CRYOPRESERVATION OF MOUSE SPERMATOZOA

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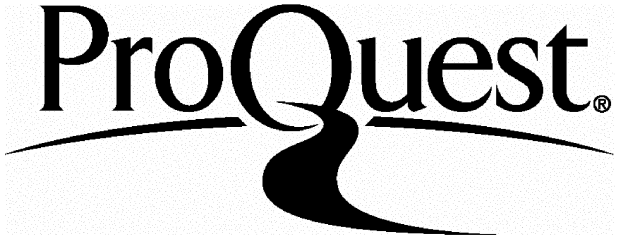

ABSTRACT

A new protocol for the cryopreservation of mouse spermatozoa was developed for the CBA strain. With the aid of a cryomicroscope, several cryoprotectants were screened. The glycerol concentration and osmolarity of an egg yolk TES/Tris diluent resulting in optimal cryoprotection were 1.25% and 675 mOsm respectively. The optimal rate of cooling was 50°C/min from 4°C to -70°C.The percentage of motile sperm following cryopreservation in the modified diluent was assessed subjectively as 55 ± 7.4% Acrosome integrity was investigated on permeabilized cells using a specific monoclonal antibody to the acrosome. The proportion of acrosome-intact spermatozoa after freezing and thawing was 55 ± 13%. The developed protocol was transferred to a cell freezer and the diluent was further modified by the inclusion of 0.1% sodium lauryl sulphate which facilitated recovery of frozen-thawed spermatozoa. This diluent was designated Mouse Sperm Cryoprotectant (MSC). Incubation of oocytes with frozen-thawed spermatozoa resulted in 51% developing to the 2-cell stage, of which 67% developed to the morula/blastocyst stage. Transfer of 2-cell stage embryos to the oviducts of pseudopregnant recipients resulted in a total implantation rate of 39%, with 16% developing to fetuses. Replacement of 2-cell stage embryos to the oviducts of pregnant recipients resulted in 17% developing to live offspring. Further experiments with cryopreserved oocytes and cryopreserved sperm from two strains of mice, CBA and (C57blxCBA) F1, resulted in fertilization rates of 5% and 13% respectively. Transfer of 2-cell stage embryos from oocytes fertilized with CBA and F1 cryopreserved spermatozoa to pseudopregnant recipients resulted in implantation rates of 67% and 92%, with 22% and 25% developing to fetuses respectively.

This protocol, employing a novel cryoprotectant, will provide a consistent and reproducible method for preserving valuable strains of mice.

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CHAPTER 1

LITERATURE REVIEW

History of semen preservation

The preservation of semen has long been realised as an effective way of maintaining valuable genetic information. Spermatozoa can be preserved at temperatures between 12°C and 25°C in simple media designed to reduce cell metabolism, or cryopreserved at temperatures below freezing, usually in liquid nitrogen at -196°C, in media containing cryoprotectants such as glycerol and dimethylsulphoxide (DMSO). Even before the development of cryopreservation this century, the concept of freezing sperm was envisaged by an Italian scientist, Mantegazza, in 1866 as a way of improving breeds of cattle and horses (Mantegazza 1866, see Watson 1990). Attempts to preserve semen started as soon as it was appreciated that artificial insemination (AI) might revolutionise animal breeding. It was discovered that fertile sperm could be recovered from the cooled epididymides of slaughtered domestic animals and sperm from several species were found to survive and remain fertile in simple media (Iwanoff, 1907). It was later found that with the addition of egg yolk as a protective agent, bull semen could be preserved for 4 days or longer at 6-10°C (Phillips and Hardy, 1940). Cryopreservation of semen initially proved difficult, since it was not understood that sperm subjected to temperatures below freezing point would suffer

irreversible damage due to ice crystal formation and thus the recovery of fertile spermatozoa after thawing was poor. The serendipitous discovery that glycerol exerted a cryoprotective effect within the sample helped to solve this problem and fowl semen was the first to be successfully cryopreserved (Polge et al., 1949). Today, the majority of research on semen cryopreservation has been carried out in domestic animals to improve artificial breeding and in a growing number of endangered species for conservation purposes (Densmore et al., 1987; Holt et al., 1988).

Cryopreservation of semen from domestic species

Bull semen can be cryopreserved with no loss of fertility, with as many calves born from inseminations with cryopreserved sperm as with fresh sperm. In most countries, inseminations are carried out almost entirely with cryopreserved semen. This has an obvious economic advantage as many cows can be inseminated from the split sample of one stud male and heritable traits such as high milk yield or a lean meat content can be conserved. However, bull semen proved to be unique, as this success is not the case for all domestic species. Ram sperm have a good post-thaw motility, but the conception rate is reduced when normal methods of insemination are used (Fiser et al., 1981). This can be overcome by increasing the numbers of sperm used in an insemination. Nevertheless, only a small percentage of conceptions in

sheep are by AI (Bonadonna and Succi, 1976). Frozen boar semen is not used routinely in Great Britain, as the spermatozoa can be preserved in an extender for up to 5 days at ambient temperature, which allows sufficient time for the samples to reach their destination through the postal system. Frozen semen is used more often in North America, where the distances are greater; however, fertility is reduced in sows inseminated with frozen semen in contrast to those inseminated with fresh semen (Reed, 1985).

Cryopreservation of human semen

Human spermatozoa are relatively resistant to the effects of freezing and thawing. Slow cooling and warming rates have been found to be most effective when cryopreserving human spermatozoa and a variety of diluents can be employed. The most common diluents used are egg yolk-citrate-glycerol and a modified Tyrode's medium containing HEPES buffer, sugars (normally glucose or fructose), glycine, glycerol and serum albumin (Mahadevan and Trounson, 1983). The use of cryopreserved semen in artificial insemination is now commonplace and in Britain and Australia it is mandatory to use quarantined frozen semen for donor inseminations. Quarantining semen provides time to investigate the sample for disease and viruses such as Human Immunodeficiency Virus (HIV) and hepatitis virus.

Cryopreservation of semen from laboratory animals

Dog semen has been cryopreserved in both pellet form and straws. Both methods use a diluent based on egg yolk and glycerol. The fertility results of inseminations using frozen semen are good, although the degree of success is dependent on the site of insemination (i.e. intracervical or intra-uterine; Farstad, 1984). Cat semen was first cryopreserved in 1978 and used in artificial insemination experiments. The fertility of frozen semen was comparable with that of fresh semen also used for artificial insemination (Platz et al., 1978). Rabbit spermatozoa were successfully cryopreserved and used in fertilization experiments in 1950. Since then, rabbit semen has been successfully preserved in a variety of extenders and, in contrast to other mammalian species, has been found to survive better in the presence of DMSO rather than glycerol (Wales and O'Shea, 1968). Until recently, there were no data available on the cryopreservation of semen from any rodent species, with the exception of an unpublished report by Rapatz and Zimmerman in 1978. This report and recent publications will be discussed more fully later in this chapter (mouse sperm cryopreservation).

Cryopreservation of semen from exotic species

Semen cryopreservation is a useful technique in captive breeding programmes and allows the introduction of genetic material from wild stock to captive gene pools. It also

helps in overcoming problems with "difficult breeders" such as the Giant Panda (Ailuropoda melanoleuca) and Clouded Leopard (Neofelis nebulosa nebulosa) by enabling provision of a semen sample for insemination precisely when the female is in season.

The semen from some exotic species has been successfully cryopreserved, such as giant panda (Moore et al., 1984), addax (Densmore et al., 1987) and blackbuck (Holt et al., 1988), but the data available for these animals are minimal compared to that of domestic species.

The main conclusion drawn from the cryopreservation of semen is that bull semen does not provide a model for the development of cryopreservation protocols, although in some instances it has been possible to use cryoprotectants and protocols developed for one species for the cryopreservation of semen from another similar species e.g. blackbuck and oryx. In the majority of cases, the development of species-specific protocols has been required for the cryopreservation of semen.

The cryopreservation of semen is not just restricted to mammals. Fish sperm from some species can be cryopreserved in diluents containing DMSO, glycerol or methanol (Stoss and Holtz, 1983) and avian sperm can be cryopreserved in diluents containing DMSO or glycerol (Lake et al., 1981), although the latter must be removed before insemination to ensure high fertility rates (Samour et al., 1988).

Mammalian sperm structure

The spermatozoon is a highly specialized cell evolved to transport genetic material from the male to the oocyte. In brief, the spermatozoon consists of a head and tail region, with the tail divided into the mid-piece, principal piece and end-piece. The difference in the size and shape of the sperm varies enormously between species; the total length of Honey possum (Tarsipes rostratus) sperm is 342.6 μm compared to that of the Cape porcupine (Hystrix africaeaustralis) which is 28.3 μm (Cummins and Woodall, 1985). The cell is organised into discreet regions, each of which has a specific function and is bounded by a plasma membrane which is also regionally differentiated. The following description of sperm morphology is restricted to that of a general eutherian mammal, unless otherwise specified (See Fawcett, 1975; Eddy, 1988)

Sperm Head. The shape of the sperm head is very diverse. In monotremes, sperm heads are vermiform, while those of eutherian mammals display a range of shapes extending from the spade or spatulate shape of the ungulates and rabbit, to the hooked head shape (falciform) of the rodent. The spermatozoon head is principally composed of the nucleus and acrosome (Figure 1). It also contains cytoskeletal components and a small amount of cytoplasm. The plasma membrane overlies the entire structure.

Nucleus. This contains DNA (Deoxyribonucleic acid) in the form of chromatin. During the development of the

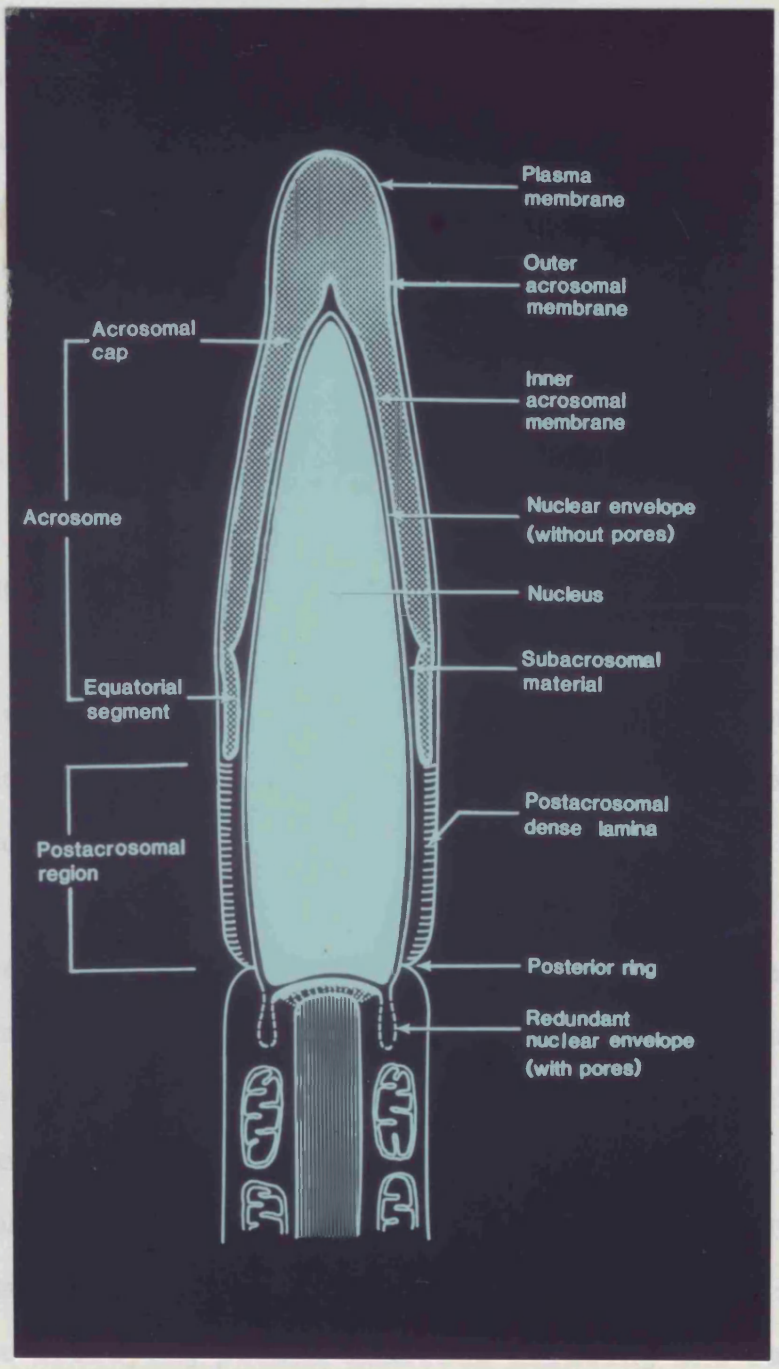


Figure 1. Diagram to show a longitudinal section through a mammalian sperm head.

spermatozoon, the chromatin undergoes condensation. The major nuclear proteins associated with DNA in the mature sperm are protamines, which are cross-linked by forming covalent disulphide linkages with adjacent DNA, rendering it metabolically inert. The decrease in volume streamlines the cell nucleus. This may aid motility and may serve to protect the DNA during sperm passage through the female tract and during fertilization. The entire structure is bounded by the nuclear membrane.

Acrosome. Covering the apical sperm head is a membrane bound, vesicular structure called the acrosome. This consists of inner and outer acrosomal membranes surrounding electron dense material rich in glycoproteins with carbohydrate residues such as galactose, mannose, fucose and sialic acid (Eddy, 1988). Acrosomal contents have been also shown to contain high amounts of a variety of enzymes including hyaluronidase, acid hydrolases and a trypsin-like protease, acrosin (Eddy, 1988) which enable the penetration of oocyte vestments. Before fertilization, sperm must first undergo the acrosome reaction. The outer acrosomal membrane of the apical region fuses in places with the overlying plasma membrane allowing the release of the acrosomal contents. The vesiculation continues until the plasma membrane and outer acrosomal membrane is lost, leaving the head covered by the inner acrosomal membrane. At this point the spermatozoa is said to be fully acrosomally reacted (see Yanagimachi, 1988).

In eutherian mammals, there is a stable area of the acrosome known as the equatorial region or segment. This region remains intact during the acrosome reaction and during sperm penetration of the zona pellucida. It is thought that the equatorial segment may serve to maintain an area of overlying plasma membrane necessary for initial fusion with the oocyte (Moore and Bedford, 1978; Bedford et al, 1979). The equatorial region varies in shape and size between species and is much larger in mouse and rat sperm than in human or bull sperm. (Figure 2, from Yanagimachi, 1988).

Mid-piece. The mid-piece of the sperm tail contains mitochondria which produce energy in the form of ATP for motility. The mitochondria are arranged helically along the longitudinal fibres of the tail. The number of 'turns' or gyres varies between species from 15 in man to as many as 300 in rodents. In some passerine birds, the mitochondria spiral along the entire length of the sperm tail (Nicander, 1970).

Axoneme. The tail contains the axoneme (or axial filament complex), which is responsible for the movement of the spermatozoa. The axoneme consists of microtubules in the 9 + 2 arrangement commonly found in the cilia and flagella throughout the plant and animal kingdom. The microtubules are composed of the protein tubulin, and are arranged as doublets consisting of two subunits; subunit A which is a complete microtubule and subunit B which is a crescent-shaped section attached to the A subunit. Two A subunits

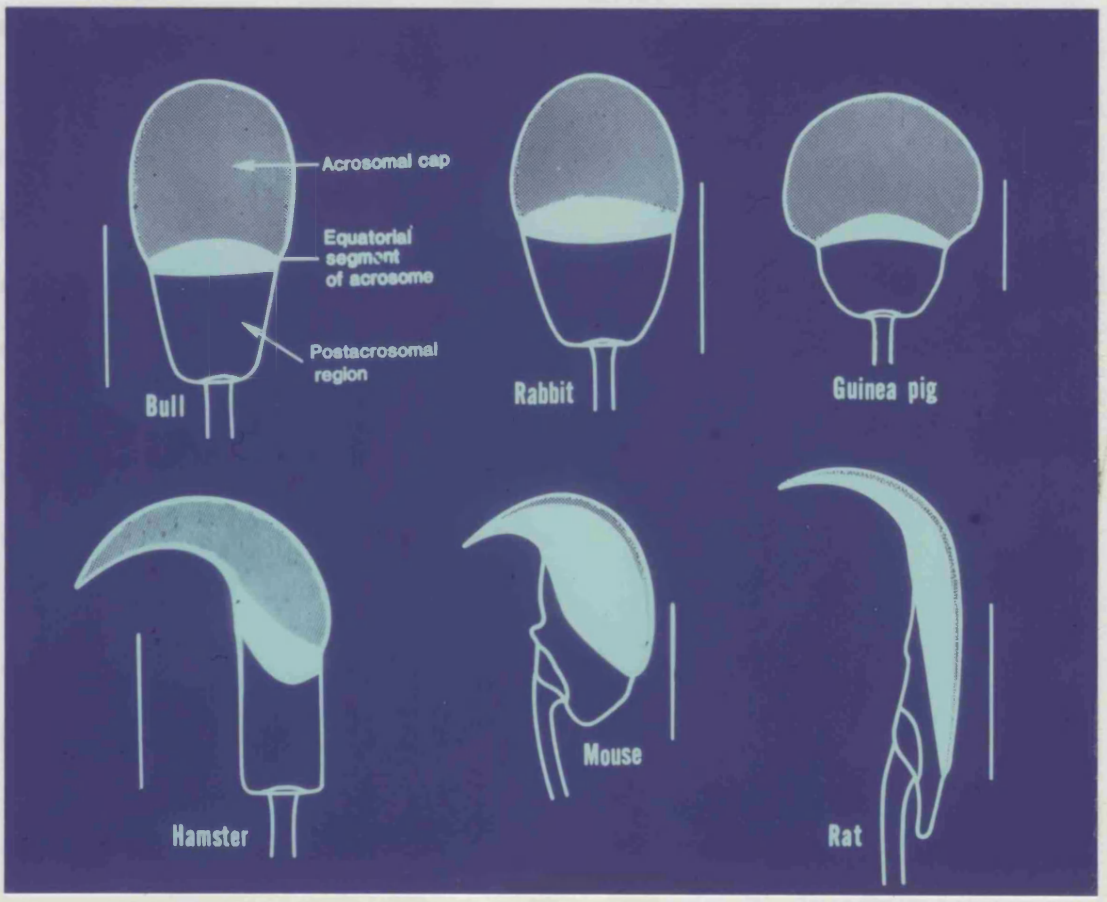


Figure 2. Diagram showing the longitudinal section of sperm heads from different species.

in the centre of the axoneme are surrounded by 9 doublets. The axonemes are connected to each other by nexin links (Stephens, 1977) and dynein arms and the structure is surrounded with a fibrous sheath. Motility is generated by the dynein converting chemical (ATP) energy using ATP-ases, to mechanical energy (movement) causing the microtubules to slide past each other. The nexin links are thought to maintain the symmetry of the structure and are elastic enough to regulate the displacement that occurs during the sliding phase (see Eddy, 1988).

Fibrous sheath. The axoneme is encased in a fibrous sheath, which runs along the outside of the spermatozoa, not quite to the end of the tail. In the rat it has been found that these fibres are probably composed of a cysteine rich polypeptide (Olson et al., 1976).

Sperm plasma membrane. The plasma membrane is a regionally differentiated, labile structure that follows the fluid mosaic model described by Singer and Nicholson (1972). Studies using membrane probes and monoclonal antibodies have shown the membrane to be made up of many discreet domains (see Friend, 1982, 1984; Eddy, 1988). Lectin staining can show the different distribution of glycoproteins on the surface of the sperm and freeze fracture studies show the arrangement of intra-membranous particles (IMPs) on the outer surface of the inner lipid bi-layer. These IMPs are thought to represent the proteins within the membrane and are able to move within the bi-layer during the fluid state at body temperature. The

different domains of the plasma membrane vary in composition and structure and reflect the specialized function of the spermatozoon. The major domains of the head region are the acrosomal cap, equatorial region and post-acrosomal region. Also situated on the head, are the anterior band, serrated band and posterior ring, which separates the head and tail region. The flagellum plasma membrane is divided into the mid-piece, posterior region and annulus. The last consists of a fibrous structure which surrounds the axoneme and is attached to the plasma membrane.

Undergoing considerable changes during maturation, capacitation and the acrosome reaction, the surface of the sperm is modified considerably between release from the testis and fertilization of the oocyte. During maturation the membrane undergoes restructuring, with surface glycoproteins laid down during the passage through the epididymis (Lea et al., 1978; Bedford and Hoskins, 1990). The surface of the sperm is coated with glycoproteins. Lectin studies have shown that the distribution of glycoproteins is not uniform over the plasma membrane. In addition, during capacitation (see chapter 3), sperm surface components acquired from seminal plasma during ejaculation, are removed and the plasma membrane is further modified (Bedford and Hoskins, 1990).

During the acrosome reaction, the highly fusogenic anterior portion of the plasma membrane of the head region fuses with the underlying acrosomal membrane to allow the

release of the acrosomal contents. The plasma membrane over the acrosomal region is eventually lost entirely at completion of the acrosome reaction.

The integrity of the plasma membrane is vital for the fertilization of the oocyte and it is well established that membrane damage leads to immotility and eventual cell death. However, during the acrosome reaction large areas of the plasma membrane are initially breached and then lost altogether, with no subsequent loss in motility. This is probably due to the posterior ring which is situated between the head and neck of the sperm. Here, the membrane is constricted and fused with the outer and inner nuclear membranes, physiologically partitioning the cell. For this reason it may be possible to have immotile sperm (damaged tail membrane) retaining the ability to fuse with an oocyte and motile sperm unable to fuse with the oocyte due to a damaged plasma membrane over the head region.

Cryopreservation and cryodamage

Why are cells cryopreserved? Cells are metabolically inactive at temperatures below -130°C, therefore the most effective way of storing sperm for long periods of time, is by cryopreservation in liquid nitrogen at -196°C. Studies have shown that the only deleterious effect to the sperm at this temperature is caused by background radiation (Ashwood-Smith et al., 1979; Mazur, 1984), but many thousands of years of exposure would be necessary before this would occur.

The development of cryopreservation techniques has enabled valuable gene lines to be maintained for the preservation of rare breeds of mammals and has revolutionized the cattle breeding industry, with the routine cryopreservation of both sperm and embryos. As mentioned earlier, cryopreservation also allows the semen sample to undergo a period of quarantine before its use, thereby preventing the transmission of disease. Cell cryopreservation techniques are not limited to gametes and embryos but are also used extensively for the preservation of erythrocytes, bone marrow and tissue culture cell lines.

How are cells cryopreserved? Sperm may be frozen in plastic straws or polypropylene cryotubes, or frozen as pellets directly on dry ice (solid carbon dioxide) and then transferred to cryotubes. Pellet and straw packaging replaced the glass ampoule that had been used previously. Straws are thin walled and allow a relatively even cooling of the sperm sample. Cryotubes have much thicker walls and during cooling there may be a time lag before the temperature inside the cryotube reaches the temperature outside, especially at faster cooling rates. It is also probable that the sperm nearest the cryotube wall will cool at a different rate to the sperm in the centre of the sample. Samples can be frozen in alcohol baths cooled with dry ice (Polge and Rowson, 1952) or by pelleting on dry ice (Nagase and Niwa, 1964). They can also be frozen in straws or cryotubes in a programmable cell freezer, which

allows for a precisely controlled rate of cooling. Straws can also be frozen in the vapour phase of liquid nitrogen (Cassou, 1964), resulting in a much faster cooling rate, the straw giving a large surface area to volume ratio, thus allowing more effective heat transference. Other cells, such as oocytes and embryos (Rall et al., 1984; Shaw et al., 1991; Nagashima et al., 1991) can be frozen almost instantly, by a method known as vitrification (Luyet et al., 1939). Vitrification, literally 'glass formation', involves the cells being frozen so rapidly (usually by immersion into liquid nitrogen) that the cell freezes instantly and intracellular water freezes into a glassy solid. An advantage of the vitrification process is that the exposure of cells to potentially toxic compounds is for a minimum amount of time. The disadvantages of vitrification include the possibility of ice crystal formation during thawing (devitrification) and the exposure of cells to high concentrations of solutes necessary for this process (McFarlane, 1986). This method has not yet been adapted for sperm.

