linear motility.

Materials and methods:

Semen was collected and incubated at 30°C as described in Chapter two. The pooled semen sample was divided in two halves. The first half was diluted with Tris egg-yolk buffer and second half was diluted with Tris egg yolk glycerol with a final glycerol concentration of 15%. The dilution were carried out at a rate of 1:2 (semen:diluent) in a water bath at 30°C. Assessments for motility and live dead ratio were carried out after ten minutes, twenty minutes and one hour using three different solutions for the suspension of the spermatozoa. These three solutions were:

A. Standard thawing solution as described in Chapter two.

B. Standard thawing solution but containing 5% glycerol.

C. Equal parts of thawing solution and one molar sucrose solution.

For the assessment, 30 µl of prepared solution A, B and C was mixed with 3 µl of diluted semen on a warm stage (37°C) slide. After thoroughly mixing by inoculator loop and leaving for one minute, the motility of the semen sample was scored.

Results: These are shown in Tables 2 and 3. The motility of all samples declined with time.

Table 2: Mean and standard error of motility of ram spermatozoa diluted in glycerolated and non-glycerolated semen after ten minutes, twenty minutes and one hour, assessed in three different solutions.

Type of semen sample	Type of solution		
	A	B	C
After ten minutes of incubation:			
Glycerolated semen	65.0 (0.00)	55.0 (1.00)	7.7 (2.08)
Non-glycerolated semen	65.0 (0.00)	55.0 (0.57)	8.7 (1.15)
After twenty minutes of incubation:			
Glycerolated semen	54.3 (1.15)	50.0 (2.00)	4.3 (1.50)
Non-glycerolated semen	61.7 (1.53)	60.3 (0.57)	4.7 (0.57)
After sixty minutes of incubation:			
Glycerolated semen	51.0 (1.00)	47.0 (2.60)	2.3 (1.30)
Non-glycerolated semen	59.0 (1.00)	57.3 (2.50)	4.0 (1.00)

A=Thawing solution.

B=Thawing solution plus 5% glycerol.

C=Equal parts of thawing solution and 1 M sucrose.

Table 3: Mean and standard error of motility of ram spermatozoa incubated up to 60 minutes in three solutions.

Treatments	Percentage of motile spermatozoa (SE)
Replications:	
1	40.561 (5.90)
2	39.33 (6.05)
3	38.89 (6.14)
F ratio	1.55
Type of solution:	
A	59.33 (1.28)a
B	54.39 (1.09)b
C	5.06 (0.65)c
F	1878.40***
Incubation times:	
10 minutes	42.78 (6.02)a
20 minutes	39.28 (6.11)b
60 minutes	36.72 (5.88)c
F ratio	19.27***

***$P \leq 0.005$

Both semen samples showed the highest motility when rediluted and assessed in thawing solution (A). Significant differences were found between using thawing solution (A) and solutions B and C ($P \geq 0.005$) with A giving greatest activity. However, using equal parts of thawing solution and one mole sucrose (C) resulted in much lower motility than either A or B (Table 3). The conclusion was that Salamon standard thawing solution was the best solution in which to assess the recovery of motility of spermatozoa.

Experiment two:

The aim of this experiment was to test the recovery of motility of ram semen diluted in Tris egg-yolk glycerol buffer incubated either at 30°C or at 5°C.

Materials and methods:

Semen collection was the same as described in Chapter two. Semen samples were pooled and divided into two halves. The first half was diluted with Tris egg-yolk buffer and second half was diluted with Tris egg yolk buffer glycerol (15% in final concentration). The dilutions was carried out at 30°C at the rate of 1:2 (semen:diluent). After transferring the semen samples to the laboratory, each sample was further divided in two equal portions. The first portion was incubated at 30°C in a water bath. The second portion was chilled to 5°C over 2 hours in a refrigerator. The motility of the semen samples was scored after 10 minutes, 20 minutes, 30 minutes, and 60 minutes as described in the previous experiment plus 2 minutes incubation on the warm stage. The live dead ratio was assessed as described in Chapter two.

Results:

The recovery rate for motility, and the percentage of live sperm, was estimated by dividing the percentages of motile or live sperm subjected to 15% glycerol by the percentage of motile or live sperm which were not subjected to glycerol. These are showed in Table 4.

Table 4: Mean and standard error of the recovery rate of percentage of motility and percentage of live ram sperm, which was incubated up to 60 minutes for semen at 30°C and at 5°C.

	Percentages of recovery	
Temperature of semen	Motility	live
30°C	82.67 (1.20)	83.17 (0.72)
5°C	83.77 (2.10)	83.40 (1.12)
F ratio	0.2	30.0
Incubation times:		
10 minutes	81.70 (1.49)	84.72 (1.72)
20 minutes	84.89 (1.37)	82.48 (0.87)
30 minutes	85.29 (1.89)	83.78 (1.34)
60 minutes	81.01 (3.13)	82.16 (1.23)
F ratio	0.89	0.60

There were no significant differences ($P \geq 0.05$) between the recovery percentages of motility and percentages of live spermatozoa for semen at 30°C and 5°C when incubated for up to 60 minutes.

Experiment three:

The aim of this experiment was to find the effect of different concentrations of the permeating solutes glycerol, dimethyl sulphoxide, acetamide, and the non-penetrating polymer propylene glycol on the motility and survival of ram spermatozoa, and to assess the effect of different methods of concentration and redilution on these characteristics.

Materials and methods:

Semen collection, handling, and dilution at 30°C with Tris egg-yolk buffer with the ratio of 1:2 was carried out as described in Chapter two. After transporting the semen samples to the laboratory, the semen was divided in two halves. The first half was incubated at 30°C and the second half was placed in refrigerator to chill to 5°C over 2 hours. Both warm and chilled semen samples were subjected to cryoprotective agents using following methods:

1:1) The concentration of cryoprotectant was raised abruptly by mixing 100 µl of semen sample with 3 ml of thawing solution A which contains 10%, 20% , or 30% by volume of cryoprotectant agent. After 1 minute and 30 minutes incubation at 30°C, the semen samples were diluted in thawing solution A to give a final concentration of 10% cryoprotectant for assessment.

1:2) The cryoprotective agent was applied as described for the first method. The redilution was carried out in stages by mixing an equal volume of concentrated semen sample with thawing solution in one stage for 20% and two stages for 30% to give a final cryoprotective agent concentration of 10% for assessment. In each stage, there was 2 minutes delay for diluted semen to equilibrate with the cryoprotectants.

2:1) The cryoprotective agents were concentrated slowly by mixing 100 µl of semen sample with 1.5 ml of thawing solution containing 10% of cryoprotective agents. This was increased to 20% or 30% after 5 minutes equilibration time for each stage. The redilution was done promptly as described for the first method.

2:2) The cryoprotective agents were applied and rediluted slowly using stages as described for the second and for the third methods.

All motility assessments and live/dead staining were carried out after 1 minute and 30 minutes of incubation with cryoprotective solutions at 30°C or 5°C. A 5 minutes incubation period was allowing after redilution at 37°C for the sperm to attain a suitable temperature for motility assessment and to recover from the osmotic pressure change.

Results:

A1: Glycerol:

The results are shown in Tables 5 and 6, and summarised in Table 7.

Table 5: Mean and standard error of motility and live percentages of spermatozoa after 1 minute and 30 minutes of incubation at 30°C in 0%, 10%, 20%, and 30% glycerol solutions, using different methods of concentration and redilution of ram semen.

Concentration of glycerol (%)	Method of glycerol concentration:dilution	Spermatozoa motility 1 minute	Spermatozoa motility 30 minutes
0	1:1	77.00 (1.53)a	73.33 (0.88)a
10	1:1	68.33 (0.88)b	63.00 (1.53)b
20	1:1	56.33 (0.88)c	36.33 (0.88)e
20	1:2	56.00 (1.15)c	50.67 (0.67)c
20	2:1	58.33 (1.67)cd	40.67 (0.67)d
20	2:2	60.33 (1.45)d	52.33 (1.45)c
30	1:1	32.33 (1.45)e	30.00 (0.00)f
30	1:2	24.67 (1.45)f	23.33 (1.20)g
30	2:1	48.00 (0.58)g	41.67 (0.88)d
30	2:2	55.00 (0.58)c	42.00 (1.15)d
F ratio		15.00***	204.04**

Concentration of glycerol (%)	Method of glycerol concentration:dilution	Spermatozoa live (%) 1 minute	Spermatozoa live (%) 30 minutes
0	1:1	79.67 (0.88)a	77.67 (0.33)a
10	1:1	73.00 (0.58)b	70.67 (0.67)b
20	1:1	69.67 (0.88)c	40.00 (0.00)g
20	1:2	68.67 (0.67)c	52.33 (0.33)e
20	2:1	61.33 (0.88)d	47.00 (0.57)f
20	2:2	61.00 (0.58)d	55.00 (0.57)c
30	1:1	50.00 (0.00)e	33.33 (0.33)h
30	1:2	27.33 (1.45)e	24.33 (0.67)i
30	2:1	50.00 (0.00)f	47.00 (0.58)f
30	2:2	60.00 (0.00)d	52.33 (0.88)d
F ratio		438.82***	849.05***

***P≤ 0.005

Increasing the glycerol level from 10% to 20% or 30% lowered the motility of spermatozoa significantly ($P \leq 0.05$). The percentage of motility decline was greatest and the percentage of live spermatozoa was lowest at higher levels of glycerol and at 30% glycerol survival was generally below 50% (Table 5). Method 2:1 (slow concentration:prompt redilution) and method 2:2 (slow concentration:slow redilution) produced the highest sperm motilities. The percentage motility of semen samples containing 30% glycerol, assessed after one minute and thirty minutes were 48%, 41% and 55%, 42% using methods 2:1 and 2:2 respectively compared with 32%, 30% and 24%, 33% using methods 1:1 and 1:2 respectively. Diluted semen samples incubated with no glycerol had average motilities of 77.0% and 73.0% after one and thirty minutes of incubation respectively. Semen samples with 10% glycerol had percentage of motilities of 68.00 and 63.00 after one minute and thirty minutes respectively. Semen samples containing 20% glycerol or 30% glycerol resulted in percentage motilities of 60.0% and 52.0% or 55.0 and 42 using method 2:2 for concentration:redilution of semen in thawing solution.

A2: Glycerol:

Table 6: Mean and standard error for percentage of motility and live percentages of spermatozoa after 1 minute and 30 minutes of incubation at 5°C in 0%, 10%, 20%, and 30% glycerol solutions, using different methods of concentration and redilution of ram semen.

Concentration of Glycerol (%)	Method of glycerol concentration:dilution	Spermatozoa motility (%) 1 minute	Spermatozoa motility (%) 30 minutes
0	1:1	72.67 (1.45)a	70.00 (0.00)a
10	1:1	----------	----------
20	1:1	52.67 (2.67)c	42.33 (1.45)c
20	1:2	28.00 (1.15)e	26.33 (0.88)d
20	2:1	57.00 (1.53)b	44.33 (2.33)c
20	2:2	58.00 (1.15)b	48.67 (0.67)b
30	1:1	22.67 (1.20)f	20.00 (0.00)e
30	1:2	16.00 (0.57)g	15.33 (0.33)f
30	2:1	48.33 (0.88)cd	45.00 (0.00)c
30	2:2	49.33 (0.67)c	46.00 (0.00)c
40	1:1	18.33 (0.88)fg	15.00 (0.00)f
40	1:2	15.33 (0.33)g	14.33 (0.33)f
40	2:1	21.33 (0.67)f	14.67 (0.33)f
40	2:2	15.00 (0.00)g	10.00 (0.00)g
F ratio		270.77***	476.64***

Concentration of Glycerol (%)	Method of glycerol concentration:dilution	Spermatozoa live (%) 1 minute	Spermatozoa live (%) 30 minutes
0	1:1	82.67 (1.45)a	72.33 (1.45)a
10	1:1	----------	----------
20	1:1	70.00 (2.89)b	45.00 (0.00)d
20	1:2	49.33 (1.76)d	28.00 (0.00)e
20	2:1	58.67 (0.67)c	48.00 (0.00)c
20	2:2	59.33 (0.67)c	53.00 (1.00)b
30	1:1	25.00 (0.58)f	21.33 (0.67)f
30	1:2	17.00 (1.00)g	19.33 (0.67)f
30	2:1	50.00 (0.00)d	47.67 (0.33)c
30	2:2	50.00 (0.00)d	47.67 (0.33)c
40	1:1	33.33 (1.67)e	17.67 (0.33)g
40	1:2	28.33 (1.67)f	15.67 (0.67)g
40	2:1	33.00 (1.00)e	17.00 (0.00)g
40	2:2	20.00 (0.00)g	12.00 (1.53)h
F ratio		218.98***	664.06***

1= Abruptly concentration or dilution.
2= Gradually concentration or dilution.

***P≤ 0.005

The results from Tables 5 and 6 summarised in Table 7, showed the survival of spermatozoa to be higher after incubation at 30°C than 5°C, although the differences were not significant at the 5% level. Therefore for the subsequent tests of vitrification incubation at 30°C was used.

Table 7: Mean and standard error of motility percentage and live percentage of spermatozoa after 1 minute and 30 minutes of incubation at 30°C or 5°C in various glycerol concentration.

Temperature of incubation	Motility percentage		Live percentage	
	1 minute	30 minutes	1 minute	30 minutes
30°C	53.6 (2.7)	45.3 (2.6)	60.0 (2.6)	49.9 (2.8)
5°C	44.9 (3.5)	39.8 (3.1)	51.3 (3.8)	42.5 (3.1)
F ratio	3.89	1.86	3.70	3.19

B: Acetamide:

The results are shown in Table 8.

Table 8 : Mean and standard error of motility percentage and live percentage of spermatozoa after 1 minute and 30 minutes of incubation at 30°C in 0%, 10%, 15%, 20%, and 30% acetamide thawing solutions, using different methods of concentration and redilution of ram semen.

Concentration of Acetamide	Method of acetamide concentration:dilution	Spermatozoa motility (%) 1 minute	Spermatozoa motility (%) 30 minutes
0	1:1	84.00 (2.08)a	77.00 (1.53)a
10	1:1	56.67 (1.67)b	52.33 (1.45)b
15	1:1	50.33 (0.88)c	49.67 (0.33)c
20	1:1	34.00 (3.06)e	20.00 (0.00)e
20	1:2	16.67 (1.67)g	13.33 (0.88)f
20	2:1	40.00 (1.15)d	30.00 (0.00)d
20	2:2	37.00 (0.57)de	18.67 (0.67)e
30	1:1	25.00 (0.57)f	0.00 (0.00)i
30	1:2	14.33 (0.67)g	5.33 (0.00)h
30	2:1	28.67 (0.67)f	8.00 (0.00)g
30	2:2	17.67 (1.45)g	5.00 (0.00)h
F ratio		209.04***	1279.81***

Concentration of Acetamide (%)	Method of acetamide concentration:dilution	Spermatozoa live (%) 1 minute	Spermatozoa live (%) 30 minutes
0	1:1	86.00 (1.00)a	85.00 (0.00)a
10	1:1	67.67 (1.45)b	63.67 (0.88)b
15	1:1	64.33 (1.20)c	60.00 (0.00)c
20	1:1	61.33 (0.67)d	45.00 (0.00)d
20	1:2	38.33 (1.76)g	28.67 (0.67)g
20	2:1	45.33 (0.88)f	33.00 (1.00)e
20	2:2	50.00 (0.00)e	30.00 (0.00)f
30	1:1	40.00 (0.00)g	5.00 (0.00)k
30	1:2	25.00 (0.00)i	11.67 (0.33)i
30	2:1	30.00 (0.00)h	14.00 (1.00)h
30	2:2	20.00 (0.00)j	10.00 (0.00)i
F ratio		711.28***	2740.62***

1= Abruptly concentration or dilution.
2= Gradually concentration or dilution.

***P≤ 0.005

There were significant ($P \leq 0.005$) differences in the survival of spermatozoa at the different levels of acetamide used, and that concentrations of acetamide over 15% reduced viability markedly. At the level of 20% of acetamide, using method three of concentration:redilution resulted in the greatest percentage of motility and the percentage of live spermatozoa (40, 50 and 30, 30) after one and thirty minutes respectively.

C: Propylene glycol:

The results are shown in Table 9.

Table 9: Mean and standard error of motility percentage and live percentage of spermatozoa after 1 minute and 30 minutes of incubation at 30°C in 0%, 10%, 20%, and 30% propylene glycol solutions, using different methods of concentration and redilution of ram semen.

Concentration of Propylene glycol (%)	Method of Propylene glycol concentration:dilution	Spermatozoa motility (%) 1 minute	Spermatozoa motility (%) 30 minutes
0	1:1	73.33 (1.67)a	70.00 (0.00)a
10	1:1	60.00 (0.00)b	52.00 (0.00)b
20	1:1	50.00 (0.00)c	40.00 (0.00)e
20	1:2	53.00 (1.00)c	45.00 (0.00)d
20	2:1	58.33 (1.67)b	46.67 (0.88)c
20	2:2	50.00 (0.00)c	40.00 (0.00)e
30	1:1	40.00 (0.00)d	24.00 (1.00)f
30	1:2	23.33 (1.67)f	20.00 (0.00)g
30	2:1	40.00 (0.00)d	24.00 (1.00)f
30	2:2	35.00 (0.00)e	15.00 (0.00)h
F ratio		205.63***	1052.43***

Concentration of Propylene glycol (%)	Method of Propylene glycol concentration:dilution	Spermatozoa live (%) 1 minute	Spermatozoa live (%) 30 minutes
0	1:1	78.67 (0.67)a	73.67 (0.88)a
10	1:1	73.67 (0.88)b	55.00 (0.00)b
20	1:1	64.33 (0.57)d	47.33 (0.33)c
20	1:2	63.00 (0.57)d	47.67 (0.33)c
20	2:1	69.33 (0.67)c	50.00 (0.00)b
20	2:2	62.00 (--)ed	45.33 (0.33)d
30	1:1	51.67 (0.88)f	25.00 (0.00)f
30	1:2	25.00 (0.00)i	20.00 (0.00)g
30	2:1	40.00 (0.00)g	30.00 (0.00)e
30	2:2	35.00 (0.00)h	10.00 (0.00)h
F ratio		953.56***	2990.27***

1= Abruptly concentration or dilution.
2= Gradually concentration or dilution.

***P≤ 0.005

The addition of propylene glycol at all concentrations significantly ($P \leq 0.005$) lowered the percentage of motility and the percentage of live spermatozoa after one minute and thirty minutes of incubation at 30°C. The motility percentage of spermatozoa using 10% propylene glycol was 60 and 52 after one minute and thirty minutes of incubation respectively. Increasing the level of propylene glycol to 20 lowered the motility percentage of sperm to 58 and 46 after one minute and thirty minutes of incubation.

D: Dimethyl sulphoxide:

The results are shown in Table 10.

Table 10: Mean and standard error of motility percentage and live percentage of spermatozoa after 1 minute and 30 minutes of incubation at 30°C in 0%, 10%, 20%, and 30% dimethyl sulphoxide solutions, using different methods of concentration and redilution of ram semen.

Concentration of Dimethyl sulphoxide (%)	Method of Dimethyl sulphoxide concentration:dilution	Spermatozoa motility (%) 1 minute	30 minutes
0	1:1	66.67 (0.88)a	59.67 (0.88)a
10	1:1	54.00 (0.88)b	35.67 (1.20)b
20	1:1	24.00 (0.57)d	9.00 (0.57)c
20	1:2	19.00 (0.57)e	6.67 (0.67)d
20	2:1	27.33 (1.45)c	10.67 (0.00)c
20	2:2	10.00 (0.00)h	4.00 (0.00)e
30	1:1	17.33 (1.20)e	9.00 (0.57)c
30	1:2	12.67 (0.33)g	6.00 (0.00)d
30	2:1	16.67 (0.33)e	8.00 (0.00)d
30	2:2	15.33 (0.33)ef	7.00 (0.00)d
F ratio		533.70***	1101.71***

Concentration of Dimethyl solhphoxide (%)	Method of D.sulphox concentration:dilution	Spermatozoa live (%) 1 minute	30 minutes
0	1:1	74.33 (0.67)a	62.00 (0.00)a
10	1:1	64.00 (0.57)b	40.00 (0.00)b
20	1:1	52.33 (1.45)c	14.33 (0.67)c
20	1:2	45.00 (0.00)d	12.00 (0.00)d
20	2:1	47.67 (0.33)d	15.00 (0.00)c
20	2:2	12.00 (0.00)g	7.33 (0.67)f
30	1:1	24.00 (0.88)e	10.00 (0.00)e
30	1:2	16.33 (0.88)f	10.00 (0.00)e
30	2:1	22.67 (1.20)e	10.00 (0.00)e
30	2:2	15.33 (0.33)f	8.00 (0.00)f
F ratio		788.00***	3406.63***

***P≤ 0.005

1= Abruptly concentration or dilution.
2= Gradually concentration or dilution.

Dimethyl sulphoxide addition greatly lowered the motility percentage and live percentage of ram spermatozoa. The motility percentage and live percentage, using

10% dimethyl sulphoxide, were 54 and 35 or 64 and 40 after one minute or thirty minutes of incubation.

Dimethyl sulphoxide was very poorly tolerated at concentration above 10% by the ram spermatozoa and could not be recommended as a component of a vitrifying solution.

Experiment four:

It was concluded from previous experiment, that glycerol, acetamide and propylene glycol were better tolerated by spermatozoa than dimethyl sulphoxide which greatly reduced the percentage of motility and the percentage of live spermatozoa.

The aim of this experiment was to examine the combined effect of the cryoprotective agents at varying concentrations 0%, 10% and 20% on the motility and survival of ram spermatozoa.

Materials and methods:

A Tris egg-yolk buffer was prepared to which was added glycerol, acetamide, and propylene glycol solution at levels of 0, 10% or 20%. Two methods of concentration and redilution of cryoprotective materials was carried out which was the same as described for the method 1:1 and method 2:1 for experiment three. The diluted chilled semen samples (5°C) were frozen and thawed for survival assessment.

Results:

The results are shown in Tables 11-16. The summary of the Anova, for the motility and survival data of the spermatozoa, was shown in Tables 11 and 13. The mean and standard error with the level of significance for the motility and survival data of the spermatozoa, was shown in Tables 12, 14 and 15, and the interactions in Appendix C.

Table 11: Summary of analysis of variance result for percentages of motility and the percentages of live ram spermatozoa incubated at 30°C up to 30 minutes.

Source of variation		Spermatozoa motility (%)				Live spermatozoa (%)			
		1 Minute		30 minutes		1 minute		30 minutes	
	DF	MS	F	MS	F	MS	F	MS	F
A	1	892.5	1142.4***	220.5	1764.0***	871.2	569.1***	116.2	744.2***
B	1	2161.5	2766.7***	544.5	4356.0***	4584.0	2993.6***	1046.5	6697.8***
C	1	1937.5	2480.0***	1200.5	9604.0***	3982.2	2601.0***	1725.7	14000.0***
D	1	3676.5	4705.9***	1800.0	14000.0***	5330.3	3481.0***	2194.5	14000.0***
A*B	1	16.5	21.1	50.0	400.0***	75.0	49.0***	270.2	1729.8***
A*C	1	30.0	38.4***	128.0	1024.0***	19.5	12.7***	69.0	441.8***
A*D	1	294.0	376.3***	264.5	2116.0***	344.5	225.0***	371.2	2376.2***
B*C	1	52.5	67.2***	162.0	1296.0***	42.8	27.9***	185.2	1185.8***
B*D	1	385.0	492.8***	220.5	1764.0***	520.2	339.6***	157.5	1008.2***
C*D	1	101.5	129.9***	612.5	4900.0***	16.5	10.8***	536.2	3432.2***
A*B*C	1	52.5	67.2***	12.5	100.0***	11.3	7.3***	124.0	793.8***
A*B*D	1	30.0	38.4***	18.0	144.0***	30.0	19.6***	42.7	273.8***
A*C*D	1	9.0	11.5***	162.0	1296.0***	42.8	27.9	195.0	1248.2***
B*C*D	1	94.5	121.0***	18.0	144.0***	166.5	108.7***	0.3	1.8
A*B*C*D	1	19.5	25.0***	0.5	4.0***	0.0	0.0	16.5	105.8***
Total	16	-----	-----	-----	-----	-----	-----	-----	-----

A=Methods of concentration and redilution of cryoprotective materials.
B=Glycerol concentrations (10% or 20%).
C=Acetamide concentrations (10% or 20%).
D=Propylene glycol concentrations (10% or 20%).

***P≤ 0.005

Table 12: Mean and standard error of the sperm motility percentages and live percentages during incubation at 30°C up to 30 minutes.

Treatments	Sperm motility (%)		Live spermatozoa (%)	
	1 minute	30 minutes	1 minute	30 minutes
Methods of concentration and redilution of cryoprotective materials (A).				
A1	13.19 (3.82)a	6.63 (2.48)a	23.62 (5.29)a	10.63 (3.33)a
A2	23.75 (4.73)b	11.88 (3.94)b	34.06 (5.94)b	14.44 (4.22)b
F ratio	1142.44***	1764.0***	569.1***	744.2***
Glycerol concentrations 10% or 20% (B).				
B1	26.69 (4.55)a	13.37 (3.76)a	40.81 (5.55)a	18.25 (3.92)a
B2	10.25 (3.33)b	5.13 (2.48)b	16.88 (4.13)b	6.81 (3.11)b
F ratio	2766.7***	4356.0***	2993.0***	6697.8***
Acetamide concentrations 10% or 20% (C).				
C1	20.25 (4.49)a	15.38 (4.04)a	40.00 (5.22)a	19.87 (4.45)a
C2	10.26 (3.53)b	3.13 (1.11)b	17.69 (4.81)b	5.19 (1.55)b
F ratio	2480.0***	9604.00***	2601.0***	11000.0***
Propylene glycol 10% or 20% (D).				
D1	29.19 (4.51)a	16.75 (3.81)a	41.75 (5.65)a	20.81 (4.22)a
D2	7.75 (2.25)b	1.75 (0.74)b	15.95 (3.58)b	4.25 (1.57)b
F ratio	4705.9***	14000.0***	3481.0***	14000.0***

A=Methods of concentration and redilution of cryoprotective materials.
B=Glycerol concentrations (10% or 20%).
C=Acetamide concentrations (10% or 20%).
D=Propylene glycol concentrations (10% or 20%).

***$P \leq 0.005$

Table 13: Summary of analysis of variance result for percentages of motility and the percentages of live ram spermatozoa incubated at 5°C up to 30 minutes.

Source of variation	DF	Spermatozoa motility (%)				Live spermatozoa (%)			
		1 Minute		30 minutes		1 minute		30 minutes	
		MS	F	MS	F	MS	F	MS	F
A	1	760.5	264.5***	338.0	2704.0***	770.2	849.9***	472.7	3025.8***
B	1	1653.1	575.0***	968.0	7744.0***	4728.7	5217.9***	504.0	3225.8***
C	1	946.1	329.0***	392.0	3136.0***	1313.2	1449.1***	0.3	1.8
D	1	1568.0	545.3***	882.0	7056.0***	2096.2	2313.1***	0.3	1.8
A*B	1	12.5	4.35	144.5	1156.0***	22.8	25.1***	11.3	72.2***
A*C	1	60.5	21.0***	84.5	676.0***	26.3	29.0***	47.5	304.2***
A*D	1	45.1	15.7***	84.5	676.0***	13.8	15.21***	0.1	0.2
B*C	1	120.1	41.7***	112.5	900.0***	225.78	249.1***	427.8	2737.8***
B*D	1	612.5	213.0***	480.5	3844.0***	1188.2	1311.2***	639.0	4089.8***
C*D	1	242.0	84.1***	112.5	900.0***	140.3	154.8***	357.8	2289.8***
A*B*C	1	24.5	8.5**	2.0	16.0***	38.3	42.2***	957.0	6125.0***
A*B*D	1	21.1	7.3	32.0	256.0***	26.3	29.0***	1212.8	7761.8***
A*C*D	1	3.1	1.1	8.0	64.0***	0.3	0.3	344.5	2205.0***
B*C*D	1	40.5	14.1***	18.0	144.0***	57.8	63.8***	236.5	1513.8***
A*B*C*D	1	1.1	0.39	4.5	36.0***	0.3	0.3	116.3	744.2***
Total	16	----	----	----	----	----	----	----	----

A=Methods of concentration and redilution of cryoprotective materials.
B=Glycerol concentrations (10% or 20%).
C=Acetamide concentrations (10% or 20%).
D=Propylene glycol concentrations (10% or 20%).

**P≤ 0.01
***P≤ 0.005

Table 14: Mean and standard error of the sperm motility percentages and live percentages during incubation at 5°C up to 30 minutes.

Treatments	Spermatozoa motility (%)		Live spermatozoa (%)	
	1 minute	30 minutes	1 minute	30 minutes
Methods of concentration and redilution of cryoprotective materials (A).				
A1	10.31 (2.90)b	4.13 (1.68)b	18.75 (4.79)b	16.12 (4.27)a
A2	20.06 (3.75)a	10.63 (3.33)a	28.56 (4.27)a	8.44 (1.40)b
F ratio	264.52***	2704.0***	849.9***	3025.8***
Glycerol concentrations 10% or 20% (B).				
B1	22.37 (3.98)a	12.88 (3.25)a	35.81 (4.58)	16.25 (2.60)a
B2	8.00 (1.76)b	1.87 (0.82)b	11.50 (1.98)	8.31 (3.65)b
F ratio	575.0***	7744.0***	5217.9***	3225.8***
Acetamide concentrations 10% or 20% (C).				
C1	20.62 (4.10)a	10.87 (3.28)a	30.06 (5.28)a	12.37 (2.96)
C2	9.75 (2.22)b	3.88 (1.71)b	17.25 (3.33)b	12.18 (3.67)
F ratio	329.09***	3136.00***	1449.1***	1.80
Propylene glycol 10% or 20% (D).				
D1	22.18 (4.11)a	12.62 (3.28)a	31.75 (5.44)a	12.19 (3.12)
D2	8.18 (1.49)b	2.12 (0.90)b	15.56 (2.46)b	12.37 (3.53)
F ratio	545.39***	7056.0***	2313.1***	1.80

A=Methods of concentration and redilution of cryoprotective materials. ***P≤ 0.005
B=Glycerol concentrations (10% or 20%).
C=Acetamide concentrations (10% or 20%).
D=Propylene glycol concentrations (10% or 20%).

Table 15: Mean and standard error of the sperm motility percentages and live percentages during incubation at 30°C up to 30 minutes.

	Spermatozoa motility (%)		Live spermatozoa (%)	
Treatments	1 minute	30 minutes	1 minute	30 minutes
Glycerol concentrations 0%, 10%, or 20% (A):				
A1	45.25 (4.50)a	29.92 (6.33)a	51.17 (4.70)a	33.67 (6.19)a
A2	33.50 (4.65)b	23.08 (6.00)b	37.50 (4.51)b	25.50 (5.80)b
A3	16.92 (3.69)c	11.42 (3.58)c	19.17 (3.69)c	14.17 (3.52)c
F ratio	2574.7***	3436.7***	928.1***	2959.1***
Acetamide concentrations 0%, 10%, or 20% (B):				
B1	45.92 (5.01)a	38.08 (6.42)a	49.58 (5.45)a	40.33 (6.30)a
B2	29.58 (4.79)b	18.17 (3.99)b	33.00 (4.97)b	20.75 (3.93)b
B3	20.17 (3.65)c	8.17 (2.31)c	25.25 (4.52)c	12.25 (2.99)c
F ratio	2156.8***	9109.1***	556.3***	6399.9***
Propylene glycol concentrations 0% or 20% (C):				
C1	40.00 (4.31)a	32.44 (4.67)a	44.78 (4.43)a	35.56 (4.37)a
C2	23.78 (3.68)b	10.50 (3.04)b	27.11 (3.95)b	13.33 (3.20)b
F ratio	2507.7***	1400.0***	842.7***	11000***

A=Glycerol concentrations (0, 10% or 20%).
B=Acetamide concentrations (0, 10% or 20%).
C=Propylene glycol concentrations (0% or 20%).

***P≤ 0.005

There were significant differences ($P \leq 0.05$) in sperm survival and activity between the two methods of dilution. A greater percentage of motility and percentage of live sperm was found when concentration and redilution were carried out using method 2:1 (staged concentration:prompt dilution). The differences between the effect of various levels of glycerol, acetamide and propylene glycol on the percentage of motility and percentage of live spermatozoa were all significant at the 5% level (Table 14). When the buffer contained 10% of each of the cryoprotective materials, the percentage of motility and the percentage of live sperm was at the highest level, but increasing the levels of any of the cryoprotective agents to 20% resulted a very poor survival of spermatozoa motility and live percentage (Table 16). The frozen-thawed sample resulted in a survival of very few spermatozoa.

Table 16: Mean and standard error of the percentage of motile and percentage of live spermatozoa for pre-freezing stage which incubation at 30°C or 5°C up to 30 minutes and for post-thawing stage.

