BIOCHEMICAL AND PHYSIOLOGICAL CHANGES IN BOVINE SPERM DURING EXTENDED INCUBATION UNDER AEROBIC AND ANAEROBIC CONDITIONS

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by

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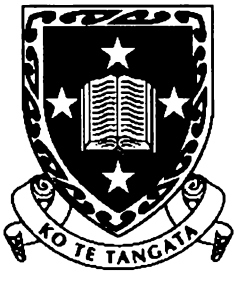

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SUMMARY

There are two main systems for storing diluted bovine semen for artificial insemination. It is either cryo-preserved in liquid nitrogen or held in liquid form at ambient temperature. During storage at ambient temperature, reactive oxygen species generated in the presence of oxygen are a major cause of cellular damage eventuating in the loss of fertility of sperm cells. This has been known for many years and current production systems for storage of semen in the liquid state attempt to minimise oxidative stress. This is achieved by reducing oxygen tension in the diluent through nitrogen gassing and by removal of hydrogen peroxide by catalase. However, the complete elimination of oxidative damage is not achieved because of diffusion of oxygen back into the diluent during re-dilution and packaging of individual insemination doses.

The present study was conducted to broaden the understanding of the biochemical mechanisms contributing to the drop in fertility during *in vitro* storage of sperm at ambient temperature, in relation to the oxidative state of the storage medium. To this aim, the survey was undertaken to determine the biochemical and physiological changes occurring in sperm during extended incubations (storage) under conditions of either complete exclusion of oxygen from the diluent, reduced oxygen tension, normal atmospheric conditions or elevated exposure to reactive oxygen species.

Velocities of bovine sperm in a medium containing glucose were found to be similar under fully anaerobic and aerobic conditions. Sperm were not able to sustain motility under anaerobic conditions when glycolysis was inhibited, but regained motility when re-aerated. This demonstrated that immobilisation when glycolysis was inhibited was

due to lack of oxygen and that conditions under which motility was analysed were truly anaerobic. Sperm motility parameters were not significantly different in the presence and absence of 4 μmol/l antimycin A and 4 μmol/l rotenone when glucose was present in the medium showing that glycolysis alone could fully sustain motility. After each incubation, functionality of sperm mitochondria was assayed by washing sperm into a medium that supported respiration but not glycolysis and motility was visually assessed. All sperm samples were highly motile in this medium indicating that their mitochondria were functional. When glycolysis was inhibited, antimycin and rotenone abolished motility immediately after addition. Thus it was concluded that bovine sperm can maintain similar levels of motility aerobically and anaerobically if a glycolysable substrate is available.

Changes in protein tyrosine phosphorylation, viability and motility were studied as a function of extended storage of bovine sperm *in vitro* at ambient temperature (18-20°C). Over a period of 8 days of storage at ambient temperature there was a time-dependent decline in sperm motility. This decline, to some extent, was reversed by incubation of stored sperm with theophylline, indicating that it was due to a decline in activation rather than the deterioration of sperm. Two soluble proteins (M_r=67 000 and M_r=36 000) displayed tyrosine phosphorylation the sum of which declined with the time of storage. Tyrosine phosphorylation of a number of proteins from whole cell extracts increased in a time-dependent manner during *in vitro* storage. The influence of the oxygenation state of the storage medium had profound effects on tyrosine phosphorylation of proteins from whole cell extracts. Tyrosine phosphorylation of proteins from whole cell extracts increased considerably during anaerobic storage while there was no significant change during aerobic storage. This increase in

phosphorylation under anaerobic conditions was reversed when sperm were transferred from an anaerobic to an aerobic environment, indicating that the oxygenation state of the medium regulates both protein tyrosine kinases and phosphatases. In addition, sperm stored under aerobic conditions for five days retained the ability to phosphorylate proteins when transferred to an anaerobic environment. Sperm motility and viability declined more rapidly under aerobic compared with anaerobic conditions.