Cryodamage of cells. As the diluent containing the sperm is cooled and reaches its freezing point, ice formation occurs in the surrounding solution. The sperm plasma membrane prevents the ice crystals from forming inside the cell and so the contents become gradually supercooled (Figure 3). This creates a difference in the chemical potential of the water resulting in its movement out of the cell down an osmotic gradient. If the cooling rate is

sufficiently slow, the cell will become progressively more dehydrated eventually resulting in an increase in intracellular solutes, decrease in intracellular water and precipitation of solutes in the surrounding solution (Figure 3). This is known as the solution effect (Lovelock, 1953). Excessive dehydration is also damaging to the plasma membrane and may affect internal structures of the cell (Clegg et al., 1982). If the rate is too high, intracellular ice will eventually form as the sperm becomes supercooled. When excessive intracellular ice formation is usually lethal (Farrant, 1977), however small amounts of intracellular ice need not necessarily damage the cell (Mazur, 1984). For successful cryopreservation, the cell should not be so shrunken that it incurs structural damage, but should avoid the formation of potentially lethal intracellular ice (see Hammerstedt et al., 1990).

Stress is also exerted on sperm during the thawing process. The cells are subjected to tremendous osmotic pressures as the cells rehydrate and there is a risk of ice formation. It was discovered that successful cryopreservation was dependent on specific thawing rates (Whittingham et al., 1972), that is, if the freezing rate was fast, a fast thawing rate would be required for cell survival. Recognizing the relation between freezing and thawing rates led to the first successful cryopreservation of mouse embryos by Whittingham et al. (1972), when they were cooled and warmed slowly. The survival of a

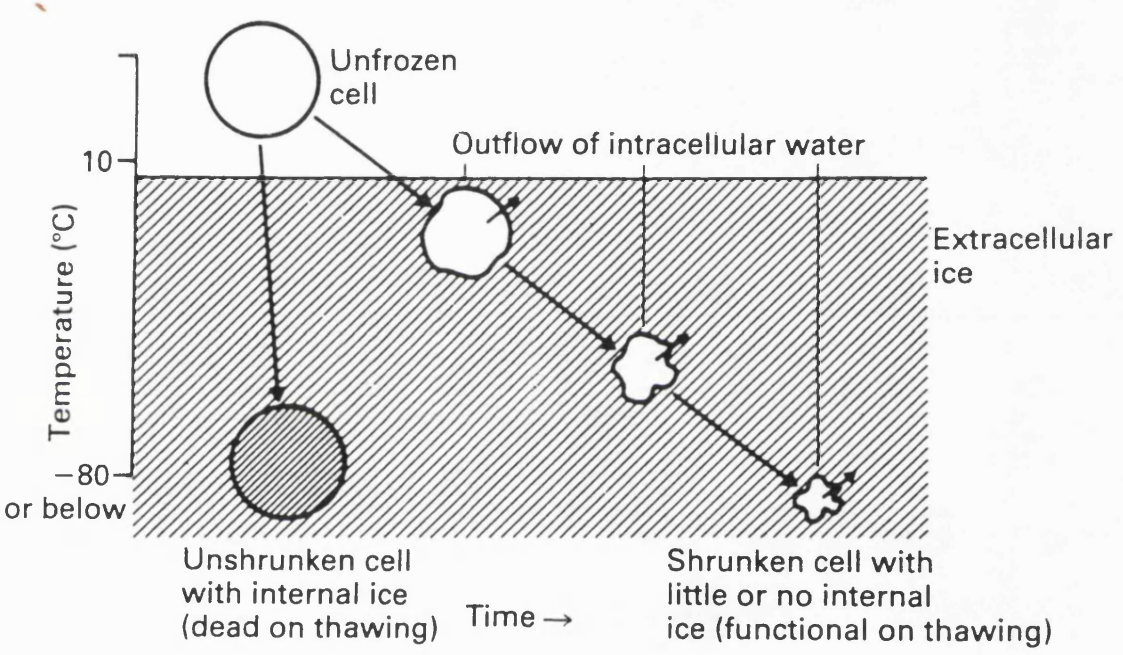


Figure 3. Diagram to show the events occurring during fast or slow cooling of a cell. (Adapted from Watson 1990)

cryopreserved cell can be increased with optimal cooling temperatures and cryoprotectants (Watson and Martin, 1975). Interestingly, it has been observed that in some instances damage to the plasma membrane after freezing does not manifest itself until thawing. Using a fluorescent probe, Holt et al. (1992) observed that the integrity of the plasma membrane of ram sperm was lost during the thawing process rather than during freezing. This was attributed to membrane lesions as a result of transversing lipid phase transitions during cooling and warming.

Changes in the plasma membrane. As the cell freezes, the plasma membrane undergoes a phase transition as it moves from a labile to a non-labile state. During the change in temperature, lipids associated with specific membrane proteins may group together, forming a gel-rich phase as the sample is further cooled. Rewarming could result in membrane proteins being left with different associated lipids. Depending on the lability of the lipids after thawing, this effect may be permanent resulting in damage to the plasma membrane. Studies on model membrane systems have attempted to understand protein-lipid interactions during freezing and thawing (Quinn, 1989; Carruthers et al., 1988).

Cold shock. Sperm from many species become irreparably damaged when subjected, even for a short time, to temperatures below 17°C and above 0°C without cryoprotectants. Boar and ram sperm are particularly

susceptible (Watson, 1981). It is thought that cold shock is dependent on the lipid composition of the cell membrane; human and monkey sperm have equal amounts of cholesterol and phospholipid, and are less susceptible to cold shock than boar and ram sperm which have proportionately less cholesterol in the membrane (Darin-Bennett and White, 1965; Watson, 1981). The acrosome is most vulnerable to cold shock and damage is seen as swelling or bubbling of the membranes. In extreme cases the acrosome is lost entirely. As a result of cold shock the plasma membrane may also be damaged. Carbohydrate metabolism is reduced and lipids are released from the plasma membrane (Blackshaw et al., 1957; Pickett et al., 1967). Membrane composition, in particular cholesterol content, may determine the susceptibility of sperm membranes to cold shock (Watson and Morris, 1987). Egg yolk is known to provide protection against cold shock (Blackshaw, 1954; Kampschmidt et al., 1953), as do milk and milk proteins. The exact action of these substances is not known, but it is thought that they may stabilise the proteins within the plasma membrane and/or alter the fluidity of the lipid bi-layer rendering it more able to withstand phase changes.

Cryoprotectants - Mechanism of action. Cryoprotectants used to protect cells during cryopreservation can be divided into two categories: those that penetrate the cell (glycerol, alcohols, DMSO) and those that do not (sugars, polymers). Substances such as glycerol, which permeate the

cell, act by binding intracellular water, reducing the amount of water for ice formation, which reduces the solution effect. Methanol and DMSO act in the same way. Non-penetrating cryoprotective agents include sugars, such as fructose and raffinose, and proteins such as those contained in egg yolk and milk. These act osmotically to dehydrate the cell during the freezing process, but they have no effect on the 'solution effect' during freezing and are more effective at fast freezing rates (see Watson, 1990). It has been indicated that cryoprotectants like the naturally occurring sugar, trehalose may hydrogen bond to the phospholipid head group region of the bi-layer, helping to stablise the cell membrane. They may also help in inhibiting the formation of a gel phase (Crowe et al., 1984). The mode of action seems to be by mixing with the bi-layer to stabilise the membrane during freezing and preventing the aggregation of intramembranous particles (Rudolph and Crowe, 1985). Glycerol, although one of the most successful cryoprotective agents discovered so far (Watson, 1990), is known to have a toxic effect on sperm (McLaughlin et al., 1992) and may have detrimental effects on the structure of the plasma membrane and metabolism of the cell (Hammerstedt et al., 1990). Permeating cryoprotectants may damage the cell by toxic effects or by osmotic stress. These effects can be lessened by adding the cryoprotectant at low temperatures, e.g. 4°C (Ashwood-Smith, 1987) when the permeability of the cell is reduced. DMSO is routinely used for transforming erythroid

leukaemic cell lines (Pulito et al., 1983) and there are many references describing the genetic and epigenetic effect of DMSO during long exposure to it (Ashwood-Smith, 1987). However, no genetic effects of DMSO have been reported for short exposure times or with the concentrations routinely used in cryoprotectants (Ashwood-Smith, 1985). The use of DMSO and polyethylene glycol (PEG) in cell fusion is also well known and may be thought to be detrimental for cell survival when included in cryoprotectants. However, their action on cell membranes may help the cell withstand the stresses of cryopreservation, and the cell fusion properties may be modified by the presence of other factors in the diluent (Fahy et al., 1984).

Detergents. These are often used to disrupt cell membranes. Alkyl ionic detergents (eg. sodium lauryl sulphate [SDS]) denature the native structure of membrane proteins and can dissociate them into their constituent polypeptides. They can also be used to remove the tail membrane for sperm motility studies. It has been found that low levels of the detergent sodium and triethanolamine lauryl sulphate (STLS), help to protect cells during cryopreservation (Arriola et al., 1987). The effect of the detergent may be exerted directly on the cell membrane or by acting on the egg yolk in the diluent, solubilising cryoprotective lipids. The inclusion of STLS, also an alkyl ionic agent, in the cryoprotectant increased

the percentage of intact acrosomes and increased the motility of bull sperm after thawing (Arriola, 1982).

Proteins and lipids. Egg yolk-citrate and egg yolk-Tris diluents are used routinely for the cryopreservation of bull semen for artificial insemination (Foote et al., 1987). Egg yolk diluents have also been found to work well in the cryopreservation of other ungulate sperm such as those of blackbuck (Holt, 1988). A disadvantage of using egg yolk in a diluent is that it provides a substrate for lipid peroxidation, potentially increasing the toxicity of the sample (Shannon et al., 1972). Other proteins often used include whole milk (Thacker, 1953; Ahmad et al., 1985), whole egg (Dunn et al., 1953) and skimmed milk (Watson, 1990). One of the components of egg yolk that has been found to have protective effects upon spermatozoa has been identified as a low density lipoprotein factor, LDF (Watson, 1975). It has a protective effect comparable to egg yolk in the preservation of bull spermatozoa, although ram spermatozoa showed greater acrosome damage after freezing with LDF alone, than with egg yolk (Watson, 1975). It is thought that the role of the phospholipid is in membrane protection, whilst the protein component helps maintain protection during cooling and storage above freezing point (Watson, 1981). The role of egg yolk therefore has a two-fold effect in protection during freezing, and during cooling and storage.

Sugars. These are ideal as cryoprotectants as they dissolve readily in water and are not toxic to the cells.

They contribute principally to the osmolarity of the diluent. Some sugars are found as naturally occurring cryoprotectants, such as fructose and trehalose (Madin and Crowe, 1975). There is evidence that trehalose may preserve membrane structure and function by inhibiting membrane mixing and preventing aggregation of intramembranous particles during the stress of dehydration and freezing (Rudolph and Crowe, 1985).

Assessment of sperm viability after freezing. Plasma membrane integrity can be assessed using fluorescent probes such as Hoescht 33258 (Centola et al., 1990) and fluorescein diacetate (McGann, 1988; Harrison, 1990; Holt, 1992). Fluorescein diacetate (FDA) crosses the cell membrane into the cytoplasm passively as a non-fluorescent esterified form. This is followed by enzymatic cleavage of the ester bonds resulting in the fluorescent form of FDA, which now cannot leave the cell unless there is a breach in the cell membrane. Studies have shown that although FDA is used to measure membrane integrity, it more accurately reflects the permeability of the cell membrane. Freeze/thaw damage can result in changes in the channels by which FDA moves across the plasma membrane, accelerating the loss of FDA even though the membrane remains intact (McGann et al., 1988). Acrosomal status can also be assessed using Hoescht 33258; by the fix vital stain method (De Leeuw, 1991); with fluorescein-labelled lectin Pisum sativum (PSA, see Mendoza et al., 1992); with chlorotetracycline (Ward and Storey, 1984) or with a

monoclonal antibody to the acrosome that can be used in conjunction with a fluorescent second antibody (Ellis et al., 1985; Moore et al., 1987). This highly specific antibody, effective in all mammalian species tested so far, is used on methanol-fixed (membrane permeable) sperm. A fluorescent second antibody is then used to allow visualization of the acrosome. In the mouse, the intact acrosome is seen as a crescent shaped structure. The monoclonal antibody binds strongly to the outer acrosomal membrane and acrosomal contents, and weakly to the inner acrosomal membrane. This enables the degree of damage to be assessed.

For cryopreserved human spermatozoa, the decline in motility after thawing is related to the fecundity of the semen sample (Holt et al., 1989). This result suggests that membrane damage due to cryopreservation is not always manifested immediately. Membrane integrity in the tail region can be assessed using the hypo-osmotic swelling test, which relies on the swelling of the tail to indicate that the membrane is still functional (Jeyendran et al., 1984).

Hamster egg penetration test. Heterologous spermatozoa bind to and fuse with zona pellucida-free hamster oocytes (Yanagimachi et al., 1976). This observation has been used as the basis for a test to assess sperm fertilizing capacity when homologous oocytes are unavailable (see Yanagimachi, 1988).

Mouse sperm cryopreservation

In contrast to the cryopreservation of mouse embryos (Whittingham, 1972) and oocytes (Whittingham, 1977), the only report of cryopreservation of mouse spermatozoa was until recently the unpublished findings by Rapatz and Zimmerman (see Graham et al., 1978). These investigators artificially inseminated 2 groups of mice; 106 females with spermatozoa cryopreserved in a skimmed milk and fructose diluent resulting in the birth of 9 young; and 84 females with spermatozoa cryopreserved in a skimmed milk and fructose diluent containing 1% bovine serum albumin and 4 mM calcium chloride, resulting in the birth of 61 young.

Electron microscope studies on mouse spermatozoa before freezing, while frozen and after thawing, in cryoprotectants containing glycerol or DMSO (Sherman and Lui, 1982) showed no evidence of deleterious effects on the ultrastructure as a result of freezing. Whole epididymides were exposed to yolk-citrate-Locke's medium containing 15% DMSO or glycerol and frozen at rates of 8°C/min and 1000°C/min. The samples were thawed at a rate of 100°C/min. Freeze substitution was used to preserve the structure of the frozen state and electron microscopy to observe any changes as a result of the freezing and thawing process. No spermatozoa survived the freezing experiments at any of the rates examined.

More recent studies have included cryopreservation of mouse spermatozoa in Dulbecco's phosphate buffered saline

(PBS) containing glycerol, DMSO or skimmed milk as cryoprotective agents (Yokoyama et al., 1990) and in physiological saline (0.86% NaCl) containing sucrose, raffinose, DMSO or glycerol, and a combination of raffinose and glycerol (Tada et al., 1990). In the first study, in vitro fertilization experiments using frozen-thawed spermatozoa cryopreserved in a 10% raffinose, 5% glycerol PBS diluent, resulted in a fertilization rate of 37% (37/100 oocytes developing to 2-cell). Twenty eight embryos at the 2-cell stage were replaced into the oviduct of pseudopregnant foster mice and 21 pups were born. For spermatozoa cryopreserved in PBS containing 10% raffinose and 10% DMSO, the fertilization rate was 19.4% (36/186 oocytes developing to 2-cell). Thirty embryos were replaced into the oviducts of pseudopregnant recipients and 5 pups were born. In the second study, spermatozoa were frozen rapidly in two steps, as pellets on solid CO_2 from 37°C to -70°C and in liquid nitrogen from -70°C to -196°C. Spermatozoa from one outbred strain (ddY) and 5 inbred strains (C57BL/6N, C3H/HeN, DBA/2N, BALB/c and kk) of mice were frozen in physiological saline containing 18% raffinose and 1.75% glycerol, with fertilization rates ranging from 13% for C57BL/6N to 64% for DBA/2N. Fifty seven 2-cell embryos from oocytes fertilized with C57BL/6N frozen-thawed spermatozoa were replaced into the oviducts of pseudopregnant foster mice and live fetuses were present when examined on day 18 of pregnancy. These methods using a raffinose and glycerol cryoprotectant have

proved difficult to repeat, although it has been possible to carry out assessment of zona binding and motility using sperm previously cryopreserved in a 10% raffinose and 5% glycerol diluent (Sztein et al., 1992). More recent studies report the use of 3% skimmed milk and 18% raffinose in distilled water as an effective cryoprotectant for mouse sperm (Takeshima et al., 1991; Nakagata and Takeshima, 1992).

PROPOSED RESEARCH

Following the tremendous success with the cryopreservation of bovine semen, protocols were developed for freezing sperm from many other species with varying degrees of success. Until recently there had been very little work carried out on rodent species due to the initial failures to find a satisfactory cryopreservation method. Considering that the mouse is one of the most popular laboratory models and embryo freezing techniques have been established for some years, it is perhaps surprising that a protocol had not been developed earlier, although with the abundance of these animals, the need to cryopreserve mouse semen may have seemed unnecessary. With the advent of transgenic mice, the need to cryopreserve mouse sperm has now become imperative. The production of transgenic mice, by microinjection of genetic material into the nucleus or by the formation of chimeras (fusion of two embryos), has increased dramatically over the last few years. Transgenic mice are proving to be invaluable

models for the study of many genetic disorders such as cancer (Cory, 1988), brain tumours (Brinster, 1984), kidney disease (Weiher, 1990) and developmental disorders (Westphal, 1990; Behringer, 1990). The ability to cryopreserve sperm from mice expressing an important transgene would eliminate the expense of maintaining large breeding colonies of animals and greatly reduce the risk of loss of the transgene through mutation.

Although glycerol is routinely used in concentrations of between 6-8% for the cryopreservation of bovine sperm, it is known to have a toxic effect on sperm (McLaughlin et al. 1992). It is this author's suggestion that glycerol may exert a toxic effect on mouse sperm. Furthermore, the cryoprotective effect of raffinose is limited to the increase in the osmolarity of the diluent and plays a minimal role in protection of the plasma membrane. Therefore, the aim of this study was to develop a diluent with i) optimal glycerol concentrations, ii) protection for the plasma membrane, resulting in a reproducible protocol for the efficient cryopreservation of mouse spermatozoa.

CHAPTER 2

INTRODUCTION

Cell injury during cryopreservation and the role of cryoprotectants in sperm survival have already been discussed in chapter 1. In developing a protocol for the cryopreservation of sperm, the principle variables to be considered are those related to the composition of the cryoprotectant and the rates at which the sample is cooled and warmed. Previous methods for mouse sperm cryopreservation are based on raffinose/glycerol (Yokoyama et al., 1991; Tada et al., 1991) and raffinose/DMSO diluents (Yokoyama et al., 1991) and involve slow cooling and warming rates (see chapter 1). To assess how consistent and reproducible they were, these methods were examined with a cryomicroscope and cell freezer as outlined below. However, it was not possible to confirm these previously published findings. Therefore, a new method for mouse sperm cryopreservation was investigated using the cryomicroscope. As a starting point, one of the most successful diluents, based on egg yolk and TES/Tris buffer (see Watson, 1990), was modified and then various cooling amd warming rates were examined. The use of an alkyl ionic detergent was also investigated. Detergent in the form of sodium and triethanolamine lauryl sulphate (STLS, Orvus ES Paste) has been used in diluents for bull sperm cryopreservation (Foote and Arriola, 1987), where its beneficial effect, when used in conjunction with egg yolk, was reported. STLS was difficult to obtain in the

United Kingdom and therefore a similar detergent of the same alkyl ionic family was used; sodium lauryl sulphate (SDS).

The role of the cryomicroscope in developing protocols for cryopreservation

Cryomicroscopy, literally the study by microscopy of events at sub-zero temperatures, provides a tremendous advantage in the development of cryopreservation protocols. The effects of different diluents and cooling rates can be examined immediately, by observing the sample during the freezing and thawing process.

The earliest form of cryomicroscopy was carried out by Chambers and Hale (1932), simply by using a microscope in a cold room. The first observations on the cryopreservation of sperm using cryomicroscopy were made by Smith et al. (1951) on fowl sperm. Details of the historical development of light cryomicroscopy have been reviewed by Diller (1982). The cryomicroscope used in this study is a standard microscope with a specially adapted stage which allows samples to be cooled to temperatures of -120°C (Figure 4). This is achieved by passing cooled liquid nitrogen vapour through a specially adapted cryostage. A small thermocouple on the stage is temperature controlled by computer, allowing the cooling and warming rates to be altered. Experiments can be carried out on samples of 1-4 μl, allowing small and precious samples to be studied. The sample can be viewed

in the conventional fashion through the eyepiece of the microscope or can be captured on a video camera for viewing on a monitor or recorded on video tape. A typical view of a frozen sample of mouse spermatozoa as seen on a cryomicroscope is shown in Figure 5.

Choice of mouse strain

The strain CBA was used for the development of a new method of mouse spermatozoa cryopreservation. This strain has a lower embryo and fetal developmental rate than the (C57blxCBA) F1 hybrid which is most commonly used for in vitro fertilization. Sperm cryopreservation will be of particular use for transgenic mice. Since these animals often have reduced embryo and fetal development rates, it was considered that the use of the CBA strain would provide a more suitable model than F1 hybrids.

Transfer of technique

The developed protocol was transferred to a cell freezer where aliquots of spermatozoa were cryopreserved using similar cooling and thawing rates. The method was repeated on two different models of cell freezer to determine the reproducibility of the protocol in different laboratories and was also tested on two additional strains of mice. Post-thaw sperm viability was assessed by examining motility, acrosome integrity and plasma membrane integrity before in vitro fertilization and embryo transfer studies (chapters 3 and 4).

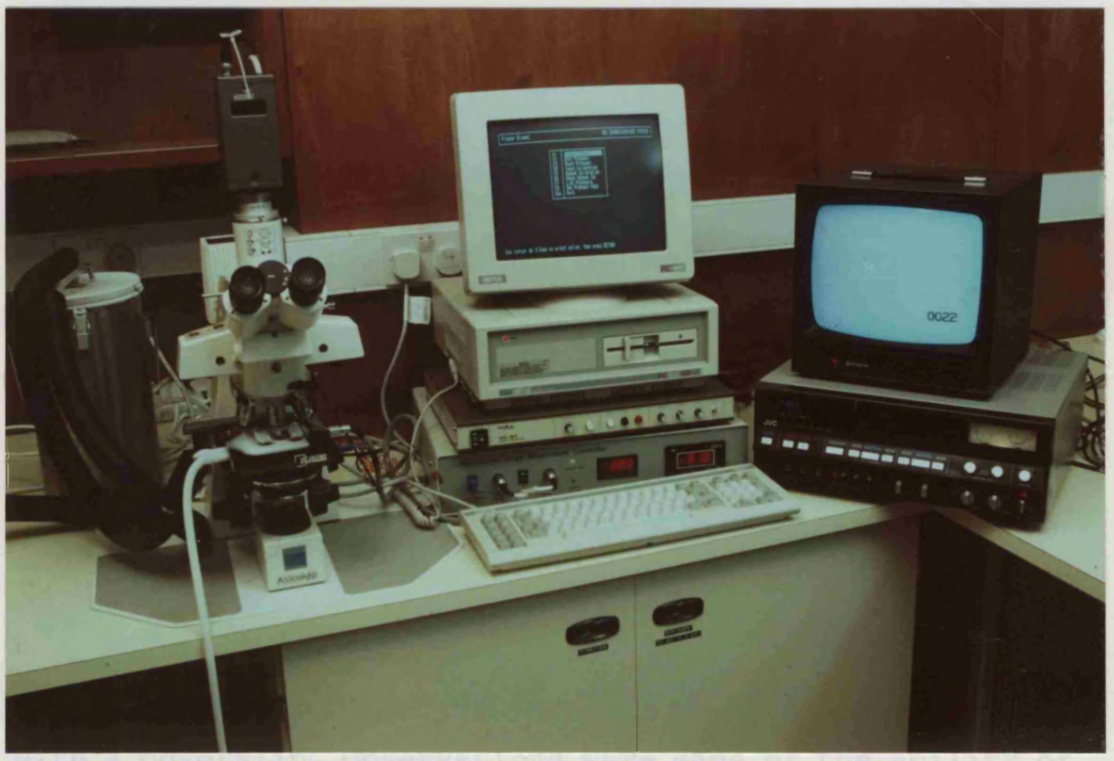


Figure 4. CM-3 Cryomicroscope.