Type of solutions	Pre-freezing assessment		Post-thawing assessment	
	1 minute	30 minutes	Frozen in dry ice (-79°C)	Frozen in L.N. (-196°C)
Percentage of motile spermatozoa				
A2*B1*C1*D1:				
Semen samples incubated:				
At 30°C	55.00(0.00)	44.00(0.00)	-----	-----
At 5°C	52.50(2.50)	40.00(0.00)	5.00(0.00)	0.00(0.00)
A2*B2*C2*D2:			Semen sample incubated:	
At 30°C	0.00(0.00)	0.00(0.00)	-----	-----
At 5°C	5.00(0.0)	0.00(0.00)	3.00(0.00)	2.00(0.00)
Percentage of live spermatozoa				
A1*B1*C1*D1:				
Semen samples incubated:				
At 30°C	73.00(0.00)	45.00(0.00)	----	-----
At 5°C	65.00(0.00)	45.00(0.00)	6.00(0.00)	0.00(0.00)
A2*B2*C2*D2:				
Semen sample incubated:				
At 30°C	1.00(0.00)	0.00(0.00)	-----	-----
At 5°C	10.0(0.00)	0.00(0.00)	3.00(0.00)	2.00(0.00)

***P≤ 0.005

A=Methods of concentration and redilution of cryoprotectants. B=Glycerol concentrations (10% or 20%).

C=Acetamide concentrations (10% or 20%). D=Propylene glycol concentrations (10% or 20%).

Discussion:

The possibility of assessing spermatozoa after subjection to high concentration of cryoprotectants was examined and it was found that by using thawing solution (Evans and Maxwell, 1987) the motility of spermatozoa could be assessed regardless of previous incubation at 30°C or at 5°C.

The staged method of raising glycerol concentration resulted in better sperm survival at 30% glycerol. But it was not beneficial at lower glycerol concentrations. The staged method of diluting the glycerol before assessment did not improve survival, hence the simple one-step diluting of sample with thawing solution was adopted.

For the purpose of developing a vitrifying solution, concentrations of 20% glycerol, or 10% acetamide or 10% propylene glycol as the sole cryoprotectants were tolerated by spermatozoa allowing the recovery of motility and survival of more than 50% of them. However, cryoprotectants at this concentration are insufficient to cause vitrification of the diluent on cooling (Fahy et al., 1984). Higher concentrations of cryoprotectants generally increased the mortality rates of the spermatozoa.

In experiment four above, it was found that the spermatozoa could only tolerate the presence of 10% of each cryoprotectants (glycerol, acetamide and propylene glycol) when used in combinations. This combination fell far short of the concentration of cryoprotectants needed to form a vitrifying solution. The concentrations of cryoprotectants for this were shown in Table 1. A solution contained 52% of vitrifying agents (20.5% (w/v) dimethyl sulphoxide, 15.5% (w/v) acetamide, 10% (w/v) propylene glycol and 6% (w/v) polyethylene glycol 8000 Mr) was used by Rall and Fahy (1985) and Rall et al. (1987). However, when ram semen samples containing either 30% or 60% of the equal parts of the above cryoprotectants were dropped into liquid nitrogen or onto dry ice, very few spermatozoa survived after warming in thawing solution (Table 16). This confirms that vitrification solution made from these components cannot be used for preserving ram semen because ram spermatozoa were not able to tolerate the necessarily high concentrations of them. Therefore vitrification cannot be recommended for cryopreservation of ram semen at this time.

CHAPTER SEVEN

DEVELOPMENT OF

A SPECTROPHOTOMETRIC METHOD

FOR THE ASSESSMENT OF VIGOUR

OF RAM SEMEN

INTRODUCTION:

Drevius (1971) introduced a sperm-rise test to estimate the motility of bull spermatozoa in different suspension using a spectrophotometric method.

Parish and Foot (1987) developed a procedure for the quantification of active bovine spermatozoa by a swim-up method, relating rate of swim-up to the sperm motility, integrity of the acrosome, sperm migration in polyacrylamide gel and fertility. The number of bovine spermatozoa separated in the swim-up procedure was quantified using an electronic cell counter. They demonstrated that this method separated spermatozoa by motility as the swim-up fraction contained 95 to 100% motile cells. The number of separated spermatozoa from semen used for artificial insemination was correlated with the *in vivo* fertility of that semen ($P \geq 0.01$).

Jasko et al. (1989) described a spectrophotometric procedure for measuring equine spermatozoan motility objectively. Their method involved the use of a specially prepared cuvette with an injection port which allowed semen to be introduced at the bottom. Changes in the light transmittance at a fixed point in the cuvette above the interface of the sperm suspension and medium were recorded on chart paper. These recordings were analysed for the height (cm), representing absorbance, and time (min) to the peak deflection.

In the present study a modification of the method of Jasko et al. (1989) was tested and applied to ram semen. Recordings of absorbance were made as sperm cells swam upward in a cuvette. The rate of increase in the concentration of spermatozoa in the light path of the cuvette was recorded as the increase of absorbance with time.

Materials and methods:

Semen collection and preparation:

Semen was collected by artificial vagina from seven mature Cambridge rams. The

number of spermatozoa per ml of fresh semen was recorded by reading the optical density and dilution was carried out by mixing 1 ml of ram semen with 2 ml of Tris-glucose egg-yolk medium as described in Chapter two. All samples of semen and diluent were maintained at 30°C in a water bath.

Spectrophotometric swim-up procedure for ram semen:

1 ml of standard thawing solution (Chapter two) an optically clear medium, was placed in a 1 cm plastic cuvette, which had been obscured by the application of pen marker to the bottom 5 mm. The cuvette was placed in the sample chamber of a Unicam SP1800 ultraviolet spectrophotometer, maintained at 37°C. The spectrophotometer was set to give full scale deflection at 0.2 absorbance units and the absorbance was set to zero. The dilueted semen sample was aspirated into a warm 100 µl glass Hamilton syringe. The needle of the syringe was passed to the base of the cuvette and the sample was gently layered at the bottom. A distinct interface was formed between the medium and the sperm suspension. The absorbance changes at 440 nm were recorded on paper running at 0.5 cm/minute as the spermatozoa swam-up inside the cuvette, using Unicam AR25 linear recorder. Recordings were analysed for rate of change of absorbance with time in minutes and is referred to in Text and Table as swim-up slope. For convenience of use the absorbance change was measured in mm on the chart paper and is recorded and presented as such in all subsequent data. Full scale deflection on the chart was 0.2 absorbance unit or 200 mm of deflection.

Swim-up slope = Amax-A0/Tmax-T0

The swim-up slope, height/time (¢ m/minute) was used as a measurement of the spermatozoa vigour in the semen sample.

Three replications of swim-up were used for each sample in these experiments.

Experiment one:

This experiment was designed to measure the relationship between the volume of diluted semen injected at the bottom of the cuvette and the rate of change of absorbance. A

range of volumes from 10 to 60 µl were used of which the number of spermatozoa per one ml was constant at $1.95*10^9$.

Results:

Figure 1 shows a typical swim-up recording. The mean change in absorbance per minute for each semen volume is shown in Table 1 and the whole data with regression line are represented in Figure 2. The correlation coefficient between slope and volume was 0.94.

Table 1. The effect of semen volume on swim-up measurement (absorbance per minute).

Volume of semen (µl)	Swim-up slope (c m/minute) Mean (Standard error)
10	0.123 (0.02)c
20	0.430 (0.05)c
30	1.316 (0.06)b
40	1.420 (0.22)b
50	1.916 (0.17)b
60	2.708 (0.35)a
F value	26.76***

***P≤ 0.001

Experiment two:

The objective of this experiment was to determine the effect of variation in the proportion of live sperm on the absorbance gradient. Pooled freshly diluted semen was divided into two portions. One portion was plunged into liquid nitrogen to kill the

spermatozoa. The two portions were mixed in varying proportion in as shown in Table 2 to produce a series of semen samples with identical sperm numbers but different numbers of live sperm. The mixtures were examined by the swim-up procedure using a constant semen volume of 25 µl containing $1.75*1o^7$ spermatozoa in each test.

Results:

The mean rate of change of absorbance per minute for each of the prepared samples is shown in Table 2.

Table 2: Mean of swim-up slope of diluted semen samples which contain various proportion of live spermatozoa.

Fresh/Frozen ratio	Swim-up slope (c m/minute) Mean (Standard error)
0/100	0.000 (0.00)
25/75	0.540 (0.14)c
50/50	0.583 (0.10)c
75/25	1.360 (0.19)b
100/0	2.097 (0.24)a
F value	21.36***

***P≤ 0.001

The complete set of data with the regression line plotted in Figure 3 shows that the rate of change of absorbance was related to the proportion of fresh semen in the sample. The correlation coefficient between slope and the proportion of fresh semen was 0.926. The results suggested that the method could be used to assess semen vigour, a quality necessary for it to swim along the reproductive tract of the ewes.

Experiment three:

This experiment was designed to examine the application of the swim-up method to the comparison of the semen of five Cambridge rams over three consecutive weeks.

Method:

The concentration and motility of sperm for each rams in each week was measured immediately after collection and the semen was diluted as described above. The spectrophotometric swim-up procedure was carried out in triplicate using 25 µl subsamples of each diluted semen.

Result:

The mean rate of change and standard error of the mean of absorbance for semen from each ram in each week, the number of spermatozoa and the active number of spermatozoa were used for each test is shown in Table 3. The number of active spermatozoa was obtained by multiplying the total number by the percentage of motility. The relationship between swim-up slope and number of total and motile spermatozoa was shown in Figures 4 and 5.

Table 3. Mean of total and active ram spermatozoa in relation to the swimming-up score (slope) for three weeks.

Ram no.	Number of total sperm*10^6 in 25µl	Number of motile sperm*10^6 in 25µl	Slope Mean (SE)
		Week one	
1	183	109	0.83 (0.06)
2	183	101	0.63 (0.09)
3	215	129	1.10 (0.22)
4	175	131	1.81 (0.59)
5	195	98	0.48 (0.08)
		Week two	
1	205	144	2.60 (0.44)
2	200	130	1.86 (0.14)
3	195	98	0.48 (0.09)
4	222	167	2.00 (0.64)
5	175	88	0.34 (0.14)
		Week three	
1	205	154	4.99 (0.62)
2	205	144	3.28 (0.40)
3	198	119	1.10 (0.22)
4	233	175	4.14 (0.09)
5	185	127	1.62 (0.20)

LSD^{*1}=1.07 LSD^{*2}=0.75 LSD^{*3}=0.19

LSD= Least significant differences.
*1= Interaction between rams and weeks.
*2= Rams.
*3= Weeks.

The probability from analysis of variance table was $P \leq 0.001$ for the effect of rams, weeks and interaction of rams and weeks.

The relationship between swim-up slope and number of motile spermatozoa shown in Figure 5 was significant ($P \geq 0.01$) with a correlation coefficient was 0.81. The correlation coefficient between swim-up slope and total number of spermatozoa was 0.59. It can be concluded that the rise in absorbance is related to the number of active spermatozoa rather than the total spermatozoa numbers. The repeatability of this technique R^2=091 was assessed using the Robinson's agreement measurement (Robinson, 1957). This indicates close agreement of replications and the method of assessment semen vigour was reproducible.

Discussion:

In this study a technique for objectively assessing the vigour of ram spermatozoa was described and evaluated using a spectrophotometer.

In the method of Jasko et al, (1989), the time of arrival of spermatozoa in the light path of spectrophotometer remote from the point of the addition of the sperm. This measurement give an indication of the swimming speed of the fastest spermatozoa. In the present method the rate of increase in absorbance is measured, as an indication of the recruitment of spermatozoa to the light path and therefore of the rate of establishment of an active population of sperm. This rate of establishment of active sperm, at a side away from deposition is considered to be a measure of vigour of semen sample. Such a quality in semen is necessary for establishing a fertilising population of sperm cells in the oviduct of the ewes. The ability to detect differences in sperm vigour with this procedure offers a simple, objective method to investigate the effect of specific treatment on sperm activity.

The existence of significant ($P \leq 0.001$) variation in the spermatozoa vigour, below rams can be attributed to the fact that there was significant differences between rams within one breed (El-Gaafary, 1987). The significant effect of weeks and the interaction

between weeks and rams was probably due to the effect of semen collection during the non-breeding season (Spring). This may have resulted in the variation of the semen vigour. Fiser and Fairfull (1983) reported that decreasing the photoperiod increased the proportion of post-thawing motility of sperm.

The mean swim-up slope within three consecutive weeks were 2.79, 1.92, 0.89, 2.98 and 0.81 for rams and 0.97, 1.45 and 3.22 for weeks respectively. This general improvement of semen with time could have resulted from the previous period of rest of the rams. The first ejaculates may have contained degenerate spermatozoa while in late ejaculates the rams were responding to stimulation.

$$\text{Swim-up slope} = \frac{\text{Height (cm)}}{\text{Time (min)}}$$

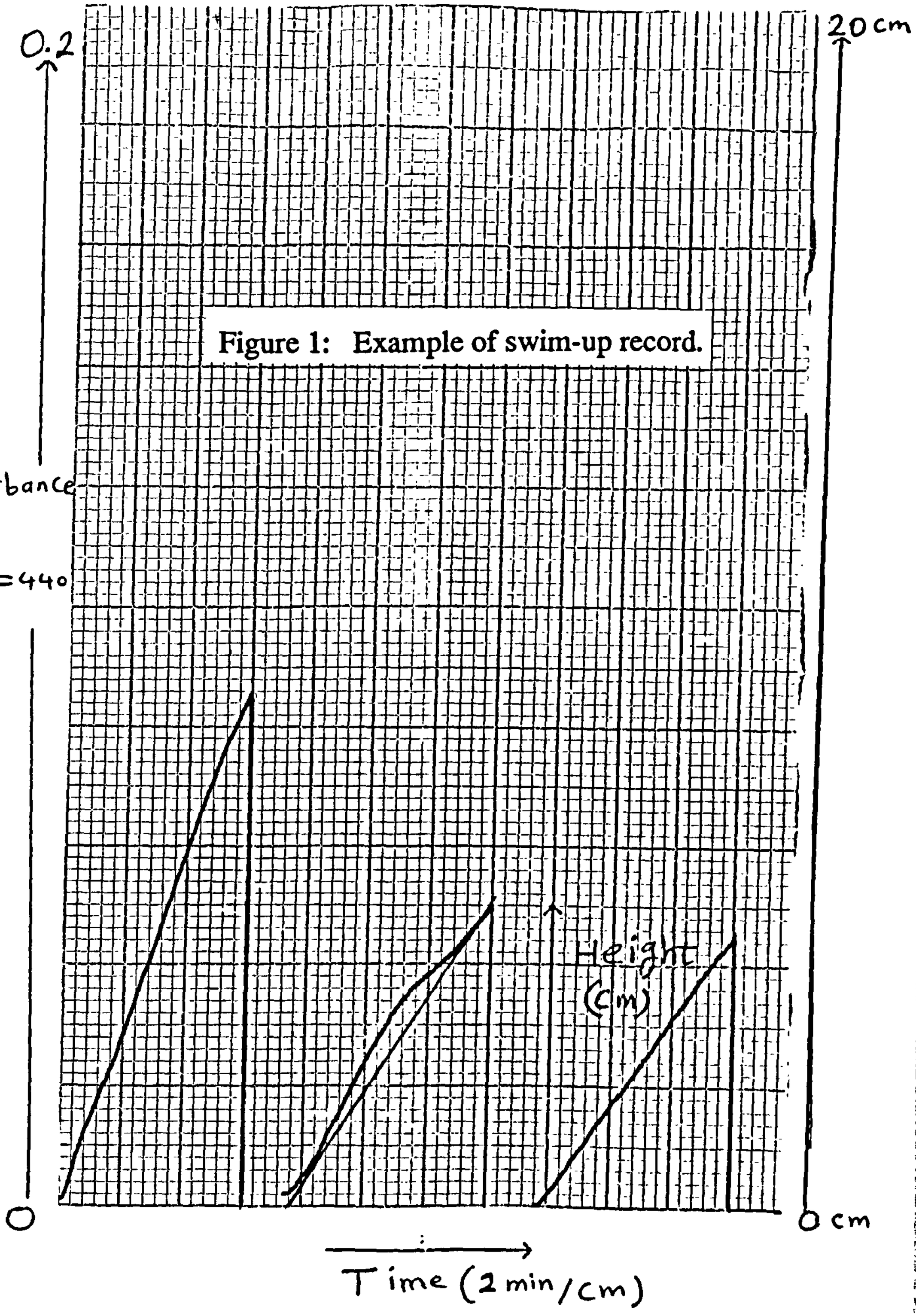


Figure 1: Example of swim-up record.

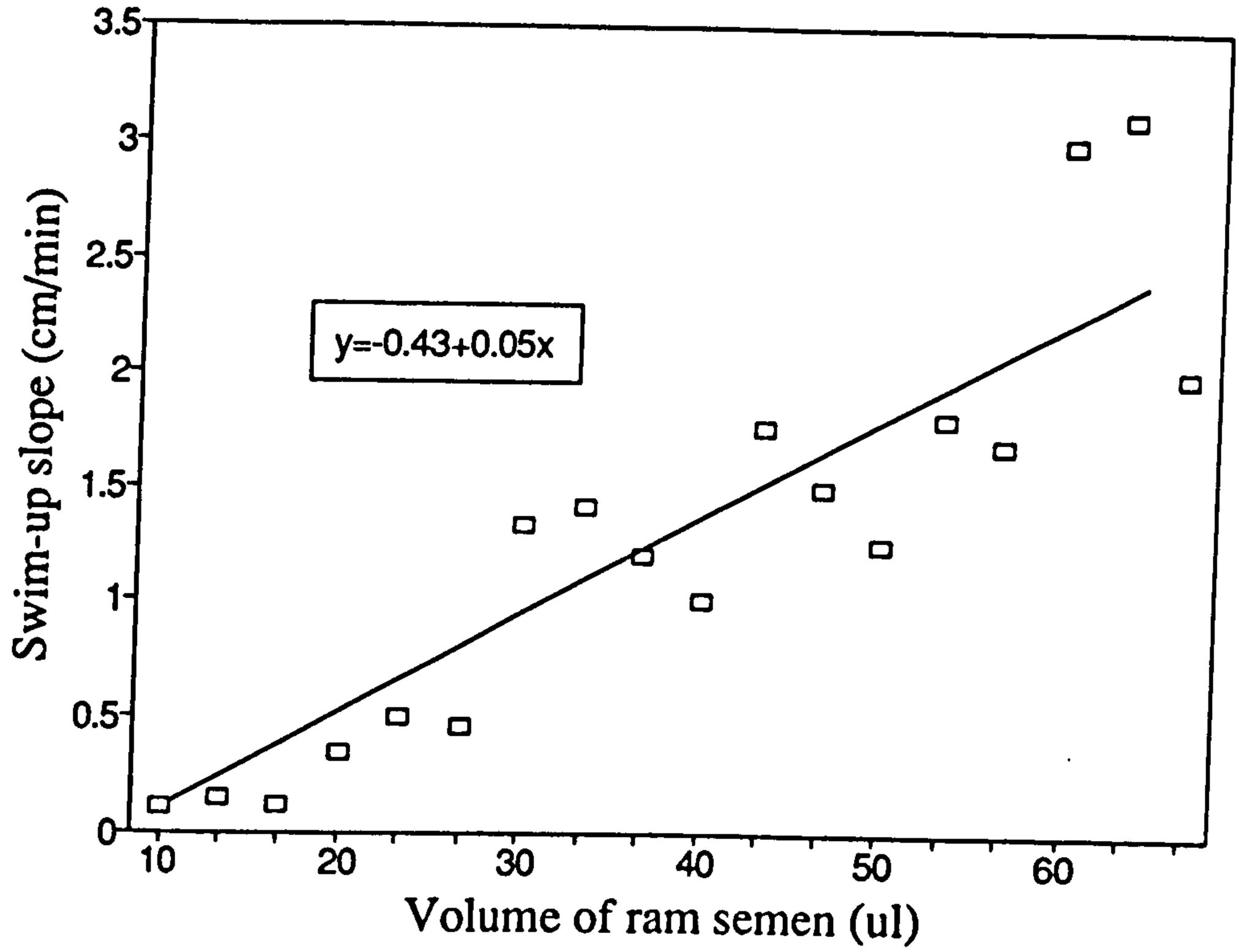


Figure 2: The relationship between volume of semen and swim-up slope (cm/min).

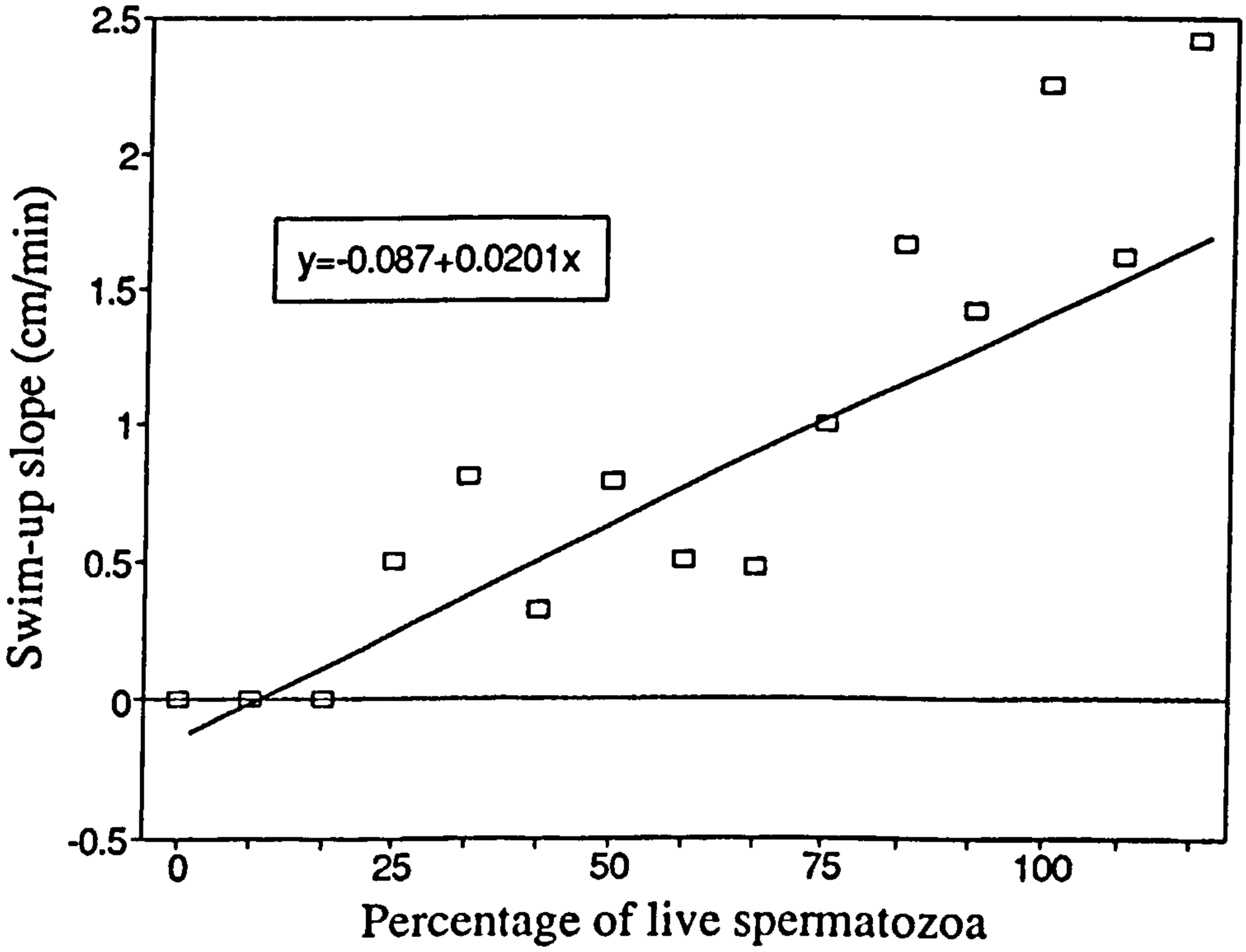


Figure 3: The relationship between percentage of live sperm and swim-up slope (cm/min).

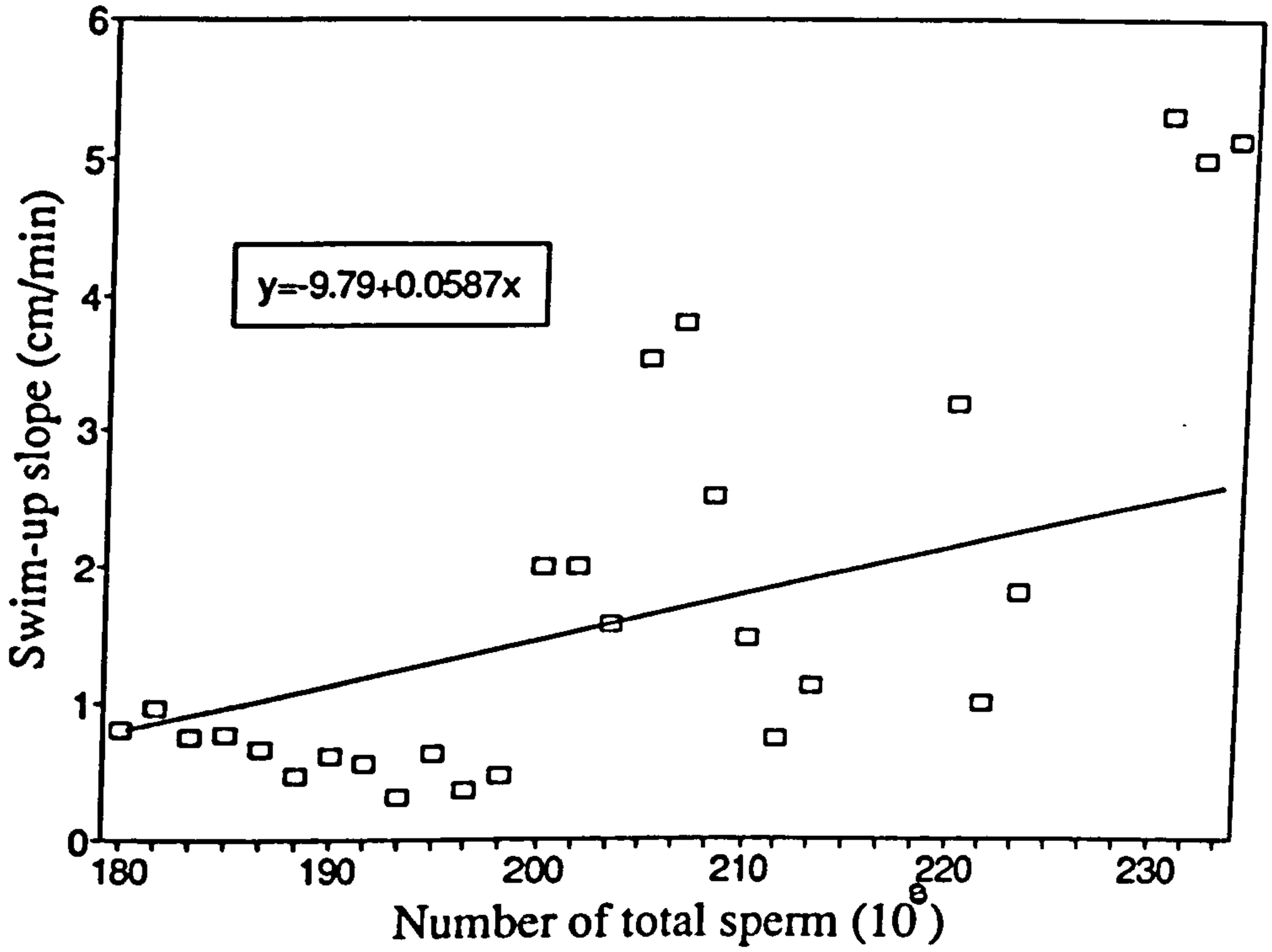


Figure 4: The relationship between the total number of sperm and swim-up slope (cm/min).

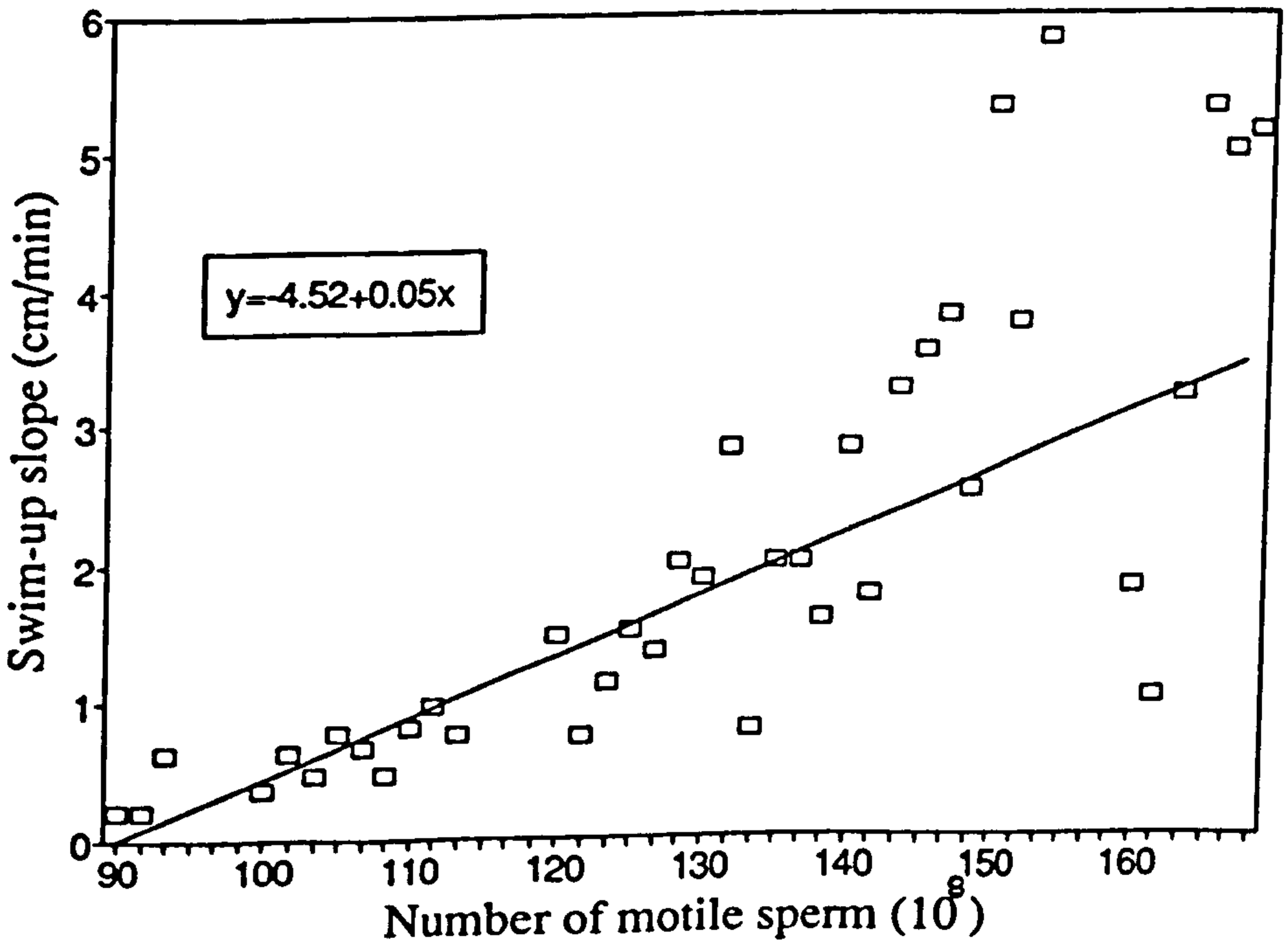


Figure 5: The relationship between the motile number of sperm and swim-up slope (cm/min).

CHAPTER EIGHT

AN INVESTIGATION OF THE EFFECT OF A HYPER-OSMOTIC DILUENT ON THE CRYOINJURY OF RAM SPERMATOZOA

Introduction:

Dehydration of cells during freezing has been proposed as a major cause of mortality in frozen-thawed spermatozoa. Cryoinjury happens if cells lose water until their capacity for shrinkage is exceeded. Further increases in the osmotic pressure gradient across the cell membrane cause membrane rupture (Meryman, 1971a and Mazur, 1970). During the freezing process ice forms first in the extracellular fluid, and water is consequently drawn from the cell increasing the degree of dehydration (Mazur, 1965 and 1970). This causes an increase in intracellular solute concentration which could be toxic for cells (Mazur, 1966). As freezing reaches completion, ice forms also inside the cell. Rapid freezing will reduce the degree of dehydration and cause the intracellular ice to form more quickly, and as smaller ice crystals (Mazur, 1980).

The most successful freezing of ram spermatozoa has been obtained by steady but rapid reduction in temperature from 5°C at the rate of between -20°C and -30°C per minute. Faster cooling rates are found to be deleterious (see Chapter four). This suggests that a certain amount of dehydration is necessary for spermatozoa to survive the freezing process (Fahy et al., 1984). Cells frozen rapidly in liquid nitrogen do not survive, possibly because no time is allowed for cell water to be lost.