Results from the flow cytometry experiments showed that the integrity of the sperm membrane was maintained better under anaerobic than under aerobic storage conditions. It was demonstrated that exogenous hydrogen peroxide either directly added to the diluent or generated through the enzymatic oxidation of phenylalanine was detrimental to sperm motility and the integrity of the plasma membrane. The assessment of sperm DNA susceptibility to *in situ* acid denaturation by the Sperm Chromatin Structure Assay did not detect any difference between sperm stored under aerobic and anaerobic conditions in a standard diluent. Exposure to exogenous hydrogen peroxide was detrimental to chromatin stability, increasing the DNA susceptibility to *in situ* acid denaturation. Inclusion of a general deoxyribonuclease inhibitor aurintricarboxylic acid in the diluent dramatically decreased sperm chromatin stability under both aerobic and anaerobic conditions, suggesting that this inhibitor had a damaging effect on sperm DNA.

The effects of storage time and the oxygenation state of the storage medium on motility, viability and *in vitro* fertility of stored sperm were investigated. *In vitro* fertility of sperm declined with the time of storage and there was no significant effect of the oxygenation state of the medium on *in vitro* fertility of stored sperm. The results of i*n*

vitro fertility trials suggests some form of sperm competition or oocyte selection of sperm based on its the ability to induce normal development.

In conclusion, the oxygenation state of the diluent in which sperm are stored *in vitro* has a significant effect on sperm metabolism, tyrosine phosphorylation signalling pathways and maintenance of viability. The impact of oxygenation state of the diluent on the *in vivo* fertility of stored sperm requires further investigation.

PUBLICATIONS

Following manuscripts resulting from the work presented in this thesis have been submitted/accepted for publication:

Krzyzosiak J., Molan P., Vishwanath R. 1998. Measurements of bovine sperm velocities under true anaerobic and aerobic conditions. *Proceedings of the Annual Conference of Australian Society for Reproductive Biology.* Pp.26. Perth, W.A., Australia.

Krzyzosiak J., Molan P., Vishwanath R. 1999. Measurements of bovine sperm velocities under true anaerobic and aerobic conditions. *Animal Reproduction Science* **55,** 163-173.

Krzyzosiak J., McMillan G., Molan P., Vishwanath R. 1999. Regulation of protein tyrosine phosphorylation, during *in vitro* ageing of ejaculated bovine spermatozoa. *Proceedings of the Annual Conference of Australian Society for Reproductive Biology.* Pp.78. Melbourne, Vic., Australia.

Krzyzosiak J., McMillan G., Molan P., Vishwanath R. 2000. Protein tyrosine phosphorylation, during prolonged *in vitro* incubation of ejaculated bovine spermatozoa is regulated by oxidative state of the medium. *Biology of Reproduction* **62,** 1615-1623.

Krzyzosiak J., Molan P., McGowan L., Vishwanath R. in press. Effect of spermatozoa number and oxygenation state of the storage media on *in vitro* fertility of bovine spermatozoa stored at ambient temperature. *Theriogenology.*

Krzyzosiak J., Evenson D., Pitt C., Jost L., Molan P., Vishwanath R. submitted. Changes in susceptibility of bovine spermatozoa to *in situ* DNA denaturation during prolonged storage at ambient temperature under conditions of exposure to reactive oxygen species and nuclease inhibitor.

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Chapter 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

Ever since artificial insemination became routine practice in the nineteen-fifties, dairy farmers around the world have benefited from this technology. The wide spread use of artificial insemination in New Zealand allows the team of ten best bulls to sire approximately 1.5 million calves each year. This contributes significantly to rapid genetic gain and would clearly be impossible if natural mating was used. World-wide, 95% of the artificial inseminations are conducted with frozen semen. Eastern Europe, The Netherlands, France and Australia make a limited use of liquid semen technology (*i.e.* semen that has not been frozen) (Vishwanath and Shannon 2000). New Zealand is the only country in the world that widely uses liquid semen technology and more than 85% of all inseminations are performed during seasonal spring mating with liquid semen (Vishwanath and Shannon 2000). Using liquid semen technology has the advantage of needing a much lower number of sperm per insemination compared to frozen semen (Vishwanath and Shannon 2000).