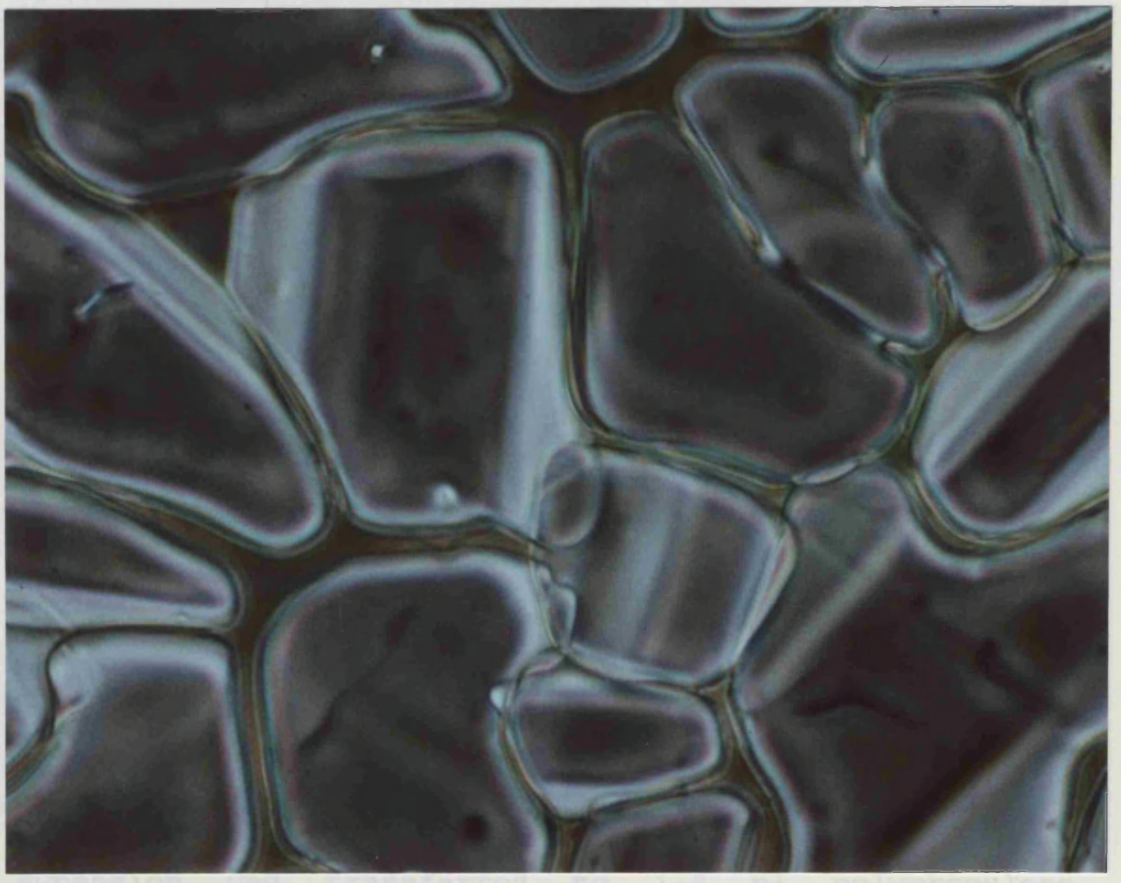


Figure 5. Mouse sperm frozen in ice crystals.

MATERIALS AND METHODS

Use of Cryomicroscope

Cryomicroscope. The model used in the following experiments was a Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, Germany) with a CM-3 cryomicroscope stage (Planer Biomed, Sunbury-on-Thames, UK). Briefly, a typical cooling experiment was carried out in the following manner. The appropriate rates of cooling and warming were programmed into the computer (IBM compatible PC). A sperm sample (1 - 4 μl) was placed on the glass embedded thermocouple in the centre of the cryostage and covered with a coverslip. Observations were made of the ability of spermatozoa to withstand the changes in temperature by direct viewing and indirectly by acrosome membrane studies. Frosting over the coverslip was minimal.

Collection and handling of spermatozoa. Epididymides from 4 CBA mice, 12 weeks old, were dissected directly into the diluent in all the following experiments unless otherwise described.

Assessment of published protocols

Diluents from published reports were assessed on the cryomicroscope and in the cell freezer.

Cryomicroscope. Diluent 1: 10 ml Dulbecco's PBS (Gibco), 500 μl glycerol and 1 g raffinose (Yokoyama et al., 1991). Epididymides from 4 CBA mice were dissected into 500 μl drop of diluent under silicon oil. The dispersed sperm supension was transferred to 1.8 ml polypropylene cryotubes (Nunc, Inter-Med) in 200 μl aliquants and cooled

at a rate of 1°C/min to 4°C in an ice and water bath and then 10°C/min to -70°C on the cryomicroscope. The sample was thawed at a rate of 10°C/min to 37°C on the cryomicroscope and post-thaw motility was assessed subjectively. The above procedure was repeated twice before transferring the method to a cell freezer.

Diluent 2: 10 ml Dulbecco's PBS (Gibco), 1.8 g raffinose, 175 µl glycerol (Tada et al., 1991).

Epididymides from 4 CBA mice were dissected into 1 ml diluent, the sperm were released by cutting the epididymis with fine scissors and agitating gently. The sample was left at room temperature (~22°C) for 15 min to equilibrate before cooling to -5°C at 1°C/min in an ice and water bath and then to -70°C at 10°C/min on a cryomicroscope. The procedure was repeated on the cryomicroscope before transferring the method to a cell freezer.

Cell freezer. Diluent 1: Sperm from 4 CBA mice were suspended in 1 ml of diluent as described previously. Aliquots of 200 µl were transferred to 1.8 ml polypropylene cryotubes (Nunc, Inter-Med) and transferred to a water bath at room temperature. The samples were cooled at a rate of 1°C/min to 4°C then 10°C/min to -70°C before plunging the sample into liquid nitrogen (-196°C) where it was stored for a minimum of 1 h. The sample was thawed at a rate of 10°C/min to 37°C by transferring to a cell freezer at -70°C for 5 min and warming at the programmed rate. The thawed sample was diluted with PBS at 37°C. The above procedure was also repeated on sperm

suspended in PBS containing 10% raffinose and with the glycerol replaced with 10% DMSO, using the same cooling and warming rates (Yokoyama et al., 1991).

Diluent 2: Sperm were suspended in 1 ml of diluent as described previously. Aliquots were transferred to 0.8 ml plastic straws and cooled in a cell freezer at a rate of 1°C/min from room temperature to -5°C and 10°C/min to -70°C. The straws were plunged into liquid nitrogen for a minimum of 1 h. Sperm were thawed by removing the straw from the liquid nitrogen and leaving at room temperature until the ice melted. The contents of the straw were then transferred to a cryotube and diluted with PBS at 37°C.

Sperm cooled to 4°C in an ice-water bath. Samples from each of the three diluents (diluent 1, diluent 1 with DMSO replacing glycerol and diluent 2) were cooled to 4°C at the rates published for each diluent. The samples were rewarmed according to the thawing protocols and samples were diluted with 200 µl PBS at 37°C.

Results

The motility of mouse spermatozoa cooled and warmed as described above is shown in Table 1 a,b, and c.

Cryomicroscope. Following cooling and re-warming to 37°C, the majority of sperm in diluents 1 and 2, displayed no progressive motility. The motility of the few motile sperm was erratic and did not show the same type of flagellar bending still observable when mouse sperm are in hyper-osmotic media. The movement of the sperm after freezing

and thawing in these diluents appeared to be restricted mainly to the head region and will be described here as 'twitching'. This movement was not the same as the movement observed in the spermatozoa in the diluent prior to cooling.

Cell freezer. Following cryopreservation in diluent 1, and dilution of the sample, between 0 and 10% of the sperm were judged progressively motile. Sperm cryopreserved in 10% raffinose, 10% DMSO in PBS, had a post-thaw progressive motility of <1% after dilution of the sample with PBS. Sperm cryopreserved in diluent 2 had a post-thaw motility of <5% after dilution with PBS. Sperm cooled to 4°C in an ice-water bath. Sperm which were cooled to 4°C in the presence of either diluent 1, 10% raffinose, 10% DMSO or diluent 2 and then re-warmed to 37°C, before dilution with PBS, showed progressive motilities of 8-30%, 10% and <5 %, respectively.

Development of new method of mouse spermatozoa cryopreservation

The following preliminary experiments were undertaken to determine whether a cryoprotectant and controlled freezing and thawing rates were necessary for mouse spermatozoa to survive crypreservation.

i) Cryopreservation of spermatozoa in PBS (no cryoprotectant). Epididymides were dissected into PBS at 37°C. Spermatozoa were released by puncturing the

Table 1. Assessment of published diluents

a) Motility of mouse spermatozoa cooled and warmed on the cryomicroscope

Experiment	Sperm motility (%)	
	10% Raffinose/ 5% Glycerol (Yokoyama *et al.*, 1991)	18% Raffinose/ 1.75% Glycerol (Tada *et al.*, 1991)
1	20 'Twitching'	10 'Twitching'
2	20 'Twitching'	1 'Twitching'

(b) Progressive motility (%) of mouse sperm cooled to -70°C in a cell freezer and stored at -196°C

10% Raffinose/ 5% Glycerol	10% Raffinose/ 10% DMSO	18% Raffinose/ 1.75% Glycerol
10	<1	5
10	<1	-
0	-	-
<1	-	-

c) Progressive motility (%) of mouse sperm cooled to 4°C in an ice-water bath

10% Raffinose/ 5% Glycerol	10% Raffinose/ 10% DMSO	18% Raffinose/ 1.75% Glycerol
8	<1	5
8	-	5
10	-	0

epididymides and agitating gently. 4 μl aliquants were cooled on a cryomicroscope at a rate of 10°C/min to 4°C and 40°C/min to -70°C. The sample was warmed at a rate of 60°C/min to 37°C.

ii) Vitrification of entire epididymides (natural cryoprotectants in epididymal plasma). Epididymides from 3 CBA mice were divided amoung petri dishes containing 1 ml of the following diluents:

a) Phosphate buffered saline.

b) Diluent 1 (10% raffinose, 5% glycerol in PBS).

c) Egg yolk, TES/Tris, 2.5% glycerol (4 ml egg yolk, 12 ml Double Distilled water, centrifuge at 20,000 g for 1 h. Into 8 ml dissolve 0.483 g TES, 0.115 g Tris, 0.04 g glucose, 0.01 g streptomycin and 2.5% glycerol).

The epididymides were left to equilibrate for 1 h at room temperature, before transferring to 1.8 ml cryotubes and plunging into liquid nitrogen (-196°C). The samples were left in liquid nitrogen for 1 h before thawing in a beaker of water at 37°C. The thawed epididymides were transferred to 500 μl T6 medium containing 4 mg/ml BSA, under silicon oil, and punctured to release the spermatozoa.

iii) Vitrification of spermatozoa (cryoprotectant - minimal toxic effect). Epididymides were dissected into 1 ml of egg yolk TES/tris diluent containing 2.5% glycerol. The spermatozoa were released by puncturing the epididymides and agitating gently. Aliquots of 200 μl were transferred to cryotubes which were plunged into liquid nitrogen (-196°C). Samples were left in liquid nitrogen

for 1 h before thawing by immersion of the cryotube in a beaker of water at 37°C. The diluent was diluted to excess with T6 medium containing 4 mg/ml BSA.

iv) Cryopreservation of spermatozoa in cryoprotectant and at a controlled rate. Epididymides were dissected into 1 ml of modified egg yolk TES/Tris diluent containing 2.5% glycerol. The spermatozoa were released by puncturing the epididymides and agitating gently. Aliquants of 4 μl were cooled on a cryomicroscope at a rate of 10°C/min to 4°C and 40°C/min to -70°C. Samples were warmed at a rate of 60°C/min to 37°C.

Results

The progressive motility of mouse sperm following cryopreservation using different techniques is presented in Table 2. From the results of the first experiment it was apparent that mouse spermatozoa did not survive cooling to sub-zero temperatures unless in the presence of a cryoprotectant and cooled at a controlled rate. Egg yolk TES/Tris diluent is one of the most widely used cryoprotectants and the cryoprotective action of glycerol has been widely documented (see chapter 1), so further experiments were carried out on modifed egg yolk TES/Tris diluent to see whether it could be adapted as a suitable cryoprotectant for mouse spermatozoa. Due to the hyper-osmolarity of the modified egg yolk TES/Tris diluent, the spermatozoa were not progressively motile in the cryoprotectant. All motility assessments of samples on the cryomicroscope were of non-progressive motility only.

Table 2. Progressive motility of mouse sperm following cryopreservation using different techniques.

Experiment	Sperm motility (%)
Sperm in PBS only (No cryoprotectant)	0
Vitrification of entire epididymis	0
Vitrification of Sperm	0
Sperm in cryoprotectant cooled at a controlled rate	10

Table 3. Post-thaw motility of sperm cooled on a cryomicroscope in clarified and non-clarified egg yolk diluent.

Experiment	Sperm motility (%)	
	Clarified	non-clarified
1	10	50
2	10	50

Comparison of the cryoprotective action of clarified and un-clarified egg yolk. Diluted egg yolk is normally centrifuged to clarify the solution for ease of visualizing the cells. However, it has been shown that whole egg yolk can have a protective effect during the cryopreservation of sperm (Arriola and Foote, 1987). Diluent was prepared as described previously and also by adding the salts and glucose to 8 ml of egg yolk and water that had not been centrifuged.

Sperm were suspended in the diluents as described above and aliquots were cooled on a cell freezer at a rate of 10°C/min to 4°C and 40°C/min to -70°C before warming at a rate of 60°C/min.

Results

Post-thaw motility of sperm cooled on a cryomicroscope in clarified and unclarified egg yolk diluent is shown in Table 3. Sperm motility was higher (50%) in the diluent containing un-clarified egg yolk than in the clarified diluent (10%). After thawing and partial dilution in T6 medium, sperm were observed surrounding large droplets of egg yolk with the head region closely affiliated with the droplet and the tail region beating outside the droplet. Transmission electron microscopy showed that egg yolk particles were in close contact with the sperm head (Figure 6).

The effect of glycerol concentration on post-thaw motility of cryopreserved mouse sperm. Although glycerol has very

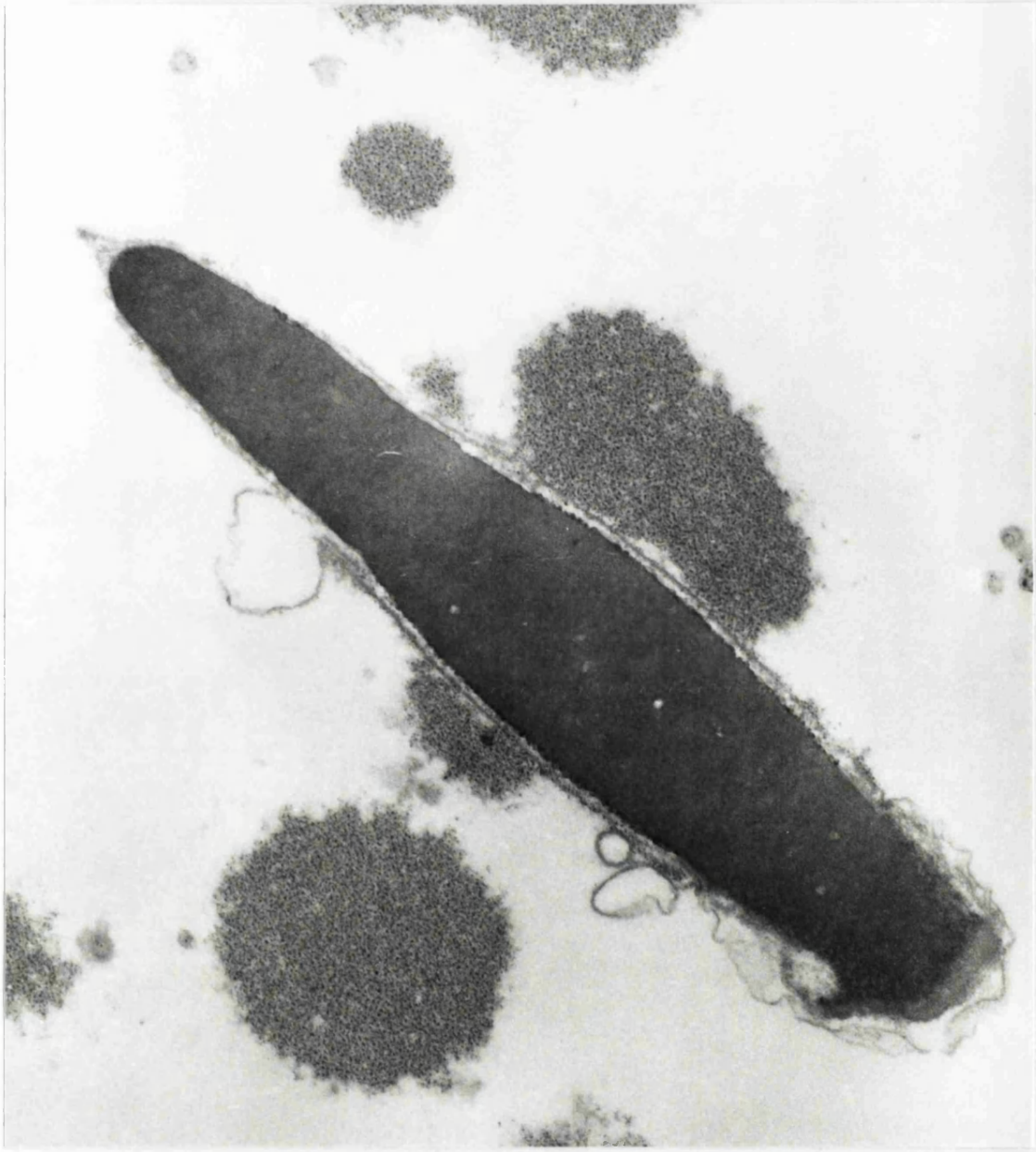


Figure 6. Transmission electron micrograph showing egg yolk particles from un-clarified diluent in close association with the sperm head.

good cryoprotective qualities it is also known to be toxic to cells (see chapter 1). Different concentrations of glycerol in the egg yolk TES/Tris diluent were tested to determine the optimum concentration for cryopreservation with a minimum toxic effect.

Epididymides were dissected into 1 ml egg yolk TES/Tris diluent containing the following concentrations of glycerol: 0%, 1.25%, 2.5%, 5%, 10%. Aliquants of 4 μl were cooled on a cryomicroscope at 10°C/min to 4°C and 40°C/min to -70°C. samples were warmed at a rate of 60°C/min to 37°C. The experiment was repeated 4 times, changing the order of the samples to take into account the effect of time.

Results

Figure 7 presents the proportion of motile sperm after cryopreservation in diluents containing different concentrations of glycerol. Glycerol proved to be an effective cryoprotectant at low concentrations (1.25%), but reduced sperm motility at concentrations of 5% or above. A small percentage of sperm retained motility after cryopreservation in diluent containing no glycerol, indicating the effectiveness of other components in the diluent, but the greatest proportion (>60%) of motile sperm was observed after cryopreservation in diluent containing 1.25% glycerol.

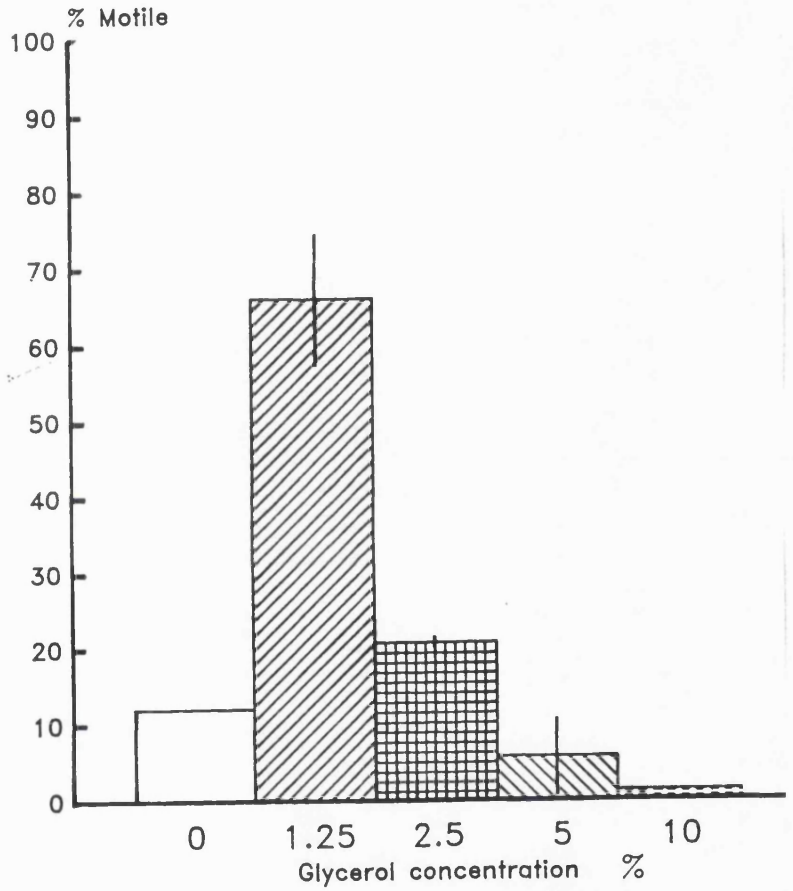


Figure 7. Proportion of motile sperm after cryopreservation in diluent containing different concentrations of glycerol.

The effect on post-thaw motility of the osmolarity of the diluent. The osmolarity of the diluent determines the degree of dehydration the spermatozoa undergoes before freezing. This also determines the amount of osmotic pressure exerted on the spermatozoa on addition and elimination of the diluent. The diluent was made up with different volumes of water to determine whether a diluent of a different osmolarity would improve the post-thaw motility of the spermatozoa.

Epididymides were dissected into 1 ml of diluent from diluent containing 4 ml, 6 ml and 8 ml of water (600, 700 and 800 mOsm respectively). Sperm were released as described previously and 4 μl aliquants were cooled on the cryomicroscope at a rate of 10°C/min to 4°C and 40°C/min to -70°C. Samples were warmed at a rate of 60°C/min.(n=3).

Results

The effect of diluent osmolarity on the post-thaw motility of mouse spermatozoa is shown in Figure 8. The modified egg yolk diluent contained 6 ml of double distilled water (~700 mOsm) instead of the usual 8 ml (600 mOsm). Neither increasing or decreasing the osmolarity of the diluent resulted in any increase in the percentage of sperm motility. The percentage of motile sperm after cryopreservation was 3 ± 1.2% for sperm in diluent containing 8 ml of water in comparison to 20 ± 5.8% of sperm cryopreserved in diluent containing 6 ml of water. Reducing the water content of the diluent to 4 ml (~800 mOsm) resulted in 5 ± 2.0% of sperm retaining motility

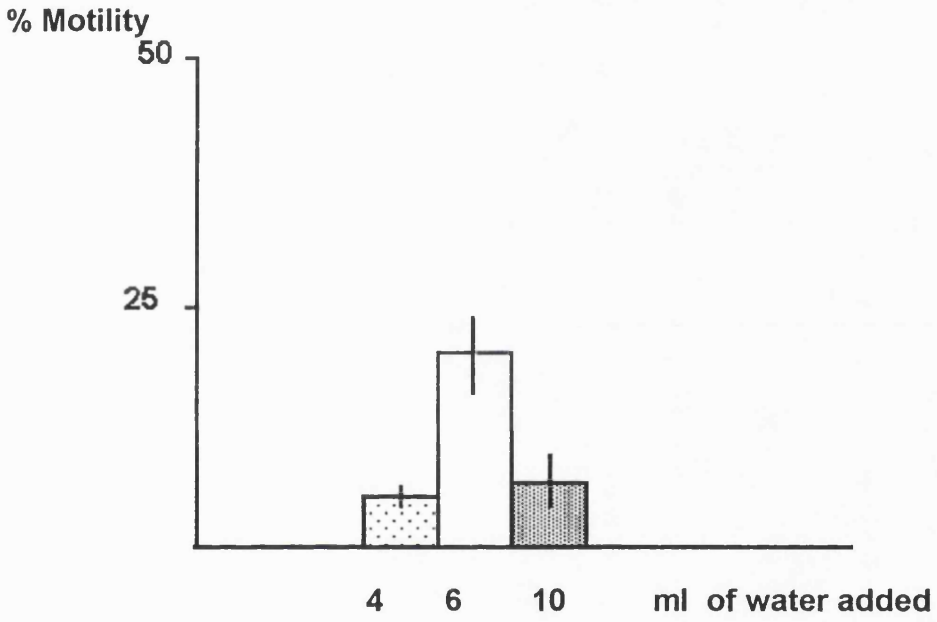


Figure 8. Post-thaw motility of spermatozoa (%SEM) in diluent made up with various amounts of water (n=3 for each value)

after thawing, indicating that further increasing the hyper-osmolarity of the diluent (and therefore increasing the dehydration of the sperm prior to cryopreservation) had a deleterious effect on the sperm.