Glycerol has been widely utilised as a cryoprotectant for the frozen storage of spermatozoa of bulls (Polge & Rowson, 1952), and rams (Colas, 1975). However, for ram semen, glycerol has not been found to provide acceptable protection for the cells at any concentration so far tested (Slavik, 1987).

For ram spermatozoa, the possible explanation for this phenomenon is that the cells fail to equilibrate sufficiently with cryoprotectants. They may then have too much free intracellular water at the time of freezing and some do not survive the freezing procedure.

Salamon (1968) examined post-thawed frozen semen variously treated and found that sugars of higher molecular weight (raffinose and lactose) provided better protection to

spermatozoa during fast freezing than do those of lower molecular weight (fructose and glucose).

Watson (1979) suggested that non-penetrating sugars such as lactose and raffinose have protective effects on spermatozoa during freezing. These sugars may have an effect on the degree of dehydration of sperm cells. Extracellular saccharides which bind water in high enough concentration could prevent the formation of ice crystals so the extracellular fluid can vitrify. This could prevent water being drawn out of cells during the freezing process. The water binding ability of these sugars may inhibit the formation of extracellular ice crystals during cooling, and as a consequence of fast freezing only small crystals of intracellular ice form. Mazur (1966; 1970 and 1980) believed that small ice crystals were not harmful for cells, but that their recrystallisation as larger sized crystals, as a result of their high surface free energies, could be damaging during thawing.

The addition of a non-penetrating solute such as raffinose to semen will cause dehydration of cells by its osmotic effect. It is possible that to survive, frozen cells must be partially dehydrated to a controlled degree; a process which will reduce the formation of intracellular ice.

Experiment one:

The objectives of this experiment were:

a) To calibrate the osmometer.

b) To investigate the effect of added raffinose on the osmotic pressure of the diluent.

c) To identify the optimal concentration of raffinose in diluted semen for sperm survival during freezing.

Materials and methods:

The linearity of the osmometer was tested on three occasions using a range of NaCl standards from 0.6-2.1 g/100 ml.

Normal Tris egg-yolk fructose (control) diluent was prepared as described in

Chapter two, and raffinose was added to make a series of diluents with final concentrations of 0 mM, 33 mM, 66 mM, 99 mM, 132 mM, 165 mM and 198 mM raffinose.

The solutions were stirred using a Whirlimix2 TM (Laboratory FSA supplies) to dissolve and distribute the raffinose. The osmolarity (mMol/kg) and pH of the diluent were recorded using a Vapour pressure osmometer (WESCORE,ING,5100C) and a pH meter (Compact pH meter C-1, HORIBA Ltd., Japan).

A bulk sample of semen were collected from 4 Cambridge rams using an artificial vagina. Motility, osmolarity and pH were recorded. The semen was diluted 1:2 (semen:diluent) with samples of each diluent. The diluted semen was cooled in a refrigerator to 5°C in two hours. The seven cooled samples were each divided into two aliquots. The first aliquot was used to measure the effect of the added raffinose on the osmotic pressure. Four percent glycerol was added to the second aliquot, and the semen samples were frozen in pellet form in dry ice as described in chapter two. The pellets were stored in liquid nitrogen for 24 hours and then thawed. Motility, osmolarity and pH of the frozen thawed semen were recorded.

Results:

a) Calibration of osmometer:

The recorded osmolarities of the saline solutions are presented in Table 1 and Figure 1.

NaCl concentrations (g/100)	Osmolarity of solutions (mMol/kg)		
	Occasion 1	Occasion 2	Occasion 3
0.60	332.00 (11.0)	230.00 (5.03)	---
0.90	352.67 (2.33)	286.00 (5.57)	290.00 (3.79)
1.20	396.67 (4.06)	343.33 (1.86)	372.33 (1.86)
1.50	453.00 (1.00)	415.33 (2.33)	460.00 (4.04)
1.80	515.67 (4.98)	495.33 (3.76)	---
2.10	575.67 (2.03)	560.33 (1.86)	---

The correlation coefficients relating osmometer reading to NaCl concentrations were 0.971, 0.994 and 0.995 for three samples occasions respectively.

b) The effect of added raffinose on the osmolarity of Tris egg-yolk diluent is shown in Table 2 and Figure 2.

Table 2: Mean and standard errors of osmolarities of diluted semen samples, contained different amount of raffinose.

Raffinose concentration (mM)	Osmolarity (mMol/kg)
0	343.33 (6.17)
33	449.00 (6.11)
66	454.70 (12.11)
99	495.70 (11.80)
132	555.00 (14.10)
165	635.30 (12.1)
198	644.67 (5.84)

These results showed an increase of osmolarity of the buffer with increase of raffinose concentration ($P \leq 0.005$). The correlation coefficient was 0.94.

c) The effect of concentration of raffinose on sperm survival during freezing.

The mean and standard error of percentages of motility, pH and osmolarity (mMol/kg) for fresh semen samples was 83 (1.66), 6.86 (0.15) and 285 (6.90) respectively.

The effect of concentrations of raffinose on percentage of motile sperm, pH and osmolarity of diluted semen is shown in Tables 3 and 4, and on the percentage of motile spermatozoa is shown in Figure 3.

Table 3: Summary of analysis of variance of semen characteristics at 5°C pre-freezing incubation and at 37°C post-frozen/thawed incubation.

	Motility (%)		pH		Osmolarity (mMol/kg)	
	MS	F	MS	F	MS	F
At 5°C pre-freezing incubation without glycerol						
Replication	1.2	1.00	0.003	0.19	87.6	1.75
Raffinose	2113.1	775.0**	0.28	187.4**	9556.1	191.5***
At 5°C pre-freezing incubation with 4% glycerol						
Replication	37.7	1.06	----	----	54064.0	5.29
Raffinose	1404.6	39.49***	----	----	14602	1.43
AT 37°C post-freeze/thawing without glycerol						
Replication	0.62	0.33	0.003	0.56	116	2.83
Raffinose	373.8	197.1***	0.197	36.6***	48955	1189.2***
At 37°C post-freeze/thawing for 4% glycerol						
Replication	27.52	1.57	----	----	102	0.04
Raffinose	485.4	27.6***	----	----	20465	7.18***

**P≤ 0.01

***P≤ 0.005

Table 4: Means and standard error of motility, and pH for chilled (5°C) pre-freezing semen samples and for post-thawing motility, for the effect of different raffinose concentration.

A:At 5°C pre-freezing incubation without glycerol

Raffinose(mM)	Motility (%)	pH	Osmolarity (mMol/kg)
0	71.67(1.67)a	6.95 (0.03)	418.33 (3.38)
33	70.0 (0.00)a	7.20 (0.00)	442.67 (1.76)
66	60.0 (0.00)b	7.40 (0.00)	469.33 (2.91)
99	40.0 (0.00)c	7.50 (0.00)	489.00 (3.79)
132	30.0 (0.00)d	7.09 (0.01)	505.33 (0.88)
165	18.0 (0.00)e	7.53 (0.03)	551.67 (1.67)
198	5.0 (0.000f	7.86 (0.03)	575.67 (9.39)
F ratio	1775.00***	187.40***	191.49***

B:At 5°C pre-freezing incubation with 4% glycerol

Raffinose	Motility (%)	pH	Osmolarity (mMol/kg)
0	70.00 (5.00)a	---------	1097.0 (72.0)
33	68.33 (1.67)a	---------	1127.0 (82.0)
66	56.67 (3.33)b	---------	1148.0 (82.0)
99	41.67 (6.01)c	---------	1205.0 (85.0)
132	30.00 (2.89)d	---------	1250.0 (100)
165	15.00 (0.00)e	---------	1290.0 (110)
198	5.00 (0.00)f	---------	1322.0 (97.0)
F ratio	39.49***	---------	1.43

C:AT 37°C post-thawing incubation without glycerol

Raffinose	Motility (%)	pH	Osmolarity (mMol/kg)
0	21.67 (0.88)c	6.61 (0.02)	442.00 (4.93)
33	28.33 (1.67)b	6.68 (0.04)	589.00 (5.57)
66	30.00 (0.00)a	6.55 (0.03)	553.00 (2.52)
99	20.00 (0.00)d	6.81 (0.01)	587.67 (0.33)
132	17.33 (0.67)e	6.37 (0.01)	572.67 (4.33)
165	3.00 (0.00)f	6.87 (0.09)	646.33 (5.93)
198	2.00 (0.00)f	7.16 90.03)	860.67 (2.33)
F ratio	197.10***	36.56***	1189.20***

D:At 37°C post-thawing incubation with 4% glycerol

Raffinose	Motility (%)	pH	Osmolarity (mMol/kg)
0	31.67 (3.33)b	---------	1214.3 (43.9)
33	40.00 (0.00)a	---------	1256.0 (29.1)
66	40.00 (0.00)a	---------	1308.3 (1.00)
99	30.00 (2.89)c	---------	1328.7 (11.3)
132	20.00 (0.00)d	---------	1378.3 (18.3)
165	11.67 (4.41)e	---------	1431.3 (45.3)
198	5.67 (2.33)f	---------	1425.7 (20.9)
F ratio	27.63***	---------	7.18***

***P≤ 0.005

The mean revival rate of post-thawing motility of sperm compared with pre-

freezing motility was 46%, 59%, 71%, 72% and 67% for 0 mM, 33 mM, 66 mM, 99 mM and 132 mM of added raffinose in semen diluent. However, 99 mM raffinose considerably reduced spermatozoa motility even before freezing.

The results showed that raffinose significantly ($P \leq 0.005$) reduced sperm motility during chilling, but that the addition of raffinose up to 66 mM increased the post-thaw motility of sperm. Further addition of raffinose resulted in significantly ($P \leq 0.05$) lower percentages of motility for both pre-freezing incubation and post-frozen/thawed incubation of ram spermatozoa.

Experiment two:

The aim of this experiment was to evaluate the effect of concentrations of raffinose and glycerol, and of pellet size on the survival of ram spermatozoa.

The experiment took the form of a 2*4*2*2 factorial test to examine the following effects:

1. Two levels of glycerol (2% and 4%) final concentration.
2. Four levels of raffinose (0, 33, 66 and 99 mM).
3. Two sizes of pellet (0.1 and 0.2 ml).
4. Two samples of semen taken in September and November.

The procedure was as described for experiment one except that the diluent contained glycerol which was added at 30°C, and all motility assessments were carried out in thawing solution. Post-thaw dilution of semen was carried out 1:1 (semen:thawing solution) as described in Chapter two.

Results:

The post-thawing motility of sperm is shown in Tables 5 and 6. The effect of pellet size (0.1 and 0.2 ml) on the percentage of motile spermatozoa up to 6 hours of post -thawing incubation at 37°C ,is shown in Figure 4. The effect of concentration of raffinose (mM) on the post-thawing motility of ram spermatozoa up to 6 hours of incubation at 37°C is shown in Figure 5.

Table 5: Summary of analysis of variance of sperm post-thawing motility during 6 hours incubation at 37°C.

		Percentage of motility of ram spermatozoa							
		0 hour		2 hours		4 Hours		6 Hours	
Source of variation	DF	MS	F	MS	F	MS	F	MS	F
A	1	2.64	0.62	132.25	14.75***	36.00	8.86*	135.14	23.19***
B	1	13.14	3.1	4.0	0.45	45.56	11.22***	97.51	16.73***
C	3	117.09	27.65	199.39	22.23***	212.04	52.19***	130.35	22.37***
D	1	129.39	30.56***	16.00	1.78	72.25	17.78***	118.27	20.30***
A*B	1	13.14	3.10	3.06	0.34	0.063	0.02	1.26	0.22
A*C	3	7.59	1.79	17.41	1.94	0.708	0.17	12.85	2.20
A*D	1	4.51	1.07	14.06	1.57	9.00	2.22	54.39	9.33***
B*C	3	38.68	9.14***	30.33	3.38*	35.10	8.64***	16.39	2.81*
B*D	1	47.26	11.16***	27.56	3.07	175.56	43.22***	97.51	16.73***
C*D	3	28.43	6.71***	13.16	1.47	8.95	2.21	49.22	8.45***
A*B*C	3	5.43	1.28	14.23	1.59	18.10	4.46*	1.89	0.32
A*B*D	1	31.64	7.47*	4.00	0.45	52.56	12.94***	70.14	12.03***
A*C*D	3	14.14	3.34*	33.23	3.70*	13.04	3.21*	15.26	2.62
B*C*D	3	9.97	2.36	5.73	0.64	11.77	2.90*	3.55	0.61A*
B*C*D	3	4.43	1.05	1.00	0.11	3.60	0.89	3.76	0.65
Total	63								

A=Sample.
B=Pellet size.
C=Raffinose.
D=Glycerol.
*=Interaction.

*=P≤0.05
***=P≤0.005

Table 6: Means of post-thawing motility of spermatozoa, which were treated with raffinose at 0 mM, 33 mM, 66 mM and 99 mM, and incubated at 37°C during 0,2,4 and 6 hours.

	Percentage of motility of ram spermatozoa			
Treatments	0 hour	2 hours	4 hours	6 hours
Samples (A)				
1	31.12 (0.77)	28.53 (0.72)a	16.87 (0.97)a	6.98 (0.04)b
2	30.72 (0.68)	25.66 (0.89)b	15.37 (0.98)b	7.01 (0.04)a
F ratio	0.62	14.75***	8.86*	23.19***
Pellet size (B)				
0.1 ml	32.47 (0.83)	28.34 (0.80)	18.00 (0.00)a	10.35 (0.04)a
0.2 ml	31.37 (0.60)	27.34 (0.81)	16.27 (0.63)b	6.94 (0.04)b
F ratio	3.10	0.45	11.22***	16.73***
Raffinose (C)				
0 mM	27.56 (1.23)c	22.37 (1.19)d	11.12 (0.85)d	7.04 (0.04)a
33 mM	30.94 (0.65)b	28.00 (0.67)b	16.12 (0.75)c	6.98 (0.05)b
66mM	35.20 (0.88)a	30.87 (0.68)a	19.70 (1.17)a	6.94 (0.04)b
99mM	31.00 (0.46)b	27.13 (1.07)c	17.56 (0.80)b	7.02 (0.06)a
F ratio	27.65***	22.23***	52.19***	22.37***
Glycerol (D)				
2%	29.50 (0.67)b	26.59 (0.81)	15.06 (0.79)b	7.02 (0.03)a
4%	32.34 (0.69)a	27.59 (0.87)	17.20 (0.86)a	6.97 (0.04)b
F ratio	30.56***	1.78	17.78***	20.30***

A=Sample (September v October).
B=Pellet size (0.1 ml v 0.2 ml.
C=Raffinose (0 mM v 33 mM v 66 mM v 99 mM).
D=Glycerol (2% v 4%).
*=Interaction.

*P≤0.05
***P≤0.005

Results:

The results in Table 6 showed that the motility of raffinose treated semen was significantly ($P \leq 0.005$) greater than of non-raffinose treated semen during post-thawing incubation for up to 4 hours. When the pellet size was reduced from 0.2 to 0.1 ml, the motility of spermatozoa was greater, though this improvement became statistically significant ($P \leq 0.005$) only after four hours of post-thawing incubation. However, the best post-thaw motility was obtained for sample one, when 4% of glycerol and 66mM raffinose were included in the diluent and the pellet size of 0.1 ml was employed. For sample two 4% glycerol, 66 mM raffinose with pellet size of 0.2 ml gave the best motility (Table 7).

Table 7: Mean and standard errors of post-thawing motility of semen samples for experiment two.

Type of solution	Percentage of motility			
	0 hour	2 hours	4 hours	6 hours
A1 B1 C2 D2	38.5(1.02)	33.5(1.50)	26.0(1.00)	18.0(1.20)
A2 B2 C2 D2	38.5(1.02)	32.0(1.50)	20.0(1.00)	13.5(1.20)
B1 C2 D2	37.25(0.51)	32.5(0.75)	23.0(0.50)	15.0(0.60)
B2 C2 D2	35.0(0.51)	32.0(0.75)	20.5(0.50)	14.0(0.60)

A=Sample (September v October).

B=Pellet size (0.1 ml v 0.2 ml).

C=Raffinose (0 mM v 33 mM v 66 mM v 99 mM).

D=Glycerol (2% v 4%).

Pellet size independently was not statistically significant at the 5% level for up to 4 hours of incubation, but it affected spermatozoa motility significantly ($P \leq 0.05$) in

interaction with the concentrations of glycerol and raffinose. The optimum solution for motility was that containing 4% glycerol with 66mM raffinose when it was frozen in 0.1 ml pellet volumes. This is shown in Table 7 as B1 C2 D2.

Experiment three:

It was concluded from experiment two that the addition of the raffinose improved spermatozoa motility and survival at post-thawing stage of ram semen frozen by the pellet method.

In Chapter four, it was found that for freezing semen in 0.25 ml French straws, a final concentration of 6% glycerol in Tris-yolk buffer, and a cooling rate of -20°C/min to -120°C before plunging into liquid nitrogen gave the best sperm survival. As the addition of 66 mM raffinose increases the osmotic pressure of the diluent and probably causes sperm dehydration, the cooling rate may play a crucial role for spermatozoa to survive in freezing procedure. Therefore different rates of cooling to freeze at -120°C before plunging to liquid nitrogen were examined.

The aim of this experiment was to investigate whether the addition of raffinose would improve semen motility and survival during post-thaw incubation when semen from individual rams was frozen using 0.25 ml French straws.

Materials and methods:

Semen samples were collected from three Cambridge rams in three consecutive weeks in February 1992 and diluted with a ratio of 1:2 (semen:buffer). The buffer solution was either Tris egg-yolk glycerol 6% or Tris egg-yolk glycerol 6% plus 66 mM raffinose. The diluted Semen samples were cooled to 5°C as described in Chapter two. They were loaded into 0.25 ml French straw in two replications. The straws were frozen at rates of cooling of -20°C, -30°C, -40°C, and -50°C per minute to -120°C using the cell freezing machine, and then plunged to liquid nitrogen and stored for 7 days. The semen samples

were thawed as described in Chapter two. The percentages of motility, of live cells and of acrosome integrity of the sperm were measured as described in Chapter two. The swim-up test was carried out as described in Chapter seven.

Results:

The results are shown in Tables 8-15, and Appendix D. These are summarised in Table 16. The effect of the rates of cooling (°C/min) on percentage of motile spermatozoa, percentage of live spermatozoa, percentage of acrosome damage and the swim-up slope (cm/min) are shown in Figures 6-9.

Table 8: Summary of analysis of variance of sperm post-thawing motility during 6 hours incubation at 37°C.

		Percentage of motility							
		0 hour		2 hours		4 Hours		6 Hours	
Source of variation	DF	MS	F	MS	F	MS	F	MS	F
A	1	3.78	0.19	0.07	0.00	0.00	0.00	0.00	0.00
B	2	283.42	14.20***	216.49	8.69***	88.50	5.53***	20.82	7.57***
C	2	238.08	11.93***	198.92	7.98***	76.63	4.77**	52.56	19.12***
D	1	536.28	26.86***	217.88	8.74***	80.79	5.05*	16.84	6.12**
E	3	621.21	31.12***	465.72	18.69***	53.62	3.35*	15.19	5.53***
D*E	3	15.45	0.77	35.26	1.41	11.32	0.71	2.62	0.95
Total	127								

A=Replications.
B=Rams.
C=Weeks.
D=Treatments.
E=Rate of cooling.
*=Interaction.

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

Table 9: Summary of analysis of variance of sperm post-thawing survival (percentage of live) during 6 hours incubation at 37°C.

		Percentage of live spermatozoa							
		0 hour		2 hours		4 hours		6 hours	
Source of variation	DF	MS	F	MS	F	MS	F	MS	F
A	1	2.82	0.06	0.64	0.02	0.07	0.00	0.11	0.01
B	2	686.32	13.51***	556.45	16.46***	116.97	4.07*	3.00	0.40
C	2	408.51	8.04***	392.45	11.61***	924.73	32.17***	841.00	112.3***
D	1	876.76	17.26***	345.37	10.22***	241.11	8.39***	38.53	5.15*
E	3	295.76	5.82***	463.10	13.70***	141.34	4.92***	4.17	0.56
D*E	3	36.24	0.71	20.06	0.59	30.59	1.06	1.21	0.16
Total	127								

A=Replications.
B=Rams.
C=Weeks.
D=Treatments.
E=Rate of cooling.
*=Interaction.

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

Table 10: Summary of analysis of variance of ram spermatozoa post-thawing acrosome damage percentage during 6 hours incubation at 37°C.

		Percentage of spermatozoa with acrosome damages							
		0 hour		2 hours		4 hours		6 hours	
Source of variation	DF	MS	F	MS	F	MS	F	MS	F
A	1	7.26	0.15	0.79	0.01	0.56	0.01	---	---
B	2	110.78	2.31	272.18	4.76**	247.25	6.09***	---	---
C	2	943.42	19.71***	1132.1	19.80***	1120.8	27.59***	---	---
D	1	1418.6	29.64***	1154.1	20.18***	892.3	21.96***	---	---
E	3	495.77	10.36***	616.20	10.77***	523.37	12.88***	---	---
D*E	3	41.34	0.86	107.75	1.88	64.41	1.59	---	---
Total	127								

A=Replications.
B=Rams.
C=Weeks.
D=Treatments.
E=Rate of cooling.
*=Interaction.

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

Table 11: Summary of analysis of variance of ram spermatozoa post-thawing slope of swimming-up test during 6 hours incubation at 37°C.

		Slope of swimming-up of spermatozoa (height/time)							
		0 hour		2 hours		4 hours		6 hours	
Source of variation	DF	MS	F	MS	F	MS	F	MS	F
A	1	0.001	0.03	0.00007	0.02	0.00	0.00	---	---
B	2	0.417	17.51***	0.0438	10.24***	0.038	5.04**	---	---
C	2	0.023	0.98	0.005	1.26	0.0007	0.10	---	---
D	1	0.024	1.02	0.012	2.79	0.009	1.20	---	---
E	3	0.009	0.38	0.01	3.05*	0.0155	2.07	---	---
D*E	3	0.026	1.11	0.003	0.62	0.0021	0.29	---	---
Total	127								

A=Replications.
B=Rams.
C=Weeks.
D=Treatments.
E=Rate of cooling.
*=Interaction.

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

Table 12: Means and standard errors of post-thawing motility of spermatozoa treated with either Tris yolk glycerol (6%) buffer or Tris yolk glycerol (6%) plus raffinose (66 mM) and frozen with various rate of cooling (-50°C/min, -40°C/min, -30°C/min and -20°C/min, incubated at 37°C up to 6 hours.

Treatments	Percentage of motility 0 hour	2 hours	4 hours	6 hours
Replications (A):				
1	19.64 (0.84)	13.97 (0.83)	6.98 (0.61)	2.45 (0.57)
2	19.98 (0.88)	14.02 (0.84)	6.98 (0.61)	2.45 (0.57)
F ratio	0.19	0.00	0.00	0.00
Rams (B):				
1	17.18 (0.95)c	12.35 (0.86)b	5.95 (0.55)b	2.15 (0.44)b
2	19.94 (0.92)b	13.17 (0.82)b	6.09 (0.63)b	1.75 (0.52)b
3	23.56 (1.14)a	17.69 (1.35)a	9.93 (1.02)a	7.50 (0.87)a
F ratio	14.20***	8.69***	5.53***	7.57***
Weeks (C):				
1	17.06 (1.29)b	11.75 (0.98)b	4.47 (0.55)c	1.53 (0.37)b
2	18.77 (0.73)b	12.83 (0.93)b	7.15 (0.59)b	—
3	22.69 (1.05)a	16.65 (0.97)a	9.00 (0.90)a	4.92 (0.70)a
F ratio	11.93***	7.98***	4.77**	19.12***
Treatments (D):				
0 mM Raffinose	17.76 (0.82)b	12.69 (0.79)b	6.25 (0.56)b	1.82 (0.47)b
66 mM Raffinose	21.86 (0.82)a	15.30 (0.84)a	7.69 (0.65)a	3.09 (0.62)a
F ratio	26.86***	8.74***	5.05*	6.12*
Rate of cooling(E):				
-50	14.40 (0.85)c	9.45 (0.59)c	5.73 (0.49)b	0.75 (0.49)b
-40	18.03 (1.10)b	13.53 (1.29)b	5.20 (0.96)b	2.95 (0.69)a
-30	23.34 (1.15)a	14.25 (1.20)b	7.66 (0.59)a	2.87 (0.69)a
-20	23.46 (0.96)a	18.75 (0.87)a	8.59 (1.14)a	2.50 (0.94)a
F ratio	31.12***	18.69***	3.35*	5.53*

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

A=Replications (1 v 2).
B=Rams (1 v 2 v 3).
C=Weeks (1 v 2 v 3).
D=Treatments (tris yolk glycerol (6%) without raffinose v the same buffer plus 66 mM raffinose).
E=Rate of cooling (-50°C/min v -40°C/min v -30°C/min v -20°C/min). *=Interaction. ---=No data was available.

The results in Table 12 shows that there was significant ($P \leq 0.005$) variation in the percentage of post-thawing motility of spermatozoa between rams during three weeks. The addition of 66 mM raffinose resulted in a significantly greater percentage of post-thawing motility during incubation up to 2 hours ($P \leq 0.005$) and for incubation up to 6 hours $P \leq 0.05$. The rate of cooling had a marked effect on post-thawing motility of spermatozoa ($P \leq 0.005$) up to 2 hours of incubation and ($P \leq 0.05$) up to 6 hours of incubation. As is shown in Figure 6, the rate of cooling of -20°C/min was more beneficial for post-thawing motility when diluent contained no raffinose. The rate of cooling of -30°C/min was more beneficial for post-thawing motility when 66 mM raffinose was added to the diluent. More rapid rates of cooling either -40°C/min or -50°C/min, resulted in a lower percentage of post-thawing motility of sperm. There was no significant ($P \geq 0.05$) interaction between treatments and the rates of cooling.

The greatest percentage of post-thawing motility was observed in the diluent containing 66 mM raffinose, and frozen at a rate of cooling of either -20°C/min or -30°C/min. The percentage of post-thawing motility of ram spermatozoa was 25.48, 15.62, 8.56 and 7.35 for D2*E3 and 24.73, 18.99, 8.81 and 5.60 for D2*E4 respectively.

Table 13: Means and standard errors of post-thawing survival (percentage of live) spermatozoa treated with either Tris yolk glycerol (6%) buffer or Tris yolk glycerol (6%) plus raffinose (66 mM) and frozen with various rate of cooling (-50°C/min, -30°C/min, -40°C/min and -50°C/min, incubated at 37°C up to 6 hours.

Treatments	Percentage of live spermatozoa 0 hour	2 hours	4 hours	6 hours
Replications (A):				
1	28.27 (1.10)	21.90 (0.98)	13.88 (0.88)	10.52
2	28.56 (1.12)	22.04 (0.98)	13.86 (0.88)	10.64
F ratio	0.06	0.02	0.00	0.01
Rams (B):				
1	24.27 (1.07)b	18.24 (0.99)c	12.52 (1.01)b	10.08
2	31.83 (1.43)a	25.33 (1.16)a	14.85 (1.21)a	10.08
3	29.50 (1.16)a	22.31 (1.15)b	14.28 (0.76)a	11.00
F ratio	13.51***	16.46***	4.07*	0.40
Weeks (C):				
1	25.06 (1.10)b	20.06 (0.74)b	9.31 (1.06)c	9.33 (0.19)b
2	27.52 (1.34)b	20.04 (1.08)b	12.70 (0.76)b	---
3	31.54 (1.30)a	25.33 (1.31)a	18.39 (0.96)a	17.00 (0.26)a
F ratio	8.04***	11.61***	32.17***	112.3***
Treatments (D):				
0 mM Raffinose	25.80 (0.95)b	20.34 (0.91)b	12.80 (0.80)b	9.45 (0.84)b
66 mM Raffinose	31.03 (1.16)a	23.66 (1.01)a	14.95 (0.94)a	11.72 (0.84)a
F ratio	17.26***	10.22***	8.39***	5.15*
Rate of cooling (E):				
-50	24.19 (1.05)b	16.78 (0.93)c	11.00 (0.70)b	11.00 (0.34)
-40	28.22 (1.31)a	23.97 (1.19)ab	14.94 (1.60)a	9.75 (0.20)
-30	30.47 (1.34)a	21.63 (1.27)b	14.21 (1.70)a	11.00 (0.34)
-20	30.78 (2.13)a	25.50 (1.59)a	15.16 (1.15)a	---
F ratio	5.82***	13.70***	4.92***	0.56

*=P≤ 0.05
**=P≤ 0.01
***=P≤ 0.005

A=Replications (1 v 2).
B=Rams (1 v 2 v 3).
C=Weeks (1 v 2 v 3).
D=Treatments (tris yolk glycerol (6%) without raffinose v the same buffer plus 66 mM raffinose).
E=Rate of cooling (-50°C/min v -40°C/min v -30°C/min v -20°/min). *=Interaction. ---=No data was available.

The result in Table 13 shows that the percentages of live spermatozoa varied significantly with weeks of collection ($P \leq 0.005$). For ram the differences were significant at the 0.5% level up to 2 hours of incubation and at 5% level up to 4 hours of incubation. Semen samples, containing 66 mM raffinose, were resulted significantly greater percentage of live spermatozoa in post-thawing incubation up to 4 hours ($P \leq 0.005$) and up to 6 hours ($P \leq 0.05$). The percentage of live spermatozoa was significantly ($P \leq 0.005$) higher up to 4 hour of post-thawing incubation when a rate of freezing of either -20°C/min or -30°/min was used. More rapid rates of cooling either -40°C/min or -50°C/min, resulted in a lower percentage of post-thawing survival of sperm. The interaction between treatment and the rate of cooling was not statically significant ($P \geq 0.05$). However, the percentage of live spermatozoa was greater with the diluent containing 66 mM raffinose and frozen with the rates of cooling either -20°C/min or -30°C/min (Table 13 D2*E3 or D2*E4). in Appendix D.

Table 14: Means and standard errors of post-thawing acrosome damages of spermatozoa, which was treated with Tris yolk glycerol (6%) buffer or Tris yolk glycerol (6%) plus raffinose (66 mM) and frozen with various rate of cooling (-50°C/min, -40°C/min, -30°C/min and -20°C/min, incubated at 37°C up to 6 hours.

Treatments	Percentage of spermatozoa with damaged acrosomes 0 hour	2 hours	4 hours	6 hours
Replications (A):				
1	47.58 (1.21)	53.24 (1.29)	55.75 (1.26)	58.64 (1.86)
2	48.06 (1.21)	53.40 (1.31)	55.61 (1.24)	58.64 (1.86)
F ratio	0.15	0.01	0.01	£
Rams (B):				
1	48.08 (1.13)	53.79 (1.48)a	57.50 (1.47)a	56.33 (1.65)
2	50.27 (1.71)	56.11 (1.64)a	57.21 (1.58)a	62.17 (2.20)
3	44.06 (1.44)	48.59 (1.25)b	50.54 (0.86)b	55.00 (0.00)
F ratio	2.31	4.76**	6.09***	£
Weeks (C):				
1	55.37 (1.63)a	59.09 (1.33)a	60.50 (1.06)a	60.75 (1.90)
2	43.87 (0.98)b	47.60 (1.03)b	50.29 (0.94)b	---
3	47.02 (1.42)ab	55.26 (1.74)c	58.76 (1.68)a	55.83 (1.35)
F ratio	19.71***	19.80***	27.59***	£
Treatments (D):				
0 mM Raffinose	51.34 (1.24)a	56.45 (1.24)a	58.83 (1.16)a	61.43 (1.76)
66 mM Raffinose	44.07 (0.96)b	50.08 (1.22)b	52.43 (1.18)b	55.86 (1.63)
F ratio	29.64***	20.18***	21.96***	£
Rate of cooling (E):				
-50	53.17 (1.77)a	58.37 (1.70)a	59.53 (1.66)a	62.25 (2.30)
-40	43.34 (1.37)c	47.62 (1.09)c	50.87 (0.81)c	55.83 (1.35)
-30	48.23 (1.38)b	53.44 (1.74)b	55.34 (1.69)b	59.25
-20	46.91 (1.85)b	54.16 (2.17)b	58.15 (2.68)a	---
F ratio	10.36***	10.77***	12.88***	£

A=Replications (1 v 2).
B=Rams (1 v 2 v 3).
C=Weeks (1 v 2 v 3).
D=Treatments (tris yolk glycerol (6%) without raffinose v the same buffer plus 66 mM raffinose).
E=Rate of cooling (-50°C/min v -40°C/min v -30°C/min v -20°C/min). *=Interaction.
£=Due to insufficient number of data, the F value could not be computed. ---=No data was available.