Inseminations with liquid semen are usually carried out within three days of collection, with one to two million sperm in a 0.25 ml dose, depending on the time between collection and insemination (Vishwanath and Shannon 2000). After three days of storage, the fertility of the sperm declines below an economically acceptable level. If the period of time during which adequate fertility of stored sperm is maintained could be extended by even one or two days the efficiency of semen utilisation could be significantly increased. This particular interest in preserving fertility of semen stored at

ambient temperature for extended periods formed the basis for the present study. The mandate of this research extended to basic investigations into biochemical and physiological changes occurring in sperm during storage at ambient temperature. The objective was to provide a foundation for the more applied studies into extending fertile life of sperm stored *in vitro* for artificial insemination.

1.1 STRUCTURE AND FUNCTION OF SPERM CELL

1.1.1 Unique features of sperm cell

The sperm cell is highly specialised to carry out the single role of passing and integrating paternal genes into the oocyte to form a zygote of the new individual. Sperm possess a number of characteristics distinct from those of other mammalian cells. They are haploid (and share this trait with oocytes) as the result of meiotic divisions. They are non-dividing cells and it is generally accepted that their genome is transcriptionally inactive, with perhaps some exceptions, so there is virtually no *de novo* synthesis of proteins in sperm cells. The DNA within the sperm nucleus is packed differently from that in somatic cells. It is not supercoiled, and is embedded in a highly ordered and cross-linked matrix of small basic proteins called protamines, rather than being wound around histones (Coelingh *et al.* 1972; Mazrimas *et al.* 1986). As the result of this, the chromatin is very condensed. During the process of spermatogenesis sperm become almost devoid of cytoplasm (Setchell 1993), and they lose the capacity for biosynthesis, growth and cell division (Hammerstedt 1993). They acquire a very characteristic morphology (Figure 1.1) which facilitates another unique trait, that is motility.

Motility is facilitated by a long flagellum, the anterior part of which called the mid-piece, is surrounded by the mitochondria. Mitochondria of sperm cells have a different appearance from that of their somatic cells counterparts. They are not spherical but are shaped in the form of an elongated twisted section of a toroid (Eddy and O'Brien 1994). They are tightly arranged around the flagellum and contained in the insoluble mitochondrial capsule rather than dispersed freely in the cytoplasm as in somatic cells and the oocyte (Eddy and O'Brien 1994). They do not divide within the life-time of the cell and are biochemicaly tagged for destruction inside the oocyte after fertilisation (Cummins *et al.* 1994; Sutovsky *et al.* 1999). Mitochondria in sperm are far less numerous than in somatic cells.

Sperm cells have to adjust to changes in the environment during their passage through the male and female reproductive tracts. They are unique in that they are the only mammalian cells that are destined to live outside the body of the individual that produced them (Salisbury *et al.* 1976). Metabolically, sperm are remarkably simple cells. Their uptake is limited to compounds that are membrane-permeable such as hexoses and three- and four-carbon carboxylic acids. These compounds enter the glycolytic pathway and tricarboxylic acid cycle to generate ATP with either lactate or CO_2 as the end-product depending on the incubation conditions (Hammerstedt 1993).

Functionally, sperm can be divided into two parts: the haploid genome contained in the nucleus (a payload or a message) and cellular machinery responsible for the safe and timely delivery of the genome to the oocyte cytoplasm during fertilisation. Morphologically, sperm are usually divided into the head, consisting of the nucleus, acrosome and small amounts of cytoplasm and cytoskeleton, and the tail or flagellum.