The effect of the rate of cooling on post-thaw motility of cryopreserved mouse sperm. Initially, the rate of cooling from room temperature to 4°C was investigated by cooling samples at a rate of 5°C, 10°C, 20°C, 40°C, 50°C, 60°C, 70°C and 80°C/min to 4°C and re-warming to 37°C. No loss of motility was observed for any of the rates investigated. The rate of cooling from 4°C to -70°C was then tested. Epididymides were dissected into 1 ml egg yolk TES/Tris diluent containing 1.25% glycerol. Spermatozoa were released as described previously and aliquants were cooled on the cryomicroscope at the following rates: 10°C/min to 4°C, followed by 20°C, 40°C, 50°C, 60°C and 70°C/min to -70°C. Samples were warmed at a rate of 60°C/min to 37°C. The experiment was repeated three times, changing the order of the different rates to take into account the effect of time.

Results

The proportion of motile sperm after freezing at different rates is shown in Figure 9. A post-thaw percentage motility of 55 ± 7.4% was observed for sperm cryopreserved at a rate of 50°C/min, in comparison to 36 ± 11%, 29 ± 9% and 20 ± 4.6% for sperm frozen at the rates of 40°C, 60°C and 70°C/min.

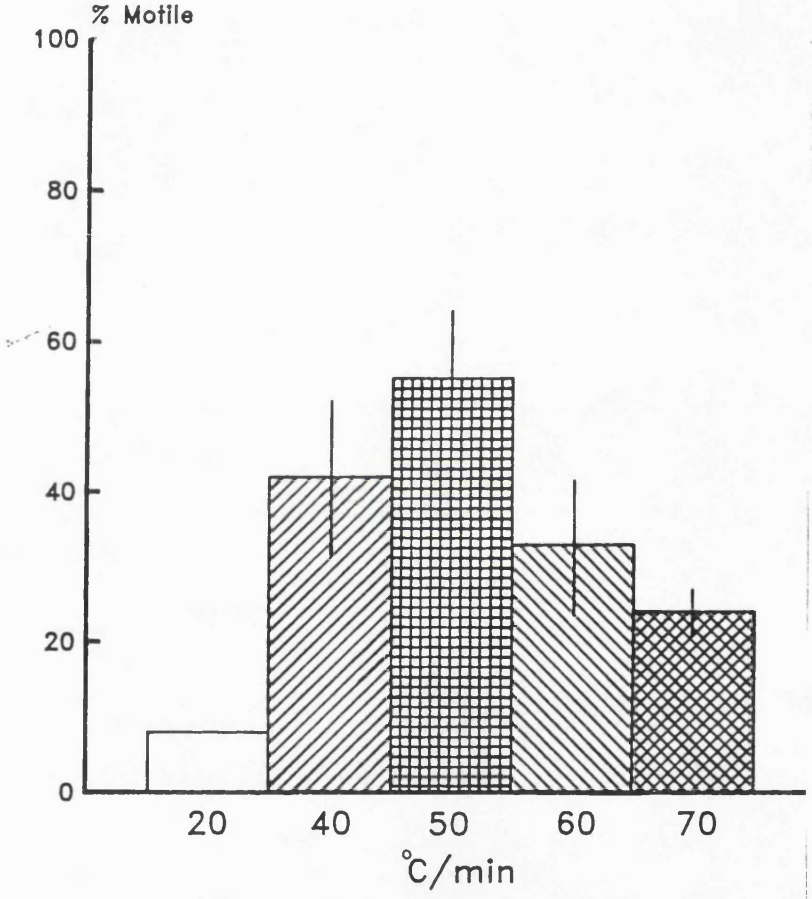


Figure 9. Proportion of motile sperm after freezing at different rates

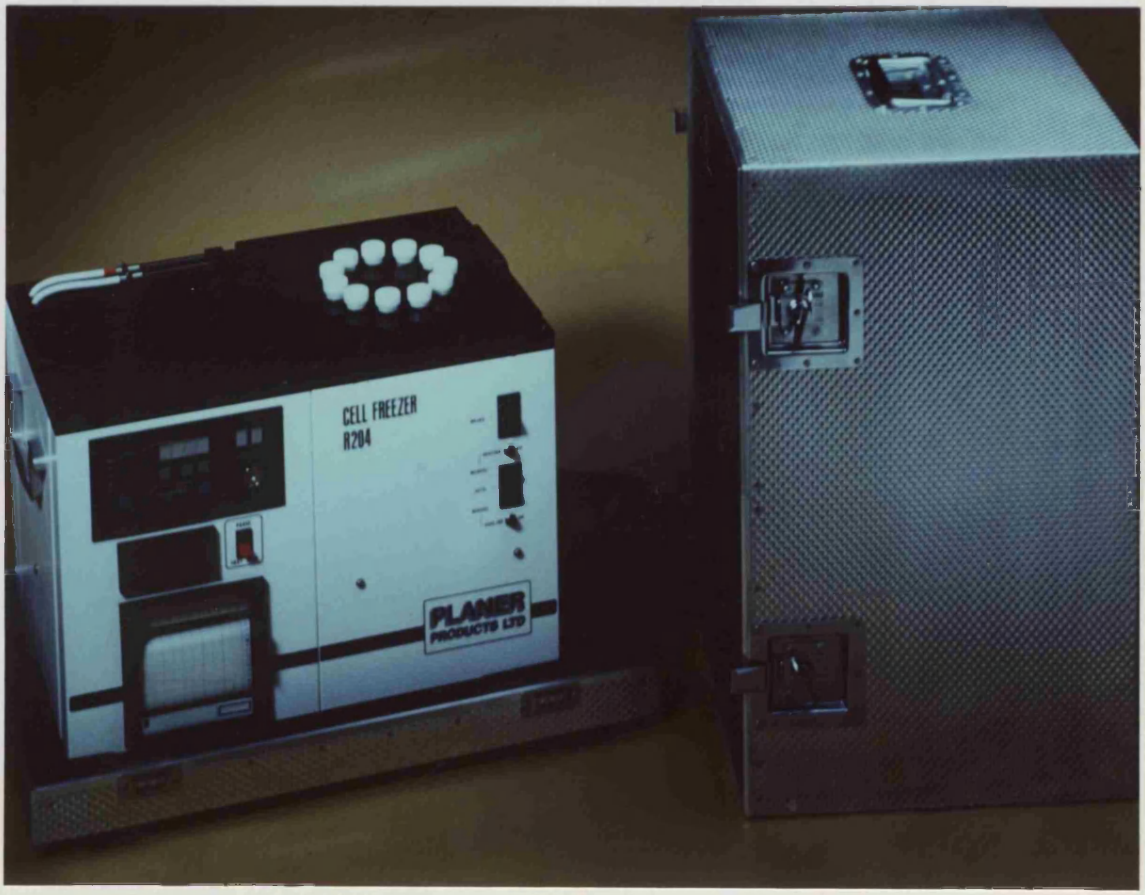


Figure 10. R204 cell freezer

Seeding temperature of samples cryopreserved on the cryomicroscope and in the cell freezer

Cryomicroscope. The temperature at which the ice front reached half-way across the thermocouple was noted in 17 experiments and was found to be -1.6 ± 0.2°C.

Cell freezer. During the experiments for the calibration of the cell freezer (see below), it was noted that -2 to -4°C, the thermometer showed readings that remained constant for up to 1 min and were often followed by a rise in temperature of between 2-5°C. This was interpreted as the 'seeding' temperature and was found to be -4 ± 0.7°C (6 experiments). The seeding temperature varied between -3 to -7°C, which was interpreted as slight supercooling of the sample.

Transfer of protocol to cell freezer

Calibration of cell freezer. The cell freezer used in these experiments is shown in Figure 10. The temperature inside the chamber of the cell freezer was measured as the chamber was cooled at a rate of 10°C/min to 4°C and 50°C/min to -70°C, measurements were made using a thin, wire thermometer and compared with the temperature readout of the cell freezer.

The process was then performed with the wire thermometer positioned inside 200 µl of egg yolk TES\Tris diluent in a 1.8 ml polypropylene cryotube.

Both protocols were repeated 6 times.

Results

The values for the two sets of temperature readouts over time were plotted on a box-whisker graph (See Figures 11, 12 and 13). From the results, it can be seen that the temperature on the readout of the cell freezer correlates closely with the thermometer readout from inside the chamber (see Figures 11 and 12). However, it can also be seen that there is a time lag between the temperature readout of the cell freezer and the actual temperature within the cryotube. At some values the temperature lag is several degrees (see Figure 13). A latent heat of freezing was also observed in the sample between the temperatures of -2°C and -7°C. This was manifested as a rise of temperature of between 2°C and 5°C.

Cryopreservation of mouse spermatozoa in a cell freezer.

Epididymides were dissected into 1 ml of egg yolk TES\Tris diluent. Spermatozoa were released into the diluent as described previously and 200 μl aliquots were transferred to 1.8 ml cryotubes. The cryotubes were immediately transferred to the chamber of the R204 cell freezer at room temperature, (~24-26°C) and cooled at a rate of 10°C/min to 4°C, held for 3 min to equilibrate and 50°C/min to -70°C, and held for 3 min to equilibrate before storing in liquid nitrogen. Samples were held in liquid nitrogen for a minimum of 1 h before thawing by immersing the cryotube in a beaker of water at 37°C. Once the sample had thawed it was transferred to an incubator at 37°C, in an atmosphere of 5% CO_2 in air, for 10 min.

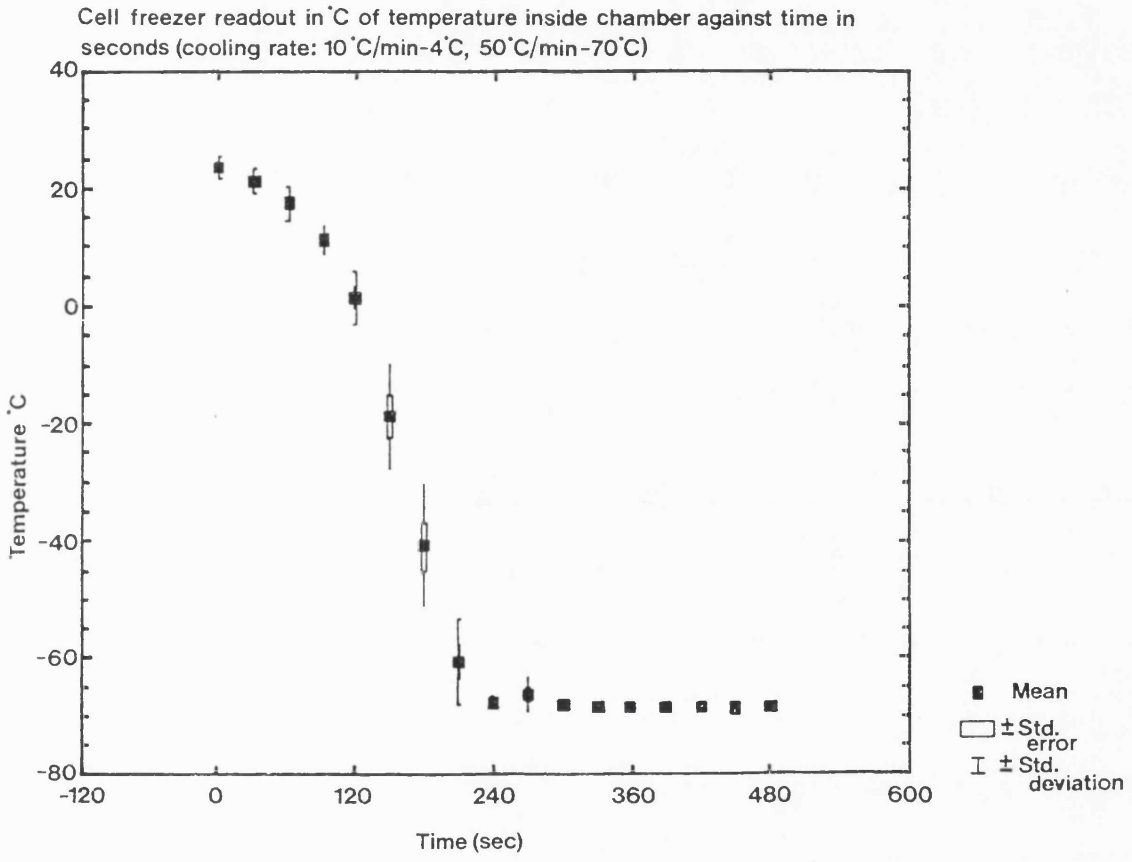


Figure 11. Cell freezer readout in °C of temperature inside chamber against time in seconds.

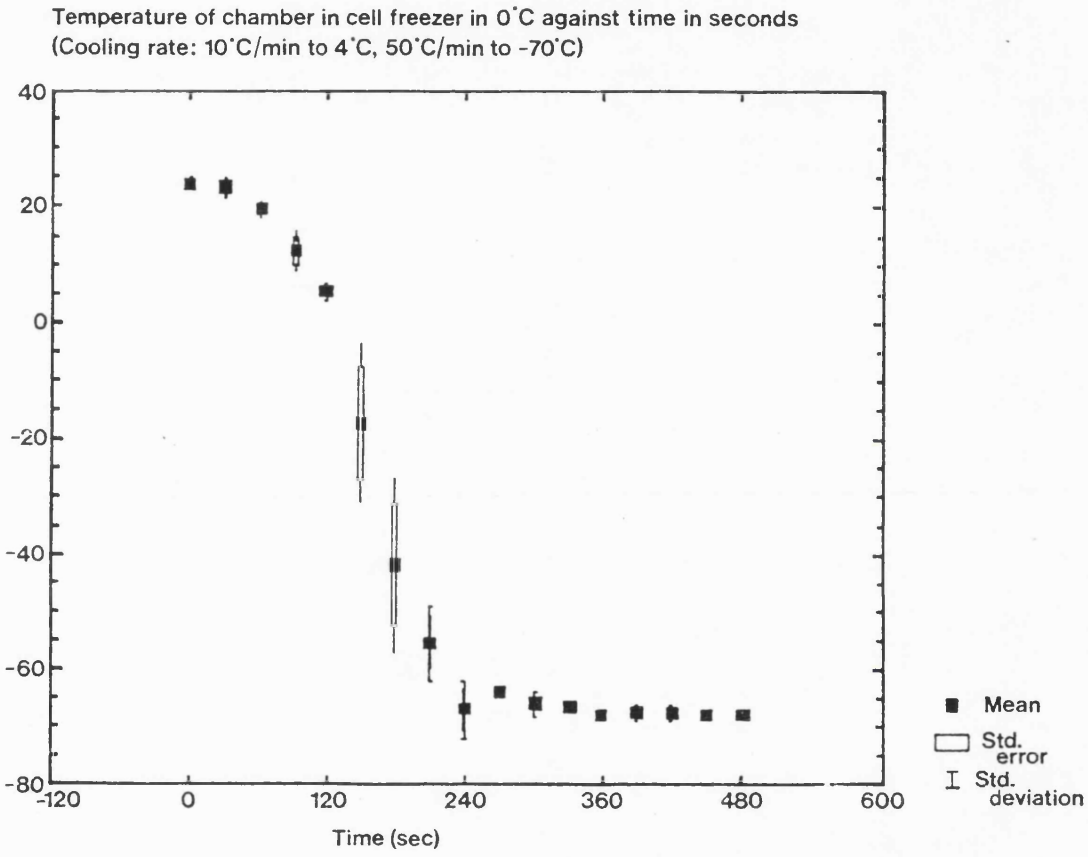


Figure 12. Temperature of chamber in cell freezer in °C against time in seconds.

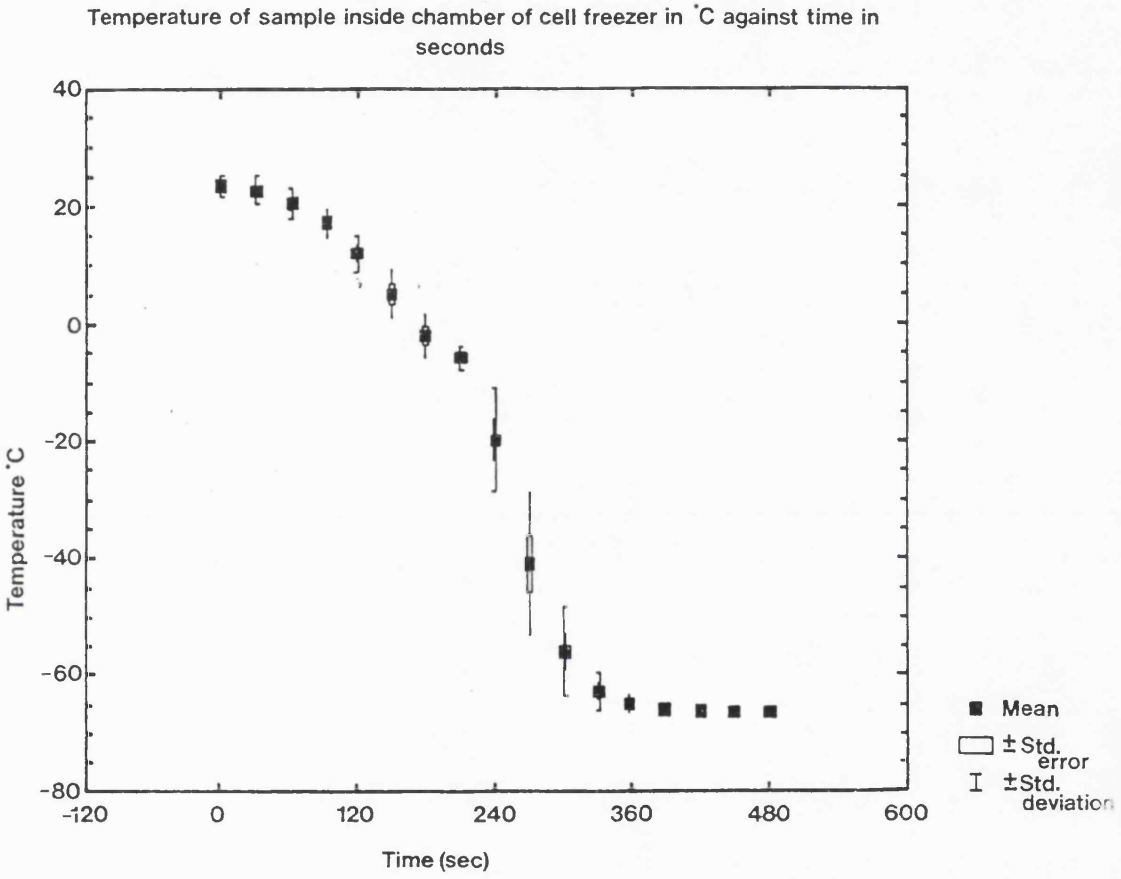


Figure 13. Temperature in °C inside sample in chamber of cell freezer against time in seconds.

The diluent was diluted slowly with T6 medium containing 4 mg/ml BSA. A summary of this procedure is shown in Figure 14.

Results

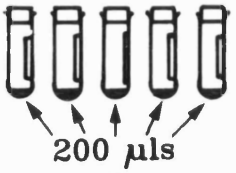
The post-thaw motility of spermatozoa in the egg yolk TES\Tris was ~30-40% and good lateral motility was displayed. However, conventional methods of sperm recovery by centrifugation of the sample and resuspension of the sperm pellet in medium proved impossible to use. This was due to the presence of large droplets from the un-centrifuged egg yolk particles in the egg yolk TES\Tris diluent which pelleted with the sperm when centrifuged. It was noted that the sperm remained motile if contact was maintained with the egg yolk particles and that a sample could be stored overnight at 4°C or room temperature and still retain this motility.

Recovery of spermatozoa from cryoprotectant. The cryopreservation in the egg yolk TES\Tris diluent resulted in a population of spermatozoa retaining motility after thawing. However, the presence of whole egg yolk in the diluent made it difficult to recover the spermatozoa from the cryoprotectant. Initial experiments indicated that the addition to mouse oocytes of sperm in cryoprotectant for *in vitro* fertilization purposes resulted in activation and degeneration of the oocytes. Therefore the following methods were investigated for the recovery of sperm from the diluent. The proportion of spermatozoa displaying

Figure 14. Summary of cryopreservation protocol

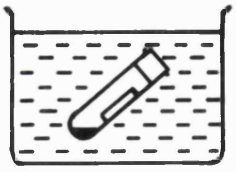
FREEZING

CAUDA EPIDIDYMIDES X 4 PAIRS
SPERM CONCENTRATION 20 X 10^6/ml
IN 1ml OF CRYOPROTECTANT



R204 CELL FREEZER (PLANER PRODUCTS LTD.)
$-10^{\circ}C/min \longrightarrow 4^{\circ}C$
$-50^{\circ}C/min \longrightarrow -70^{\circ}C$
STORE IN LIQUID N_2

THAWING



IMMERSED IN WATER AT
37°C UNTIL THAWED

EQUILIBRATED IN INCUBATOR
37°C, 5% CO_2
FOR 15 MINS

CENTRIFUGE AT 800g FOR 4 MINS.
RESUSPEND PELLET IN T6 MEDIUM
AND 15mgs/ml BSA.

progressive motility after elimination of the cryoprotectant was assessed.

Dialysis. a) A thawed aliquot was transferred to a 0.45 μm culture plate insert (Millipore Products Division, Bedford, MA 01730) which was placed in a petri dish containing 3 ml of T6 medium containing 4 mg/ml BSA and transferred to an incubator at 37°C in an atmosphere of 5% CO_2 in air, for 30 min.

b) A thawed aliquot was transferred to a 0.45 μm culture plate insert which was placed in a petri dish containing 3 ml of T6 medium containing 4 mg/ml BSA, the osmolarity of which had been adjusted to 700 mOsm (equivalent to the osmolarity of the cryoprotectant) with sucrose. The petri dish was transferred to an incubator at 37°C, in an atmosphere of 5% CO_2 in air, for 30 min.

Dilution to excess. a) 1 ml of T6 medium containing 4 mg/ml BSA was added directly to a thawed aliquot of spermatozoa in diluent.

b) 1 ml of T6 medium containing 4 mg/ml BSA was added to a thawed aliquot of spermatozoa in diluent dropwise over a period of 30 min.

Dispersal in medium. A thawed aliquot was transferred to a drop of T6 medium containing 4 mg/ml BSA under silicon oil and allowed to disperse.

Solubilizing egg yolk diluent with detergent. Sodium tri-ethanolamine lauryl sulphate is well documented as increasing the post-thaw survival of bull spermatozoa (Arriola and Foote, 1987). At the time of the experiment

this detergent was not available in the UK, so sodium lauryl sulphate (SDS), from the same family of alkyl ionic detergents was used. SDS was added to 4 ml of egg yolk in 12 ml distilled water in the following concentrations: 1%, 0.5%, 0.25% and 0.1%. The solution was left at room temperature for 30 min, agitating occasionally to mix. The egg yolk mixture was then clarified by centrifuged at 20,000 g for 1 h. To 8 ml of supernatant was added 0.483 g TES, 0.115 g Tris. 0.04 g glucose, 0.01 g streptomycin and 1.25% glycerol. Epididymides were dissected into 1 ml of each diluent and the sperm were released as described previously. Aliquots of 200 μl were transferred to 1.8 ml cryotubes and cooled at a rate of 10°C/min to 4°C and 50°C/min to -70°C, before storing in liquid nitrogen. Samples were thawed by immersing the cryotube in a beaker of water at 37°C. The thawed sample was transferred to an incubator and equilibrated for 10 min at 37°C, in an atmosphere of 5% CO_2 in air. The cryoprotectant was eliminated by centrifuging at 400 g for 3 min and resuspending the sperm pellet in 500 μl T6 medium containing 4 mg/ml BSA (Fraction V). (Following this experiment, all samples of mouse sperm cryopreserved in egg yolk TES\Tris diluent were resuspended in T6 medium containing 15 mg/ml BSA [Fraction V], for use in the <u>in vitro</u> fertilization experiments).

Results

<u>Dialysis</u>. a) After a period of 30 min the sperm were

examined under the microscope. There were very few motile sperm in the sample (< 1%) although these displayed progressive motility as dialysing had decreased the osmolarity of the diluent. Many sperm had tails that were bent at the mid-piece region or were coiled up. This was interpreted as an osmotic shock effect. However, the filter unit was not able to dialyse out the egg yolk particles.

b) Dialysing against medium adjusted to the osmolarity of the diluent resulted in no sperm showing bent or coiled tails, but the percentage of progressive motile sperm was very low. The egg yolk particles were still present in the sample.