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

The result in Table 14 shows that the addition of raffinose led to a significantly lower incidence of acrosome damage during post-thawing incubation up to 6 hours ($P \leq 0.005$). The week of collection had a significant ($P \geq 0.05$) effect on percentage of spermatozoa with damaged acrosomes in post-thawing incubation up to 6 hours (Table 14). The acrosome integrity of ram spermatozoa was significantly ($P \leq 0.005$) effected by the ra tes of cooling. The rapid rate of cooling of -50°C/minute resulted a greater percentage of spermatozoa with damaged acrosomes while a cooling rate of -40°/minute resulted the lowest percentage of spermatozoa with damaged acrosomes. But using a rate of cooling of -30°C/minute or -20°C/minute resulted a similar percentage of spermatozoa with damaged acrosomes (Figure 8). The interaction between treatment and the rate of cooling was not statically significant at the 5% level. However, the lowest percentage of spermatozoa with damaged acrosome was observed with diluent containing 66 mM raffinose and frozen with the rate of cooling of -40°C/min (Table 14).

Table 15: Means and standard errors of post-thawing slope of swimming-up test of ram spermatozoa, which was treated with Tris yolk glycerol (6%) buffer or tris yolk glycerol (6%) plus raffinose (66 mM) and frozen with various rate of cooling (-50°C/min, -40°C/min, -30°C/min and -20°C/min, incubated at 37°C up to 6 hours.

Treatments	Swimming-up slope (c m/min) of ram spermatozoa. 0 hour	2 hours	4 hours	6 hours
Replications (A)				
1	0.23 (0.03)	0.10 (0.01)	0.07 (0.01)	0.10 (0.05)
2	0.23 (0.03)	0.10 (0.01)	0.07 (0.01)	0.10 (0.05)
F ratio	0.03	0.02	0.00	£
Rams (B)				
1	0.11 (0.01)a	0.07 (0.01)a	0.02 (0.00)a	0.01 (0.00)
2	0.26 (0.03)b	0.09 (0.01)a	0.10 (0.02)b	0.10 (0.05)
3	0.34 (0.03)c	0.14 (0.01)b	0.08 (0.02)b	0.19 (0.06)
F ratio	17.51***	10.24***	5.04**	£
Weeks (C)				
1	---	---	---	---
2	0.22 (0.03)	0.11 (0.00)	0.07 (0.01)	---
3	0.25 (0.03)	0.09 (0.01)	0.07 (0.01)	0.10 (0.03)
F ratio	0.98	1.26	0.10	£
Treatments (D)				
0 mM Raffinose	0.22 (0.03)	0.09 (0.01)	0.06 (0.01)	0.11 (0.06)
66 mM Raffinose	0.25 (0.03)	0.11 (0.01)	0.08 (0.01)	0.09 (0.04)
F ratio	1.02	2.79	1.20	£
Rate of cooling (E)				
-50	0.25 (0.03)	0.12 (0.01)	0.08 (0.02)	---
-40	0.21 (0.04)	0.07 (0.01)	0.01 (0.00)	---
-30	0.23 (0.03)	0.10 (0.02)	0.07 (0.01)	---
-20	0.24 (0.24)	0.12 (0.01)	0.09 (0.02)	---
F ratio	0.38	3.05*	2.07	£

A=Replications (1 v 2).
B=Rams (1 v 2 v 3).
C=Weeks (1 v 2 v 3).
D=Treatments (tris yolk glycerol (6%) without raffinose v the same buffer plus 66 mM raffinose).
E=Rate of cooling (-50°C/min v -40°C/min v -30°C/min v -20°C/min). *=Interaction.
£=Due to insufficient number of data, the F value could not be computed. ---= No data was available.

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

The result in Table 15 shows that the differences between replications for post-thawing swimming-up slope of ram spermatozoa was not statistically significant ($P \leq 0.005$). The effect of rams was significant ($P \geq 0.005$). But the effect of weeks were not significant ($P \geq 0.05$). Semen samples containing 66 mM raffinose produced a greater mean of the swimming-up slope during post-thawing incubation but the difference was not statistically significant ($P \geq 0.05$). The rate of cooling had a conflicting results on the swimming-up test. The greater slope was obtained (Figure 9) with more rapid and less rapid rates of cooling (-50°C/min and -20°C/min). However, the effect of the rates of cooling on post-thawing swimming-up slope of spermatozoa was only significant ($P \geq 0.05$) after 2 hours of incubation. Although, the interaction between treatment and the rate of cooling on post-thawing swimming-up slope was not significantly ($P \leq 0.05$) different, the greatest score for swimming-up slope was obtained with diluent containing 66 mM raffinose and frozen with the rate of cooling of -20°C/minute.

Table 16: A summary of the results of experiment three.

Type of diluent vs Freezing rate	Results of experiment four			
	0 hour	2 hours	4 hours	6 hours
	Percentage of motility:			
D2*E3	25.48 (1.85)	15.62 (1.76)	8.56 (1.76)	7.35 (0.50)
D2*E4	24.73 (1.51)	18.99 (1.60)	8.81 (0.85)	5.60 (0.60)
	Percentage of live spermatozoa:			
D2*E3	32.93 (3.10)	23.37 (1.80)	17. 39 (1.37)	12.2 (2.45)
D2*E4	32.18 (1.35)	25.85 (2.49)	14.93 (1.10)	---
	Percentage of spermatozoa with damaged acrosome:			
D2*E2	42.06 (2.09)	45.02 (1.37)	48.49 (0.91)	---
	Swim-up slope (mm/min) of ram spermatozoa:			
D2*E4	0.31 (0.07)	0.13 (0.02)	0.11 (0.03)	---

D=Treatments (Tris yolk glycerol (6%) without raffinose v the same buffer plus 66 mM raffinose).

E=Rate of cooling (-50°C/min v -40°C/min v -30°C/min v -20°C/min).

*=Interaction.

Discussion:

Raffinose raised the external osmotic potential of diluted semen and therefore would withdraw water from the cells. Assuming raffinose to be totally excluded from the sperm cells, the concentration used would provide an excess osmotic pressure outside the cells of about 66 mOsmo which should result in a compensatory loss of about 15% of the cell water. In experiment one, ram spermatozoa tolerated quite a wide range of osmolarity

(300 to 1200 mMol/kg). Although the revival rates of motility was maximal (72%) with 99 mM of raffinose, it was not proposed for use due to greater reduction of pre-freezing motility. The concentration of 66 mM raffinose with the revival rate of motility of 71% was subsequently used. However, the presence of 66 mM raffinose in semen diluent increased the post-thaw motility of spermatozoa. This agrees with the report of Salamon and Lightfoot (1969) that the lower pre-freezing motility of spermatozoa diluted in hypertonic media was reversed after thawing. Cells regained motility on thawing in hypertonic sodium citrate. They found that relatively hypertonic diluents are required for maximal post-thawing activity of ram spermatozoa frozen by the pellet method. The revival rate was significantly ($P \leq 0.005$) affected by the type of sugar used in the diluent, sugar concentration (from 111.0 to 277.5 mM), and concentration of sodium citrate (from 34 to 170 mM) in the diluent. Means revival rate of motility for sperm frozen in diluents containing arabinose, glucose, lactose, and raffinose were 29.3, 70.3, 78.6, and 84.6% respectively.

Fiser et al. (1981) investigated the effect of osmolarity of skim milk as a diluent for ram spermatozoa and concluded that cells survived better in hypertonic diluents than in isotonic or hypotonic diluents.

The results of experiment two indicated that higher molecular weight sugars like raffinose 66mM could protect spermatozoa when used in combination with glycerol 4%, rather than glycerol alone. This is in agreement with Salamon (1968); and Tada et al. (1990) who measured the fertilising ability of mouse spermatozoa when raffinose and glycerol were used as cryoprotectants. Their results suggest that when spermatozoa were recovered after freezing and thawing, only raffinose and sucrose afforded protection as indicated by motility, but spermatozoa frozen with sucrose were incapable of fertilising oocytes. The highest fertilisation rate was obtained with 18% raffinose and 1.75% glycerol. Lightfoot and Salamon (1969b) showed that when freezing ram semen using the pellet method, the volume of the pellet could be increased from 0.30 to 0.80 ml without

reducing the proportion of spermatozoa that survived the process of freezing and thawing. They obtained no interactions between pellet volume and either diluent tonicity or glycerol concentration on the spermatozoa survival. However, the results of present experiments disagree with that claim and show that there was an interaction effect between pellet size, diluent, tonicity and cryoprotectant levels.

For freezing ram semen in 0.25 ml French straws after raffinose treatment, a rapid rate of cooling (-30°C/minute) was beneficial for post-thawing survival. This agrees with the earlier results (Chapter five) that post-thaw survival of sperm was improved at rates of cooling of -20°C/min to -30°C/min.

The fluctuations in the percentage of sperm motility obtained between experiments clearly indicated that there was an effect of season on ram semen quality. The post-thaw motility of raffinose treated semen for experiments 1, 2 and 3 were 35%, 35% and 22% respectively. The lower rate of post-thawing motility in experiment 3 was due to semen collection during the anoestrous season (El-Gaafary, 1987).

Experiment four:

The effect of semen diluent on the rate of oxidation of glucose by ram spermatozoa.

Materials and methods:

The rate of glucose oxidation by ram spermatozoa was measured using [U-14C] glucose (sp.act.286mci/mmole, Radiochemical centre, Amersham, U.K.). The CO2 evolved was collected and counted in a scintillation counter.

Semen was collected from 5 Cambridge rams during winter 1991 (exp. 4a, 4b and 4c) and Spring 1992 (exp. 4d). The number of spermatozoa per ml were estimated by reading the optical density of semen using the spectrophotometry method as described in Chapter two and pH was recorded as described for experiment one. Semen was pooled and diluted 1:2 (semen:diluent) with Tris yolk buffer, Tris yolk glycerol buffer, or Tris yolk glycerol raffinose buffer of the compositions shown in Table 17.

Table 17: The composition of three buffers were used.

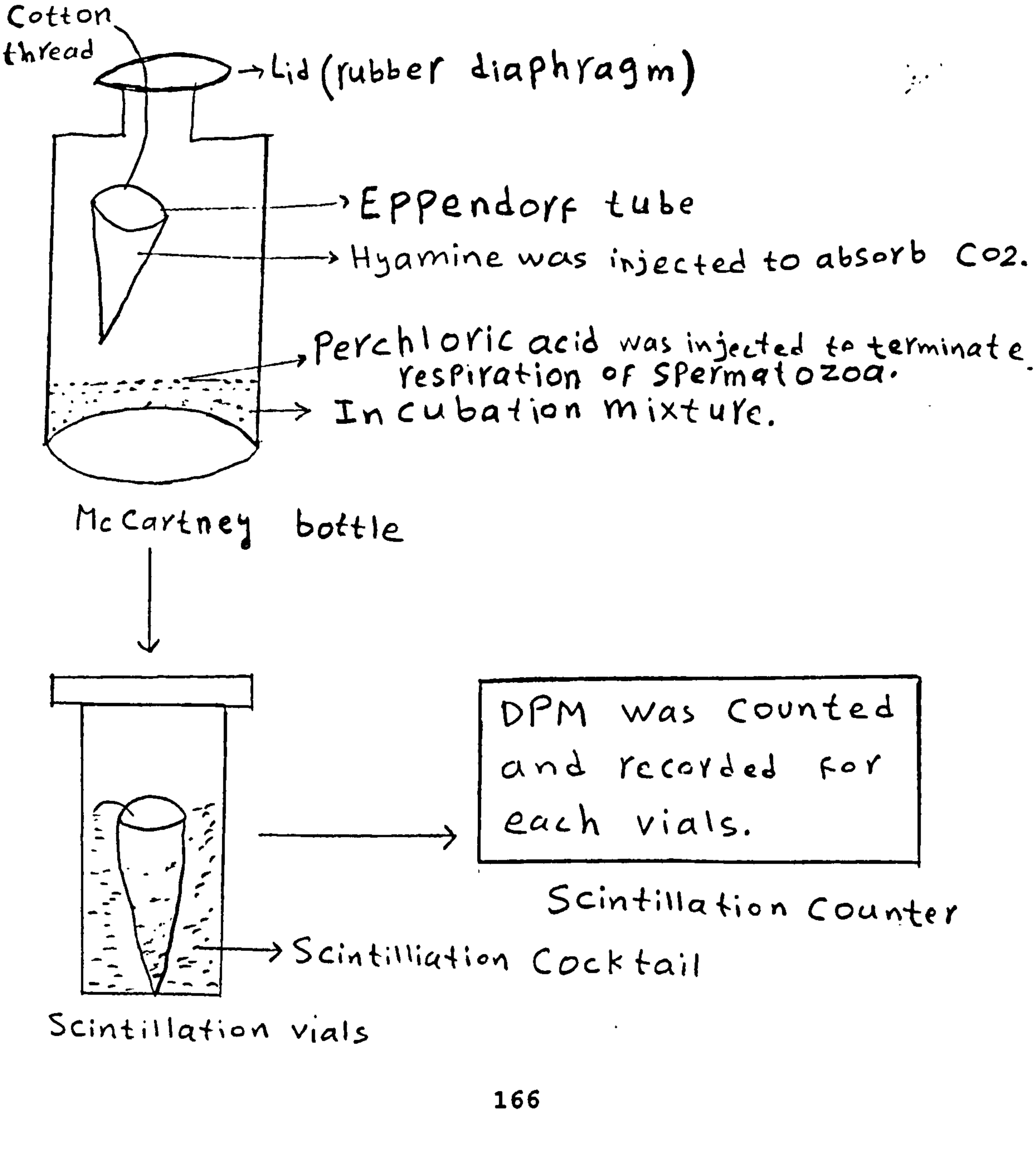
Materials	Tris	Glycerol	Raffinose
Tris	4.36 g	4.36 g	4.36 g
Citric acid	2.4 g	2.4 g	2.4 g
Glucose*	17 mM	17 mM	17mM
Streptomycin	0.1 g	0.1 g	0.1 g
Penicillin	0.06 g	0.06 g	0.06 g
Egg-yolk	18.0 ml	18.0 ml	18.0 ml
Glycerol	------	6.0 ml	6.0 ml
Raffinose	------	------	66 mM
Distilled water to	100 ml	100 ml	100 ml

*The glucose concentration was increased from 17 mM on 21.11.91 to 33 mM on

27.11.91, 29.11.91 and 6.12.91.

McCartney bottles were used to incubate the semen samples. These had previously been rinsed with repelcoat (Silicon solution) and then dried in an oven at 100°C for one hour to prevent the sperm sticking to the sides of the bottle.

The top of an Eppendorf tube was cut-off and the tube was retained for use. The side of each tube was pierced to allow it to be suspended in a McCartney bottle by a cotton thread (Diagram 1).



20 µl of 14C-glucose was mixed with 2 ml of cold 2 mM glucose. 0.1 ml of this solution was added to each McCartney bottle to which 1.4 ml of Krebs Ringer Hepes buffer (Table 18) has already been added as the incubation medium for the spermatozoa. 0.5 ml of diluted semen were dispensed into McCartney bottles. Three bottles without semen served as blanks. The bottles were closed with screw-on lids which incorporated rubber diaphragms. The components of the incubation mixtures are shown in Table 19.

Table 18: Composition of Krebs Ringer Hepes buffer.

NaCl	1.84 g	125 mM
KCl	0.08 g	4.7 mM
CaCl2.2H2o	0.046 g	1.3 mM
KH2Po4	0.040 g	1.2 mM
MgSO4	0.036 g	1.2 mM
Hepes	1.46 g	25 mM

Distilled water to 100 ml

The pH was adjusted to 6.8 using HCl.

Table 19: The components of the incubation mixtures (ml) for measuring the glucose respiration rate of ram spermatozoa using 14C-labelled glucose.

Diluted semen sample	0.0	0.50
Krebs Ringer Hepes	1.4	1.4
Selected buffer from Table 1	0.5	0.0
Labelled glucose	0.1	0.1
Final volume in McCartney bottle	2.0	2.0

The incubation of the bottles was started at one minute interval at 37°C in a shaking water bath. After one hour 0.2 ml perchloric acid was injected through the diaphragm tops into each incubation mixture to terminate the respiration of the spermatozoa and

displace the CO2. 0.5 ml of hyamine was injected into the suspended eppendorf tubes to absorb the CO2 released. The bottles were left for a further hour to complete the CO2 absorbtion. After two hours, the bottles were removed from the water bath and opened. The eppendorf tubes were removed from the bottles and their content placed in scintillation vials. 3 ml of Scintillation Cocktail T., BDH, UK., was added to each vial, shaken, and labelled before being taken to the scintillation counter. Disintegrations per minute (DPM) were recorded for each vial. 0.1 ml of incubation mixtures was counted from each of the blank (no semen) bottles to determine the specific activity of the glucose.

For experiment 4a: The rate of glucose oxidation by ram spermatozoa in Tris buffer, Glycerol buffer and Raffinose buffer was measured.

For experiment 4b: The semen samples in the three buffers were centrifuged at laboratory temperature (25°C) at 1000 g for 10 minutes (Maxi-fuge, Whyteleafe Scientific Ltd) as described by Slavik (1987). Immediately, after centrifugation, the supernatant was removed using a plastic pipette and the cells were resuspended in the original volume of Tris yolk buffer and the rate of glucose oxidation was measured.

For experiment 4c: Semen samples in the three buffers were frozen as 0.20 ml pellets on dry ice in a 4°C cold room and then transferred to liquid nitrogen as described in Chapter two. After one week, 5 pellets from each of the three treatments of semen samples were thawed in a 37°C water bath. Measurements of the glucose oxidation rates, motility and pH assessment were carried out on 0.5 ml sample of the frozen thawed semen.

For experiment 4d: The dilution of the pooled semen was carried out with following buffers:

1. Tris yolk buffer contain 17 mM glucose.

2. Tris yolk buffer contain 33 mM glucose.

3. Tris yolk glycerol (4%) buffer contain 17 mM glucose.

4. Tris yolk glucose (4%) buffer contain 33 mM glucose.

Semen diluted with each of these four buffers was divided into two portions. One portion was washed by centrifugation and resuspended in Tris egg-yolk buffer as described in experiment 4b. The rate of glucose oxidation was measured in the uncentrifuged (control) semen samples and in the centrifuged resuspended semen samples in two replications.

Experiment 4a: The aim was to measure the effect of glycerol and raffinose on the rate of glucose oxidation by ram spermatozoa.

Results:

The dates of experiments, the characteristics of the fresh semen samples, and the percentage of motility of diluted semen after one hour of incubation is shown in Table 20.

Table 20: Characteristics of semen samples when freshly collected and after one hour of incubation.

	Fresh semen			Diluted semen (after one hour of incubation)		
Date of test	Motility%	pH	Number of cells*10^9/ml	Motility % Tris	Glycerol	Raffinose
21.11.1991	75	6.5	2.85	50	50	50
27.11.1991	75	6.75	2.7	50	50	50
6.12.1991	75	6.6	2.4	40	20	20

The DPM obtained from the evolved CO2 and the specific activity of the glucose (DPM/μmoles of glucose) in the incubation mixture are shown in Table 21. The mean amounts of glucose metabolised by each diluted sample per 10^8 total sperm cells and 10^8 motile spermatozoa were calculated.

Table 21: Mean and standard error of the corrected* DPM of the evolved CO2 from the three differently diluted semen samples.

Date of test	Sample	Tris	Glycerol	Raffinose	Specific activity (DPM/μmole)
21.11.1991	1	6333(950)	3597(575)	3555(787)	44624
27.11.1991	2	2209(---)	240(222)	669(522)	46460
6.12.1991	3	1406(566)	275(90)	539(214)	43750

*. The correction was done by subtracting DPM of the blank sample recorded value from the DPM of the semen sample.

The mean rates of oxidation of glucose by the semen diluted with the three buffers is presented in Table 22 together with the statistical analysis.

Table 22: Mean and standard error of the rate of glucose oxidation, nmole/10^8 total cells and nmole/10^8 motile spermatozoa for three buffer treatments, combined for three successive dates.

	Glucose oxidised (nmole/hour)	
	Per 10^8 total cells	Per 10^8 motile cells
Treatments:		
Tris	15.10 (3.3)a	26.22 (7.3)a
Glycerol	5.95 (3.3)b	12.37 (4.2)b
Raffinose	7.32 (2.5)b	15.11 (4.5)b
F ratio	7.6**	4.11*
Samples:		
21.11.1991	15.02 (2.16)a	28.57 (4.17)a
27.11.1991	3.85 (0.9)b	7.71 (3.9)b
6.12.1991	4.30 (1.9)b	7.80 (1.4)b
F ratio	17.64***	13.76***

*P≤ 0.05 **P≤ 0.01 ***P≤ 0.005

The amount of glucose oxidised by ram spermatozoa (Table 22) decreased with the addition of 4% glycerol from a mean rate of 15.10 nmole/hour/10^8 cells to 5.94 nmole/hour/10^8 cells. There was no further reduction due to the addition of 66 mM raffinose (7.32 nmole/hour/10^8 cells). The mean of glucose oxidised per hour/10^8 motile cells in treatment three (glycerol plus raffinose) was greater than in treatment two (glycerol), 15.11 nmole/hour/10^8 cells and 12.37 nmole/hour/10^8 cells respectively, but this difference was not significant at the 5% level.

The first semen sample collected oxidised glucose at a much higher rate (P≤ 0.005) than did the subsequent two samples for both total and motile spermatozoa (Table 22).

Experiment 4b: The aim was to investigate the reversibility of the reduction of glucose oxidation by ram spermatozoa in glycerol and glycerol raffinose containing buffers.

Results:

The dates of experiments, the characteristics of the fresh semen samples, and the percentages of motility of diluted centrifuged semen samples after one hour of incubation at 37°C was shown in Table 23. The effect of washing by centrifugation on the rate of glucose oxidation (nmole/hour/10^8 total and motile spermatozoa) is shown in Figure 10.

Table 23: Semen characteristics.

	Fresh semen		Diluted centrifuged semen			
	Motility (%)	pH	Number of Cells/ml *10^9	Motility (%) Tris	Glycerol	Raffinose
Dates of test						
27.11.1991	75	6.75	2.7	35	35	35
6.12.1991	75	6.6	2.4	33	20	20

The DPM obtained from the evolved CO2 and the specific activity of the glucose (DPM/μmoles of glucose) in the incubation mixture are shown in Table 24. The calculated amounts of glucose metabolised per 10^8 total sperm cells and 10^8 motile spermatozoa by each sample is shown in Table 25.

Table 24: Mean and standard error of the corrected DPM of the evolved CO2 from resuspended semen samples that has previously been subjected to three different buffer treatment.

Date of test	Sample	Tris	Glycerol	Raffinose	Specific activity (DPM/μmole)
27.11.1991	1	1157(249)	2315(1700)	385(67)	46460
6.12.1991	2	702(158)	456(101)	485(180)	43750

Table 25: Mean and standard error of the rate of glucose oxidation, nmole/10^8 total cells and nmole/10^8 motile spermatozoa for three buffer treatments, combined for two successive dates.

	Glucose oxidised (nmole/hour)	
Treatments:	Per 10^8total cells	Per 10^8 motile cells
Tris	13.93 (2.37)	40.35 (6.66)a
Glycerol	20.10 (12.4)	30.42 (3.21)a
Raffinose	5.15 (0.69)	17.23 (2.49)b
F ratio	1.17	6.33**
Samples:		
27.11.1991	18.45 (8.22)	29.81 (5.12)
6.12.1991	7.30 (1.36)	28.86 (3.98)
F ratio	1.82	0.03

**$P \leq 0.01$

The results showed that after removal of the glycerol by centrifugation and resuspension of the cells in glycerol free diluent the oxidation rate of glucose per 10^8 total spermatozoa was indistinguishable from that of the control spermatozoa (Figure 10). There was no significant ($P \geq 0.05$) difference between the amount of glucose oxidised (nmole/10^8 total and motile cells/hour) of Tris yolk treated and glycerol treated semen samples (Table 25). The mean of glucose oxidised per hour/10^8 motile spermatozoa was 40.35 and 30.42 for Tris and Glycerol respectively. The mean oxidation rate of glucose (nmole per hour/10^8 motile cells) for Raffinose treated semen samples was significantly lower than for the Tris sample ($P \leq 0.05$), but not for the Glycerol diluted semen (Table 25).

Experiment 4c: The aim was to measure the rate of glucose oxidation by frozen/thawed ram spermatozoa and the effect upon this of added glycerol and raffinose.

Results:

Table 26: The characteristics of the fresh semen samples.

Date of test	Sample	Motility (%)	pH	Number of cell *10^9/ml
29.11.1991	1	75	6.5	2.85
6.12.1991	2	75	6.6	2.4

Table 27: The post-thaw motility, live percentage and pH of semen samples, before and after one hour of incubation was presented.

Date of test	No. of sample	Incubation (minutes)	Tris			Glycerol			Raffinose		
			M*	L*	P*	M*	L*	P*	M*	L*	P*
29.11.1991	1	1	5	8	6.97	36	38	6.84	40	42	6.97
	1	60	4	5	6.25	33	35	6.65	40	40	6.74
6.12.1991	2	1	5	8	---	37	38	---	40	42	---
	2	60	4	7	---	34	35	---	40	40	---

M*=Motility.

L*=Live percent.

P*=pH.

Table 28: Mean and standard error of the corrected DPM of the evolved CO2 from the three different diluted frozen-thawed ram semen samples.

Date of test	Sample	Tris	Glycerol	Raffinose	Specific activity (DPM/μmole)
29.11.1991	1	522 (170)	360 (70)	654 (145)	45647
6.12.1991	2	229 (32)	146 (13.4)	142 (23)	43750

The mean rate of oxidation of glucose by the thawed ram semen is presented for the three diluents used in Table 29 together with the statistical analysis.

Table 29: Mean and standard error of the rate of glucose oxidation, nmole/hour/10^8 total cells and nmole/hour/10^8 motile spermatozoa for three buffer treatments, combined for two successive dates.

	Glucose oxidised (nmole/hour)	
	Per 10^8 total cells	Per 10^8 motile cells
Treatments:		
Tris	3.73 (0.88)	44.9 (13.3)a
Glycerol	2.58 (0.5)	7.2 (1.3)b
Raffinose	3.83 (1.26)	9.6 (3.1)b
F ratio	1.23	5.88*
Samples:		
29.11.1991	4.85 (0.8)a	30.3 (11.0)a
6.12.1991	1.91 (0.2)b	10.7 (3.3)b
F ratio	15.46***	9.18**

*P≤ 0.05 **P≤ 0.01 ***P≤ 0.005

The results showed that there was no significant (P≥ 0.05) differences for the n mole/10^8 total cell of glucose oxidised for three differently treated ram semen samples which was subjected to freezing (Table 29).

The first semen sample collected had a much higher rate of glucose oxidation (P≤ 0.05) than the subsequent sample for both total and motile spermatozoa (Table 29).

Experiment 4d: During the 14C-glucose oxidation tests of ram spermatozoa, differences of the rates of glucose oxidation between semen collections had been observed. The aim of this experiment was to find if different concentrations (17 mM or 33 mM) of glucose in diluted semen samples affected the rate of glucose oxidation and reexamine the effect of glycerol on the rate of glucose oxidation of spermatozoa.

Results:

The pH of the freshly pooled semen sample was 6.80 and optical density was 0.70 representating $2.20*10^9$ spermatozoa per ml.

The total number, and motile number, of ram spermatozoa in 0.5 ml of diluted semen sample for uncentrifuged and centrifuged samples for two treatments are shown in Table 30. The rate of glucose oxidation (nmole/hour/10^8 cells) in the diluents containing 17 mM glucose or 33 mM glucose is shown in Table 31.

Table 30: Characteristics of diluted semen sample before and after centrifugation.

Treatments	Number of spermatozoa $*10^8$			
	Total cells		Motile cells	
	Uncentrifuged	Centrifuged	Uncentrifuged	Centrifuged
Glucose 17 mM:				
Tris	3.66	1.22	2.19	0.61
Glycerol	3.66	1.22	2.01	0.43
Glucose 33mM				
Tris	3.66	1.22	2.56	0.61
Glycerol	3.66	1.22	2.01	0.40

Table 31: The mean and standard error of the DPM value of the evolved 14-Co2, glucose oxidised (nmole/hour of incubation, nmole/hour/10^8 of total) spermatozoa and glucose oxidised (nmole/hour/10^8 motile spermatozoa.

Treatments	Centrifugation of diluted semen samples	
	Control	Centrifuged
DPM:		
17 mM glucose:		
Tris	27551 (1193)	8739 (889)
Glycerol	29022 (852)	7041 (909)
33 mM glucose:		
Tris	15744 (651)	3862 (555)
Glycerol	14179 (449)	3477 (389)
Glucose oxidised nmole/hour:		
17 mM glucose:		
Tris	180.95 (7.83)	57.39 (5.84)
Glycerol	190.55 (5.58)	46.24 (5.97)
33 mM glucose:		
Tris	197.63 (8.17)	48.47 (6.96)
Glycerol	177.98 (5.63)	43.63 (4.89)
Glucose oxidised nmole/hour/10^8 total spermatozoa:		
17 mM glucose:		
Tris	49.44 (2.14)	47.04 (4.79)
Glycerol	52.06 (1.52)	37.91 (4.90)
33 mM glucose:		
Tris	53.99 (2.23)	39.73 (5.70)
Glycerol	48.63 (1.52)	35.78 (4.02)
Glucose oxidised nmole/hour/10^8 motile spermatozoa:		
17 mM glucose:		
Tris	82.25 (3.56)	94.08 (9.57)
Glycerol	94.80 (2.78)	107.50 (13.9)
33 mM glucose:		
Tris	77.19 (3.19)	79.50 (11.40)
Glycerol	88.54 (2.80)	90.90 (10.20)

Table 32: Mean and standard error of the rate of glucose oxidation, nmole/10^8 total cells and nmole/10^8 motile spermatozoa incubated in either Tris or Glycerol buffer containing 17 mM glucose or 33 mM glucose for control (uncentrifuged) and centrifuged semen.

	Glucose oxidised (nmole/hour)	
	Per 10^8 total cells	Per 10^8 motile cells
Treatments:		
Tris	47.55 (2.24)	83.25 (3.88)b
Glycerol	43.59 (2.33)	95.44 (4.38)a
F ratio	2.31$	6.05*
Centrifugation:		
Control	51.03 (1.01)a	85.70 (2.20)
Centrifuged	40.11 (2.44)b	92.99 (5.71)
F ratio	25.20***	2.17
Glucose:		
17 mM	46.61 (2.14)	94.67 (4.54)a
33 mM	44.53 (2.50)	84.02 (3.85)b
F ratio	0.91	4.61*
Replication:		
1	44.15 (2.73)	85.85 (4.36)
2	46.99 (1.79)	92.84 (4.31)
F ratio	1.71	1.99

$=The effect of treatment was significant at 10% level for 10^8 total sperm.

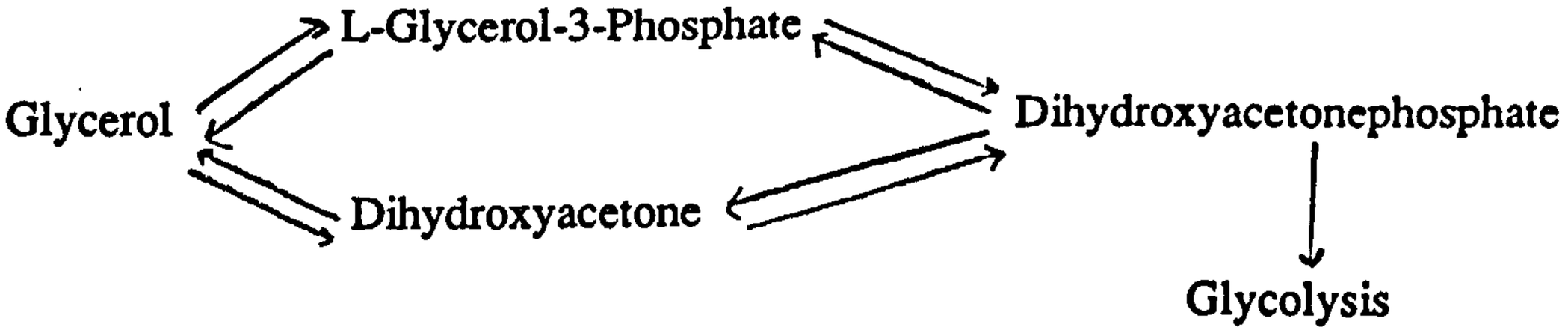
*$P \leq 0.05$ ***$P \leq 0.005$

The results in Table 32 show that varying the glucose concentration from 17 mM to 33 mM did not affect the rate of glucose oxidation per 10^8 total spermatozoa. Glycerol

lowered the rate of glucose oxidation per 10^8 total sperm ($P \leq 0.1$). The glucose oxidation rate per 10^8 motile sperm was increased significantly after glycerol treatment ($P \leq 0.05$). Centrifugation and resuspension of the semen samples lowered the rate of glucose oxidation per 10^8 of ram spermatozoa significantly ($P \leq 0.05$). But this effect was only significant ($P \geq 0.161$) per 10^8 motile spermatozoa. Increasing the level of glucose in semen diluent reduced the rate of glucose oxidation, but this was only significant per 10^8 motile cells ($P \leq 0.05$).