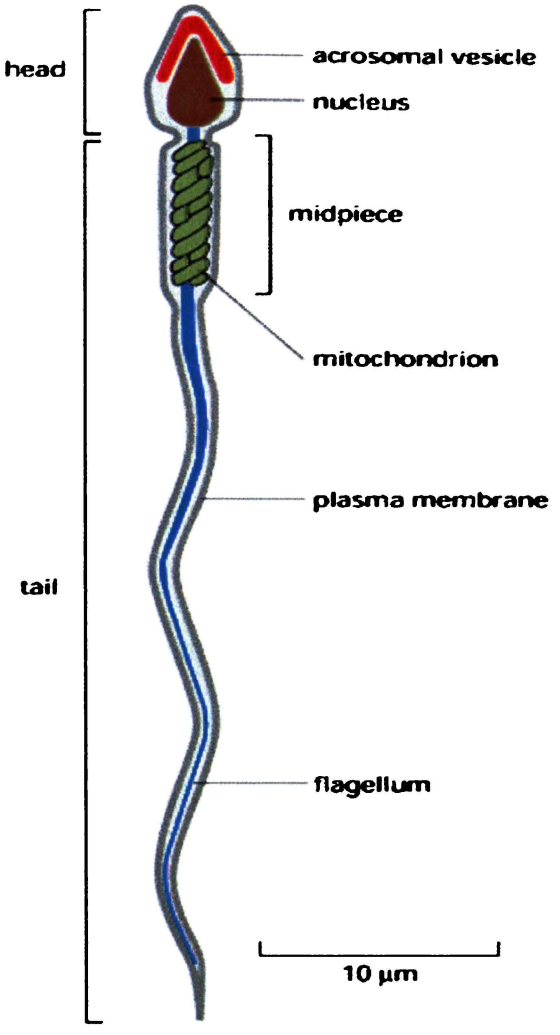


Figure 1.1 Schematic representation of longitudinal section of mammalian sperm (from: Alberts *et al.* 1994).

1.1.2 Nucleus

The function of the nucleus is to hold the genetic payload until it is delivered to the oocyte cytoplasm where the merging of male and female haploid pronuclei occurs creating the zygote. The bovine sperm nucleus has a symmetrical spatula-like shape flattened in the plane of the anterior-posterior axis (Eddy and O'Brien 1994). The nucleus contains a highly condensed haploid chromatin with the DNA and nucleoproteins arranged in a unique manner (Ward and Coffey 1991). The volume of

the sperm nuclear chromatin is much smaller than the volume of nuclear chromatin of somatic cells. The protamines in the chromatin undergo extensive disulphide cross-linking during maturation in the epididymis (Calvin and Bedford 1971). Balhorn and colleagues have demonstrated that in mature bull sperm all seven of the protamine cysteines are cross-linked as disulphides, four of them are intra-molecular disulphide bridges and three cross-link neighbouring protamine molecules (Balhorn *et al.* 1991). This results in the formation of a very rigid regular structure consisting of protamines interconnected across the entire sperm chromatin complex. According to the model proposed by Ward and Coffey (1991), DNA is packed within the protamine matrix as a side-by-side linear array and is not supercoiled. This very tight arrangement of mammalian sperm chromatin makes it resistant to physical and chemical disruption (Meistrich *et al.* 1976; Yanagida *et al.* 1991; Yanagimachi *et al.* 1992) providing the DNA some protection from the fragmentation caused by reactive oxygen species (Hughes *et al.* 1998). It is especially important as sperm unlike other cells are devoid of any DNA repair enzymes (Van Loon *et al.* 1991). This highly cross-linked structure also imparts mechanical stiffness, considered to be helpful during penetration of the zona pellucida (Yanagimachi 1994).

Abnormalities in the composition and packing of nuclear proteins can cause sub-fertility or infertility in affected individuals (Mann and Lutwak-Mann 1981).