Dilution to excess. When the sample was diluted to excess with the addition of 1 ml of medium, no sperm showed any motility and the majority of the sperm were bent or coiled up, characteristic of osmotic shock. Addition of 1 ml of medium slowly, drop by drop, resulted in ~30-40% motile sperm, very few were observed as having bent tails or coiled up. However, the egg yolk particles were still present.

Dispersal in medium. When examined under the microscope, it was observed that as the sperm left the diluent and became dispersed in the medium they became bent or coiled and all motility ceased. No progressive motility was observed when the entire sample was dispersed throughout the medium.

Solubilizing the egg yolk with detergent. Sperm remained motile in the diluent after cryopreservation. The percentage motility was reduced from 30-40% in diluent with un-solubilized egg yolk to 25-30% motility in solubilized egg yolk. However, using the latter diluent, the sperm could be recovered by centrifugation and resuspension of the sperm pellet in medium.

The resulting modified egg yolk TES\Tris diluent containing 1.25% glycerol and 0.1% SDS was designated Mouse sperm Cryoprotectant (MSC).

Transfer of protocol to Kryo 10:17 cell freezer (Planer Prods. Ltd.). Epididymides were dissected into 1 ml of MSC. Spermatozoa were released as described previously and 200 μl aliquots were transferred to cryotubes before cooling at a rate of 10°C/min to 4°C and 50°C/min to -70°C. Samples were thawed as described above.

Results

After resuspending the sperm pellet in T6 medium, the percentage of progressive motile sperm was <5%. Many of the sperm appeared damaged with bent tails.

Transfer of protocol to modified (for faster freezing rate) Kryo 10:17 cell freezer. The cell freezer was modified by the manufacturers by adjusting the inlet valve for the liquid nitrogen vapour entering the chamber. The above procedure for sperm preparation was repeated for the modified 10:17 cell freezer.

Results

After modification of the cell freezer, a post-thaw progressive motility of 10-15% sperm was observed. The motility was vigorous and comparable with the motility of fresh sperm.

Isolation of motile spermatozoa

A post-thaw motility of 25-30% was obtainable after elimination of the cryoprotectant. However, the sample still contained residual MSC and many dead sperm which resulted in a mean parthenogenetic activation rate of 17% following incubation with unfertilized oocytes (see chapter 3). Further centrifugation and resuspension of the cryopreserved sperm in fresh T6 medium resulted in a very low percentage of motile sperm. Different methods were investigated to determine an effective way to isolate the apparently fragile frozen-thawed motile sperm.

Swim-up. The frozen-thawed sperm were resuspended in T6 medium as described previously and gently overlaid with 1 ml of fresh T6 medium containing 15 mg/ml BSA. The sample was incubated at 37°C, in an atmosphere of 5% CO_2 in air for 30 min, before examining the top layer for the presence of motile sperm.

Result

Very few motile sperm were collected in the overlying layer of T6 medium. The non-motile and dead sperm accumulated in the bottom of the sample and seemed to trap the motile cells, as many motile sperm were also found at the bottom.

Glass bead column. Resuspended sperm were passed through a glass bead column according to the method of McGrath et al. (1977) for the isolation of a motile population of mouse sperm.

Result

No motile sperm were collected after passing the sample through the column. Many of the eluted cells were without heads.

Percoll gradient. Resuspended frozen-thawed sperm were centrifuged on a Percoll gradient of 2.5 ml isotonic Percoll and 7 ml of T6 medium.

Result

Both motile and non-motile sperm were collected at the same band in the gradient.

Drop of medium under oil. An aliquot of resuspended frozen-thawed sperm was transferred to a drop of T6 medium containing BSA under oil and incubated at 37°C, in an atmosphere of 5% CO_2 for 30 min. It was hoped that the non-motile cells would sink to the bottom of the drop and that the motile cells could be collected from the top of the drop.

Result

The non-motile sperm appeared to trap the motile sperm, and most of the cells had sunk to the bottom of the drop.

Assessment of motility, plasma membrane and acrosome integrity of mouse sperm cryopreserved in MSC.

Sperm samples cryopreserved in the developed diluent, Mouse Sperm Cryoprotectant (MSC), were compared with freshly collected samples, for post-thaw motility, acrosome and plasma membrane integrity.

Motility. Motility was assessed subjectively for freshly collected sperm and sperm frozen on a cryomicroscope and in a cell freezer. The percentage of motile cells was assessed in freshly collected sperm from 10 individual animals. Experiments on the cryomicroscope and the cell freezer were carried out on pooled samples from 4 animals and were repeated 6 and 20 times respectively. On the cryomicroscope the motility displayed by spermatozoa was non-progressive. Subjective assessments were by counting ~ 100 cells for each experiment.

Acrosome integrity. Sperm cryopreserved in a cell freezer and freshly collected sperm were assessed for acrosome integrity according to the method described in the appendix. Samples of sperm frozen on the cryomicroscope were obtained by removing the cover slip covering the sperm sample on the cryostage and fixing it in methanol. The coverslip was then treated according to the above method.

In each experiment for acrosome assessment, 20 fields were counted (~10 cells/field) and the experiment was repeated three times.

Plasma membrane integrity. Plasma membrane integrity was examined in freshly collected sperm and sperm frozen in a cell freezer. The protocol above was repeated, but sperm were fixed in glutaraldehyde fixative instead of methanol. In each experiment for plasma membrane assessment, 20 fields were counted (~10 cells/field) and the experiment was repeated three times.

Scanning electron microscopy of frozen-thawed spermatozoa.

Small pieces of glass coverslip (~4 mm x 4 mm) were flooded with a 1% solution of poly-L-lysine and left to dry overnight. A sample of cryopreserved sperm was thawed and the MSC eliminated as described previously. A drop of sperm suspension was placed on the coated coverslip and left for 10 min. The coverslip was transferred to 25% glutaraldehyde for fixation for 2 h and was processed for scanning electron microscopy as described in the Appendix.

Results

Motility assessment. The percentage of motile cells in freshly collected and frozen sperm (cryomicroscope and cell freezer) is shown in Figure 15. In 10 animals studied, the mean percentage of motile freshly collected sperm was 71 ± 7.8%, with individual samples ranging from between 44% and 100%. Pooled samples from the 4 animals used for cryopreservation experiments on the cryomicroscope and cell freezer resulted in an average post-thaw percentage motility of 39 ± 4.5 % and 24 ± 1.7% respectively. The experiments were repeated 6 times on the cryomicroscope and 20 times on the cell freezer.

Acrosome integrity. Intact acrosomes were observed as uniformly fluorescent, crescent-shaped structures, as shown in Figure 16. The percentage of intact acrosomes in freshly collected sperm and sperm cryopreserved on a cryomicroscope and cell freezer is presented in Figure 17. The percentage of intact acrosomes in freshly collected sperm was 90 ± 2.48%. Acrosome integrity for sperm frozen on the cryomicroscope and cell freezer was assessed at 55 ± 13.5% and 29 ± 1.9% respectively.

Plasma membrane integrity. Antibody could only bind to the acrosome through a breach in the plasma membrane. Sperm with fluorescing acrosomes were assessed as plasma membrane damaged, while non-fluorescing sperm were assessed as plasma membrane intact (see Figure 18). The percentage of plasma membrane intact freshly collected sperm was assessed as 95 ± 1.3% and is shown in Figure 19. 18 ± 4.3% spermatozoa had intact plasma membranes following cryopreservation in the cell freezer (Figure 19).

Scanning electron microscopy of frozen-thawed spermatozoa. Scanning electron micrographs of frozen-thawed mouse spermatozoa are presented in Figure 20. The micrographs show spermatozoa with: a) plasma membrane intact, b) plasma membrane damaged and c) plasma membrane damaged due to the fixation process. Small egg yolk droplets were observed closely affiliated with the head of the sperm. It was noted that where the plasma membrane was missing there

were fewer egg yolk droplets associated with the sperm surface.

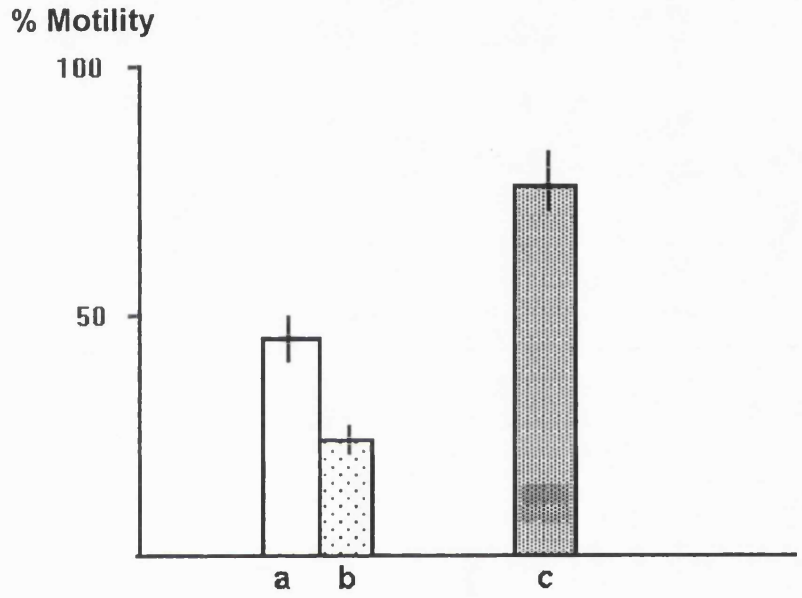


Figure 15. The motility of mouse spermatozoa (% ± SEM) after freezing-thawing in: (a) cryomicroscope (n=6); (b) cell freezer (n=22). (c) motility of fresh, unfrozen spermatozoa (n=10)

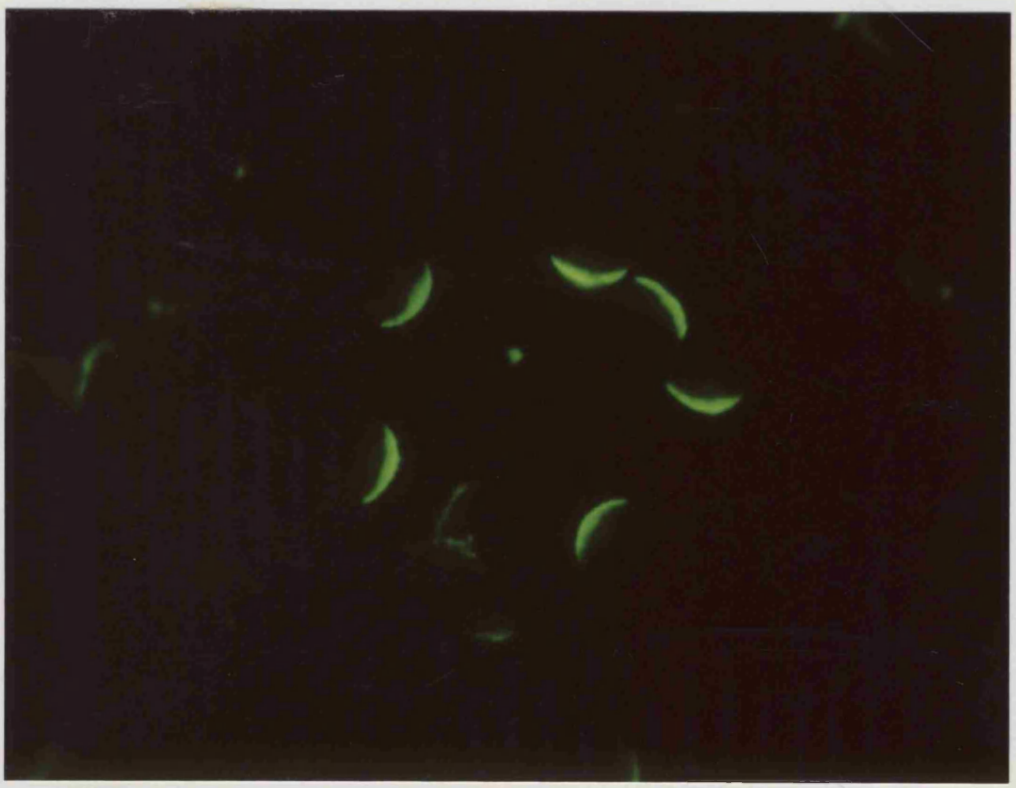


Figure 16. Immunolocalization of the acrosome. Intact acrosomes are seen as uniformly stained, crescent-shaped structures.

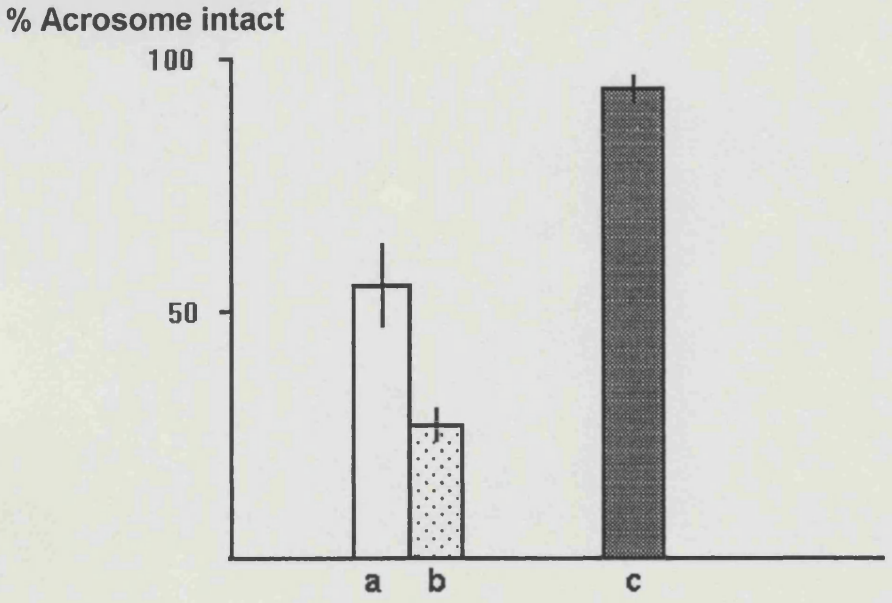
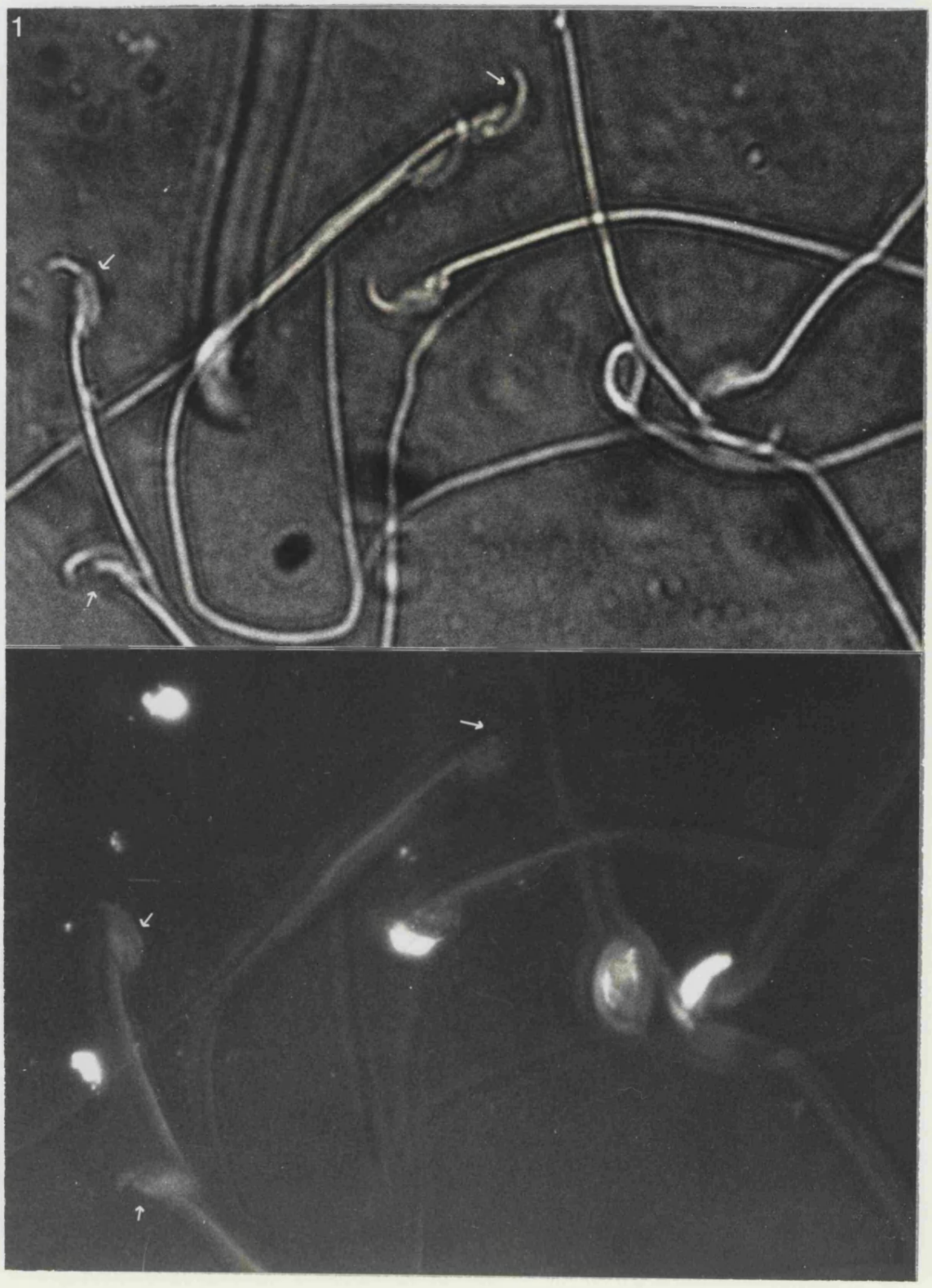


Figure 17. Acrosome integrity of mouse spermatozoa (% ±SEM) after freezing- thawing in: (a) a cryomicroscope (n=); (b) a cell freezer (n=9). (c) Acrosome integrity of fresh unfrozen spermatozoa (n=5).

Figure 19. Cryopreserved mouse sperm. Arrows show intact plasma membrane.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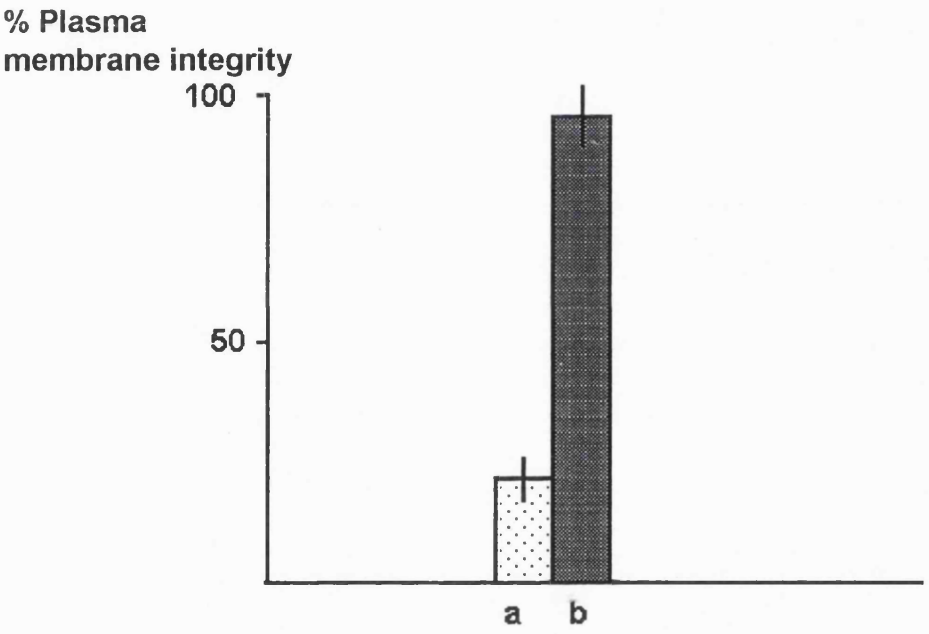

Figure 18. The proportion of spermatozoa with intact plasma membranes (% SEM): (a) after cryopreservation in a cell freezer; (b) fresh, unfrozen cells.

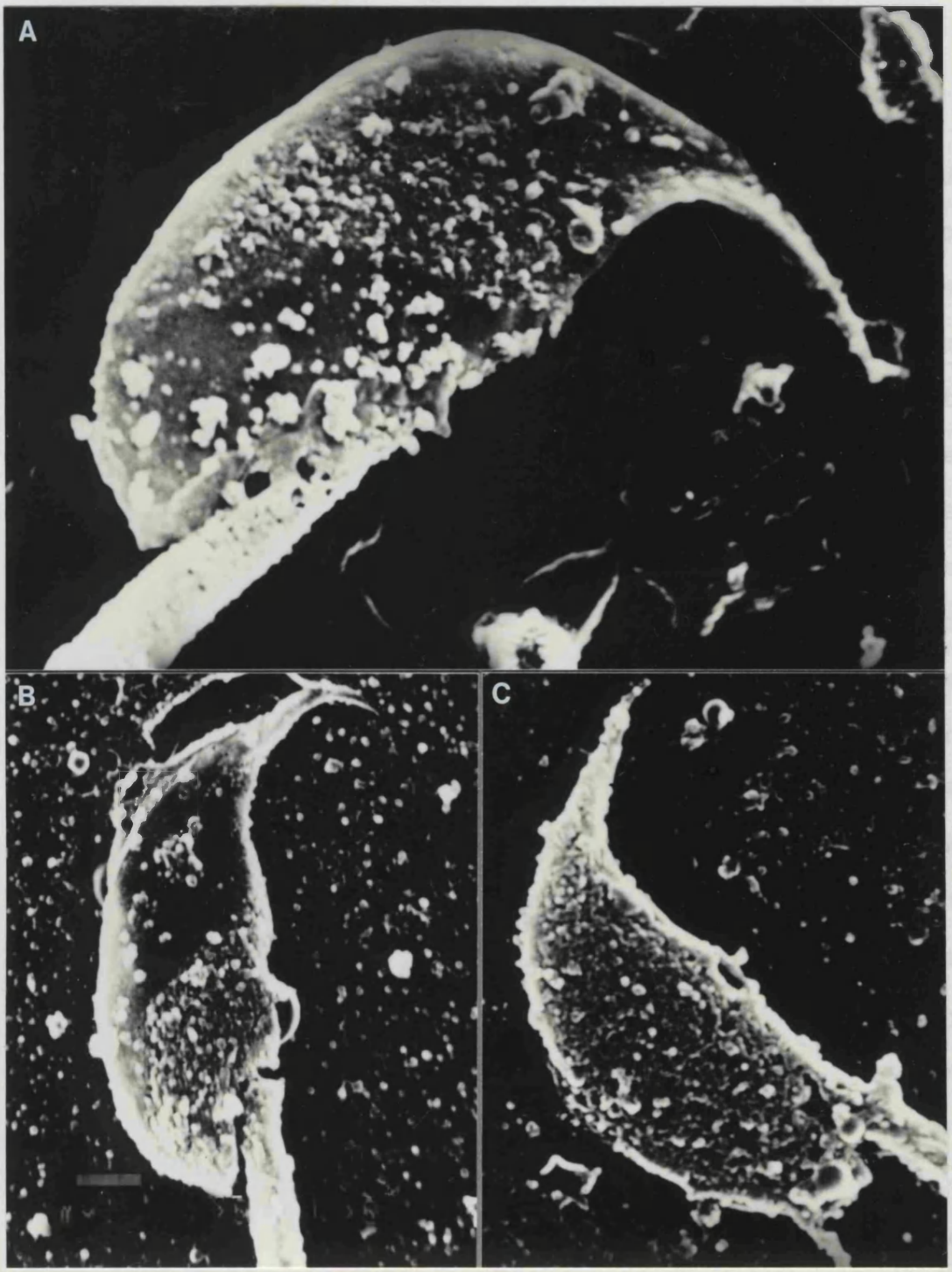

Figure 20. Scanning electron micrograph showing cryopreserved mouse sperm with plasma membranes that are a) intact, b) freeze-damaged and c) damaged from the fixation procedure

CONCLUSION

The published protocols based on raffinose/glycerol and raffinose/DMSO proved inconsistent and difficult to repeat. Cooling to 4°C resulted in the majority of the sperm losing motility. The percentage of motile sperm after cryopreservation in these diluents (<10%) was not considered to be sufficient for the method to be a viable option. A novel method for the cryopreservation of mouse sperm was developed using a cryomicroscope to investigate the effects of glycerol concentration, osmolarity and cooling rate on post-thaw motility of sperm. No motile sperm were observed when samples were cryopreservation occurred in diluent containing concentrations of 5 and 10% glycerol, although 12% of sperm were observed to be motile in diluent containing no glycerol. It would appear that concentrations of glycerol routinely used in cryoprotectants for other species (6-8% in the bull) may exerted a toxic effect on mouse sperm. Low concentrations of glycerol (1.25%) resulted in a population of sperm retaining post-thaw motility, combining a cryoprotective action with a minimal toxic effect. The osmolarity of the diluent was found to be most effective between ~650 and 700 mOsm. How this affects the post-thaw motility of mouse sperm is unclear. It is possible that the sperm may be partially dehydrated before freezing, reducing the risk of intra-cellular ice formation, although it is not known whether mouse sperm cytoplasm has sufficient water content

for this to be a problem. Alternatively, the osmolarity of the diluent may be critical for the temperature at which the sperm freeze. Supercooling may impose stresses that the sperm cannot withstand. The rate of cooling of mouse sperm in MSC from room temperature to 4°C did not appear to be critical, as no appreciable loss of motility was observed in sperm cooled to 4°C at rates of between 5°C and 80°C/min. This would suggest that MSC protects mouse sperm from cold shock. However, the highest percentage of motile sperm were observed after cryopreservation at a rate of 50°C/min from 4°C to -70°C. During the freezing process, the solutes in the diluent become concentrated as water in the solution freezes. It is possible that 50°C/min is the optimum rate for the sperm to equilibrate with the change in solute concentration.