Discussion:

The addition of glycerol to the diluent reduced the rate of oxidation of glucose by the spermatozoa. The addition of raffinose did not further reduce it. Washing the sample by centrifugation and resuspension reduced or eliminated this effect. This suggested that the initial inhibition was due to metabolic replacement and not the result of toxicity. Mohri and Masaki (1967) showed that glycerol affected aerobic glucose utilisation through the glycerokinase enzyme which exists in the mitochondria of ram and bull sperm. This enzyme phosphorylates glycerol as the first step of its utilisation. Glycerol-3-phosphate is then oxidised by mitochondrial enzymes to dihyroxyacetonephosphate, which in turn enters the glycolytic pathway as shown in the diagram below:



When spermatozoa presented in glycerol, are inseminated, they would be expected to metabolise glucose like fresh sperm once they swim clear of the insemination fluids.

The mean (standard error) of the rates of glucose oxidised (nmoles/hour/10^8 cells) reported in the literature are 67 (1.9) for turkey spermatozoa (Pinto et al., 1984); 3.2 (1.1)

for boar spermatozoa (Ford and Harrison, 1987); 12.9 (1.6) for rat spermatozoa (Ford et al., 1981); 32 for ram spermatozoa (Voglmayr et al., 1967); 31.5 for ram spermatozoa (Voglmayr et al., 1977); 83 for ram spermatozoa (Murdoch and White, 1966) and 54 (Mountford, 1989).

In this study, the mean rate of glucose oxidised by ram semen diluted in a Tris buffer was 25.5 (11.0) nmoles/10^8 spermatozoa. This compares reasonably with the data of Voglmayr et al. (1967) who obtained a mean of 32 nmoles/hour/10^8 using ejaculated ram semen and Voglmayr et al. (1977) who obtained 31.5 nmoles/10^8 using washed ram sperm collected from the epididymis is slightly low. It is lower than the rate of glucose oxidised (83 nmoles/hour/10^8 cells) was reported by Murdoch and White (1966). The lower value of glucose oxidation obtained in this experiment compared with the result of above authors could be due to the use of diluted semen which contained other metabolites, such as glucose, while they measure the rate of glucose oxidised using washed sperm collected from epididymis.

The differences between the rate of glucose oxidation (nmole/hour/10^8 cell), obtained within samples could be due to change of the glucose concentration from 17 mM (21.11.91) to 33 mM (27.11.91 to 6.12.91). This would agree with Watson (1979) who concluded that the components of the extracellular medium influences the biochemical activity of semen. However, the overall higher rate of glucose oxidation (nmole/hour/10^8 cells) for experiment 4d (45.57), compared with experiment 4a and 4b (10.29), may indicate that spermatozoa utilised more glucose through glycolysis during the breeding season and used oxidative means during the non-breeding season.

The mean rate of glucose oxidised by frozen-thawed ram sperm in three buffers was 3.8 (1.2) nmole/hour/10^8cells. The rate of oxidation of 14C-Glucose did not differ for the different buffers.

Experiment five:

In the experiments reported above, it was shown that ram semen survived freezing better and had greater motility when 66 mM raffinose was added to the diluent. The use of 14C-glucose showed that the addition of raffinose to hyper-osmotic levels did not upset the energy metabolism of the sperm as measured by its rate of glucose utilisation.

The aim of this experiment was to compare semen frozen in the presence of raffinose either in 0.1 ml pellets or in 0.25 ml straws, with semen frozen in pellets by the standard method of Evans and Maxwell (1987). The comparison was made on the thawed semen by a fertility test and by a range of *in vitro* tests carried out on the same batches of frozen semen.

Materials and methods:

Nine Texel rams were used in the experiment. They were trained to serve into the A.V. as described in Chapter two. Two rams showed no interest in the teaser ewe at all, and two others were insufficiently tame for collection to be made, even after several days of training. Semen samples were collected from the other 5 Texel rams twice in two consecutive weeks. Pooled semen from each collection was divided into three aliquots and diluted in the ratio of 1:2 with the three different diluents. The composition of these three diluents is shown in Table 33.

Table 33: The composition of the diluents used in experiment four.

	Diluent one	Diluent two	Diluent three
Tris	4.361 g	4.361 g	4.361 g
Glucose	0.600 g	0.600 g	0.600 g
Citric acid	2.388 g	2.338 g	2.338 g
Egg-yolk	18 ml	18 ml	18 ml
Glycerol	6 ml	6 ml	9 ml
Raffinose	-----	66 mM	66 mM
Penicillin	100,000 IU	100,000 IU	100,000 IU
Streptomycin	100 IU	100 IU	100 IU

Distilled water was added to 100 ml and pH was adjusted to 6.8 using HCl (1 M).

The diluted semen samples were cooled to 5°C over 2.5 hours and frozen as follows:

1) For treatment one the semen was extended with diluent one and frozen as 0.20 ml pellets by the standard method of Evans and Maxwell (1987).

2) For treatment two the semen was extended with diluent two and frozen similarly on dry ice, but in 0.1 ml pellets.

3) In treatment three the semen was extended with diluent three and frozen in 0.25 ml French straw in a programmable cooler using a cooling rate of -30°C/minute to a temperature of -120°C.

Frozen semen samples were stored in liquid nitrogen (-196°C) until the day of use.

***In vitro* assessment:**

Motility percentage, live percentage, acrosome integrity and osmotic resistance was assessed as described in Chapter two. The slope of swimming-up was measured as described in Chapter seven. The ATP content of the spermatozoa was measured as

described below:

ATP determination:

The ATP was assayed in the ram semen using the bioluminescence method similar to that described by Grudova et al, (1988). This is based on the property of luciferase to trigger the oxidation of the specific substrate luciferin by air oxygen in the presence of ATP and Mg^{2+}. This reaction shown below, is absolutely specific for ATP.

Luciferase

ATP + luciferin + O_2 ---------- Oxyluciferin + Mg^{2+} AMP + CO_2+ PPi + quantum light (hv).

ATP reacts with firefly luciferin to form adenylluciferin. This is oxidised by air with light emission. Both reaction are catalysed by firefly luciferase. Conditions were chosen so that:

a) ATP was well below its Km, so that the rate of light emission was proportional to its concentration.

b) Small amounts of enzyme was used, and competitive inhibitors of luciferase included, so that the amount of ATP used up in the reaction was negligible, and the light output was constant during the determination.

Materials for ATP determination:

1. Reaction buffer: This is composed of 0.1 M tris, 10 mM of Magnesium chloride, and 2mM of Ethylenediaminetetra-acetic acid (EDTA). For each of the above substance 1.21 g 0.203 g and 0.07 g were weighed out, respectively and the whole made up to 100 ml using distilled water. The pH of reaction buffer was adjusted to 7.5 using hydrochloric acid 5 M. This solution is stable at 4°C for months. On the day of ATP measurement, 1mM of dithiotheritol (DTT) was added to the reaction buffer to stabilise the enzyme.

2. Tetrasodium pyrophosphate 20 mM was made up in reaction buffer. On the day of use, it was further diluted to 40 μM solution.

3. 1 ml of stock ATP solution 1 mM, was diluted fifty times to prepare a standard solution containing 20 µM ATP per ml. It was necessary to make this on the day of use.

4. Luciferin: 0.5 mg/ml of luciferin was dissolved in reaction buffer containing 5 mg/ml bovine serum albumin (BSA). This was stored in 0.1 ml aliquots at -20°C and diluted to 2 ml before use with reaction buffer containing 5 mg/ml BSA.

5. Luciferase: 1 mg/ml of luciferase was dissolved in reaction buffer and was stored in 0.1 ml aliquots at -20°C, while protected from light by wrapping the tube in foil. Luciferase loses activity on freeze-thawing, and should be frozen in aliquots, to be thawed not more than twice each. The enzyme was diluted to 2 ml before use with reaction buffer containing 5 mg/ml BSA.

6. The diluted luciferin and luciferase were mixed in equal volumes before use within the next 2 hours.

Preparation of semen for ATP assay:

Diluted semen samples were boiled in a test tube to extract ATP from the sperm and then plunged into liquid nitrogen to preserve the ATP. On the day of ATP measurement, the semen was thawed at 37°C, centrifuged and the supernatant taken for analysis.

The composition of assay mixture:

10 µl of reaction buffer, 10 µl of tetrasodium pyrophosphate, and 60 µl of supernatant from a boiled semen sample in conjunction with either 10 µl reaction buffer or with 10 µl ATP standard in reaction buffer were mixed in an assay tube. Then 40 µl of luciferin luciferase mixture was injected into the assay tube using a Hamilton syringe. These were mixed well before being placed in the luminometer (Luminescence Biometer, Dupont Instruments). Peak heights relative to the amount of ATP present were recorded on a chart recorder.

a) Calibration:

A standard curve was obtained using various volumes of ATP standard (from 0 µl to 60 µl) in place of the semen extract.

Determination of ATP contents in a semen samples:

A standard curve relating ATP content (0-60 µl) with peak height, was prepared. The peak height produced by a 60 µl sample of semen supernatant was then determined with and without the addition of 10 µl of standard ATP as an internal standard so that allowance could be made for quenching.

ATP determination were carried out in triplicate at the pre-freezing (5°C) stage, and again at the post-thawing stage. Semen samples were thawed using a 37°C water bath and then diluted with thawing solution (Chapter two) at a ratio of 1:1 for assessment.

Fertility test:

108 Welsh Mountain ewes were synchronised by progestagen impregnated sponges followed by the intramuscular injection of 320 IU pregnant mare serum gonadotrophin (PMSG) on the day of sponge removal. 5 Harnessed vasectomised rams were introduced on the day of sponge removal. After 56 hours, the ewes were cervically inseminated using 0.20 ml of thawed ram semen, which contained $124*10^6$ spermatozoa. The operation was carried out at pasture in a trailer under farm conditions. Insemination was carried out giving semen from each treatment in turn to groups of three ewes. The treatment order was established at random. On the day of insemination, ewes mated and marked by the teasers before 9.00am were inseminated first, followed by the rest of the flock. Harnessed fertile rams were introduced 10 days after insemination. The non-return rates for ewes were recorded when they had not returned to service and been marked by the rams within 34 days of artificial insemination (Salamon and Lightfoot, 1970).

Results:

a) *In vitro* assessment:

The results of *in vitro* assessment of the semen samples at the pre-freezing stage is summarised in Tables 34, 35, and at the post-thawing stage in Tables 36 and 37.

Table 34: Means, standard errors and Anova of motility, live percentage, and percentage of acrosome damage for chilled (5°C) pre-freezing semen samples, for the standard pellets, raffinose pellets and raffinose straws in two consecutive weeks.

	Motility (%)	Live (%)	Acrosome damage (%)
Treatments (A):			
Standard pellet	63.00(0.45)a	64.50(0.22)a	27.83(0.98)b
Raffinose pellet	59.00(0.68)b	59.67(0.33)b	28.00(0.89)b
Raffinose straw	60.00(0.00)b	60.00(0.00)b	31.50(1.57)a
F	32.50***	196.75***	337.00***
Weeks (B):			
1	60.22(0.76)	61.11(0.75)	31.67(0.83)a
2	61.11(0.61)	61.67(0.83)	26.55(0.37)b
F	4.41	6.25	2116.0***
Interactions (A*B):			
A1*B1	62.67(0.33)	64.00(0.00)	30.00(0.00)b
A1*B2	63.33(0.88)	65.00(0.00)	25.67(0.33)d
A2*B1	58.00(1.15)	59.00(0.67)	30.00(0.00)b
A2*B2	60.00(0.00)	60.00(0.00)	26.00(0.00)d
A3*B1	60.00(0.00)	60.00(0.00)	35.00(0.00)a
A3*B2	60.00(0.00)	60.00(0.00)	28.00(0.00)c
F ratio	1.94	1.75	73.00***

***P≤ 0.005

Table 35: Means, standard errors and Anova of osmotic resistance, swimming-up slope, and the content of ATP (nmoles/10^8 live spermatozoa) for chilled (5°C) pre-freezing semen samples, for the standard pellets, raffinose pellets, and raffinose straws in two consecutive weeks.

	Osmotic resistance (%)	Swimming-up slope (cm/min)	ATP (nmoles/ 10^8live cells)
Treatments (A):			
Standard pellet	42.50(1.12)a	1.77(0.15)	13.20(1.08)c
Raffinose pellet	40.33(1.05)b	1.37(0.09)	21.48(0.42)a
Raffinose straw	39.00(0.47)c	1.50(0.17)	18.94(0.96)b
F	337.00***	1.53	36.34***
Weeks (B):			
1	38.67(0.33)b	---	16.68(1.68)b
2	42.56(0.73)a	1.54(0.09)	19.08(0.86)a
F	1225.00***	---	8.66*
Interactions (A*B):			
A1*B1	40.00(0.00)c	---	10.80(0.00)
A1*B2	45.00(0.00)a	1.76(0.14)	15.60(0.00)
A2*B1	38.00(0.00)d	---	22.44(0.00)
A2*B2	42.67(0.33)b	1.36(0.09)	20.54(0.00)
A3*B1	38.00(0.00)d	---	16.80(0.00)
A3*B2	40.00(0.00)c	1.50(0.17)	21.08(0.00)
F ratio	73.00***	---	---

*P≤ 0.05 ***P≤ 0.005

Table 36: Means, standard errors and Anova of motility, live percentage, and percentage of acrosome damage for post-thawing frozen semen samples at 37°C, for the standard pellets, raffinose pellets and raffinose straws in two consecutive weeks.

	Motility (%)	Live (%)	Acrosome damage (%)
Treatments (A):			
Standard pellet	36.67(0.76)b	38.67(0.67)b	53.00(1.29)a
Raffinose pellet	40.17(2.32)a	41.50(2.06)a	48.67(0.61)b
Raffinose straws	40.00(2.24)a	41.50(2.01)a	47.67(1.52)b
F	191.36***	20.35***	20.19***
Weeks (B):			
1	35.00(0.00)b	37.11(0.35)b	50.67(0.62)a
2	42.89(1.15)a	44.00(1.00)a	48.89(1.60)b
F	2291.36***	270.70***	5.95*
Interactions (A*B):			
A1*B1	35.00(0.00)c	37.33(0.67)c	51.00(2.08)b
A1*B2	38.33(0.33)b	40.00(0.00)b	55.00(0.00)a
A2*B1	35.00(0.00)c	37.00(1.00)c	50.00(0.00)b
A2*B2	45.33(0.33)a	46.00(0.00)a	47.33(0.33)c
A3*B1	35.00(0.00)c	37.00(0.00)c	51.00(0.00)b
A3*B2	45.00(0.00)a	46.00(0.00)a	44.33(0.67)c
F ratio	191.36***	24.42***	18.23***

*P≤ 0.05 ***P≤ 0.005

Table 37: Means, standard errors and Anova of osmotic resistance, the swimming-up slope, and the amount of ATP (nmoles/10^8 live spermatozoa) for post-thawing frozen semen samples at 37°C, for the standard pellets, Raffinose pellets, and raffinose straws in two consecutive weeks.

	Osmotic resistance (%)	Swimming-up slope (cm/min)	ATP (nmoles/ 10^8live cells)
Treatments (A):			
Standard pellet	10.33(0.33)a	0.17(0.02)b	10.34(0.66)a
Raffinose pellet	6.83(0.54)b	0.14(0.01)c	5.50(0.25)b
Raffinose straw	7.00(0.45)b	0.20(0.00)a	12.50(1.03)a
F	72.59***	147.57***	6.30**
Weeks (B):			
1	8.89(0.48)	0.18(0.02)a	10.36(0.84)
2	7.22(0.70)	0.16(0.01)b	8.54(0.65)
F	38.79***	36.57***	1.24
Interactions (A*B):			
A1*B1	10.67(0.67)	0.23(0.00)a	7.40(0.00)b
A1*B2	10.00(0.00)	0.12(0.00)d	13.30(0.00)a
A2*B1	8.00(0.00)	0.11(0.00)d	6.60(0.00)b
A2*B2	5.67(0.33)	0.17(0.00)c	6.40(0.00)b
A3*B1	8.00(0.00)b	0.20(0.01)b	17.10(0.00)a
A3*B2	6.00(0.00)c	0.20(0.00)b	7.90(0.00)b
F ratio	3.62	284.71***	----

***P≤ 0.005

Pre-freezing assessment of semen samples showed that the percentage of motility, percentage of live spermatozoa, the osmotic resistance, and swimming-up vigour were significantly ($P \leq 0.05$) greater for the sperm in the standard buffer than in the raffinose containing buffers. But the ATP content per 10^8 live spermatozoa for standard buffer was significantly ($P \leq 0.05$) lower than for the raffinose containing buffers.

Post-thawing assessment of semen samples showed that the raffinose containing pellets and straws had significantly ($P \leq 0.05$) higher percentages of motile and live spermatozoa than did the standard pellets (Table 36). The amount of acrosome damage was greater ($P \leq 0.05$) for the standard pellets than the raffinose containing pellets and straws. However, the spermatozoa extended with diluent one and frozen as standard pellets were more resistant to the hypo-osmotic solution than were spermatozoa in diluents two or three, when frozen as raffinose containing pellets or straws. Raffinose containing straws showed a significantly ($P \leq 0.05$) higher swimming-up slope than raffinose contained pellets. Thawed standard pellets contained significantly greater amounts of ATP per 10^8 live spermatozoa than the raffinose pellets (Table 37).

The slope of the swimming-up test was 14%, 25%, and 27% of the pre-freezing value for standard pellets, raffinose pellets and raffinose straws respectively. The post-thawing recovery percentage of swimming-up for raffinose treated semen samples was significantly ($P \leq 0.01$) greater than for semen samples in the standard diluent. A comparison of the post-thawing recovery of spermatozoa for the ability to survive and the ability to swim-up shows that although 60% and 69% of spermatozoa extended with standard diluent and raffinose containing diluent survived, only a smaller fraction (14% and 26% of spermatozoa respectively) could swim-up into the spectrophotometer light path. This impairment of the spermatozoa suggests that there are differences between the activities of cells before freezing and after undergoing freezing procedure which can be called the freezing effect.

b)Fertility results:

The fertility results are shown in Tables 38, 40 and 42. The results of fertility analysis are shown in Tables 39, 41 and 43.

The effect of the teaser rams on marking the ewes and the interval from sponge removal/PMSG injection to insemination, on non-return rate of the ewes cervically inseminated are shown in Figures 11 and 12.

Table 38: Fertility results for ewes inseminated with ram semen frozen in standard pellets, raffinose pellets or raffinose straws.

Methods of freezing	Number of ewes inseminated	Number of ewes fertilised*	Percentage of ewes fertilised*
Standard pellets	34	7	21
Raffinose pellets	35	7	20
Raffinose straws	39	12	31

*Number of ewes fertilised is equal to the number of ewes that did not return to service after two consecutive oestrous cycles.

Table 39: Chi-square analysis of fertility results.

Standard pellets	DF	Chi-square value for fertility data
vs		
Raffinose pellets		
vs	2	1.50*
Raffinose straws		

*This is significant at the 53% level.

For $P \leq 0.05$ Chi-square is 5.99.

Table 40: Fertility results for ewes marked or not marked by the teasers, and inseminated cervically using frozen ram semen.

Methods of grouping	Number of ewes inseminated	Number of ewes fertilised*	Percentage of ewes fertilised*
Marked by 9.0am	43	8	19
Marked by 4.0pm	9	2	22
Unmarked	56	16	28

*Number of ewes fertilised is equal to the number of ewes that did not return to service after two consecutive reproduction cycles.

Table 41: Chi-square analysis of fertility results for the effect of the marking by teaser rams.

Marked by 9.0am	DF	Chi-square value for fertility data
vs		
Marked by 4.0pm		
vs	2	1.34*
Unmarked		

*This is significant at the 49% level.

For $P \leq 0.05$ Chi-square is 5.99.

Table 42: The fertility results of the ewes were inseminated cervically after 56 hours, 57 hours and 58 hours of sponge removal and PMSG injection using frozen ram semen.

Time of insemination (Hours)	Number of ewes inseminated	Number of ewes fertilised*	Percentage of ewes fertilised*
56 hours	36	7	19
57 hours	27	6	22
58 hours	45	13	29

*Number of ewes fertilised is equal to the number of ewes did not return to service after two consecutive reproduction cycles.

Table 43: Chi-square analysis of fertility results for the effect of the time of insemination (hours) after sponge removal/PMSG injection.

56 hours	DF	Chi-square value for fertility data
vs		
57 hours		
vs	2	1.044*
58 hours		

*This is significant at the 41% level.

For $P \leq 0.05$ Chi-square is 5.99.

The non-return rates of the ewes were 21, 20 and 31% for the standard pellets, raffinose pellets and raffinose straws respectively (Table 38). The differences between these rates was not significant at the 5% level of probability.

Discussion:

The percentage of motility and percentage of live spermatozoa and the swimming-up slope were significantly ($P \leq 0.05$) lower for the raffinose treated spermatozoa than spermatozoa diluted in buffer one at the pre-freezing stage. This could have been due to the concentration of solute which made the diluent more viscous or to dehydration of the cells by the raffinose. At that stage the concentration of ATP (nmoles per 10^8 live spermatozoa) was significantly higher for spermatozoa extended in diluents two and three than diluent one. This suggested that the reduction of spermatozoa activity due to the hyper-osmotic environment could have spared ATP. At the post-thawing stage, when the osmotic pressure was reduced by the addition of thawing solution, the percentage of motile and of live sperm were significantly ($P \leq 0.05$) greater for raffinose pellets and straws than standard pellets. The swim-up test showed that the raffinose straws treated spermatozoa were more vigorous than those from the standard pellets and raffinose pellets ($P \leq 0.05$). However, the post-thaw recovery rate for motility was 57%, 67%, and 66% for standard pellets, raffinose pellets and raffinose straws respectively. The mean recovery rates for motility in two consecutive weeks was 57% and 70% respectively. The swimming-up recovery rate was 14%, 25% and 27% for standard pellets, raffinose pellets and raffinose straws respectively. The recovery rate of swimming-up was significantly ($P \leq 0.05$) higher for raffinose treated semen samples.

The post-thawing swim-up test was significantly correlated with the ATP content/10^8 live sperm ($P \leq 0.05$), with the ATP content/10^8 total sperm ($P \leq 0.02$), and with the percentage non-return rate ($P \leq 0.1$). However, there were no significant correlations between percentage of motile sperm, ATP content and non-return rate (Table 44).

Table 44: The correlation coefficient (r) for the post-thawing *in vitro* and *in vivo* tests.

Relationship	Correlation coefficient (r)
Swim-up slope * ATP content/10^8 live sperm	0.971**
Swim-up slope * ATP content/10^8 total sperm	0.983***
Non-return rate% * ATP content/10^8 live sperm	0.775
Non-return rate% * ATP content/10^8 total sperm	0.810
Non-return rate% * Swim-up slope	0.904*
Motility% * ATP content/10^8 live sperm	-0.241
Motility% * Swim-up slope	0.000
Motility% * Non-return rate%	0.427

*P≤ 0.1

**P≤ 0.05

***P≤ 0.02

Although the fertility test using the limited number of ewes available showed no significant benefit from the addition of raffinose to the diluent, it did confirm that raffinose treated spermatozoa and frozen in straws had retained their fertility capacity. This, taken in association with the grater vigour and activity of sperm frozen with raffinose, suggests that raffinose used in this way could be a useful component of a freezing diluent.

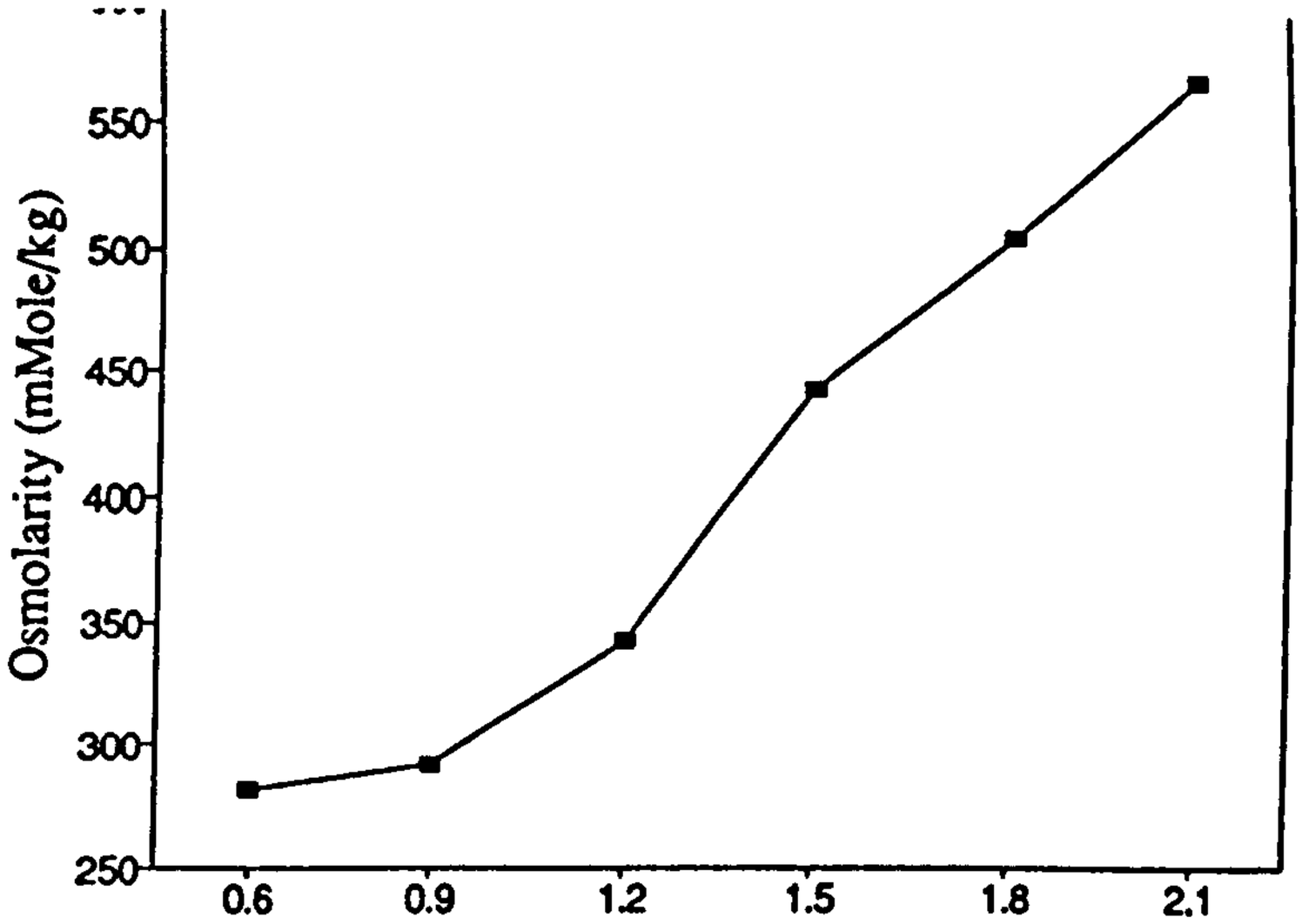


Figure 1: Standard curve relating osmolarity (osmometer reading) to concentration of NaCl (g/100ml).

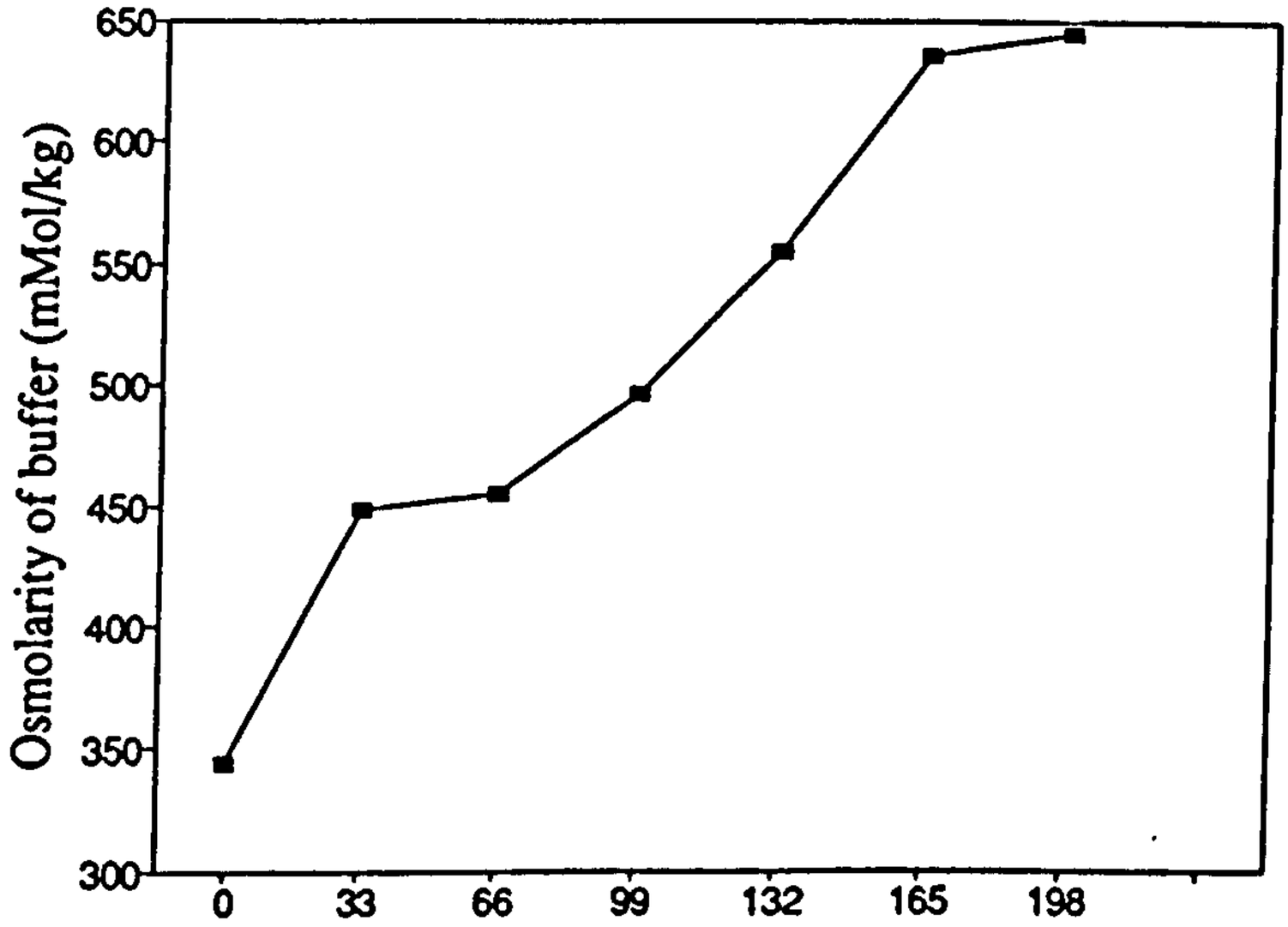


Figure 2: The effect of raffinose concentration (mM) on the osmolarity of Tris buffer.

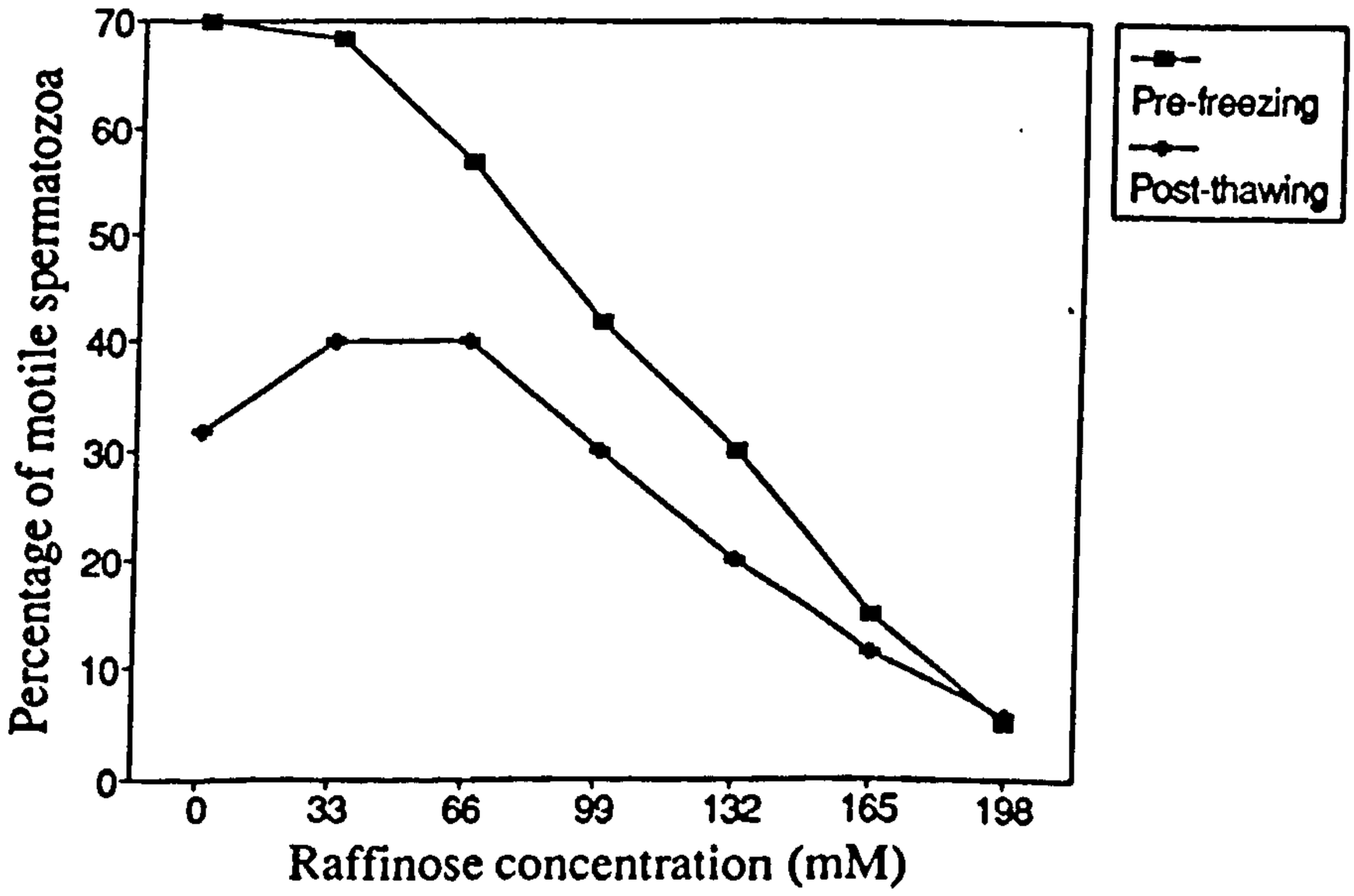


Figure 3: The effect of raffinose concentration on the percentage of motile spermatozoa at the pre-freezing (—■—) and post-thawing (—•—) stage.