From the results of the validation of the cell freezer, it was observed that the freezing curve of the cell freezer readout closely followed the curve of the temperature inside the chamber. It was also noted that the freezing curve of the temperature inside the chamber varied by as much as 20°C between 4°C and -60°C between experiements. The freezing curve of the temperature inside a cryotube was consistent between experiments, although there was a time lag in the temperature decrease of ~2 min between the inside and the outside. This time lag is due to the relatively thick polypropylene walls of the cryotube which may act as a partial insulator. The insulating action may have a beneficial effect in

buffering the sample as it is cooled, minimizing the effects of adverse temperature differences inside the chamber. The thick-walled cryotube may contribute therefore to the effectiveness of the cryopreservation protocol.

Recovery of the sperm from the diluent was necessary for in vitro fertilization experiments. The presence of residual cryoprotectant and sperm contents resulted in activation and degeneration of oocytes when incubated with frozen-thawed sperm in diluent (see chapter 3). The presence of large particles of egg yolk made conventional methods for the elimination of cryoprotectant, such as centrifuging and resuspension of the pellet or dialysis, ineffective. Much lower concentrations of SDS were used (0.1%), in comparison to STLS (5%; Arriola and Foote, 1987) but the protective egg yolk particles were still solubilized and sperm could be recovered from the diluent by centrifugation. It is not known whether the detergent affects the sperm membrane in any way, although it is possible that it may make the membrane more permeable and therefore less sensitive to glycerol or osmotic shock during freezing and thawing.

The difference in post-thaw motility may be attributed to a number of factors. The motility of sperm cryopreserved on the cryomicroscope was assessed in the diluent. Although this motility was not progressive, comparison before and after freezing provided a good indicator of the effectiveness of the protocol. Sperm

frozen in a cell freezer were removed from the diluent before motility assessments were made and cells may have been osmotically stressed during the elimination of the MSC. From cryomicroscope studies it was apparent that samples froze at -1.6 ± 0.2°C, however, temperature readings from inside a sample in a cryotube showed that the sample froze at a lower temperature of -4 ± 0.7°C. It is possible that samples frozen in a cell freezer may have been supercooled, resulting in a proportion of sperm being damaged. The larger volume in the cryotube of 200 μl, compared to 4 μl on the cryomicroscope, may have also resulted in a temperature gradient across the sample. Sperm in the centre of the sample may not have been cooled at the same rate as sperm on the periphery of the sample.

Isolation of a motile population of sperm after cryopreservation would have enabled the percentage of motile sperm with intact acrosomal and plasma membranes to be determined. The recovery of the motile spermatozoa, without residual cryoprotectant, proved difficult. Conventional washing techniques (by repeated centrifugation and resuspension of the sperm pellet in fresh medium) resulted in a substantial reduction in the percentage of motile spermatozoa. Cryopreserved spermatozoa appeared to be fragile and more susceptible to damage and loss of viability than fresh spermatozoa. The presence of large numbers of dead and immotile cells prevented the separation of the population by 'swim-up' methods and by centrifugation on a Percoll gradient.

Assessment of post-thaw acrosome and plasma membrane integrity. TES/Tris buffer is known to have the effect of loosening the acrosomal cap. Cryopreservation of mouse sperm in diluent containing citrate as the buffer, resulted in an increase in the number of sperm with intact acrosomes, however, there were no motile sperm after thawing (data not shown). This emphasises the problem in developing cryoprotectants for spermatozoa. Something which may be an effective cryoprotectant for one structure may be deleterious to another.

Acrosome integrity was examined after thawing by fixing the sperm in methanol. This has the effect of permeabilising the plasma membrane and allowing antibody to bind to the acrosome. When incubated with a fluorescent second antibody, the acrosome can be seen as a bright crescent shaped structure. The antibody binds strongly to the outer acrosomal membrane and acrosomal contents, and weakly to the inner acrosomal membrane. Acrosomes were observed to be either intact, damaged or missing, with a greater proportion of intact acrosomes (91%) observed in freshly collected sperm when compared to frozen-thawed sperm (55 ± 24%, $P \leq 0.05$). Plasma membrane integrity was assessed using the same antibody on glutaraldehyde fixed sperm. In this experiment, the plasma membrane was fixed and the antibody only able to bind to the acrosome if there was a breach in the plasma membrane. Since the inner acrosomal membrane bound antibody weakly, and therefore showed faint fluorescence, it was possible to distinguish

between plasma membrane intact sperm and those with missing acrosomes. Scanning electron microscopy also confirmed the presence of plasma membrane intact cryopreserved sperm, although it is always possible that the membrane may have been damaged on the area of sperm that was not visible. It was interesting to note that the egg yolk in the diluent appeared to be closely affiliated with the plasma membrane. This effect was also seen in transmission electron microscopy, although it was not possible to determine whether the egg yolk was actually binding to the membrane. The close affiliation of the egg yolk to the sperm membrane may help in stabilizing the membrane during the freezing and thawing process. Scanning electron micrographs showed that in areas where the plasma membrane was missing, there was no egg yolk visible and a clear demarcation was observed between areas of intact and missing plasma membrane.

Cryopreservation in MSC resulted in a population of mouse sperm retaining motility after thawing. Fluorescent antibody and electron microscopy confirmed the presence of plasma membrane and acrosome intact cells. However, as the motile population of sperm were not able to be isolated after thawing, it was not possible to confirm that the motile sperm were also membrane intact. Therefore, <u>in vitro</u> fertilization studies were undertaken to determine the presence of motile, membrane-intact sperm that were capable of fertilization and normal embryo development.

CHAPTER 3

INTRODUCTION

The mammalian spermatozoon has two main components, the head and tail or flagellum. The presence of potential membrane barriers, such as the posterior ring, has already been discussed with regard to the role they may play in maintaining sperm-surface domains (chapter 1). The posterior ring also acts as an ultrastructural barrier between the head and tail region. Due to compartmentalisation of the spermatozoon, it is possible for it to remain motile, but to have lost fertilizing ability due to damaged membranes over the head. Conversely, the plasmalemma might be damaged in the tail region whilst remaining intact over the head, resulting in a non-motile sperm that retains the potential for binding to the oocyte surface. Although motility is one of the parameters for viable sperm, it is important that it should not be relied on as the only assessment of viability. It is crucial therefore, that spermatozoa fertilizing ability is measured directly. In vitro fertilization and embryo culture methods enable the developmental potential of a population of spermatozoa to be fully assessed up to blastocyst, and with embryo transfer techniques, to full term and beyond. Hence, the fecundity of a population of spermatozoa before and after cryopreservation can be accurately assessed.

Development of *in vitro* fertilization and embryo culture

In vitro fertilization

Mouse embryos were first cultured *in vitro* from the 8-cell stage to blastocyst stage in a simple chemically defined medium (Whitten, 1956. See below). From these pioneer studies, early mouse development was studied in cultures containing oviductal explants (Biggers *et al*., 1962, Whittingham and Biggers, 1967; Whittingham 1968) and the nutritional requirements for early development were investigated using chemically defined media (Biggers and Brinster, 1965; Brinster, 1965; Biggers *et al*., 1967, Biggers *et al*., 1971). The first partially defined *in vitro* fertilization of mouse oocytes was developed using *in vivo* capacitated spermatozoa in the presence of cultured mouse oviduct (Whittingham, 1968). Fertilization of mouse oocytes with *in vitro* capacitated sperm (but without oviduct) was achieved finally by including bovine follicular fluid in the culture system (Iwamatsu and Chang, 1969). Mouse *in vitro* fertilization is used routinely to study sperm capacitation, early embryo development (large numbers of synchronously dividing early cleavage embryos can be produced using IVF) and oocyte cryopreservation.

Fertilization media

Two types of media are used for *in vitro* culture, chemically defined and biological. Chemically defined media are composed of highly purified chemicals, that enable the medium to be reproduced consistently, at

different times and in different laboratories and also allow the medium to be varied in a controlled manner. Chemically defined media are also free of any enzyme activity which may affect the culture. Biological media contain substances such as serum or other biological fluids (skim milk, egg yolk). The first chemically defined medium for embryo culture was described by Whitten (1956), who cultured 8-cell stage embryos to blastocyst. The most commonly used medium for in vitro fertilization is T6, a modified Tyrode's solution (Quinn et al. 1982). The preferred medium for embryo culture from one-cell to blastocyst is M16 medium, also based on a Krebs-Ringer bicarbonate solution. This is similar to Whittens medium (1971), but contains different energy substates, pyruvate and lactate. For a detailed reference on M16 see Whittingham (1971).

Spermatozoa capacitation

Capacitation is the term used to describe the physiological changes spermatozoa undergo in the female tract that renders it capable of fertilization (Austin, 1952). The time required for sperm capacitation varies between species. Studies where sperm was preincubated in either the female tract of the rabbit, golden hamster and mouse, or in artificial media, have found that the time required for capacitation to be about 5-6 h, 2 h and 1 h respectively (see Yanagimachi, 1988). The capacitation time is influenced by factors such as the hormonal state of the female and the composition of the

medium. Mammalian spermatozoa were first capacitated entirely *in vitro* by culturing unfertilized oocytes with hamster cauda epididymal spermatozoa in Tyrode's solution or TC 199 (Yanagimachi and Chang, 1963). Mouse spermatozoa were first capacitated *in vitro* in cultures containing bovine follicular fluid (Iwamatsu and Chang, 1969) and later in a chemically defined medium (see Yanagimachi, 1988). The medium commonly used today for the capacitation of mouse spermatozoa is a modified Krebs-Ringer solution.

Capacitation involves the removal and/or re-distribution of sperm surface components acquired during passage through the epididymis. This process can be reversed (decapacitation) by incubation with seminal plasma (Chang, 1957). In the mouse, capacitated sperm may be distinguished by assessing the binding pattern of fluorescent antibiotic chlortetracycline to the sperm head (Ward and Storey 1984). A decapacitation factor associated with epididymal mouse spermatozoa has been partially characterised (Fraser *et al*., 1990).

Calcium ions (Ca^{2+}) are required for capacitation, hyperactivation and the acrosome reaction. In brief, sperm require micromolar concentrations of extracellular Ca^{2+} for the early stages of capacitation and millimolar concentrations for the later stages including hyperactivated motility and eventually the acrosome reaction (Yanagimachi and Usui, 1974; Fraser, 1977).

Hyperactivation of spermatozoa

Spermatozoa recovered from the female tract following ovulation of several species display a vigorous form of motility associated with capacitated sperm, known as hyperactivation. In contrast to human and boar spermatozoa, high percentages of mouse spermatozoa will hyperactivate in vitro (Fraser and Quinn, 1981; Suarez et al., 1992). Freshly collected epididymal sperm from the mouse shows a vigorous, progressive form of motility (Fraser, 1977), however, after incubating for 1 h in medium sperm show an overall increase in flagellar bend amplitude and beat asymmetry, characteristic of hyperactivation (Fraser, 1977). In medium, this motility is less progressive than non-capacitated spermatozoa, but experiments have shown that this motility enables sperm to progress more efficiently in a viscous environment similar to that found in the oviduct, and therefore may be important for transport along the oviduct and penetration of the oocyte vestments (Suarez et al. 1992). In contrast, recent studies on a mutant strain of mouse, t^{w32} /+ suggest that hyperactivated mouse sperm have a decreased progressive motility in vivo but are less likely to move up the oviduct, and that this could therefore be detrimental to fertilization (Olds-Clarke and Sego 1992a, 1992b).

Acrosome reaction

Following capacitation, the acrosome reaction, a Ca^{2+} dependent event (see Yanagimachi and Usui, 1974), renders the spermatozoa capable of penetrating the oocyte vestments and fusing with the oolemma. In mammalian spermatozoa, the outer acrosomal membrane fuses with the overlying plasma membrane in many places, allowing the release of the acrosome contents. The natural inducer of the acrosome reaction has not yet been fully defined, but in the mouse the zona pellucida is capable of inducing it (Wassarman et al., 1985). The cumulus oophorus may also be beneficial for induction of the acrosome reaction although it is not essential (see Yanagimachi, 1988).

As mentioned previously, millimolar levels of Ca^{2+} are required for the acrosome reaction (Fraser, 1982). In the mouse, potassium ions (K^+) are also thought to be necessary for the acrosome reaction and absence of extracellular K^+ results in its inhibition (Fraser, 1983).

Sperm concentration

One of the most important factors in determining fertilization is the spermatozoa/oocyte collision rate (Siddiquey and Cohen, 1982). High rates of fertilization (80-94%) can be obtained with concentrations of 2.5-9.0 X 10^5 sperm/ml, whereas lower rates (43-64%) of fertilization are achieved with lower concentrations 0.3-1.25 X 10^5 sperm/ml (Fraser and Drury, 1975). In

large volumes of medium (10 - 100 μl), the overall sperm concentration (number of spermatozoa/ml of fertilization media) seems to be the most important criterium, whereas in smaller volumes (1-5 μl) the number of spermatozoa per oocyte determines the fertilization rate (Siddiquey and Cohen, 1982).

Cumulus oophorus

The follicular cells surrounding the oocyte are transformed into the cumulus oophorus at ovulation. Cumulus cells are important in the fertilization of mouse oocytes in albumin-free media (Fraser, 1985) but are probably not essential for fertilization or embryo development in vivo. However, they may provide the ovum with the energy substrate, pyruvate, from the metabolism of glucose or lactate in the female tract (Leese and Barton, 1984). Although the energy substrates in chemically defined media are in excess of the requirements of the oocyte, it was found that the percentage of oocytes fertilized in vitro, was greater for oocytes with cumulus oophorus than those in which the cumulus has been removed (Miyamoto and Chang, 1972). The authors concluded that the capacitation of spermatozoa and fertilization of mouse oocytes in vitro may be more dependent on the medium than on the presence of cumulus cells.

Oocyte aging and activation

Many mammalian oocytes undergo degenerative changes with age that may appear as though the oocyte had been fertilized (i.e. formation of pro-nuclei, cleavage). The length of time between oocyte collection and incubation with spermatozoa increases the possibility of spontaneous activation. This problem can be reduced by pre-incubating the spermatozoa to allow capacitation before oocyte collection.

Strain variation

Reproductive performance is greater in hybrid mice than in inbred strains and therefore the use of F1 mice as oocyte and sperm donors is most prevalent for in vitro fertilization experiments. It has been reported that fertilization rates differ for oocytes incubated with spermatozoa from different strains (Tada et al., 1990) and there may be variations in embryo survival survival following cryopreservation of spermatozoa between different strains (Tada et al. 1990).

Embryo culture

Pre-implantatiom embryos were first cultured in simple, chemically defined medium under controlled conditions (Whitten 1956, Brinster, 1963). The medium used for most mouse oocyte and embryo culture is a modified Krebs-Ringer bicarbonate solution supplemented with lactate, BSA and antibiotics. It was established

that the successful culture of mouse embryos was dependent on factors such as the purity and chemical composition of the medium, the temperature, which should be maintained at 37°C, and the pH (7.2 - 7.4) (Whittingham 1971). Two-cell embryos will not develop *in vitro* unless lactate is present as an energy substrate and development of two-cell embryos can be enhanced by the addition of pyruvate to the medium (Brinster, 1965). Although the inclusion of glucose in the medium does not increase the number of embryos developing to the blastocyst stage (glucose cannot be utilised by the embryo until the four-cell stage, Brinster, 1965), it was included to provide an alternative energy source should the lactate and pyruvate become depleted. Two-cell stage embryos from all strains of mice can be successfully cultured to the blastocyst stage in chemically defined media, although the embryos from most strains do not develop past the two-cell stage when cultured in the same medium from the one-cell stage. This is characteristically described as the " *in vitro* two-cell block". Only embryos from some inbred strains and their F1 hybrids are able to develop through to the blastocyst stage. Embryos from these strains are preferably used for the study of *in vitro* fertilization. Recent studies have shown that embryos from random bred MF1 mice develop to morulae when cultured in a modified medium containing lactate and pyruvate, but lacking glucose (Brown and Whittingham, 1992). Although embryos

can be successfully cultured in vitro, optimal embryo development will only be achieved when in vitro systems mimic the changing environment of the oviduct culture.

MATERIALS AND METHODS

In vitro fertilization experiments were carried out using fresh and frozen spermatozoa prepared according to the methods described in the Appendix. The in vitro fertilization method was based on the method of Glenister et al. (1987) and is described in detail in the Appendix.

In vitro fertilization - Validation of the system

Fresh sperm from CBA mice and oocytes collected from superovulated (C57blxCBA) F1 females were used for in vitro fertilization experiments (see Appendix).
Once consistent results were obtained (fertilization rates more than 66%), in vitro fertilization experiments using frozen-thawed spermatozoa were carried out.

In vitro fertilization - Frozen-thawed spermatozoa

In vitro fertilization experiments were carried out using fresh spermatozoa as a positive control and supernatant obtained from the cryoprotectant removed from the frozen-thawed sperm as a negative control. The latter was prepared by taking supernatant from the (centrifuged) thawed sample and diluting it with the same volume of T6 + BSA used to resuspend the sperm

pellet. The concentration of frozen-thawed sperm was adjusted to 1 x 10^6 sperm/ml by determining the concentration of the sample and transferring an aliquot to a fertilization drop. An equivalent volume of diluted supernatant was added to the negative control fertilization drop in order to determine any eggs had been activated by the residual cryoprotectant and spermatozoa contents released during cryopreservation. Oocytes were also incubated in T6 + BSA medium only, to determine the background activation rate.

RESULTS

Preliminary validation of the <u>in vitro</u> fertilization and embryo culture system.

In 20 experiments, 66 ± 4.1% of oocytes incubated with fresh spermatozoa developed to the 2-cell stage. These results are shown in Table 4a. The embryos cleaved to 2-cell stage, 24 ± 1 h after incubating with the sperm. Of the 2-cell stage embryos, 68 ± 6.6% developed to the morula/blastocyst stage (Table 4b).

Oocytes incubated with freshly collected spermatozoa.

The proportion of oocytes developing to the 2-cell stage following incubation with freshly collected sperm is shown in Table 5. 78 ± 3.7% developed to the 2-cell stage. The increase in fertilization rate in comparison to those previously (Table 4a) may be attributed to the validation experiments containing results from early

experiments before the technique had been fully mastered by the author. Of 2-cell embryos, 56 ± 10% developed to the morula/blastocyst stage. These results are shown in Table 6a.

In vitro fertilization using frozen-thawed spermatozoa

The percentage of oocytes which developed to the 2-cell stage following incubation with cryopreserved sperm is shown in Table 5b. The fertilization rate was reduced in comparison to that of oocytes fertilized with freshly collected sperm, although the percentage of 2-cell embryos developing to the morula/blastocyst stage was comparable between oocytes fertilized with freshly collected and frozen sperm. These results are shown in Table 6a and b.

Of oocytes incubated with frozen spermatozoa, 55 ± 4.6% developed to the 2-cell stage, of which 67 ± 11% developed to the morula/blastocyst stage.

Oocytes incubated with diluted supernatant

The percentage of oocytes developing to the 2-cell stage after incubation with diluted diluent is shown in Table 7. Of oocytes incubated with diluted supernatant from the frozen-thawed sperm sample, 16 ± 3.4% developed to the 2-cell stage, with no 2-cell embryos developing to the morula/blastocyst stage.

Table 4 Validation of In vitro fertilization with fresh spermatozoa

(a) Development of oocytes to 2-cell embryos

Experiment	Number of oocytes	Number of 2-cell embryos	%
1	20	19	95
2	11	9	82
3	10	7	70
4	30	24	80
5	36	27	75
6	49	30	61
7	31	20	65
8	39	31	79
9	29	19	66
10	20	11	55
11	32	24	75
12	25	12	48
13	40	35	88
14	39	24	62
15	26	16	62
16	40	33	83
17	36	20	56
18	39	7	18
19	24	8	33
20	23	17	74
Total	599	393	66±4.1

(b) Development to morula/blastocyst

Experiment	Number of 2-cell embryos	Morula /blastocyst	%
1	24	12	50
2	12	7	58
3	35	17	49
4	24	19	79
5	16	14	88
6	33	30	91
7	20	13	65
8	7	2	29
9	8	5	63
10	17	15	88
Total	196	134	68±6.5

Table 5. Development of oocytes fertilized *in vitro* with fresh or frozen-thawed spermatozoa to the 2-cell stage

(a) Fresh spermatozoa

Experiment	Number of oocytes	Number of 2-cell embryos	%
1	29	27	93
2	46	34	74
3	72	58	81
4	47	29	62
5	35	21	60
6	25	19	76
7	19	17	89
8	10	9	90
9	24	22	92
10	56	45	80
11	27	14	52
12	49	44	90
13	51	37	72
Total	490	375	77±3.7%

(b) Frozen-thawed spermatoza

Experiment	Number of oocytes	Number of 2-cell embryos	%
1	57	35	61
2	115	30	26
3	54	20	37
4	105	50	48
5	34	25	74
6	50	24	48
7	51	36	71
8	25	19	76
9	30	12	40
10	44	17	37
11	32	11	34
12	25	13	52
13	38	28	74
14	17	14	82
15	102	60	59
Total	779	394	51 ± 4.6%

Table 6. Development of oocytes fertilized *in vitro* with fresh or frozen-thawed spermatozoa from 2-cell to morula/blastocyst

(a) Fresh spermatozoa

Number of 2-cell embryos	Morula /blastocyst	%
22	11	50
45	22	49
14	2	14
44	28	64
37	27	73
Total 162	90	56±10

(b) Frozen-thawed spermatozoa

Number of 2-cell embryos	Morula /blastocyst	%
12	12	100
17	11	65
11	2	18
13	4	31
28	21	75
14	6	43
60	46	77
155	102	67±11

Table 7. Development of oocytes incubated with diluent supernatant

Experiment	Number of oocytes	Number of 2-cell embryos	%
1	35	11	31
2	12	2	17
3	61	2	3
4	53	14	26
5	20	3	15
6	47	11	23
7	52	6	12
8	34	7	21
9	10	0	0
Total	324	56	17±3.4

Background activation rate

Of oocytes incubated with medium only, 1.8 ± 1.4% developed to the 2-cell stage, with none developing to the morula/blastocyst stage. This background activation rate was significantly less than for oocytes incubated with diluted supernatant ($p \leq 0.05$)

CONCLUSION

After freezing and thawing in MSC medium, a proportion of spermatozoa from CBA mice retained their motility and fertilizing capacity. The fertilization rate for oocytes incubated with frozen sperm was reduced in comparison to oocytes incubated with freshly collected sperm, although the developmental rate of 2-cell embryos to the morula/blastocyst stage was comparable for both freshly collected and frozen sperm. The fertilization rate of oocytes incubated with cryopreserved sperm may be reduced as a result of the dead and immotile sperm in the sample. Although the concentration of sperm in the fertilization drop is adjusted for motility to 1 x 10^6 sperm/ml, the presence of large numbers of dead sperm may exert a toxic effect on the remaining sperm, reducing their life span (Shannon, 1965), and may also have an effect on the oocyte. Following sperm death, an amino acid oxidase becomes active and peroxide is released as a toxic metabolic by-product (Shannon and Curzon, 1972; Alvarez and Storey, 1992). The toxic effect is increased by the presence of egg yolk, which provides a substrate for

peroxide formation (Shannon and Curzon, 1972). Sperm lipid peroxidation is also thought to be enhanced as a result of cryopreservation, and that this enhancement is partly mediated through a loss of peroxidation protective enzyme activity (Alvarez and Storey, 1992).

The presence of a large proportion of dead spermatozoa may also reduce the fertilization rate by preventing live sperm from reaching the surface of the oocyte. Isolation of a population of motile sperm proved difficult as the dead sperm prevented an effective swim-up method and column isolation techniques (McGrath et al., 1977) damaged already fragile sperm.

A high level of background activation occurred when unfertilized oocytes were mixed with cryoprotectant recovered from the freezing preparation. The activation rate can be reduced by more efficient removal of the cryoprotectant (see chapter 5), however this led to increased damage of the frozen-thawed spermatozoa and a decrease in the recovery of motile cells, and was not carried out in this series of experiments.

CHAPTER 4

INTRODUCTION

In vitro fertilization and embryo culture experiments, as described in chapter 3, demonstrated that after cryopreservation in MSC diluent, a population of mouse spermatozoa was capable of fertilizing oocytes *in vitro*, some of which developed to the morula or blastocyst stage in culture. The transfer of pre-implantation embryos was carried out to determine whether such oocytes fertilized with cryopreserved spermatozoa were capable of further development to fetuses and live offspring.

Transfer of embryos

The percentage of embryos developing to fetuses is similar for all stages transferred, with the exception of one-cell embryos which have a lower fetal development rate (Bronson and McLaren, 1970). Pre-implantation embryos from one-cell to morulae stage are transferred to the ampullae of 0.5 day pseudopregnant females while blastocyst stage embryos are transferred to the uterine horn of 2.5-3 day pseudopregnant females (Hogan *et al.*, 1986). The advantage of replacing embryos at the two-cell stage is that they are returned to an *in vivo* environment soon after fertilization which provides the optimum conditions for development prior to implantation. Development of embryos *in vitro* is slightly slower than the development of embryos *in vivo* and transfer of two-cell stage embryos

allows them the chance to 'catch-up' with development (Hogan et al., 1986).

Number of embryos transferred

In general, for oviductal and uterine transfers, 50-75% of unmanipulated embryos develop to term (Hogan et al., 1986). The number of embryos transferred should give a litter size of 5-7. Two or three embryos replaced to a suitable recipient may result in the formation of fetuses that are too large to be born without being damaged. If the litters are too large, the young may be born small with a risk of sterility. Transfer of seven embryos per ampulla/uterus is normally the accepted amount to transfer.

Recipient females

(C57blxCBA) F1 hybrid females are often used as foster mothers as they maintain pseudopregnancy well and are good mothers (Hogan et al., 1986). MF1 females can also be used as they have large fimbril ends to the oviduct which facilitates the introduction of the transfer pipette. The females should be at least 6 weeks old and ideally should be experienced mothers.

Assessment of implantation sites, fetuses and live offspring

The fetal development rate is assessed by killing the animal on day 15 of pregnancy and counting implantation

sites and fetuses. Embryos will have implanted into the uterine wall by day 10 and can be counted as fetuses if they have continued to develop or as implantation sites if they have not. Occasionally there may be be more implantation sites than embryos replaced. Avertin anaesthetic can activate oocytes already in the female tract of the pseudopregnant recipient and these may continue to develop up to the implantation stage (Kaufman, 1977). Transferred embryos can also be left to develop to live offspring. In this case, the embryos are usually from a coloured strain and replaced into true albino recipients. Brown experimental embryos can then be distinguished from amongst albino litter mates.

MATERIALS AND METHODS

Controls for technique

The oviductal transfer of 2-cell stage embryos is described in full in the appendix.

F2 (C57bl/CBAxC57bl/CBA) and CBA (C57bl/CBAxCBA) 2-cell embryos were flushed from the oviducts of naturally mated, superovulated three week old mice and placed into the oviducts of pseudopregnant recipients. Animals were killed on day 15 of pregnancy and implantation sites and fetuses were counted.

Embryo transfer to pseudopregnant animals

Up to eight embryos were replaced into each oviduct with the average number being seven per oviduct. Control

embryos from oocytes fertilized with fresh spermatozoa were placed into one oviduct and experimental embryos were placed into the contralateral oviduct. The side of the oviduct in which the experimental embryos were placed into (ie. left or right), was alternated to compensate for any technical bias. Animals were killed by cervical dislocation on day 15 of pregnancy, with day 1 as the day of transfer and implantation sites and fetuses were counted.

Embryo transfer to pregnant animals

Between 4 and 7 embryos were placed in the oviducts of MF1 females that had been naturally mated with intact MF1 males. Embryos were only replaced in females where a vaginal plug was observed on the morning after mating. Pregnant females were allowed to go to term and brown offspring were counted amongst their albino litter mates (Figure 21).

Live offspring developed to puberty and, when they were mated with each other, produced normal size litters.

RESULTS

Technique control

The results of replacing 2-cell embryos into the oviducts of pseudopregnant recipients are shown in Table 8. The transfer of two-cell embryos from natural matings resulted in an implantation rate of 89 ± 1.1% for (C57bl/CBAxC57bl/CBA) F1xF1 embryos and 85 ± 0.96% for (C57bl/CBAxCBA) F1xCBA embryos. The percentage of embryos

Figure 21. Live offspring (amongst albino litter-mates) from cryopreserved CBA sperm following <u>in vitro</u> fertilization and embryo transfer.

developing to fetuses was 64 ± 0.89% for F1xF1 embryos and 68 ± 0.66% for F1xCBA embryos.

Implantation and fetal development rate of oocytes fertilized with fresh spermatozoa

The results of the transfer of 2-cell embryos (C57bl/CBAxCBA) from oocytes fertilized with freshly collected sperm are shown in Table 9. Of 93 embryos replaced in the oviducts of pseudopregnant recipients, a total of 68 ± 0.95% implanted and 37 ± 0.52% developed to fetuses.

Implantation and fetal development rate of oocytes fertilized with cryopreserved spermatozoa.

The results of the transfer of 2-cell embryos (C57bl/CBAxCBA) derived from oocytes fertilized with cryopreserved sperm are shown in Table 10. Of 98 embryos replaced into the oviduct of pseudopregnant recipients, a total of 39 ± 0.71% implanted and 16 ±0.25% developed to fetuses. This result was significantly reduced ($p \leq 0.005$) in comparison to embryos from oocytes fertilized with fresh sperm. Of 58 embryos replaced into pregnant MF1 recipients, 17 ± 0.53% developed to live offspring.

In one experiment, the number of implantation sites and fetuses exceeded the number of embryos replaced. This was probably due to activation of an oocyte released during a natural cycle by the anaesthetic (Avertin) (Kaufman, 1977).

Table 8. Transfer of two-cell stage embryos flushed from the oviducts of naturally mated females.

F1xCBA embryos

Embryos transferred	Implantation sites	Fetuses
15	15	11
14	9	6
13	13	9
13	12	9
Total 55	49 (89 ± 1.1%)*	35 (64 ± 0.89%)*

F1xF1 embryos

Embryos transferred	Implantation sites	Fetuses
14	9	6
13	13	9
13	12	9
10	8	8
6	6	5
12	10	9
Total 68	58 (85±0.96%)*	46 (68±0.66%)*

* %±SEM

Table 9. Transfer of 2-cell embryos fertilized *in vitro* with fresh spermatozoa.

C57BL/CBAxCBA embryos

embryos transferred	Implantation sites	Fetuses
7	4	1
7	3	3
7	7	2
7	5	5
7	5	4
7	6	1
8	8	5
7	3	3
14	14	6
7	4	3
8	0	0
7	4	1
93	63 (68±0.95%)*	34 (37±0.52%)*

* %±SEM

Table 10. Transfer of 2-cell embryos fertilized *in vitro* with frozen-thawed spermatozoa to pseudo-pregnant recipients and pregnant recipients

a) Pseudopregnant recipients

C57BL/CBAxCBA embryos

Embryos transferred	Implantation sites	Fetuses
7	3	2
7	2	1
7	0	0
7	3	2
7	2	2
6	1	1
6	4	2
7	0	0
14	1	0
5	0	0
5	4**	2
13	10	2
7	2	2
98	32 (33±0.71%)*	16 (14±0.25%)*

b) Pregnant recipients

C57/BL/CBAxCBA embryos

Embryos transferred	Live offspring
6	0
7	0
5	1
7	3
9	4
11	2
5	0
8	0
58	10 (17±0.52%)*

*%±SEM
** Activated implantation

CONCLUSION

Normal implantation and fetal development rates were obtained with the transfer of embryos from natural matings. This result demonstrated that any decrease in implantation and fetal development was a result of the experimental conditions and not the technique itself.

Embryos from oocytes fertilized *in vitro* with fresh sperm showed a significantly ($p \leq 0.05$) lower implantation rate (68%) and overall fetal development rate (37%), than embryos from natural matings (85% and 68%). This is because fertilization and developmental rates are less efficient in *in vitro* systems when compared to those *in vivo* (Hogan *et al.*, 1986). Embryos from oocytes fertilized with frozen sperm resulted in a reduced implantation rate in comparison to embryos from oocytes fertilized with fresh spermatozoa ($p \leq 0.05$). The fetal formation rate (16% and 17%) was also reduced in comparison to embryos derived from oocytes fertilized with freshly collected sperm (37%, $p \leq 0.05$).

The percentage of implantated embryos that continued to develop to fetal stages was comparable for oocytes fertilized with both freshly collected and cryopreserved sperm. This indicated that the majority of embryo loss was occurring prior to implantation with a minimal loss post-implantation. It was concluded that the transfer of embryos from oocytes fertilized with cryopreserved sperm had a decreased implantation rate, although the proportion of implanted embryos that developed to fetuses was

comparable to control embryos. The reason for this was unclear, but it is feasible that residual cryoprotectant and spermatozoa contents acting on the oocyte/zygote could have an effect on subsequent implantation events.

Chapter 5

INTRODUCTION

The first protocol for the cryopreservation of mouse oocytes was based on methods developed for embryos (Whittingham, 1972; Wilmut, 1972). The procedure involved exposure to molar concentrations of DMSO at 0°C, seeding at -7°C and slow cooling to -80°C before transfer to liquid nitrogen. Warming was slow (8-20°C/min) and DMSO diluted at 0°C (Whittingham, 1977). At present the development of protocols for oocyte cryopreservation is still largely based on methods devised for embryos and include slow cooling in DMSO to -40°C combined with rapid thawing (Whittingham et al., 1979), ultra-rapid freezing (Szell et al., 1987) and vitrification (Rall and Fahy, 1984).

Aneuploidy

One of the major problems in devising protocols for oocyte cryopreservation is that the mature oocyte is arrested at the metaphase II stage of meiosis. At this period, the chromosomes are arranged on a microtubular spindle which is extreme ly sensitive to temperature changes. It has been suggested that during cooling the microtubule spindle may become disrupted due to this sensitivity, resulting in chromosome loss or non-disjunction (aneuploidy). With frozen-thawed oocytes, a small increase in the frequency was reported in studies on 272 chromosome spreads (Glenister et al., 1987). However,

Kola et al. (1988) noted a 3-fold increase in the frequency of aneuploidy in 47 chromosome spreads studied. The problem is that DMSO at ambient temperatures initiates the formation of microtubule asters which recruit microtubules from the spindle. This can be overcome by the addition of DMSO at 4°C. At low temperatures DMSO has the effect of stabilizing the microtubule spindle, by favouring the polymerisation of tubulin (Vincent et al., 1979). The observation that chromosome scattering can be prevented by the addition of DMSO at low temperatures suggests that aneuploidy can be prevented in the cryopreservation of mouse oocytes.

Polyploidy

In contrast to the relatively low incidence of aneupoidy, the level of polyploidy has been shown to increase after fertilization with frozen-thawed oocytes (Carroll et al., 1989). In the mouse, this is due to an increase in the digynic polyploidy caused by retention of the second polar body, rather than as a result of polyspermy (Carroll et al., 1989). In this case, DMSO may be disrupting the formation of actin microfilaments required for polar body formation.

Zona hardening

One of the major reasons for the failure of oocyte to undergo development following cryopreservation is an inability to fertilize (Carroll et al., 1989). This has

been identified as a result of zona hardening due to spontaneous maturation and has been correlated ~~as~~ WITH a conversion of the zona binding protein ZP2 into the inactive form ZP2f (Moller and Wassarman, 1989). The presence of macromolecules during cryopreservation has been found to affect the fertilization rate. Low rates of fertilization occur after freezing in diluent containing polyvinyl alcohol (PVA) and with some batches of BSA. In contrast, the presence of fetal calf serum (FCS) results in a fertilization rate comparable with that for freshly collected oocytes (Wood et al., 1989). The effect of FCS in the diluent does not appear to affect the ability of embryos to develop after transfer (Schroeder et al., 1990). This suggests that oocytes cryopreserved in the presence of FCS are able to undergo fertilization and their rates of development are comparable to controls.

Whereas the cryopreservation of spermatozoa and oocytes has been well documented for several species (Watson 1990, Whittingham and Carroll, 1992), the birth of offspring from cryopreserved oocytes fertilized with cryopreserved sperm has not been reported*. The ability to reproduce offspring from frozen oocytes and frozen sperm would greatly increase the flexibility of maintaining gene lines and would play an important role in conservation by providing a wider gene pool. The advantage of freezing gametes over embryos is that combinations of genetic material can be chosen whereas in the embryo the genetic constitution is already established. In the mouse, this

has important implications for those working with valuable mutant, inbred and transgenic lines, as the cryopreservation of gametes would eliminate the need for large breeding colonies and may prevent the loss of valuable genes through natural mutation in the breeding process.

MATERIALS AND METHODS

Cryopreservation of spermatozoa

Spermatozoa from CBA and (C57BlxCBA) F1 hybrid mice were cryopreserved in MSC (0.1% SDS and 1.25% glycerol) according to the method described in the Appendix. In brief, the samples were cooled at a rate of 10°C/min to 4°C and then 50°C/min to -70°C, before storing in liquid nitrogen (-196°C) for a minimum of 24 h. After thawing at ~1000°C/min, the sample was equilibrated in an incubator at 37°C, in an atmosphere of 5% CO_2 in air, for 10 min before centrifuging at 1000 g for 1 min (see activation rate below). The sperm pellet was resuspended in T6 medium containing 15 mg/ml BSA (Fraction V, Sigma). The concentration was adjusted to 1 x 10^6 sperm/ml and the samples incubated at 37°C, in an atmosphere of 5% CO_2 in air, to allow capacitation to occur.

Cryopreservation of oocytes

Cumulus free oocytes were cryopreserved according to the method of Carroll et al. (1989) in 1.5 M DMSO in M2 medium containing 10% fetal bovine serum. Oocytes were

loaded into straws and held at 4°C in an ice bath for 10-12 min before cooling in a cell freezer at 2°C/min to -7°C. After "seeding", samples were cooled at 0.3°C/min to -40°C, before transfer into liquid nitrogen for storage. Straws were warmed in air at room temperature for 40 sec and then transferred to water at 30°C until the sample melted. DMSO was diluted from the sample at room temperature before fertilization *in vitro*.

In vitro fertilization

Fertilization *in vitro* was carried out according to the method of Wood *et al.* (1987). Frozen and freshly collected oocytes were incubated with capacitated frozen and freshly collected spermatozoa in T6 medium containing 15 mg/ml BSA (Fraction V). After 4 h, oocytes were washed three times in M16 medium containing 4 mg/ml BSA before culturing overnight in the same medium at 37°C, in an atmosphere of 5% CO_2 in air.

Embryo Transfer

Next day two-cell embryos were transferred to the oviducts of pseudopregnant (C57BlxCBA) F1 hybrid recipients on Day 1 of pseudopregnancy according to the method described in the Appendix. Recipients were examined on Day 15 of pregnancy for implantation sites and fetuses.

RESULTS

Activation rate

Incubation of freshly collected oocytes with diluted supernatant from cryopreserved sperm resulted in <2% developing to 2-cell stage embryos (background activation). This was in contrast to 17% activation rate previously recorded (chapter 3). The difference in activation rate between the two experiments can be attributed to the difference in the removal of the MSC from the spermatozoa after thawing. The experiments in this chapter were carried out in a different laboratory (MRC, Experimental Embryology and Teratology Unit, St George's Hospital, London) and centrifugation of the thawed sample was undertaken at 1000 g. This caused the spermatozoa to form a discreet pellet, thereby allowing the majority of the MSC to be removed from the sample. However, the rate of centrifugation also resulted in a loss of sperm motility and only 10-15% motile sperm were recovered, in contrast to 25-30% in previous experiments.

<u>In vitro</u> fertilization of frozen-thawed and freshly collected oocytes with freshly collected sperm

The rate of fertilization was similar for frozen-thawed and fresh oocytes incubated with freshly collected spermatozoa. Frozen oocytes incubated with freshly collected sperm resulted in 81% developing to 2-cell stage embryos, as shown in Table 11. Freshly collected oocytes

incubated with freshly collected sperm resulted in 79% developing to 2-cell stage embryos (Table 11).

In vitro fertilization of frozen-thawed and fresh oocytes with frozen-thawed sperm

The rate of fertilization was similar for frozen-thawed and fresh oocytes incubated with frozen-thawed spermatozoa (Table 11). Frozen-thawed oocytes incubated with F1 and CBA frozen-thawed sperm resulted in 13% and 5% respectively developing to 2-cell stage embryos. Fresh oocytes incubated with frozen-thawed F1 and CBA sperm resulted in 12% and 6% developing to 2-cell stage embryos. Fertilization rates using frozen-thawed spermatozoa were reduced significantly ($p \leq 0.05$) compared to fresh spermatozoa (Table 11).

Oviductal transfer of 2-cell stage embryos to pseudopregnant recipients

After transfer to pseudopregnant recipients, implantation was similar in all treatment groups.
20 - 25% of embryos derived from frozen-thawed oocytes fertilized by frozen-thawed spermatozoa developed to late-stage fetuses, compared to 40 - 60% in all other groups.

Implantation and fetal development rates are shown in Table 12.

Table 11. Fertilization of frozen-thawed and fresh oocytes incubated with frozen-thawed and fresh spermatozoa from different strains of mouse

Spermatozoa		Oocytes			
		Frozen-thawed		Fresh	
Treatment	Strain	Number of oocytes*	% Fert[a]	Number of oocytes*	% Fert[a]
Frozen	F1	192	13	177	12
	CBA	464	5	438	6
Control	F1	245	81	234	79

* Totals of 3-7 replicate experiments

[a] % oocytes forming 2-cell stage embryos

Table 12. Implantation and fetal development rates of frozen-thawed oocytes fertilized by frozen-thawed spermatozoa

Spermatozoa		Oocytes					
		Frozen-thawed			Fresh		
Treatment	Strain	No. of oocytes*	%[a] Imp.	%[b] Fet.	No. of oocytes	% Imp.	% Fet.
Frozen-thawed	F1	12	92	25	22	68	41
	CBA	18	67	22	24	79	50
Fresh	F1	62	84	55	88	75	60

[a] % of embryos implanted
[b] % of embryos developing to fetuses
* Totals of 3-7 replicate experiments

CONCLUSION

For the first time, viable fetuses have been produced by *in vitro* fertilization of cryopreserved oocytes with cryopreserved (C57blxCBA) F1 and CBA sperm followed by transfer of 2-cell embryos to recipients. The ability to produce offspring from cryopreserved gametes will greatly increase the flexibility in maintaining valuable gene lines and may eliminate the need for large breeding colonies of animals. From the results it can be concluded that cryopreservation reduces the capacity of a population of mouse sperm to fertilize both frozen-thawed and fresh (control) oocytes. Finally, although the rate of implantation for 2-cell stage embryos was similar for all treatment groups, the rate of development to fetuses was reduced for cryopreserved oocytes fertilized with cryopreserved sperm ($p \leq 0.05$). The reason for this is unclear. Possibly the cryoprotectant for the spermatozoa had an adverse effect of frozen-thawed oocytes which due to their treatment tended to be more fragile than fresh oocytes, although why an effect was only apparent at a later stage of embryo development is not known.

* Since the preparation of this chapter, a poster presentation at the American Association of Laboratory Animals, 1992 entitled "*In vitro* fertilization results between cryopreserved mice gamete (Cryopreserved unfertilized eggs and cryopreserved sperm)". Nakagata *et al*, has been brought to the Authors attention.

CHAPTER 6

DISCUSSION

The development of protocols for sperm cryopreservation has been largely based on empirical studies, whereby individual protocols are tailored to meet the needs of a specific species, rather than developing standardised procedures for a group of species. This is perhaps justified, as the spermatozoon is composed of highly differentiated domains (acrosome, nuclueus mid-piece, flagellum etc.), each of which may vary in structure and composition between species and in the way they endure cryopreservation. However, the approach in developing cryopreservation protocols is not entirely empirical. It is now recognised that there is a relationship between cooling and warming rates (Leibo, 1981), and a relationship between cryoprotectant concentration and cooling rate (Watson and Martin, 1975). An understanding of the relationship between these variables, together with the characterization of cryoprotective compounds, provide a good basis from which to develop a cryopreservation protocol.

Assessment of sperm viability after thawing can include tests on membrane integrity, motility and fertilizing capacity. However, it is important to remember that although membrane integrity and motility are good indicators of sperm survival after cryopreservation, they will not affirm whether a population of sperm remain able to fertilize an oocyte. It is well documented that fowl

spermatozoa suffer a total loss of fertility in the presence of glycerol concentrations as low as 1% (Lake et al., 1990) even though they remain motile. As yet, there is no definitive method for determining whether a population of sperm retain their fertilizing capacity after thawing, although there is now strong indication of a link between types of motility and fertility (see Chapter 1).

In the development of the protocol described in this study, principle variables in cryobiology were considered; namely, cooling and warming rate of the sample, composition and concentration of cryoprotectant (including osmolarity) and elimination of the cryoprotectant after thawing. The use of the cryomicroscope enabled freezing parameters to be defined, while allowing continual monitoring of a sperm sample during freezing and thawing. The close contact of the sample with the stage, and the small volumes involved, meant that physical factors such as temperature gradients across samples or supercooling did not have to be considered. Furthermore, several diluents and cooling rates could be screened in a comparatively short space of time, and the post-thaw motility assessed immediately. A disadvantage of using the cryomicroscope was that it was not possible to remove the diluent from the sample and therefore assessment of motility had to be made in situ. It was important therefore to transfer the protocol developed on the cryomicroscope to a cell freezer, since for practical

purposes sperm would normally be cryopreserved in a cell freezer for storage. The protocol was also transferred to another model of cell freezer, the Kryo 10:17. It was discovered that modifications in the design of the newer model changed the way nitrogen vapour was introduced into the cooling chamber. This resulted in a decrease in the percentage of motile sperm when compared to aliquots that were cryopreserved in the R204 model. Further modification of the Kryo 10:17 to alter the delivery of the nitrogen vapour into the cooling chamber resulted in an increase in the percentage of motile sperm after cryopreservation.

The success of MSC can be attributed to the importance of the role played by each component in the protection of the spermatozoa during cryopreservation. Low concentrations of glycerol (1.25%) combined a cryprotective action with a minimal toxic effect. The effects of glycerol toxicity on mouse spermatozoa were seen in diluents containing higher concentrations of glycerol. The presence of motile spermatozoa after cryopreservation in a diluent containing no glycerol demonstrated the effectiveness of other cryoprotectant compounds in the diluent. However, the increase in the number of motile spermatozoa after cryopreservation in a diluent containing 1.25% glycerol clearly shows the importance of this compound as a cryoprotective agent. Egg yolk was found to be an effective cryoprotective agent for mouse spermatozoa with whole egg yolk being more effective than non-solubilized clarified egg-yolk. Mouse

sperm appeared to become osmotically shocked as they were recovered from the diluent, unless the sample was slowly diluted to excess. Recovery of the sperm from the egg yolk in the diluent was achieved by the inclusion of low concentrations (0.1%) of SDS, which allowed recovery of the sperm by straightforward centrifugation and resuspension of the pellet, with no subsequent osmotic shock. Although the SDS was included to solubilise the egg yolk, it is possible that the detergent is also acting on the sperm plasma membrane, rendering it more able to withstand cryopreservation. This is the first time that SDS has been used in a cryoprotectant with any success. Scanning electron microscopy showed the egg yolk particles closely affiliated with the spermatozoa plasma membrane, but it was not possible to discern whether the particles were bound to the plasmalemma. The mechanism of the cryoprotective action of egg yolk is unclear. It may be interacting with the plasma membrane providing it with stability as it undergoes phase transitions during the freezing and thawing process. Following thawing and recovery of the spermatozoa from the MSC, the motility of the sperm declined rapidly and 4-6 hours later only a few motile spermatozoa remained. Egg yolk is known to provide a substrate for lipid peroxidation and the presence of free radicals as a result of this may be damaging to spermatozoa (Alvarez and Storey, 1992). The release of cell contents from large numbers of spermatozoa may also affect the viability of the remaining motile population.