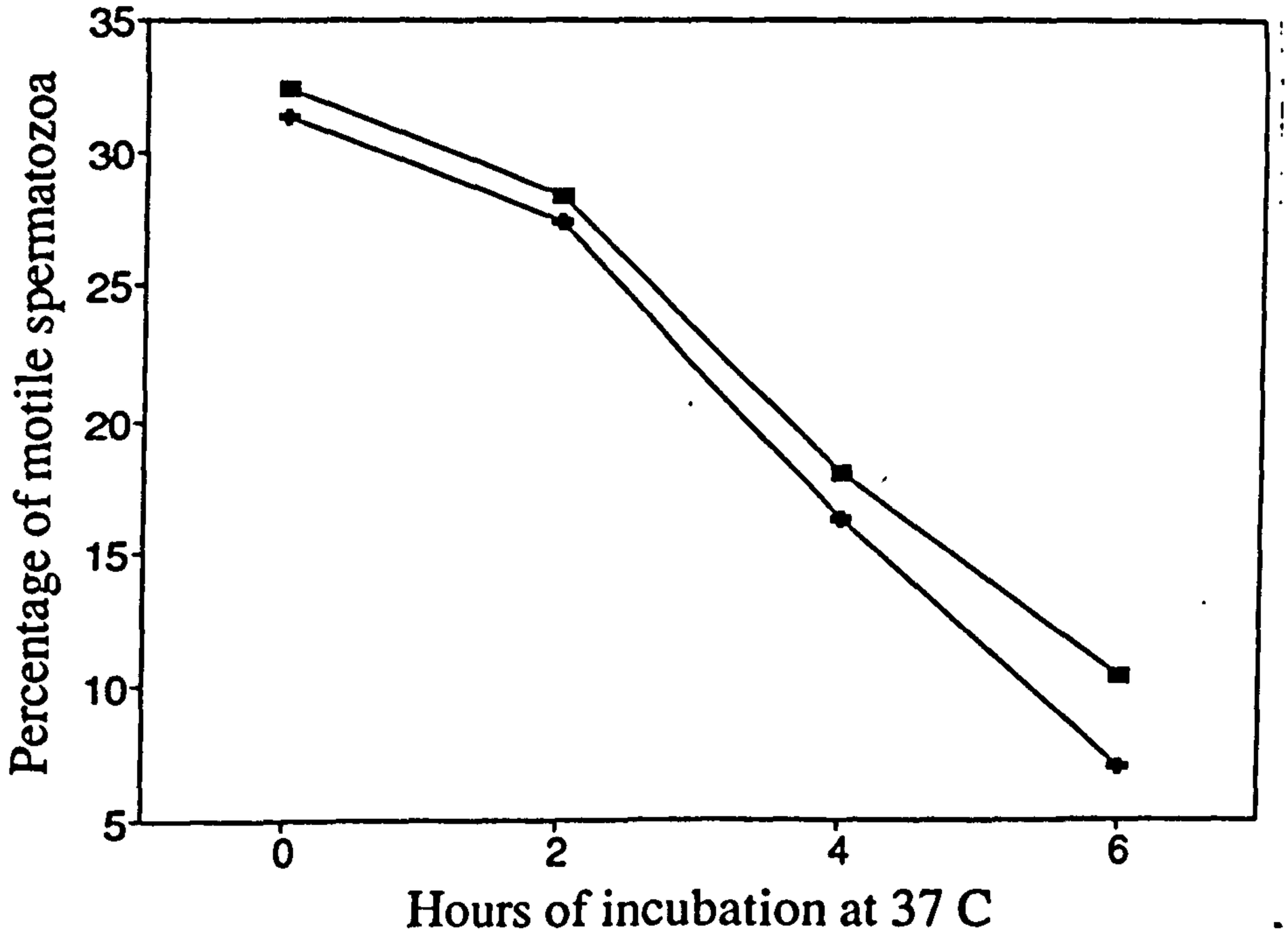


Figure 4: The effect of pellet sizes, 0.1ml (—■—) and 0.2ml (—•—) on the percentage of motile spermatozoa up to 6 hours of post-thawing incubation at 37°C.

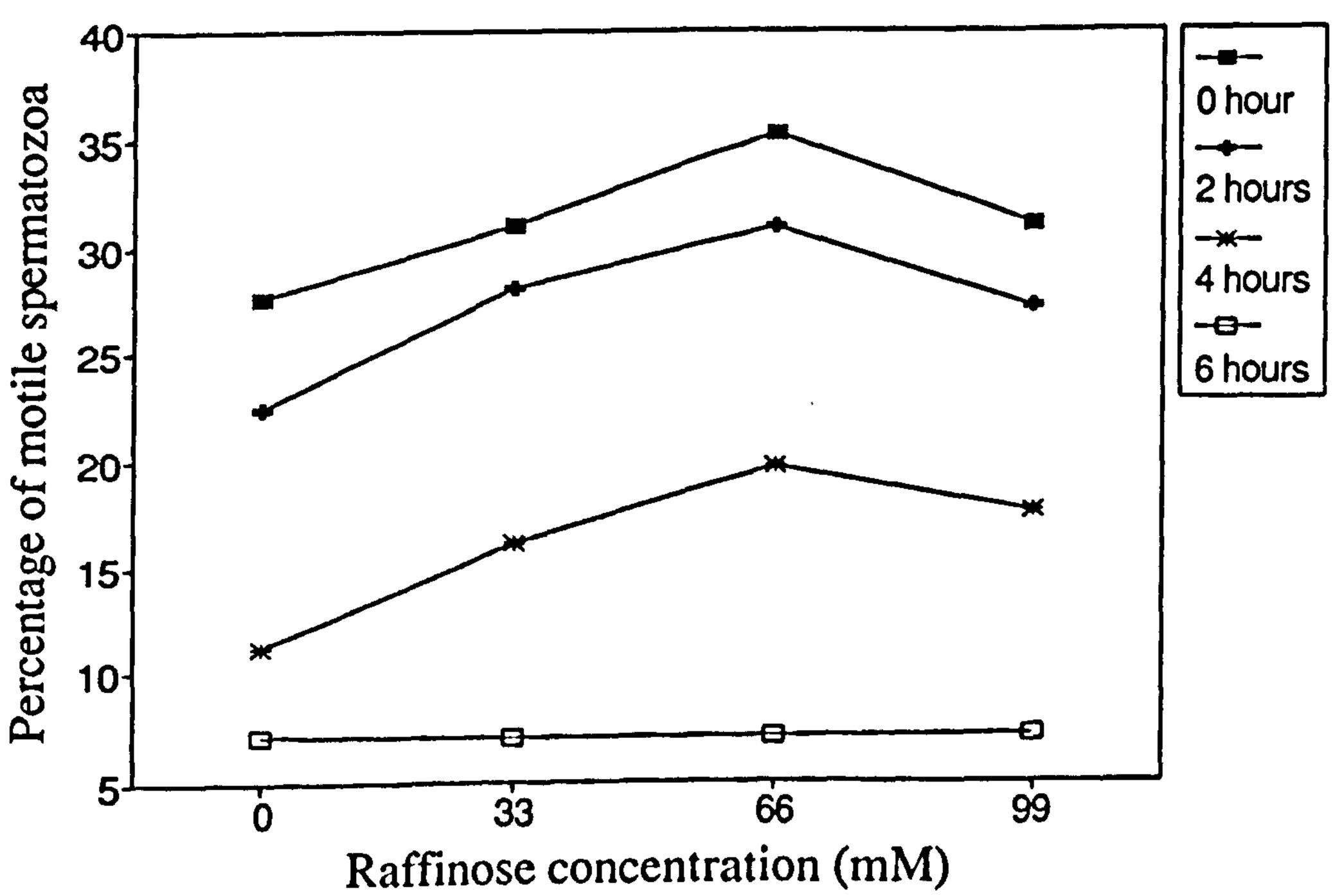


Figure 5: The effect of concentration of raffinose (mM) on the post-thawing motility of ram spermatozoa up to 6 hours of incubation at 37°C.

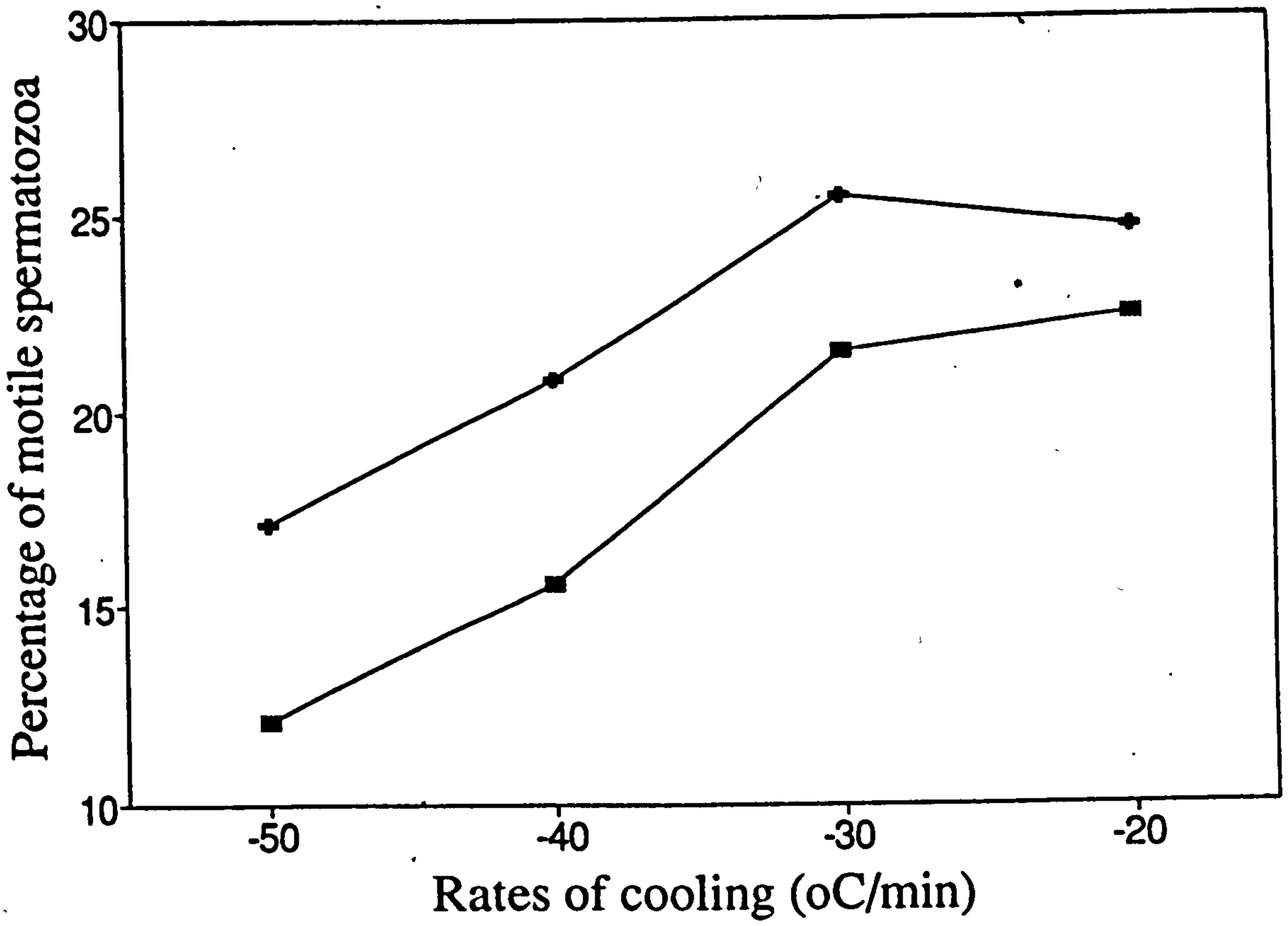


Figure 6: The effect of rate of cooling on post-thawing motility of ram sperm frozen with (—+—) and without (—■—) raffinose.

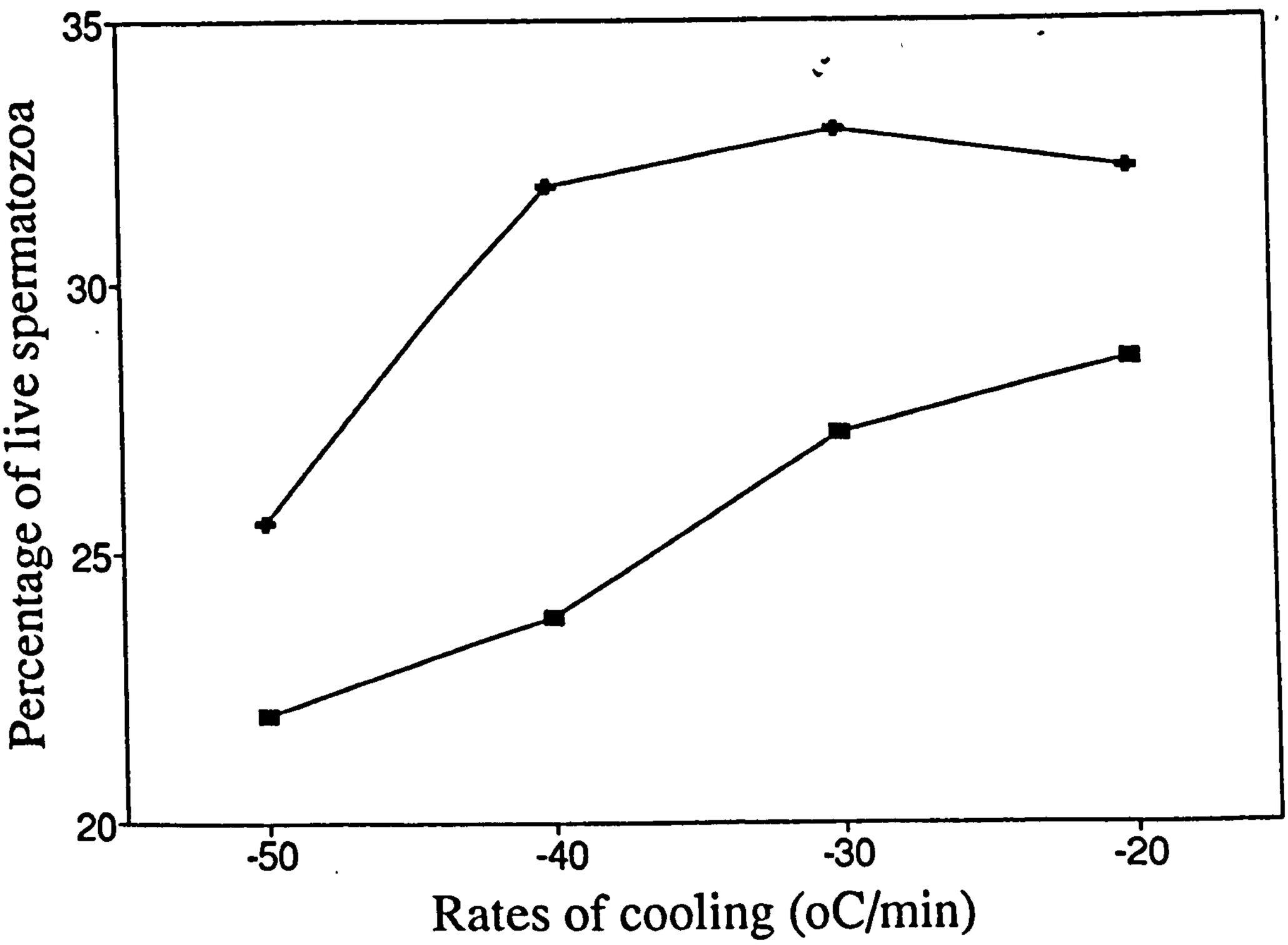


Figure 7: The effect of rate of cooling on post-thawing survival of ram sperm frozen with (—+—) and without (—■—) raffinose.

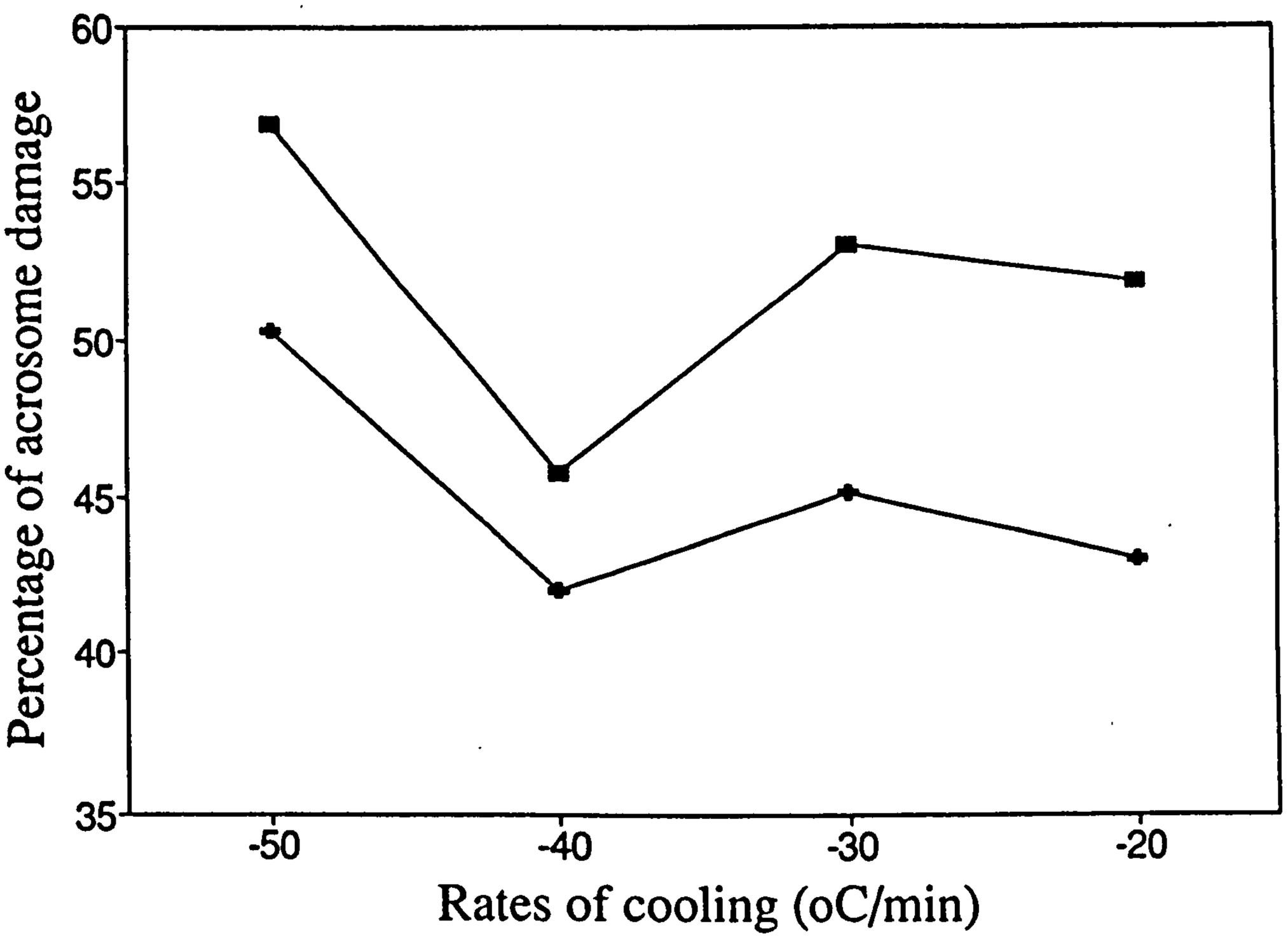


Figure 8: The effect of rate of cooling on acrosome damage to spermatozoa in ram semen frozen with (—●—) and without (—■—) raffinose.

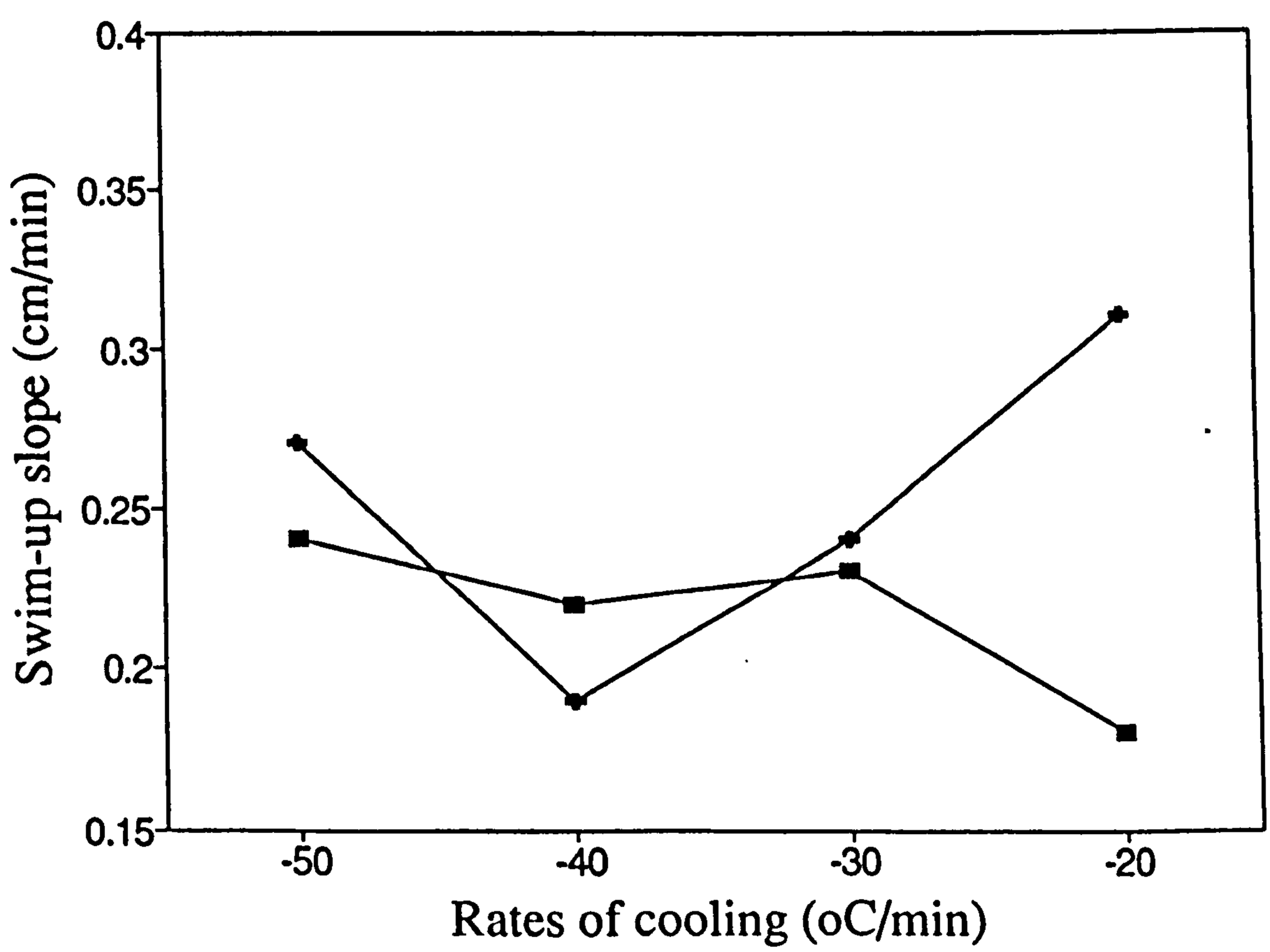


Figure 9: The effect of rate of cooling on post-thawing swimming-up slope (cm/min) of ram sperm frozen with (—●—) and without (—■—) raffinose.

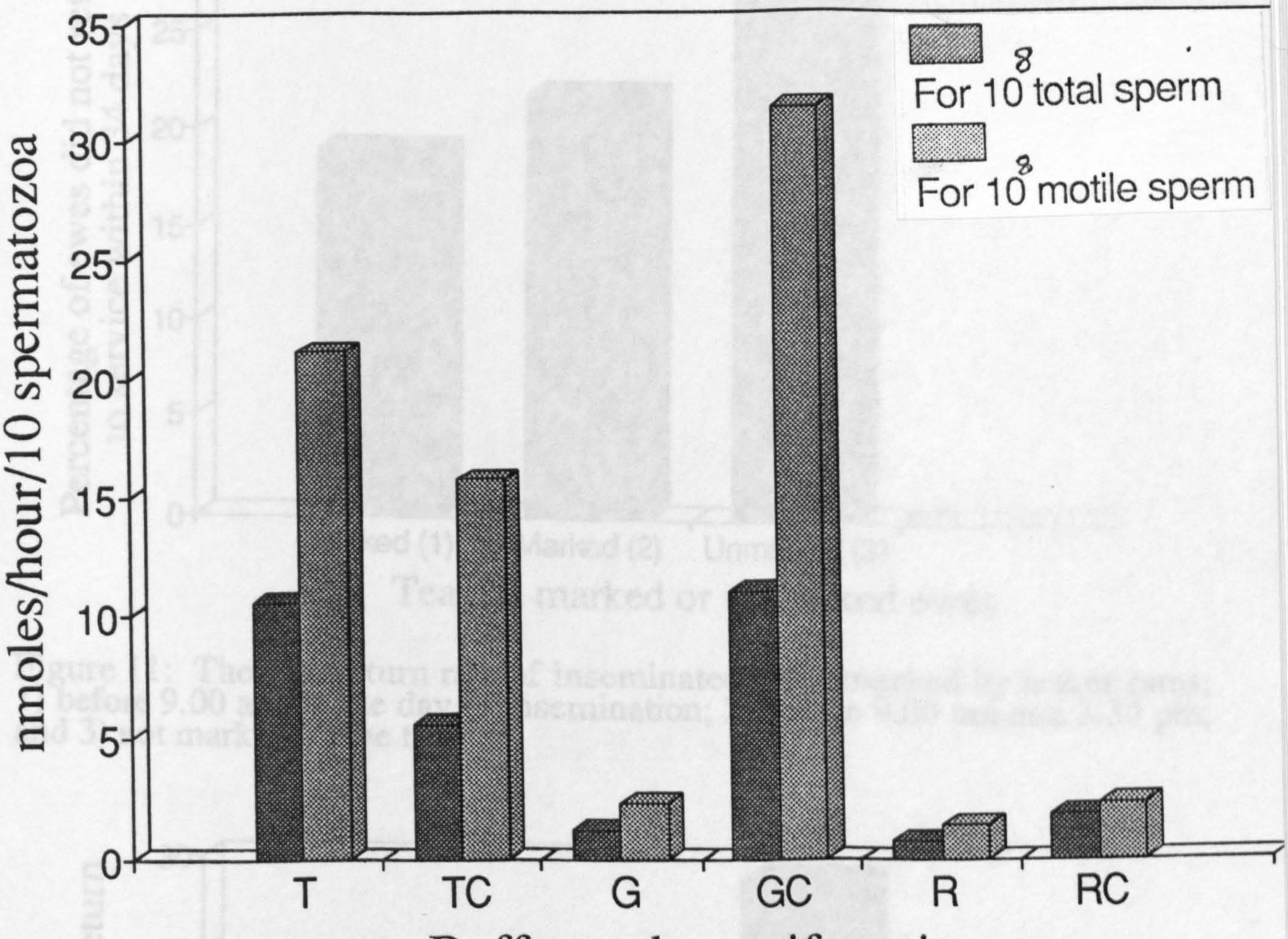


Figure 10: The effect of washing by centrifugation on the rate of glucose oxidation (nmol/hour/10^8 cells) by the total and motile ram spermatozoa.

T: Semen in Tris buffer (control).
G :Semen in Tris buffer with 4% glycerol.
R :Semen in Tris buffer with 4% glycerol and 66mM raffinose.
Tc, Gc, and RC: Semen as above after centrifugation twice and resuspension in Tris buffer.

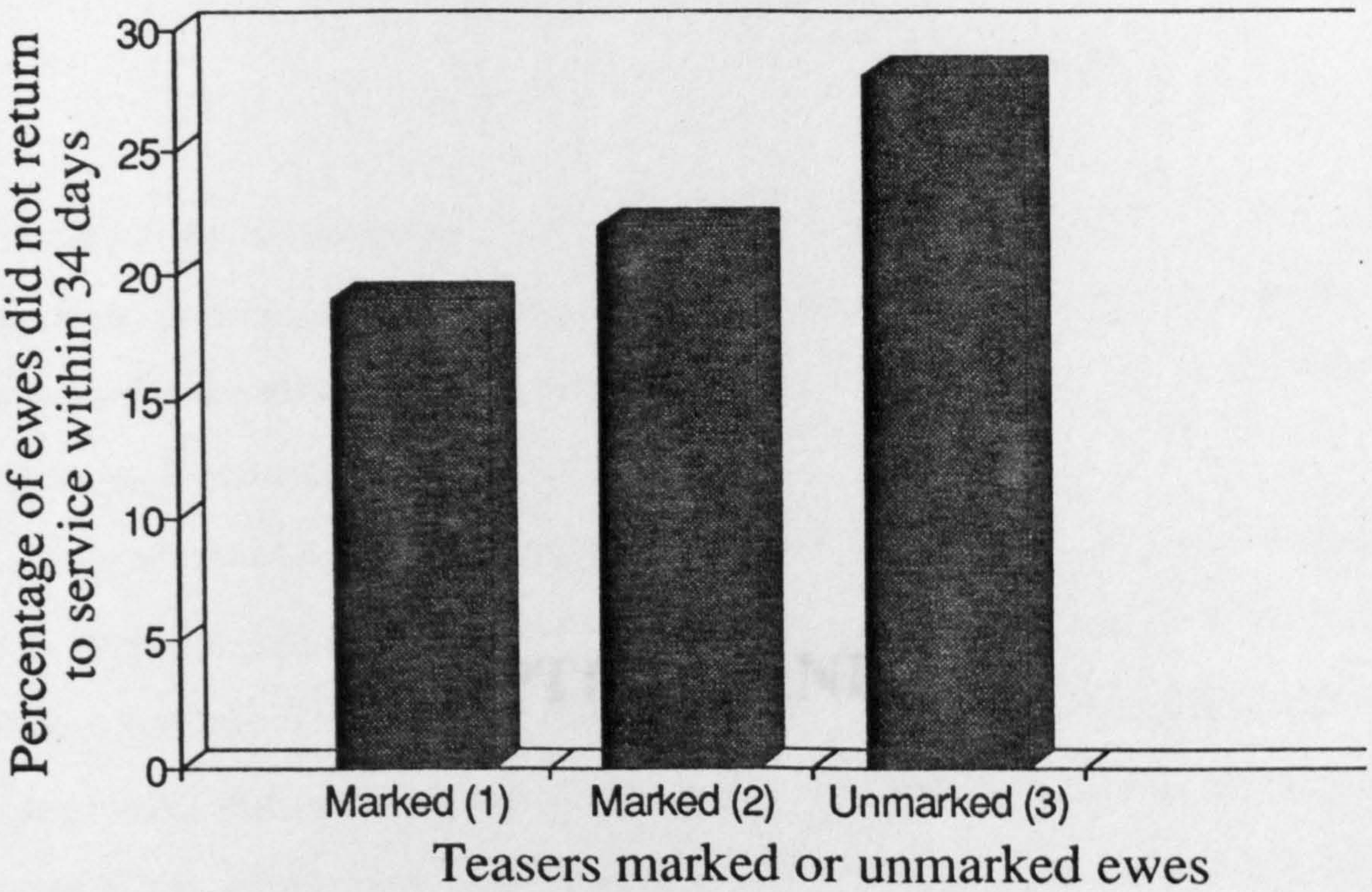


Figure 11: The non-return rate of inseminated ewes marked by teaser rams; 1) before 9.00 am on the day of insemination; 2) before 9.00 am and 3.30 pm; and 3) not marked by the teasers.