Dead bull sperm cause an increase in amino acid oxidase activity (Shannon and Curzon, 1971). Since egg yolk provides a substrate for peroxide formation, the inclusion of catalase or EDTA in the cryoprotectant alleviated this effect (Shannon and Curzon, 1971). Cryoprotectant containing egg yolk and glycerol in a citrate buffer has been shown to result in a decline in human sperm motility before freezing and a further decline after cryopreservation (McLaughlin et al., 1992). The toxicity of the diluent was responsible for at least half of the decrease in the percentage of motile cells.

A monoclonal antibody (18.6) generated against the acrosome provided a good marker for the assessment of acrosomal integrity. This antibody binds to the outer acrosomal membrane and acrosomal contents, and binds weakly to the inner acrosomal membrane. With the use of a fluorescent second antibody, acrosomes were assessed as intact, damaged or missing. It was not possible to determine the difference between fully acrosome reacted spermatozoa and spermatozoa with acrosomes missing as a result of freezing damage, but as an intact acrosome is a pre-requisite for fertilization in the mouse, it was possible to assess a post-thaw percentage of potentially viable spermatozoa.

In vitro fertilization was initially validated using fresh spermatozoa until a consistent fertilization rate was obtained. The fertilizing capacity of spermatozoa is strain dependent and the use of CBA spermatozoa resulted

in a lower fertilization rate and embryo development rate than in experiments where F1 spermatozoa were used, as the CBA strain has a poorer fertilization and embryo development rate. Controls demonstrated that residual cryoprotectant and/or dead spermatozoa contents resulted in a 17% activation rate. As described previously, the elimination of all the cryoprotectant by several washes was not possible due to the fragile nature of the cryopreserved spermatozoa. The effective isolation of a motile population of cryopreserved spermatozoa, containing no residual cryoprotectant or dead spermatozoa contents, may result in the elimination of the activation rate. If the residual cryoprotectant was affecting the motile spermatozoa an increase in the fertilization rate might also be expected. Where the process for the elimination of the cryoprotectant was more vigorous (See Chapter 5), the activation rate was reduced to background levels. However, this was accompanied by a dramatic decrease in the number of motile cells and subsequent reduction in the fertilization rate. This problem could be overcome using techniques such as sub-zonal microinjection (Mann, 1988). This technique would enable valuable gene lines to be maintained, even if the percentage of motile sperm was low after cryopreservation. Immotile spermatozoa prevent motile cells from reaching the oocyte, a higher fertilization rate might be expected if only motile sperm were recovered. Despite attempting a variety of procedures this was not achieved.

The percentage of 2-cell embryos developing to the morula or blastocyst stage was comparable for oocytes fertilized with both fresh and frozen spermatozoa. This suggested that although the initial fertilization rate was reduced, embryos derived from frozen sperm were capable of normal development to the morula or blastocyst stage. The timing of the successive cell divisions of the embryos derived from oocytes fertilized with frozen spermatozoa was comparable with the cleavage times of oocytes fertilized with fresh spermatozoa. As failure of embryos to cleave on time may be an indication of parthenogenetic activation, this was an indication that normal development was occurring (Hogan, et al., 1986).

In contrast, although the implantation rate was comparable following transfer of 2-cell stage embryos from both fresh and frozen sperm, the percentage of embryos developing to fetuses was reduced for embryos derived from cryopreserved sperm. Therefore, embryo loss is occurring at the post-implantation stage. The reason for the failure of some embryos to develop to the fetal stage was unclear, but could be partly accounted for by the fact that a percentage of the two-cell stage embryos transferred may have been parthenogenetically activated oocytes. It is also conceivable that exposure of the spermatozoa to MSC may affect the genome which is affecting the developmental capacity of the embryo. Studies investigating the effects of incubating human sperm in the presence of TES/Tris and egg yolk found structural chromosome abnormalities already

present after 1 day (Martin et al. 1992). However, earlier findings had reported no significant variations in the frequency and type of chromosome abnormalities (Benet et al., 1991). Chromosome studies on embryos derived from oocytes fertilized with frozen-thawed spermatozoa would be one way to examine the effects of MSC on embryo development. Fetuses that developed to term showed normal growth and development to adulthood, and when mated proved fertile, giving birth to normal size litters. However, only two generations were examined and long term studies through several generations would need to be examined to eliminate any long term genetic effect of the MSC on the germ line.

Cryopreservation of mouse oocytes did not appear to affect the developmental capacity of the embryos, and fetal development was comparable with that of embryos derived from fresh oocytes incubated with fresh spermatozoa. However, fertilization of both fresh and frozen oocytes with cryopreserved spermatozoa again resulted in a reduction in the fetal development rate. The combination of cryopreserved oocytes and cryopreserved sperm resulted in a further reduction in the fetal development rate.

The difference in the fertilization and embryo development rates between mouse strains has been well documented (Hogan et al. 1986). The post-thaw survival of mouse embryos following cryopreservation is dependent on genotype (Schmidt, et al., 1987). Semen characteristics

are also related to genotype (Wildt, et al., 1992), therefore it might be expected that there will be differences in the survival of the spermatozoa from different mouse strains following cryopreservation. The resistance of the spermatozoa from some strains to freezing damage may be due to differences in the protein and lipid composition of the plasma membrane (see Chapter 1). The cryopreservation protocol that was developed in this study, provided protection for a specific strain (CBA). However it may not be appropriate for a different strain. The most effective cryoprotectant and freezing protocol for each strain may involve identifying the composition of the sperm membranes. The characterisation of lipid and protein ratios in sperm membranes may give a greater insight into the development of effective cryoprotectants. With the use of transgenic mice as models it may be possible to identify lipids or proteins within the membrane that confer a resistance to cryo-damage.

The cryopreservation of mouse spermatozoa in conjunction with the established techniques for oocytes and embryos will provide greater flexibility for genetic management of laboratory mouse strains. Recently, transgenic mice were produced from cryopreserved fertilized ova (Leibo et al., 1991). The cryopreservation of mouse spermatozoa completes the range of techniques at our disposal for storing the genome in this species.

APPENDIX

T6 Culture medium (Quinn et al., 1982)

Chemical	mM	MW	g/100 ml
NaCl	124.23	58.45	0.726
$NaHCO_3$	25.0	84.0	0.21
KCl	2.68	74.55	0.02
NaH_2PO_4	0.36	84.0	0.0056
Penicillin Na	100 units/ ml		0.006
Streptomycin			0.005
H_2O			to 100 ml
$MgCl_2$	0.49		0.01
$CaCl_2$	1.80	147.2	0.0264
Phenol Red			0.01
Glucose	5.56	179.8	0.10

Weigh out all chemicals except $CaCl_2$, dissolve in ~70 ml H_2O. Dissolve $CaCl_2$ in ~15 ml H_2O, add to solution and make up to 100 ml in a volumetric flask. Filter through a 0.22 μm filter unit and store at 4°C for up to 1 week.

Reagents

Penicillin, streptomycin and phenol red from Sigma Chemical Co. Ltd., Fancy Rd, Poole, Dorset, BH17 7TG.
All other chemicals from BDH Merck Ltd. Hunter Boulevard, Magna Park, Lutterworth, Leics. LE17 4XN.

M16 Culture Medium (Whittingham et al. 1971)

Chemical	mM	MW	g/100 ml
NaCl	94.66	58.45	0.5533
KCl	4.78	74.55	0.0356
$CaCl_2.2H_2O$	1.71	147.2	0.0252
KH_2PO_4	1.19	136.0	0.0162
$MgSO_4.7H_2O$	1.19	246.5	0.0293
$NaHCO_3$	25.0	84.0	0.21
Na lactate(60%)	23.28	112.1	0.434
Na Pyruvate	0.33	110.0	0.0036
Glucose	5.56	179.8	0.100
Penicillin			0.006
Streptomycin			0.005
Phenol Red			0.001
H_2O			to 100 ml

Weigh out penicillin and streptomycin, dissolve in double distilled water (DDW). Weigh out $CaCl_2$, dissolve in DDW. Weigh out remaining chemicals except Na lactate, dissolve in ~50 ml DDW. Add pen/strep. and $CaCl_2$ solutions. Weigh out Na lactate syrup and add to solution. Make up to 100 ml in a volumetric flask. Filter through a 0.22 μm filter unit and store at 4°C for up to 1 week. (pH 7.4, 288-292 mOsm)

Reagents

Na lactate, penicillin, streptomycin and phenol red from Sigma Chemical Co. Ltd. All other chemicals are Analar grade from BDH Merck Ltd.

M2 Medium (Quinn et al., 1982)

Chemical	mM	MW	g/100 ml
NaCl	94.66	58.45	0.5533
KCl	4.78	74.55	0.0356
	1.71	147.2	0.0252 aCl .2H
KH_2PO_4	1.19	136.0	0.0162
$MgSO_4.7H_2O$	1.19	246.5	0.0293
$NaHCO_3$	4.15	84.0	0.0349
HEPES	20.85	238.0	0.4969
Na lactate (60%)	23.28	112.0	0.434
Na Pyruvate	0.33	110.0	0.0036
Glucose	5.56	179.86	0.100
Penicillin			0.006
Streptomycin			0.005
Phenol Red			0.001
H_2O			to 100 ml

285-287 mOsm

Weigh out the HEPES and dissolve in 10 ml distilled water. Adjust pH to 7.4 with 0.2 N NaOH. Make up remainder of medium according to the method described previously for T6. Add to HEPES solution. Make up to 100 ml in a volumetric flask. Filter through a 0.22 μm filter unit and store at 4°C for up to 1 week.

Reagents

Na lactate, penicillin, streptomycin and phenol red from Sigma Chemical Co. Ltd. All other chemicals are Analar grade from BDH, Merck Ltd.

Mouse Sperm Cryoprotectant (MSC)

Chemical	mM	MW	g
TES*	21.07	229.2	0.483
Tris**	9.49	121.14	0.115
Glucose	2.22	180.16	0.04
Glycerol		92.09	100 µl
Egg Yolk			25%
Sodium Lauryl Sulphate (SDS)			0.1%
H_2O			6 ml

1) Add 12 ml H_2O to 4 ml egg yolk and 0.1% SDS. Leave for 30 min at room temperature (~22-24°C).
2) Centrifuge at 20,000g for 1 h.
3) Add remaining chemicals to 8 ml of supernatant.
4) Store at 4°C for up to 3 days.

675-680 mOsm.

Reagents

Tris** Tris[hydroxymethyl]methylamine

TES* N-Tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid.

TES from Sigma Chemical Co. Ltd. All other chemicals from BDH Merck Ltd.

Avertin Anaesthetic

Stock Solution. Dissolve 10 g 2,2,2-tribromoethanol ($C_2H_3Br_3O$) (Aldrich Chemical Co. Ltd., Gillingham, Dorset, England) in 10 ml butan-2-ol (BDH Merck Ltd.). Store in the dark in a tightly capped bottle at 4°C (NB. Strength of the stock solution may increase with storage).

Dose. Add 0.12 ml stock solution to 10 ml 0.9% saline, prepare in a glass bottle, warm the solution under tap water and shake vigorously to mix. Store at 4°C for several days. Inject 0.02 ml/g body weight intra-peritoneally.

Animals

Sperm Donors: 10 week old CBA/CaBl, Bantin and Kingman Ltd., Grimston Aldrough, Hull, HU11 4QE.

Oocyte Donors: 3 week old (C57BlXCBA) F1, Bantin and Kingman Ltd., Grimston Aldrough, Hull, HU11 4QE

Foster recipients: 8-12 week old (C57Bl/CBA), Bantin and Kingman Ltd. 8-12 week old MF1, Harlan Olac Ltd., Shaw's Farm, Bicester, Oxon, OX6 OTP.

All animals were housed at 22 ± 2°C, 50 ± 2% Relative Humidity, 14 h light / 10 h dark cycle and were fed ad libitum on Rat and Mouse Diet No.1 (S.D.S., Witham, Essex.)

Superovulation

1000 i.u. Folligon (Pregnant Mares Serum Gonadotrophin, PMSG, Intervet UK Ltd., Science Park, Milton Rd, Cambridge CB4 4FP) was diluted with 2 ml solvent and made up to 20 ml with sterile saline to make a working dilution of 5 i.u./0.1 ml.

1000 i.u. Chorulon (Human Chorionic Gonadotrophin, hCG, Intervet UK Ltd.) was diluted with 2 ml solvent and made up to 20ml with sterile saline to make a working dilution of 5 i.u./0.1 ml.

3 week old female mice were injected intraperitoneally with 5 i.u. Folligon (PMSG) followed by 5 i.u. Chorulon (hCG) 48 h later. Oocytes were collected from the oviduct 14-16 h post-hCG.

Collecting 2-cell embryos from naturally mated mice

Superovulated mice (see above) were housed overnight with stud males after the HCG injection. On day 2 following hCG injection, females with vaginal plugs on day 1 were killed by cervical dislocation and the oviducts were dissected into M2 medium. The oviducts were flushed through with M2 using a fine needle, holding the oviduct carefully around the needle with fine forceps. Flushed 2-cell embryos were transferred to a drop of M16 + BSA under silicon oil (Dow Corning, 200/200cs, BDH, Merck Ltd. UK) and placed in the incubator at 37°C, in an atmosphere of 5% CO_2 in air.

Pseudopregnant foster mothers

8-12 week old female mice, in natural oestrus, were mated with vasectomised males. Plugged females were used and oviductal transfers were carried out on the day of plugging (day 1 of pseudopregnancy).

Preparation of vasectomised males

10 week old male mice were anaesthetised with an inter-peritoneal injection of 0.015-0.017 ml 2.5% Avertin per gram of body weight. The animal was laid on its back and the abdomen was swabbed with 70% alcohol. All instruments used in surgical procedures were sterilised. The skin was cut transversly to 1-1.5 cm in the lower abdomen, taking care not to damage the penis. A similar transverse incision was made in the body wall. (To help locate the body wall later, a small piece of suture thread was stitched into each side of the incision). Using blunt ended forceps, the fat pad of the testis was carefully

pulled out and the testis, epididymis and vas deferens identified. Sharp forceps were used to pierce the membrane beneath the vas deferens and the suture thread was passed through and tied in a double knot. This was repeated further along the vas deferens, with the knots ~5 mm apart. The vas deferens between the knots was cut and the testis replaced into the body wall, handling the fat pad only. The procedure was repeated on the other vas deferens. The body wall was sewn up using 2-3 sutures followed by the suturing of the skin.

Testing vasectomised males

Vasectomised males were housed individually and mated with superovulated 3-week old female mice. The oviducts from females with vaginal plugs on day 1 were flushed 24 h later and the oocytes checked for fertilization. No 2-cell embryos were seen indicating a successful operation. This procedure was repeated with 2-3 female mice for each vasectomised male.

Mouse sperm cryopreservation

Freezing protocol. Four adult male mice (10 weeks old) were killed by cervical dislocation and their cauda epididymides without vas deferens, were removed and placed into 1 ml of cryoprotectant at 30°C. Spermatozoa were released by cutting the epididymal tubules with fine scissors and agitating gently. The tissue was removed and the spermatozoa dispersed throughout the cryoprotectant by gently pipetting up and down using a fine pasteur pipette for 30 sec. Aliquots of 200 μl were transferred to 1.8 ml polypropylene cryotubes (Nunc, Inter-Med) which were then placed in a programmable cell freezer (R204, Planer Products Ltd.) at 30°C. Each sample was frozen at a rate of 10°C/min to 4°C, followed by 50°C/min to -70°C. The cryotubes were immersed directly in liquid nitrogen within the freezer and transferred to a storage vessel containing liquid nitrogen for a minimum of 24 h.

Thawing protocol. Cryotubes were immersed in water at 37°C until just thawed and the sample was transferred to an incubator at 37°C, in an atmosphere of 5% CO_2 in air, for 10 min. After centrifugation at 800 g for 4 min the supernatant was removed and the sperm pellet resuspended in 500 μl T6 medium containing 15 mg/ml BSA. This sperm suspension was then placed in an incubator at 37°C, in an atmosphere of 5% CO_2 in air.

In vitro fertilization

Media preparation. T6, T6 + 15 mg/ml BSA and M16 + 4 mg/ml crystalline BSA were equilibrated overnight in the incubator at 37°C, in an atmosphere of 5% CO_2 in air. The pH of T6 + 15 mg/ml BSA was adjusted the following morning using NaOH solution to 7.2-7.4 and fertilization drops of 300 μl were prepared under silicon oil (Dow Corning 200/200cs. BDH Merck Ltd.) in 33 mm petri dishes (BDH, Merck Ltd. Leics. LE17 4XN) and returned to the incubator.

Fresh sperm preparation. A male mouse was killed by cervical dislocation and the epididymides dissected into 300 μl T6 medium (pH 7.4) under silicon oil. The epididymides were punctured with fine forceps and gently squeezed to release the sperm. The epididymides were removed from the drop and the sperm suspension was replaced in the incubator and allowed to disperse for 10 min. The concentration of the sperm preparation was assessed using a double Neubauer haemocytometer and aliquots of sperm were added to the fertilization drops to a final concentration of 1 x 10^6 sperm/ml. The sperm were left to capacitate for 1 h from the time of dissection until incubation with the oocytes.

Frozen sperm preparation. Sperm aliquots were thawed according to the method described earlier. The concentration of the sperm was assessed using a haemocytometer and aliquots of sperm were added to a fertilization drop to a final concentration of 1 x 10^6

sperm/ml, adjusting for motility. The sperm were left to capacitate for 1 h from the time of thawing.

Oocyte collection and incubation with sperm. 3 week old females were injected with PMSG and hCG for superovulation (see Superovulation). Females were killed 14-16 h post hCG injection by cervical dislocation and the oviducts were dissected into T6 + BSA under oil. The cumulus masses were released by gently tearing the oviducts with fine forceps and were then transferred to fertilization drops containing fresh or frozen sperm. Control dishes contained T6 + 15 mg/ml BSA only.

The oocytes and sperm were incubated for 4 h at 37°C, 5% CO_2. The oocytes were transferred to M16 + 4 mg/ml BSA (pH 7.4) after washing in three separate drops of M16 + BSA before incubating overnight at 37°C, in an atmosphere of 5% CO_2 in air.

(See Wood et al., 1987)

Oviductal embryo transfer

Embryo preparation. 2-cell embryos were washed in three changes of M2 medium containing 4 mg/ml BSA before transferring to a drop of fresh M2 + BSA.

Surgical procedure. Recipients (see Pseudopregnant Foster Mothers) were mated the evening before the transfer and those plugged the following morning were used in transfer experiments. The recipients were anaesthetised with 0.015-0.017 ml of 2.5% Avertin per gram of body weight. The animal was placed abdomen down on a pad of clean tissue and the back was swabbed with alcohol. The hair was parted using fine forceps down the lower half of the back and a lateral incision of 2.5 cm was made in the skin. The skin was carefully separated from the body wall underneath and the incision was moved to one side until the ovary and associated fat pad could be seen beneath the surface of the body wall. A small incision of 0.5 cm was made in the body wall and blunt forceps were used to pull out the fat pad and associated ovary, oviduct and uterus. Care was taken to avoid touching the ovary or uterus. A Serafin (Holburn Surgical and Veterinary Instruments Ltd., Broadstairs, Kent, UK) clip was attached to the fat pad and the ovary, oviduct and uterus were laid across the back of the animal on a small square of sterile gauze. Under a dissecting microscope, the bursa surrounding the oviduct was carefully torn away using two pairs of watchmakers forceps. The fimbril end of the oviduct was located with minimal amount of handling. A transfer

pipette, pulled in advance from 145 mm Volac preplugged glass pipette (John Poulten Ltd., 77-93 Tanner St., Barking, Essex, IG11 8QD) was loaded with embryos. The end of the pipette was carefully introduced into the fimbril end of the oviduct and the embryos delivered into the oviduct by very gently blowing into the mouth piece connected to the transfer pipette. When the two air bubbles behind the embryos were seen just inside the oviduct, the pipette was carefully removed and the fimbril end of the oviduct was gently clamped using forceps for a few seconds. The Serafin clip was removed and the ovary, oviduct and uterus were replaced into the body cavity handling the fat pad only. The body wall was left to heal naturally with no suturing. The process was repeated for oviductal transfer on the opposite side. Michelle clips (Holburn Surgical and Veterinary Instruments Ltd.) were used to close the skin wounds.

Immunofluorescence visualization of the acrosome with monoclonal antibody. (Ellis et al, 1985)

1) Disperse sperm throughout T6 medium containing 4 mg/ml BSA (A-4503, Sigma Chemical Co. Ltd.) and smear onto slides.

2) Air dry and fix in methanol (BDH Merck Ltd.) for 30 sec.

3) Flood slide with antibody* (Supernatant from monoclonal cell culture).

4) Flood control slide with 0.9% saline.

5) Incubate for 30 min at 37°C, 5% CO_2.

6) Rinse slides x 3 with 0.9% saline.

7) Flood slide with FITC anti-mouse second antibody (10 µls in 400 µls saline, F-9137, Sigma Chemical Co. Ltd.)

8) Incubate for 15 min at 37°C, 5% CO_2.

9) Rinse slides x 3 with saline, blot gently with tissue paper.

10) Add a drop of Citifluor (antifade compound, Citifluor Ltd., Connaught Building, City University, Northampton Sq. London, EC1V 0HB.), cover with a coverslip and examine under oil immersion on a microscope.

*Monoclonal antibody, 18.6, Institute of Zoology, Zoological Society of London, NW1 4RY.

Scanning electron microscopy

Phosphate buffer

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
H_2O	800 ml

Adjust pH to 7.2 and make up to 1000 ml

Glutaraldehyde fixative

0.05M phosphate buffer	92 ml
25% glutaraldehyde	8 ml
Sucrose	1.71 g

Osmium tetroxide fixative

0.05M phosphate buffer	100 ml
Sucrose	8.55 g
Osmium tetroxide	1 g

Dissolve the sucrose in the phosphate buffer. Break the vial containing the osmium tetroxide (in fume hood) and add to buffer. Sonicate, filter and store at 4°C.

Tissue fixation for scanning electron microscopy

Fix the sample in glutaraldehyde fixative for a minimum of 2 h. Decant off the glutaraldehyde fixative and cover the sample with osmium fixative. Leave overnight at 4°C.

Tissue dehydration. Dehydrate in 2 x 5 min changes in each of the following: 10%, 30%, 40%, 70%, 80%, 96% and 100% acetone. Critical point dry in CO_2 and gold sputter coat at 30 mA for 1.5 min in argon. Examine under electron microscope.

Transmission electron microscopy.

Epon/Araldyte Resin

Epon	1.55 ml
Araldyte	2.0 ml
DDSA	2.5 ml
DBPH	0.1 ml
DMP 30	0.2 ml

Sample preparation for transmission electron microscopy.

Fix sample as for scanning electron microscopy.

Process as follows:

1. Place in 70% ethanol for minimum 2 h.
2. 90% ethanol for 30 min x 2.
3. 100% ethanol for 30 min x 2.
4. Propylene oxide for 10 min x 2.
5. Resin/propylene oxide 50/50 for 30 min.

Evacuate remaining resin for 30 min whilst tissue is in 50/50 resin/propylene oxide. Transfer sample using a small stick with a blob of resin on the end and place in the oven for 48 h.

Section, stain and examine under electron microscope.

Reagents

Osmium tetroxide and all reagents for resin from Bio-Rad, Maylands Ave, Hemel Hempstead, HP2 7TD. Gluteraldehyde from Agar Scientific Ltd., 66a Cambridge Rd, Stanstead, Essex, CM24 8DA. All other reagents from BDH Merck Ltd.

Statistics

All results were calculated as percentages ± standard error of the mean (SEM).

Student T test was used to determine significance of result (Arkin and Colton, 1970).

Box-Whisker graphs were obtained using CSS: Statistica Software (StatSoft, Inc. Tulsa, OK 74104, USA)

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