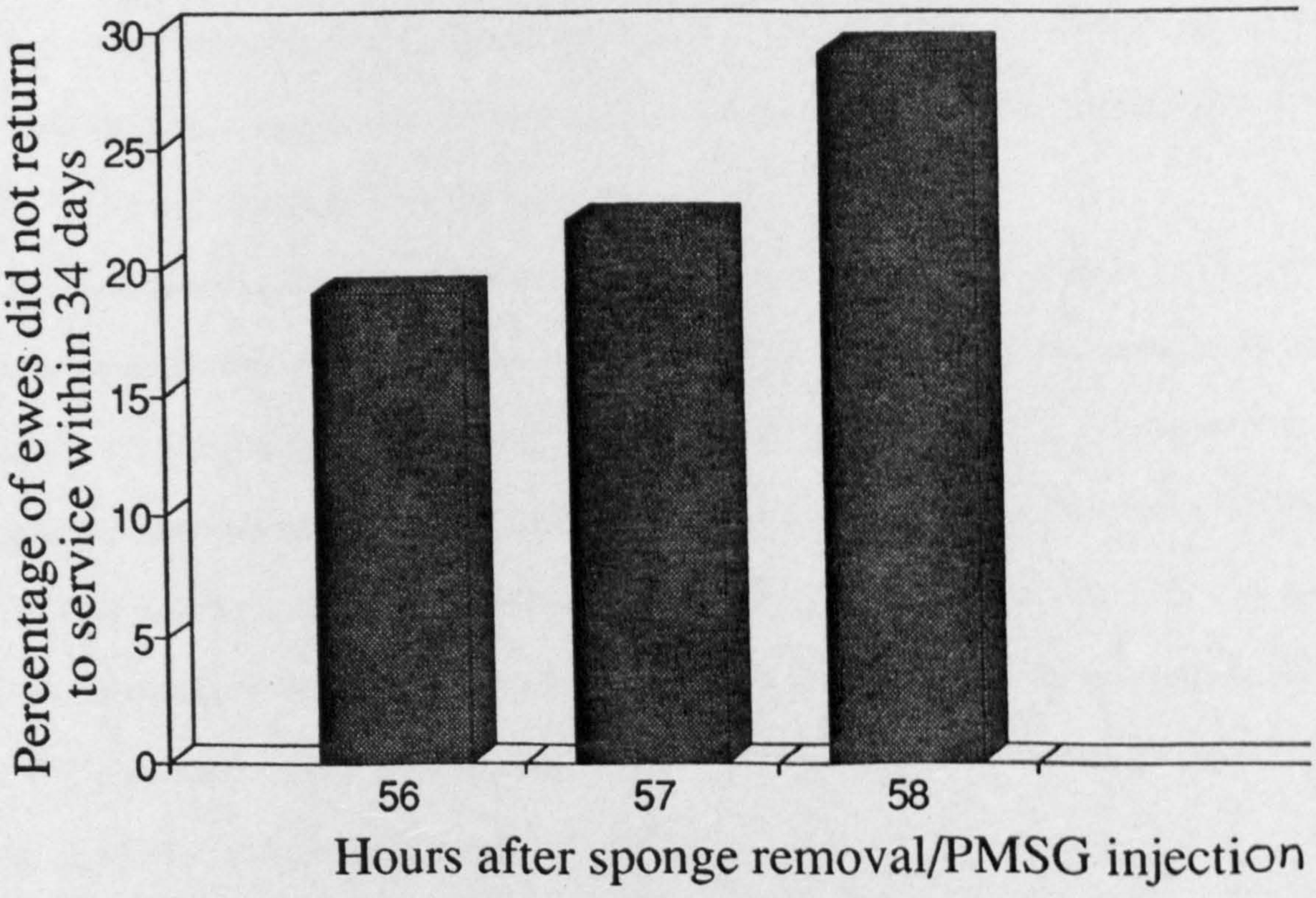


Figure 12: The relationship between the interval from sponge removal/PMSG injection to insemination and non-return rate of ewes inseminated with frozen-thawed semen.

CHAPTER NINE

GENERAL DISCUSSION

Artificial insemination has great potential for increasing the genetic response by enabling ram(s) to serve more ewes than in natural mating. It was shown theoretically in Chapter three that the effectiveness is greatly dependent on the fertility. Thus the fertility should be maintained at the highest levels.

The addition of 4% (v/v) glycerol to the diluted semen in Tris egg-yolk buffer increased the percentage of sperm motility for the first two hours. But subsequently the motility and survival of the sperm in glycerol declined faster than for untreated sperm. This was possibly due to the entry of glycerol into the metabolic pathway of the sperm (Mann and White, 1957). The lambing percentage following insemination using chilled ram semen was lower for the glycerol containing semen (59% cf 73%) although not significantly so. The lambing rate following insemination of chilled ram semen diluted in Tris egg-yolk buffer without glycerol (73%) was higher than that reported by Dziuk et al. (1972) and lower than reported by Maxwell et al. (1980) who injected Suffolk ewes with 600 IU PMSG upon sponge removal and administered double cervical insemination after 50 and 60 hours and obtained 82% lambing rate.

In the current study, the effect of temperature and solutions in which sperm could survive freezing-thawing procedures were examined in order to obtain a greater survival and fertility after insemination. *In vitro* investigation on the effect of temperature on freezing ram semen revealed that an almost similar post-thaw survival rates were obtained when diluted semen was cooled to 5°C, -1°C, and -5° before starting fast freezing. Therefore the most convenient temperature was adopted as routine. Semen was chilled to 5°C in a refrigerator before undergoing the faster freezing procedures as suggested by Quinn et al. (1968) and Evans & Maxwell (1987).

In the study on the rate of freezing, it was found that post-thaw revival of ram sperm was improved at rates of freezing faster than -20°C/min. When the freezing rate was slower fewer sperm survived. This is in agreement with the results of Patt and Nath

(1969). However, Colas (1972) as quoted by Colas and Courot (1977) obtained 33% and 36% lambing rate respectively using freezing rate of -10°C to -15°C/min. The difference between these rates was not significant ($P \leq 0.05$).

If during or after freezing, the temperature was held for a period at -45°C fewer sperm survived than at a cooler stage. The optimum intermediate point of freezing in this work was found to be colder than -65°C with the best survival obtained by freezing to -120°C without staging at an intermediate temperature. Salamon (1971) however obtained similar survivals on thawing of semen frozen to -79°C and -140°C.

Semen in straws, frozen to -100°C and -120°C before being plunged into liquid nitrogen survived better than semen frozen to -60°C or -80°C. This is in agreement with Fiser et al. (1987) who achieved a 73% cf 67% pregnancy rate using thawed semen, previously frozen to -100°C and -79°C respectively.

There are few reports (Lightfoot and Salamon, 1969a; Jones and Martin, 1965) on the influence of the pre-freezing dilution ratio on post-thawing survival of ram semen. Lightfoot and Salamon (1969a) observed that two to fourfold dilution of sperm in sodium citrate based buffer was superior to lower or higher rates of dilution at which comparatively poorer post-thawing survival was obtained. This work showed that the rate of dilution (1:1, 1:2, 1:3, and 1:4) did not affect ($P \geq 0.05$) post-thawing survival of sperm. Ejaculates could therefore be safely diluted, within the above range, to the required sperm concentration rather than by a constant ratio, as fertility depends on the number of sperm to be inseminated (Colas, 1975).

There is little agreement on the optimum pH for a semen diluent and a variety of pH levels are reported in the literature eg pH 6.65-6.70 (Fiser et al., 1981); pH 6.8 (El-Gaafary, 1987); pH 7.0 (Dziuk et al., 1972; Salamon and Visser, 1972; Lapwood and Martin, 1972). In the present work ram semen was found to be tolerant of a wide range of pH levels, but that a pH between 6.60 to 7.0 resulted in the best post-thaw survivals. Therefore a pH 6.80 was subsequently used.

The positive role of egg-yolk on post-thawing survival of ram semen was

confirmed by using various concentration of egg-yolk (v/v) in combination with glycerol. 12% (v/v) egg-yolk was found to be the most satisfactory final concentration. This is in agreement with the report of Salamon and Lightfoot (1969) and Watson and Martin (1975b).

For freezing ram semen in straws or as pellets different final concentrations of glycerol were shown to be required. It was found that for straws a glycerol concentration of 6% (v/v) and for pellets a glycerol concentration of 4% (v/v) resulted the highest percentage of post-thawing motility. Salamon (1968) found that 5% (v/v) glycerol was the optimum final concentration for pellets. Fiser and Fairfull (1984) obtained the best post-thaw survival with a final glycerol concentration of 4% to 6% for semen frozen in 0.50 ml straws.

The immediate addition of glycerol in the diluent at 30°C resulted in a greater post-thaw motility than did the slow addition of glycerol as the semen cooled from 30°C to 5°C. This is not in agreement with Colas and Courot (1977). However, Salamon (1968) found that the type of sugar in the diluent determined whether the glycerol should be added at 30°C or at 5°C. He obtained poorer survival with glucose after the addition of glycerol at 30°C. But lactose and raffinose yielded best survival with the addition of glycerol at 30°C.

An equilibration time of chilled semen at 5°C for 2.5 hours was found to be sufficient. Therefore, there was no advantage for chilled semen to be equilibrated more than 2.5 hours. This was supported by Colas (1975) and Fiser and Batra (1984) who observed a constant post-thaw motility, when semen was frozen between 2.0 to 6.0 hours of equilibration at 5°C. As glycerol has a small but consistent damaging effect on semen, equilibration should not be unnecessary extended.

In the fertility trial (Chapter five) a slightly lower lambing rate were obtained following cervical insemination of semen frozen in straws than semen frozen as pellets (14% cf 18%). But the difference was not significant at the 5% level.

As a radical departure from normal freezing routine an attempt was made to

formulate a solution for preserving ram semen in a vitrifying solution. Despite all the precautions taken to concentrate the cryoprotective agents slowly and redilute the semen promptly, ram sperm did not tolerate the concentrations of the cryoprotective agents (glycerol, acetamide and propylene glycol) necessary to make a vitrifying solution (Fahy et al., 1984).

An attempt was made to develop a swim-up test for assessing ram semen vigour objectively. The results, in Chapter seven, indicated significant positive correlations between the swim-up slope (change in absorbance per minute) and each of the volumes of semen used (r=0.94), the percentage of live sperm in it (r=0.926), the number of total (r=0.59) and motile (r=0.81) sperm*10^8 respectively. With the use of small volumes, (60 µl) of each diluted semen sample, semen vigour could be assessed objectively. In the 1992 fertility trial, the correlation coefficient between sperm vigour shown in the swim-up test and the non-return rate was 0.904 ($P \leq 0.1$). In a similar trial Suttiyotin et al. (1992) obtained a correlation coefficient of 0.483 ($0.1 \geq P \geq 0.05$) between a 48 days non-return rate and a 60 days conception rate with a similar test of swim-up velocity.

Preliminary experiments (Chapter eight) examined the effect of raffinose addition on the inhibition of the cryoinjury of ram spermatozoa. The osmolarity of Tris buffer solution was increased using different concentration of raffinose from 0 mM to 198 mM in normal Tris buffer. Raffinose significantly ($P \leq 0.005$) reduced spermatozoa motility during chilling. However, the addition of raffinose up to 66 mM increased the post-thaw motility and survival of spermatozoa. Further *in vitro* tests showed that semen frozen as pellets survived best when diluted with two volumes of Tris egg-yolk buffer containing 6% (v/v) glycerol with 66 mM raffinose. For semen frozen in 0.25 ml French straws, the best diluent was Tris buffer contained 9% (v/v) glycerol with 66 mM raffinose.

Measurement of the rate of glucose oxidation using 14C-labelled glucose as a tracer confirmed that raffinose addition did not adversely effect glucose oxidation by the semen samples. In comparison with the glycerol, it might have allowed less glycerol to enter into the cell, due to the increased osmolarity of solution, so using glucose as a source of energy

could be maintained.

In the fertility trial, reported in Chapter eight, raffinose containing semen frozen in straws resulted in the higher (31%) non-return rate after 34 days post-insemination compared with 21% and 20% for standard pellets and raffinose pellets respectively. It is possible that, the ease of use and speed of thawing of semen in straws contributed to the higher sperm survival. The fertility difference was however not significant at the 5% level. The time delay to inseminate thawed pellet might have played some roles in a lower non-return rate for pelleted semen samples. In the glucose oxidation test, it was found that the removal of the glycerol by centrifugation and resuspension of the sperm increased the rate of glucose oxidation. Insemination would allow the sperm to swim from glycerol and raffinose and a normal oxidative phosphorylation rate should be reestablished. For future use of AI, raffinose containing straws can be recommended due to rapid operation needed to prevent more damage to spermatozoa.

Cervical insemination using frozen-thawed ram semen 56 hours after sponge removal and PMSG injection produced a lower non-return rate than after 57 or 58 hours. The non-return data were 19 (7/36), 22 (6/27) and 29 (13/45) for 56, 57 and 58 hours respectively. Findlater et al. (1991) estimated that mean time of ovulation to be 61.8 (s.d:5.3) hours after sponge removal and PMSG injection. If this is correct then inseminating frozen semen later than 56 hours is likely to be beneficial. When the semen is of low viability its survival time may be less than that of ovum, so later insemination may be indicated. In the fertility trial in Chapter eight a higher percentage of ewes did not return to service following cervical insemination 58 hours after sponge removal and PMSG injection rather than 57 and 56 hours. This may be a sign of the respectively short life of frozen-thawed ram semen.

However, Chi-squared analysis showed there were no significant ($P \leq 0.05$) effects of methods of freezing, of teaser marking, or of time of insemination after sponge removal and PMSG injection.

The average fertility rates obtained during this study for frozen-thawed ram semen were 16% and 24% in 1990 and 1992 respectively (Figure 1). However, the best fertility rate were 73% using freshly diluted ram semen in 1989, and 31% using frozen-thawed ram semen in 1992 (Figure 2).

There is no previous record of the use of raffinose as a supplementary cryoprotective agent in the way reported here, for ram semen. However, Visser and Salamon (1973) used Tris-glucose-yolk buffer and Raffinose-citrate-yolk buffer to freeze ram semen. They obtain a lambing rate of 40.0% (28/70) and 44.8% (30/67) following insemination of frozen-thawed semen respectively. Lightfoot and Salamon (1969a) obtained the best post-thawing survival by using a moderately hypertonic raffinose-yolk-citrate diluent with 3-4% glycerol in the diluted semen. But there was no fertility study was reported in this respect.

In this study, post-thaw survival of ram spermatozoa was improved using a hypertonic raffinose diluent with no additional loss of fertility.

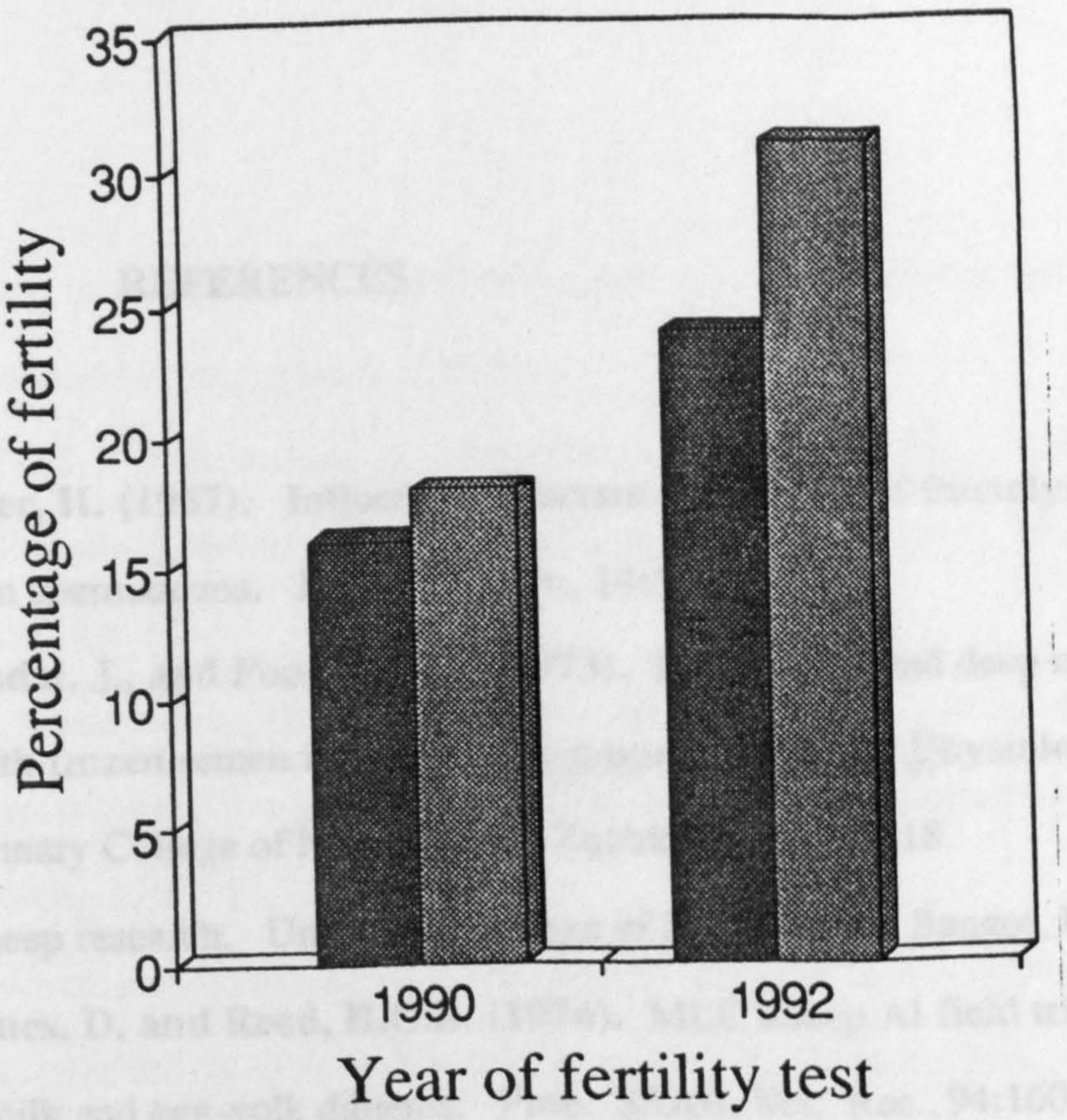


Figure 1: A comparison of the mean ( ) and best ( ) fertilities obtained following insemination with frozen-thawed ram semen in 1990 (lambing percentage) and in 1992 (non-return rate).

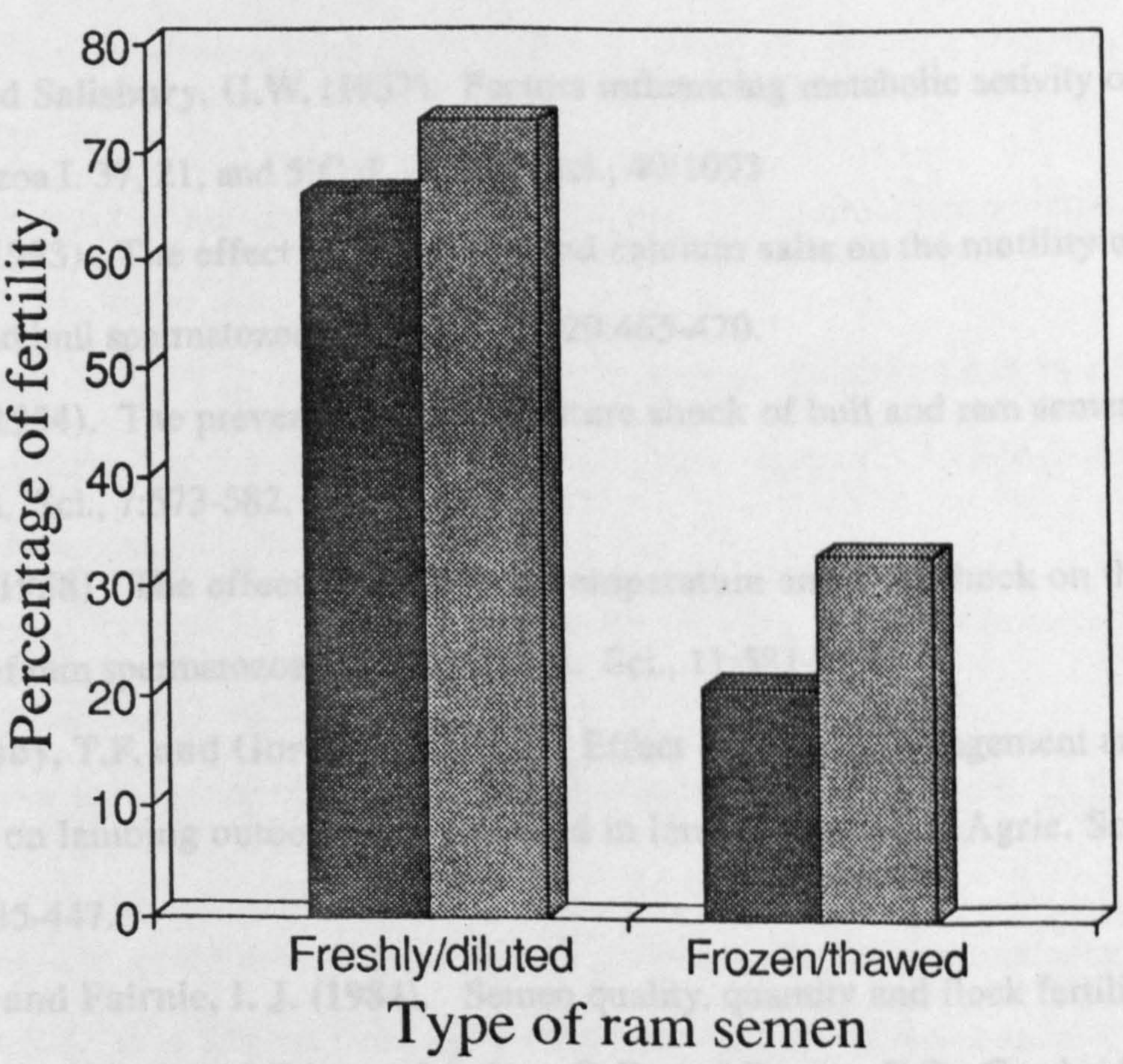


Figure 2: A comparison of the mean ( ) and best ( ) fertilities obtained during this study using cervical AI with freshly-diluted ram semen (lambing rate) and frozen-thawed ram semen (non-return rate).

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APPENDICES:

Appendix A for Chapter 3:

****==========****

Spreadsheet to calculate genetic gain
in a self contained sheep flock

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Assumptions:

a) For a mating system

Number of ewes	100
Number of rams	1
Ram to ewes ratio	0.01
Ewe to ram ratio	100
Ewes mated or inseminated lambi	60%
Number of males born	30
Number of females born	30
Number of males selected	1
Number of females selected	25
Proportion of ram lamb selected	0.0333
Proportion of ewe lamb selected	0.8333
Age at first lambing (ewes)	1
Generation length (ewes)	2.50
Age at birth 1st progeny (rams)	1
Generation length (rams)	1

	v (*1)	i (*2)	h2	p	vih2p	L
Rams to offsrings	0.033	2.231	0.20	1.50	0.67	1
Ewes to offsrings	0.83	0.305	0.20	1.50	0.09	2.5

Response per annum for lamb weaning weight (kg) 0.217

(*1) v is the proportion of either rams or ewes selected from those born.
(*2) Values of i (selection intensity) have to be inserted from Standard Tables.

The assumptions and formulae used in the spreadsheet are described in Chapter 3.

Appendix B for Chapter 5:

Table B11: The number of each table correspond to the number of the Table in each Chapter.

Interactions:				
A1*B1	37.67 (0.93)	24.00 (1.50)	8.42 (0.54)b	3.17 (0.30)
A1*B2	28.92 (1.03)	18.00 (1.80)	5.92 (0.50)c	1.92 (0.23)
A2*B1	35.83 (1.08)	27.17 (1.04)	12.75 (0.81)a	4.33 (0.41)
A2*B2	30.25 (1.54)	17.33 (1.16)	6.68 (0.80)c	2.25 (0.22)
F ratio	2.14	3.07	7.31*	3.88
A1*C1	34.83 (1.74)a	24.33 (2.04)a	7.25 (0.75)	2.83 (0.34)
A1*C2	31.75 (1.40)b	17.67 (0.97)c	7.08 (0.53)	2.25 (0.28)
A2*C1	31.75 (1.47)b	20.25 (1.67)b	8.75 (1.05)	3.17 (0.49)
A2*C2	34.33 (1.58)a	24.25 (1.82)a	10.67 (1.30)	3.42 (0.42)
F ratio	6.85*	23.78***	2.47	3.88
A1*D1	34.92 (1.23)	22.67 (1.95)a	7.42 (0.68)	3.08 (0.29)
A1*D2	31.67 (1.84)	19.33 (1.68)b	6.92 (0.60)	2.00 (0.27)
A2*D1	32.67 (1.66)	21.08 (2.11)b	9.17 (1.17)	3.75 (0.57)
A2*D2	33.42 (1.42)	23.42 (1.46)a	10.25 (1.24)	2.83 (0.24)
F ratio	3.41	6.71*	1.43	0.16
B1*C1	36.75 (1.09)	26.33 (0.77)	10.50 (0.62)	4.08 (0.30)a
B1*C2	36.75 (0.99)	24.83 (1.75)	10.67 (1.19)	3.41 (0.47)a
B2*C1	29.83 (1.49)	18.25 (2.03)	5.50 (0.48)	1.92 (0.26)b
B2*C2	29.33 (1.12)	17.08 (0.65)	7.08 (0.74)	2.25 (0.18)b
F ratio	0.05	0.02	1.14	5.59*
B1*D1	37.83 (0.82)	25.42 (1.62)	10.90 (0.70)	4.66 (0.28)a
B1*D2	35.67 (1.14)	25.75 (1.02)	10.25 (1.14)	2.83 (0.30)b
B2*D1	29.75 (0.93)	18.33 (1.83)	5.67 (0.48)	2.17 (0.24)b
B2*D2	29.42 (1.62)	17.00 (1.09)	6.92 (0.78)	2.00 (0.21)b
F ratio	0.72	0.58	2.24	15.52***
C1*D1	33.33 (1.51)	23.75 (2.13)a	8.25 (1.06)	3.42 (0.45)
C1*D2	33.25 (1.82)	20.83 (1.67)b	7.75 (0.79)	2.58 (0.36)
C2*D1	34.25 (1.48)	20.00 (1.78)b	8.33 (0.92)	3.42 (0.47)
C2*D2	31.83 (1.51)	21.92 (1.69)a	9.42 (1.28)	2.25 (0.18)
F ratio	1.16	4.88*	1.43	0.62

A=Methods of packaging semen (pellet or straw).
B=Methods of addition of glycerol (at once or slowly).
C=Percentages of glycerol (4% or 6%).
D=Equilibration time (2.5 hours or 3.5 hours).

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

Appendix C for Chapter 6:

Table C12:

Interactions:				
A1*B1	22.12 (5.88)a	12.00 (4.17)b	37.12 (7.19)b	19.25 (5.05)a
A1*B2	4.25 (2.37)d	1.25 (0.82)d	10.12 (4.04)d	2.00 (0.89)d
A2*B1	31.25 (6.99)b	14.75 (6.52)a	44.50 (8.74)a	17.25 (6.34)b
A2*B2	16.25 (5.57)c	9.00 (4.65)c	23.62 (6.60)c	11.62 (5.82)c
F ratio	21.16***	400.0***	49.0***	1729.8***
A1*C1	20.00 (5.92)b	10.75 (4.34)b	34.00 (7.31)b	16.50 (5.70)b
A1*C2	6.37 (3.76)d	2.50 (1.64)d	13.25 (5.98)d	4.75 (2.27)d
A2*C1	32.50 (6.34)a	20.00 (6.72)a	46.00 (7.27)a	23.25 (7.01)a
A2*C2	15.00 (5.82)c	3.75 (1.57)c	22.12 (7.59)c	5.62 (2.25)c
F ratio	38.44***	1024.0***	12.7***	441.8***
A1*D1	20.87 (6.16)b	11.25 (4.30)b	33.25 (8.41)b	15.50 (5.67)b
A1*D2	5.50 (2.77)d	2.00 (1.31)c	14.00 (4.79)d	5.75 (2.93)c
A2*D1	37.50 (5.43)a	22.25 (5.92)a	50.25 (6.74)a	26.12 (6.00)a
A2*D2	10.00 (3.54)c	1.50 (0.78)d	17.87 (5.55)c	2.75 (1.18)d
F ratio	376.36***	2116.0***	225.0***	2376.2***
B1*C1	35.75 (5.64)a	21.75 (6.08)a	53.12 (6.02)a	28.00 (5.72)a
B1*C2	17.00 (5.80)b	5.00 (1.89)c	28.50 (7.24)b	8.50 (2.47)c
B2*C1	16.75 (5.37)b	9.00 (4.64)b	26.87 (5.59)c	11.75 (5.77)b
B2*C2	3.75 (2.42)c	1.25 (0.82)d	6.87 (3.63)d	1.87 (1.03)d
F ratio	67.26***	1296.0***	27.9***	1185.8***
B1*D1	40.87 (4.32)a	23.50 (5.44)a	57.75 (4.69)a	28.75 (5.24)a
B1*D2	12.50 (3.54)c	3.25 (1.29)c	23.87 (5.30)c	7.75 (2.64)c
B2*D1	17.50 (5.43)b	10.00 (4.43)b	25.75 (6.46)b	12.87 (5.54)b
B2*D2	3.00 (1.56)d	0.25 (0.16)d	8.00 (2.94)d	0.75 (0.31)d

F ratio	492.84***	1764.0***	339.6***	1008.2***
C1*D1	38.75 (5.57)a	27.25 (5.34)a	53.62 (6.54)a	32.25 (5.81)a
C1*D2	13.75 (3.28)c	3.50 (1.21)c	26.37 (4.56)c	7.50 (2.71)c
C2*D1	19.62 (5.47)b	6.25 (1.57)b	29.87 (7.33)b	9.37 (2.25)b
C2*D2	1.75 (0.77)d	0.00 (0.00)d	5.50 (1.70)d	1.00 (0.46)d
F ratio	129.96***	4900.0***	10.8***	3432.8***
A1*B1*C1*D	45.00 (1.00)b	30.00 (1.00)	63.50 (4.50)	40.00 b
A1*B1*C1*D2	18.00 (1.00)e	8.00 (0.00)	35.00 (2.00)	19.00 d
A1*B1*C2*D1	23.50 (2.50)d	10.00 (0.00)	40.00 (1.00)	15.00 e
A1*B1*C2*D2	2.00 (0.00)i	0.00 (0.00)	10.00 (0.00)	3.00 h
A1*B2*C1*D1	15.00 (1.00)f	5.00 (0.00)	27.00 (1.00)	6.00 g
A1*B2*C1*D2	2.00 (0.00)i	0.00 (0.00)	10.00 (0.00)	1.00 j
A1*B2*C2*D1	0.00 (0.00)j	0.00 (0.00)	2.00 (0.00)	1.00 j
A1*B2*C2*D2	0.00 (0.00)j	0.00 (0.00)	1.00 (0.00)	0.00 k
A2*B1*C1*D1	55.00 (1.00)a	44.00 (2.00)	73.00 (0.00)	45.00 a
A2*B1*C1*D2	25.00 (2.00)d	5.00 (1.00)	40.50 0.50)	8.00 f
A2*B1*C2*D1	40.00 (1.00)c	10.00 (0.00)	54.00 (0.00)	15.00 e
A2*B1*C2*D2	5.00 (0.00)h	0.00 (0.00)	10.00 (0.00)	1.00 j
A2*B2*C1*D1	40.00 (1.00)c	30.00 (1.00)	50.00 (1.00)	38.00 c
A2*B2*C1*D2	10.00 (0.00)g	1.00 (0.00)	20.00 (0.00)	2.00 i
A2*B2*C2*D1	15.00 (0.00)f	5.00 (0.00)	23.50 (1.50)	6.50 g
A2*B2*C2*D2	0.00 (0.00)j	0.00 (0.00)	1.00 (0.00)	0.00 k
F ratio	25.00***	4.00 (0.00)	0.02	105.8***

A=Methods of concentration and redilution of cryoprotective materials. ***P≤ 0.005
B=Glycerol concentrations (10% or 20%).
C=Acetamide concentrations (10% or 20%).
D=Propylene glycol concentrations (10% or 20%).

Table C14:

Interactions:				
A1*B1	16.87 (4.72)	7.50 (2.95)b	31.75 (7.01)b	19.50 (5.05)a
A1*B2	3.75 (1.20)	0.75 (0.31)d	5.75 (0.98)d	12.75 (7.04)c
A2*B1	27.87 (6.03)	18.25 (5.32)a	39.87 (6.01)a	13.00 (0.76)b
A2*B2	12.25 (2.25)	3.00 (1.56)	17.25 (2.36)c	3.87 (1.38)d
F ratio	4.35	1156.0***	25.1***	72.2***
A1*C1	14.37 (5.24)b	6.00 (3.07)b	24.25 (8.14)b	15.00 (5.79)b
A1*C2	6.25 (1.95)c	2.25 (1.26)c	13.25 (4.84)d	17.25 (6.67)a
A2*C1	26.87 (5.77)a	15.75 (5.48)a	35.87 (6.59)a	9.75 (1.41)c
A2*C2	13.25 (3.72)b	5.50 (3.18)b	21.25 (4.42)c	7.12 (2.44)d
F ratio	21.04***	675.0***	29.0***	304.2***
A1*D1	16.12 (4.96)b	7.75 (2.86)b	27.50 (8.29)b	16.00 (5.93)
A1*D2	4.50 (1.32)d	0.50 (0.33)d	10.00 (2.79)d	16.25 (6.56)
A2*D1	28.25 (6.11)a	17.50 (5.59)a	36.00 (7.29)a	8.37 (1.57)
A2*D2	11.87 (1.96)c	3.75 (1.62)c	21.12 (3.06)c	8.50 (2.44)
F ratio	15.70***	676.0***	15.2***	0.20
B1*C1	29.75 (6.26)a	18.25 (5.32)a	44.87 (6.86)a	20.00 (4.43)a
B1*C2	15.00 (3.54)c	7.50 (2.95)b	26.75 (4.42)b	12.50 (2.32)b
B2*C1	11.50 (2.99)c	3.50 (1.45)c	15.25 (3.12)e	4.75 (1.10)d
B2*C2	4.50 (0.87)d	0.25 (0.16)d	7.75 (1.50)d	11.87 (7.23)c
F ratio	41.78***	900.0***	249.1***	2737.8***
B1*D1	33.75 (5.15)a	22.00 (4.34)a	50.00 (4.82)a	20.62 (4.45)a
B1*D2	11.00 (1.92)b	3.75 (1.62)b	21.62 (3.05)b	11.87 (1.94)c
B2*D1	10.62 (2.78)b	3.25 (1.50)b	13.50 (2.94)c	3.75 (1.29)d
B2*D2	5.37 (1.80)c	0.50 (0.33)c	9.50 (2.49)d	12.87 (7.04)b
F ratio	213.04***	3844.0***	1311.2***	4089.8***
C1*D1	30.37 (6.21)a	18.00 (5.37) a	40.25 (8.70)a	15.62 (5.42)a
C1*D2	10.87 (2.50)c	3.75 (1.62)c	19.87 (3.74)c	9.12 (2.24)b
C2*D1	14.00 (3.85)b	7.25 (3.02)b	23.25 (5.38)b	8.75 (3.01)
C2*D2	5.50 (1.09)d	0.50 (0.33)d	11.25 (2.58)d	15.62 (6.73)a
F ratio	84.17***	900.0***	154.7***	2289.8***
A1*B1*C1*D1	37.50 (2.50)	20.00 (1.45)b	60.00 (3.45)	40.00 (3.05)b

A1*B1*C1*D2	10.00 (0.00)	2.00 (0.00)f	22.00 (1.50)	15.00 (1.30)d
A1*B1*C2*D1	15.00 (1.00)	8.00 (0.00)e	35.00 (2.20)	20.00 (2.20)c
A1*B1*C2*D2	5.00 (0.00)	0.00 (0.00)h	10.00 (0.60)	3.00 (0.00)g
A1*B2*C1*D1	9.00 (0.00)	2.00 (0.00)f	10.00 (0.60)	3.00 (0.00)g
A1*B2*C1*D2	1.00 (0.00)	0.00 (0.00)h	5.00 (0.00)	2.00 (0.00)h
A1*B2*C2*D1	3.00 (0.00)	1.00 (0.00)g	5.00 (0.00)	1.00 (0.00)i
A1*B2*C2*D2	2.00 (0.00)	0.00 (0.00)h	3.00 (0.00)	4.00 (0.00)f
A2*B1*C1*D1	52.50 (2.00)	40.00 (3.40)a	65.00 (0.00)	45.00 (0.90)a
A2*B1*C1*D2	19.00 (2.00)	11.00 (1.00)c	32.50 (2.50)	15.00 (0.00)d
A2*B1*C2*D1	30.00 (2.50)	20.00 (1.50)b	40.00 (2.10)	20.50 (2.50)c
A2*B1*C2*D2	10.00 (0.00)	2.00 (0.00)f	22.00 (1.70)	4.50 (0.50)f
A2*B2*C1*D1	22.50 (2.50)	10.00 (1.00)d	26.00 (1.00)	13.00 (0.00)e
A2*B2*C1*D2	13.50 (1.50)	2.00 (0.00)f	20.00 (1.00)	4.50 (0.50)f
A2*B2*C2*D1	8.00 (1.00)	0.00 (0.00)h	13.00 (0.50)	1.50 (0.50)h
A2*B2*C2*D2	5.00 (0.00)	0.00 (0.00)h	10.00 (0.00)	0.00 (0.00)j
F ratio	0.39	36.00***	0.31	744.2***

A=Methods of concentration and redilution of cryoprotective materials.
B=Glycerol concentrations (10% or 20%).
C=Acetamide concentrations (10% or 20%).
D=Propylene glycol concentrations (10% or 20%).

***P≤ 0.005

Table C15:

Interactions:				
A1*B1*C1	72.50 (0.50)a	67.50 (0.50)	81.00 (5.00)a	70.00 (0.00)a
A1*B1*C2	50.00 (0.00)c	40.00 (0.00)	54.50 (0.50)c	44.00 (1.00)c
A1*B2*C1	50.00 (0.00)c	35.00 (0.00)	53.50 (0.50)c	38.00 (0.00)d
A1*B2*C2	40.00 (0.00)d	20.00 (0.00)	44.50 (0.50)d	25.00 (0.00)g
A1*B3*C1	33.00 (0.00)f	15.00 (0.00)	42.50 (0.05)d	20.00 (0.00)h
A1*B3*C2	26.00 (1.00)gh	2.00 (0.00)	31.00 (0.00)f	5.00 (0.00)l
A2*B1*C1	60.00 (0.00)b	60.00 (0.00)	60.00 (0.50)b	60.00 (0.00)b
A2*B1*C2	29.00 (1.00)g	18.00 (0.00)	35.00 (0.00)e	20.00 (0.00)h
A2*B2*C1	41.00 (1.00)d	35.00 (0.00)	44.00 (0.00)d	35.00 (0.00)e
A2*B2*C2	27.00 (0.00)g	4.50 (0.50)	31.00 (1.00)f	7.00 (1.00)k
A2*B3*C1	35.00 (0.00)e	20.00 (0.00)	44.00 (0.00)d	29.00 (1.00)f
A2*B3*C2	9.00 (1.00)j	1.00 (0.00)	11.00 (1.00)i	2.00 (0.00)m
A3*B1*C1	37.50 (0.50)e	36.00 (0.00)	40.00 (0.00)d	38.00 (0.00)d
A3*B1*C2	26.50 (1.50)gh	7.00 (1.00)	27.00 (1.00)g	10.00 (0.00)j
A3*B2*C1	15.50 (0.50)i	12.50 (0.50)	20.00 (0.00)h	15.50 (0.50)i
A3*B2*C2	4.00 (1.00)k	2.00 (0.00)	5.00 (0.00)j	4.00 (0.00)l
A3*B3*C1	15.50 (0.50)i	11.00 (1.00)	18.00 (0.00)h	14.50 (0.50)i
A3*B3*C2	2.50 (0.50)k	0.00 (0.00)	5.00 (0.00)j	3.00 (0.0)m
F ratio	38.72***	39.64	18.63***	6.71***

***P≤ 0.005

A=Glycerol concentrations (0, 10% or 20%).
B=Acetamide concentrations (0, 10% or 20%).
C=Propylene glycol concentrations (0% or 20%).

Appendix D for Chapter 8:

Table D6:

Interactions:				
A1*B1	32.12 (1.33)	28.50 (1.15)	18.00 (1.75)	11.45 (1.68)
A1*B2	31.12 (0.83)	28.56 (0.89)	17.75 (0.85)	11.62 (0.92)
A2*B1	32.81 (1.01)	28.18 (1.25)	18.00 (1.01)	9.25 (0.75)
A2*B2	31.62 (0.89)	26.12 (1.30)	16.18 (0.90)	9.00 (0.94)
F ratio	3.10	0.34	0.02	0.22
A1*C0	27.87 (2.12)	24.27 (1.88)	12.12 (1.59)	5.25 (1.11)
A1*C1	30.75 (1.16)	28.25 (1.06)	16.62 (1.43)	10.87 (1.78)
A1*C2	34.75 (1.28)	31.75 (0.56)	20.50 (2.21)	12.62 (1.57)
A1*C3	32.12 (0.66)	29.37 (0.68)	18.25 (1.35)	13.37 (1.92)
A2*C0	27.25 (1.41)	20.00 (0.94)	10.12 (0.58)	4.62 (0.94)
A2*C1	31.12 (0.69)	27.75 (0.88)	15.62 (0.60)	8.00 (1.40)
A2*C2	34.62 (1.28)	30.00 (1.22)	18.87 (0.89)	9.50 (1.40)
A2*C3	29.87 (0.29)	24.87 (1.73)	16.87 (0.87)	8.40 (0.80)
F ratio	1.79	1.94	0.17	2.20
A1*D1	29.44 (1.13)	27.56 (1.11)	15.44 (1.34)	8.25 (1.08)
A1*D2	32.81 (0.90)	29.50 (0.87)	18.31 (1.34)	12.81 (1.39)
A2*D1	29.56 (0.77)	25.62 (1.18)	14.69 (0.87)	7.19 (0.83)
A2*D2	31.87 (1.07)	25.69 (1.38)	16.06 (1.05)	8.06 (1.00)
F ratio	1.07	1.57	2.22	9.33***
B1*C0	25.00 (1.61)	20.50 (1.07)	8.75 (0.80)	3.25 (0.53)
B1*C1	32.00 (0.82)	29.12 (0.85)	15.87 (1.48)	7.00 (1.52)
B1*C2	33.62 (1.46)	30.00 (1.22)	18.00 (2.14)	10.50 (1.91)
B1*C3	31.25 (0.80)	27.75 (1.59)	18.50 (1.30)	10.62 (2.11)
B2*C0	30.12 (1.42)	24.25 (1.97)	13.50 (0.94)	6.62 (1.03)
B2*C1	29.87 (0.910	26.87 (0.910	16.37 (0.50)	11.87 (1.30)
B2*C2	34.75 (0.49)	31.75 (0.56)	21.37 (0.71)	11.62 (1.18)
B2*C3	30.75	26.50 (1.50)	16.62 (0.88)	11.12 (1.27)
F ratio	9.14***	3.38*	8.64***	2.81*
B1*D1	28.19 (1.01)	25.69 (1.21)	12.56 (0.98)	5.25 (0.57)

B1*D2	32.75 (1.06)	28.00 (1.27)	18.00 (1.48)	10.44 (1.58)
B2*D1	30.81 (0.78)	27.50 (1.08)	17.56 (0.89)	10.19 (0.87)
B2*D2	31.94 (0.92)	27.18 (1.24)	16.37 (0.88)	10.44 (1.10)
F ratio	11.16***	3.07	43.22***	16.73***
C0*D1	24.50 (1.30)	21.50 (1.25)	10.75 (1.56)	5.37 (1.24)
C0*D2	30.62 (1.46)	23.25 (2.06)	11.50 (0.82)	4.50 (0.76)
C1*D1	30.75 (0.37)	28.75 (0.67)	15.50 (0.78)	9.12 (1.99)
C1*D2	31.12 (1.30)	27.25 (1.15)	16.75 (1.32)	9.75 (1.31)
C2*D1	32.25 (0.88)	29.50 (1.09)	17.62 (1.93)	7.62 (0.98)
C2*D2	36.12 (1.22)	32.25 (0.56)	21.75 (0.98)	14.50 (0.87)
C3*D1	30.50 (0.60)	26.62 (1.72)	16.37 (0.71)	8.75 (0.65)
C3*D2	31.50 (0.68)	27.62 (1.36)	18.75 (1.35)	13.00 (2.10)
F ratio	6.71***	1.47	2.21	8.45***
A1*B1*C0	25.00 (3.00)	21.75 (1.65)	8.50 (1.55)	3.25 (0.95)
A1*B1*C1	32.50 (1.44)	30.25 (1.03)	16.50 (2.99)	8.50 (3.01)
A1*B1*C2	34.25 (2.46)	31.75 (1.03)	18.50 (4.37)	12.50 (3.20)
A1*B1*C3	32.75 (1.03)	30.25 (0.85)	20.50 (1.72)	13.50 (3.77)
A1*B2*C0	30.75 (1.80)	27.75 (2.14)	15.75 (1.36)	7.25 (1.06)
A1*B2*C1	29.00 (0.94)	26.25 (0.87)	16.75 (0.65)	13.25 (1.61)
A1*B2*C2	33.25 (1.04)	31.75 (1.16)	22.50 (1.25)	12.75 (1.34)
A1*B2*C3	31.50 (0.60)	28.50 (1.43)	16.00 (0.98)	13.25 (1.39)
A2*B1*C0	25.00 (1.66)	19.25 (1.49)	9.00 (0.48)	3.25 (1.58)
A2*B1*C1	31.50 (1.11)	28.00 (1.44)	15.25 (0.71)	5.50 (2.18)
A2*B1*C2	33.00 (1.49)	28.25 (1.03)	17.50 (0.63)	8.50 (2.10)
A2*B1*C3	29.75 (0.00)	25.25 (2.63)	16.50 (1.11)	7.75 (1.29)
A2*B2*C0	29.50 (0.90)	20.75 (0.75)	11.25 (1.50)	6.00 (1.05)
A2*B2*C1	30.75 (0.76)	27.50 (1.09)	16.00 (0.76)	10.50 (0.90)
A2*B2*C2	36.25 (1.45)	31.75 (2.20)	20.25 (0.96)	10.50 (1.05)
A2*B2*C3	30.00 (1.20)	24.50 (0.90)	17.25 (1.80)	9.00 (1.18)
F ratio	1.28	1.59	4.46*	0.32
A1*B1*D1	27.87 (1.80)	27.12 (1.81)	12.00 (1.70)	4.87 (0.92)
A1*B1*D2	34.37 (1.16)	29.87 (1.36)	20.00 (2.37)	14.00 (2.29)
A1*B2*D1	31.00 (0.85)	28.00 (1.12)	18.87 (1.08)	11.62 (0.97)
A1*B2*D2	31.25 (0.98)	29.12 (1.19)	16.64 (0.960	11.62 (1.20)

A2*B1*D1	28.50 (1.03)	24.25 (1.72)	13.12 (1.20)	5.62 (1.32)
A2*B1*D2	31.12 (1.44)	26.12 (2.02)	16.00 (1.43)	6.87 (1.44)
A2*B2*D1	30.62 (0.86)	27.00 1.20)	16.25 (1.25)	8.75 (1.45)
A2*B2*D2	32.62 (1.50)	25.25 (1.10)	16.12 (1.80)	9.25 (1.15)
F ratio	7.47*	0.45	12.94***	12.03***
A1*C0*D1	23.25 (2.14)	21.50 (2.20)	11.25 (2.12)	5.50 (2.05)
A1*C0*D2	32.50 (1.44)	28.00 (2.12)	13.00 (1.22)	5.00 (1.22)
A1*C1*D1	30.50 (1.16)	28.25 (1.56)	15.00 (1.25)	9.50 (1.98)
A1*C1*D2	31.00 (1.63)	28.25 (2.07)	18.25 (1.90)	12.25 (1.70)
A1*C2*D1	32.50 (0.78)	30.75 (0.57)	17.50 (1.81)	9.00 (1.20)
A1*C2*D2	35.00 (1.37)	32.75 (1.38)	23.50 (1.70)	16.25 (0.98)
A1*C3*D1	31.50 (0.70)	29.75 (1.00)	18.00 (0.80)	9.00 (1.20)
A1*C3*D2	32.75 (1.13)	29.00 (0.92)	18.50 (1.25)	17.75 (1.65)
A2*C0*D1	25.75 (0.50)	21.50 (2.60)	10.25 (0.50)	5.25 (0.50)
A2*C0*D2	28.75 (0.25)	18.50 (2.40)	10.00 (0.58)	4.00 (0.75)
A2*C1*D1	31.00 (0.30)	29.25 (2.25)	16.00 (1.50)	8.75 (1.02)
A2*C1*D2	31.25 (0.75)	26.25 (1.75)	15.25 (1.20)	7.25 (1.00)
A2*C2*D1	32.00 (0.80)	28.25 (2.15)	17.75 (1.75)	6.25 (0.85)
A2*C2*D2	37.25 (1.12)	31.75 (1.65)	20.00 (1.45)	12.75 (0.50)
A2*C3*D1	29.50 (0.45)	23.50 (2.05)	14.75 (0.75)	8.50 (0.25)
A2*C3*D2	30.25 (0.56)	26.25 (1.50)	19.00 (1.00)	8.25 (0.45)
F ratio	3.34*	3.70*	3.21*	2.62
B1*C0*D1	22.00 (1.47)	19.75 (1.18)	7.25 (1.03)	2.75 (0.75)
B1*C0*D2	28.00 (2.00)	21.25 (1.89)	10.25 (0.630	3.75 (0.750
B1*C1*D1	30.50 (0.90)	28.75 (1.43)	14.00 (0.81)	4.75 (1.00)
B1*C1*D2	33.50 (0.78)	29.50 (2.03)	17.75 (1.66)	9.25 (1.52)
B1*C2*D1	30.00 (0.33)	27.50 (0.92)	13.00 (0.98)	6.00 (1.77)
B1*C2*D2	37.25 (1.85)	32.50 (1.47)	23.00 (1.64)	15.00 (1.30)
B1*C3*D1	30.25 (0.96)	26.75 (1.69)	16.00 (1.40)	7.50 (0.80)
B1*C3*D2	32.25 (1.25)	28.75 (1.00)	21.00 (0.72)	13.75 (1.77)
B2*C0*D1	27.00 (0.75)	23.25 (2.22)	14.25 (0.850	8.00 (0.82)
B2*C0*D2	33.25 (0.75)	25.25 (2.35)	12.75 (1.70)	5.25 (2.40)
B2*C1*D1	31.00 (1.10)	28.75 (0.99)	17.00 (1.20)	13.50 (1.20)
B2*C1*D2	28.75 (1.25)	25.00 (1.00)	15.75 (1.750	10.25 (1.50)

B2*C2*D1	34.50 (0.50)	31.50 (2.15)	22.25 (2.15)	9.25 (0.40)
B2*C2*D2	35.00 (1.00)	32.00 (1.85)	20.50 (0.90)	14.00 (1.00)
B2*C3*D1	30.75 (0.75)	26.50 (1.45)	16.75 (0.50)	10.00 (0.50)
B2*C3*D2	30.75 (0.75)	26.50 (2.10)	16.50 (0.75)	12.25 (0.70)
F ratio	2.36	0.64	2.90*	0.61
A1*B1*C0*D1	20.00 (2.00)	19.00 (1.00)	6.00 (1.00)	2.00 (0.00)
A1*B1*C0*D2	30.00 (0.00)	24.50 (0.50)	11.00 (1.00)	4.50 (1.50)
A1*B1*C1*D1	30.00 (1.00)	29.00 (1.00)	13.00 (2.00)	3.50 (1.50)
A1*B1*C1*D2	35.00 (0.00)	31.50 (1.50)	20.00 (0.00)	13.50 (1.50)
A1*B1*C2*D1	30.00 (2.50)	30.00 (0.00)	11.00 (1.00)	7.00 (1.00)
A1*B1*C2*D2	38.50 (0.50)	33.50 (0.50)	26.00 (1.00)	18.00 (0.00)
A1*B1*C3*D1	31.50 (1.00)	30.50 (0.50)	18.00 (1.00)	7.00 (1.00)
A1*B1*C3*D2	34.00 (1.00)	30.00 (0.00)	23.00 (2.00)	20.00 (0.00)
A1*B2*C0*D1	26.50 (1.50)	24.00 (4.00)	16.50 (1.50)	9.00 (1.00)
A1*B2*C0*D2	35.00 (0.00)	31.50 (0.50)	15.00 (0.00)	5.50 (2.50)
A1*B2*C1*D1	31.00 (1.00)	27.50 (2.50)	17.00 (1.00)	15.50 (0.50)
A1*B2*C1*D2	27.00 (2.50)	25.00 (0.00)	16.50 (1.50)	11.00 (1.00)
A1*B2*C2*D1	35.00 (0.00)	31.50 (1.50)	24.00 (1.00)	11.00 (1.00)
A1*B2*C2*D2	31.50 (1.50)	32.00 (0.00)	21.00 (1.00)	14.50 (0.00)
A1*B2*C3*D1	31.50 (1.50)	29.00 (1.00)	18.00 (1.00)	11.00 (1.00)
A1*B2*C3*D2	31.50 (1.50)	28.00 (2.00)	14.00 (0.00)	15.50 (2.50)
A2*B1*C0*D1	24.00 (1.00)	20.50 (2.50)	8.50 (1.50)	3.50 (0.50)
A2*B1*C0*D2	26.00 (4.00)	18.00 (0.00)	9.50 (0.50)	3.00 (0.00)
A2*B1*C1*D1	31.00 (1.50)	28.50 (1.50)	15.00 (0.00)	6.00 (0.00)
A2*B1*C1*D2	32.00 (2.00)	27.50 (2.50)	15.50 (0.50)	5.00 (0.00)
A2*B1*C2*D1	30.00 (0.00)	25.00 (0.00)	15.00 (0.00)	5.00 (0.00)
A2*B1*C2*D2	36.00 (2.00)	31.50 (1.50)	20.00 (0.00)	12.00 (0.00)
A2*B1*C3*D1	29.00 (1.00)	23.00 (5.00)	14.00 (0.00)	8.00 (0.00)
A2*B1*C3*D2	30.50 (0.50)	27.50 (2.50)	19.00 (1.00)	7.50 (2.50)
A2*B2*C0*D1	27.50 (2.50)	22.50 (1.50)	12.00 (0.00)	7.00 (1.00)
A2*B2*C0*D2	31.50 (1.50)	19.50 (1.50)	10.50 (0.50)	5.00 (0.00)
A2*B2*C1*D1	31.00 (1.00)	30.00 (0.00)	17.00 (1.00)	11.50 (4.50)
A2*B2*C1*D2	30.50 (2.50)	25.00 (0.00)	15.00 (0.00)	9.50 (2.50)
A2*B2*C2*D1	34.00 (1.00)	31.50 (1.50)	20.50 (1.50)	7.50 (2.50)

A2*B2*C2*D2	38.50 (1.50)	32.00 (2.00)	20.00 (0.00)	13.50 (1.50)
A2*B2*C3*D1	30.00 (0.00)	24.00 (1.00)	15.00 (0.00)	9.00 (1.00)
A2*B2*C3*D2	30.00 (0.00)	25.00 (1.00)	19.00 (1.00)	9.00 (1.00)
F ratio	1.05	0.11	0.89	0.65

A=Sample (September v October).
B=Pellet size (0.1 ml v 0.2 ml.
C=Raffinose (0 mM v 33 mM v 66 mM v 99 mM).
D=Glycerol (2% v 4%).
*=Interaction.

Table D12:

Interactions:				
D1*E1	12.04 (1.20)	8.12 (0.80)	5.04 (0.60)	3.10 (1.10)
D2*E1	17.10 (0.76)	11.24 (1.58)	6.31 (0.80)	4.60 (0.50)
D1*E2	15.60 (1.40)	11.24 (0.80)	4.49 (0.58)	2.55 (1.44)
D2*E2	20.79 (1.40)	16.30 (1.89)	8.09 (1.40)	3.65 (1.18)
D1*E3	21.54 (1.25)	13.37 (1.64)	6.87 (0.80)	4.60 (0.50)
D2*E3	25.48 (1.85)	15.62 (1.76)	8.56 (1.70)	7.35 (0.50)
D1*E4	22.54 (1.15)	18.99 (0.70)	8.50 (0.82)	5.60 (0.80)
D2*E4	24.73 (1.51)	18.99 (1.60)	8.81 (0.85)	5.60 (0.60)
F ratio	0.77	1.41	0.00	0.95

A=Replications (1 v 2).
B=Rams (1 v 2 v 3).
C=Weeks (1 v 2 v 3).
D=Treatments (tris yolk glycerol (6%) without raffinose v the same buffer plus 66 mM raffinose).
E=Rate of cooling (-50°C/min v -40°C/min v -30°C/min v -20°C/min).
*=Interaction.
---=No data was available.

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

Table D13:

Interactions:				
D1*E1	22.00 (1.60)	14.92 (1.37)	9.36 (0.72)	10.25 (1.15)
D2*E1	25.62 (1.25)	18.10 (1.18)	10.74 (1.17)	11.75 (2.25)
D1*E2	23.81 (1.72)	21.17 (1.32)	12.49 (1.70)	8.35 (0.30)
D2*E2	31.87 (1.40)	26.23 (2.45)	15.87 (1.83)	11.15 (2.10)
D1*E3	27.25 (1.40)	19.60 (1.70)	11.87 (1.70)	9.75 (2.40)
D2*E3	32.93 (3.10)	23.37 (1.80)	17.39 (1.37)	12.25 (2.45)
D1*E4	28.62 (2.40)	24.60 (2.04)	13.87 (1.45)	---
D2*E4	32.18 (1.35)	25.85 (2.49)	14.93 (1.10)	---
F ratio	0.71	0.59	1.06	0.16

*=P≤ 0.05
**=P≤ 0.01
***=P≤ 0.005

A=Replications (1 v 2).
B=Rams (1 v 2 v 3).
C=Weeks (1 v 2 v 3).
D=Treatments (tris yolk glycerol (6%) without raffinose v the same buffer plus 66 mM raffinose).
E=Rate of cooling (-50°C/min v -40°C/min v -30°C/min v -20°/min).
*=Interaction.
---=No data was available.

Table D14:

Interactions:				
D1*E1	56.88 (2.26)	61.83 (2.01)	62.93 (2.04)	---
D2*E1	50.30 (2.88)	55.39 (2.52)	56.12 (2.32)	---
D1*E2	45.75 (1.78)	50.52 (1.43)	52.99 (1.11)	---
D2*E2	42.06 (2.09)	45.02 (1.37)	48.49 (0.91)	---
D1*E3	53.06 (1.98)	58.89 (2.55)	59.93 (2.36)	---
D2*E3	45.12 (2.09)	48.27 (1.49)	50.49 (1.80)	---
D1*E4	51.94 (1.70)	55.14 (1.43)	61.24 (3.31)	---
D2*E4	43.00 (0.70)	53.45 (1.37)	59.14 (4.37)	---
F ratio	0.86	1.88	1.59	---

A=Replications (1 v 2).
B=Rams (1 v 2 v 3).
C=Weeks (1 v 2 v 3).
D=Treatments (tris yolk glycerol (6%) without raffinose v the same buffer plus 66 mM raffinose).
E=Rate of cooling (-50°C/min v -40°C/min v -30°C/min v -20°C/min).
*=Interaction.
£=Due to insufficient number of data, the F value could not be computed.
---=No data was available.

*P≤ 0.05
**P≤ 0.01
***P≤ 0.005

Table D15:

Interaction				
D1*E1	0.24 (0.05)	0.11 (0.03)	0.08 (0.04)	---
D2*E1	0.27 (0.05)	0.11 (0.02)	0.08 (0.03)	---
D1*E2	0.22 (0.08)	0.06 (0.02)	0.00 (0.00)	---
D2*E2	0.19 (0.04)	0.08 (0.07)	0.02 (0.01)	---
D1*E3	0.23 (0.04)	0.07 (0.02)	0.06 (0.02)	---
D2*E3	0.24 (0.04)	0.12 (0.04)	0.09 (0.02)	---
D1*E4	0.18 (0.03)	0.11 (0.01)	0.06 (0.03)	---
D2*E4	0.31 (0.07)	0.13 (0.02)	0.11 (0.03)	---
F ratio	1.11	0.62	0.95	---

A=Replications (1 v 2).
B=Rams (1 v 2 v 3).
C=Weeks (1 v 2 v 3).
D=Treatments (tris yolk glycerol (6%) without raffinose v the same buffer plus 66 mM raffinose).
E=Rate of cooling (-50°C/min v -40°C/min v -30°C/min v -20°C/min).
*=Interaction.
£=Due to insufficient number of data, the F value could not be computed.
---= No data was available.

Addendum to Chapter 8. Experiment 5:

The full 1993 lambing data for the 108 ewes inseminated is still incomplete, but the ewes lambing to artificial insemination have been identified and their data are shown in Table 45.

Table 45: Lambing results for ewes inseminated with ram semen frozen in standard pellets, raffinose pellets or raffinose straws.

Methods of freezing	Number of ewes inseminated	Number of ewes lambed to AI	Percentage of ewes lambed to AI
Standard pellets	34	4	12
Raffinose pellets	35	5	14
Raffinose straws	39	6	15

Chi-square analysis indicated that there were no differences between treatments (Chi-square = 0.157).

These results confirm that the raffinose treatment of the semen did not adversely affect its fertility.

The lambing rates for ewes showing oestrus or not showing oestrus at the time of artificial insemination were 15% (8/52) and 13% (7/56) respectively.

Discussion:

The fertility of frozen-thawed ram semen, after single cervical insemination, has been reported to be generally low. Some reports showing this are listed in Table 46.

Table 46: Fertility reported from pellet frozen ram semen, inseminated cervically in a single dose.

Author	Non-return rate
Salamon & Lightfoot (1967)	15%
Salamon & Lightfoot (1970)	5.6%
	Lambing rate
Salamon (1967)	6.1%
Platov (1968)	13%
Loginova (1968)	27%
Fraser (1968)	31%
Volkov (1968)	16%
Salamon (1971)	47%

The impairment of sperm transport through the cervix, resulting in failure to establish an adequate sperm reservoir in the anterior cervix adjacent to the uterus is the main cause of reduced fertility (Lightfoot & Salamon, 1970a). Quinlivan and Robinson (1969) found residues left in the vagina from the synthetic progestagens impaired sperm transport and survival. In the current fertility trial, progesterone sponges and PMSG were used to synchronise oestrus. These could have been contributed to the low

fertility of 14%.

A mean lambing rate of 45.1% with a range of 17% to 76% was obtained in 21 flocks following intrauterine insemination with frozen-thawed Texel semen by laparoscopy (Alwyn Phillips, personal communication). If this can be fairly compared with the 90% lambing using frozen Suffolk semen (Findlater et al., 1991), it suggests that Texel ram semen may loose much fertility on freezing.

Possible reasons for the discrepancy between non-return rate and lambing data:

1. Out of season breeding:

One recognised disadvantage of early season, or out of season artificial insemination is that non-return to service does not necessarily indicate pregnancy. It is possible that some of the ewes were not ready to become sexually active, and some of these may have reverted to anoestrus and consequently had not been recognised by the ram. Such a likelihood does not invalidate the comparison between the treatments, although it makes a significant effect more difficult to detect. However, for commercial reasons, artificial insemination had to completed before the normal tupping season.

2. Season and embryonic loss:

Embryo mortality was reported to be heavier in ewes mated early in the breeding season (Cooper, 1982).

Insemination was carried out very early in the breeding season in the present experiment. The mean onset of the breeding season for Welsh Mountain ewes is 25th October with a range from 15th October to 11 November (Hafez, 1942), while artificial insemination was performed on 17th of September. Under the normal commercial management of mature Welsh Mountain sheep at the College farm at Aber, tupping starts about 1st of October.

3. Frozen semen and embryonic loss:

There have been reports (Langford et al, 1979 and Lightfoot & Salamon, 1970a) that embryonic mortality increased when frozen semen was used rather than fresh ram semen.

Conclusion:

The object of the present study, which was to evaluate the use of hypertonic raffinose diluent was partially achieved. Although the superiority of raffinose treated semen was not demonstrated in artificial insemination, it was satisfactorily shown in the survival rate of spermatozoa *in vitro* and did not impair its fertility after insemination.

Addendum to Chapter 8. Experiment 5:

The full 1993 lambing data for the 108 ewes inseminated is still incomplete, but the ewes lambing to artificial insemination have been identified and their data are shown in Table 45.

Table 45: Lambing results for ewes inseminated with ram semen frozen in standard pellets, raffinose pellets or raffinose straws.

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Chi-square analysis indicated that there were no differences between treatments (Chi-square = 0.157).

These results confirm that the raffinose treatment of the semen did not adversely affect its fertility.

The lambing rates for ewes showing oestrus or not showing oestrus at the time of artificial insemination were 15% (8/52) and 13% (7/56) respectively.