1.1.3 Acrosome

The sperm acrosome is a unique organelle analogous to lysosome (Allison and Hartree 1970) or a zymogen granule of the pancreatic cells (Friend 1977). It is formed from the Golgi apparatus during the later stages of spermatogenesis. Mammalian acrosomes

vary in shape and size between species. The bovine acrosome (Figure 1.2) can be described as a vesicle-like structure made of continuous lipid bilayer membrane with the internal space filled with sialoglycolipoprotein matrix and sandwiched between cell plasma membrane and the outer membrane of the nucleus (Mann and Lutwak-Mann 1981).

The outer membrane of the acrosome is fragile and easily disrupted. It lies immediately underneath the plasma membrane of the anterior part of sperm head, and continues as the inner acrosomal membrane that surrounds the anterior part of the outer nuclear membrane (Eddy and O'Brien 1994; Mann and Lutwak-Mann 1981). The acrosomal matrix contains multiple enzyme activities necessary for penetration of oocyte investments by sperm during fertilisation (Eddy and O'Brien 1994; Yanagimachi 1994). The acrosome can be divided into two distinct regions, acrosomal cap and the equatorial segment. The function of the acrosome during fertilisation will be discussed in Section 1.1.5.

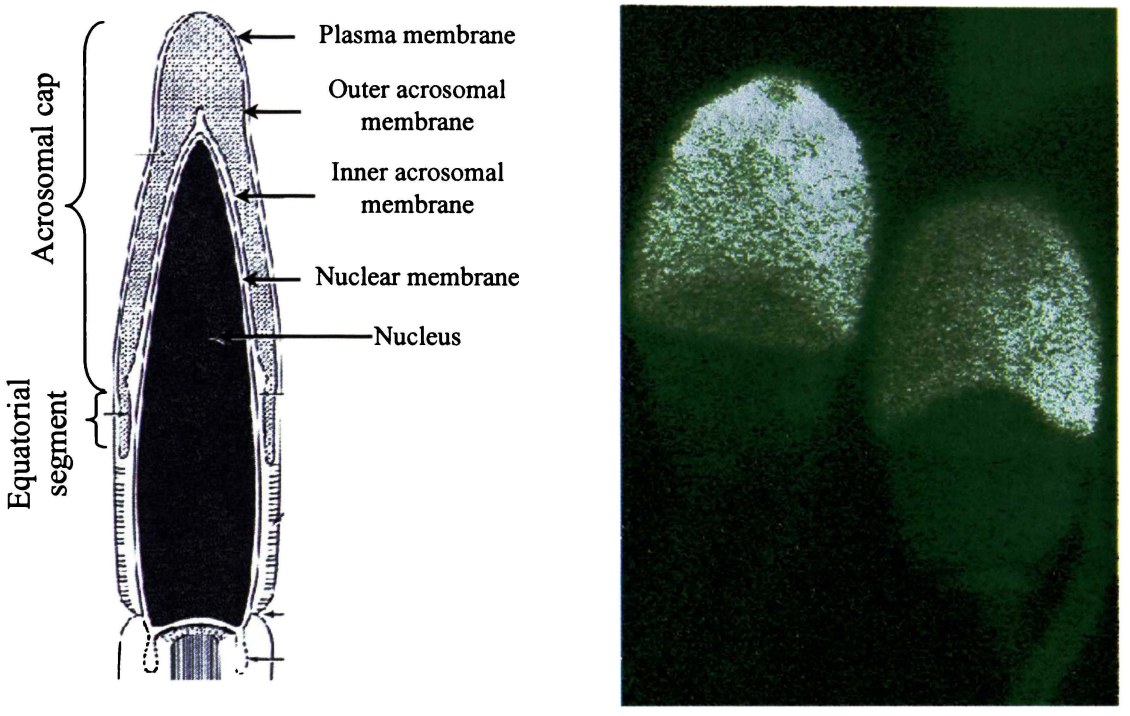


Figure 1.2 Schematic representation of the sagittal section through sperm head showing details of the acrosome (from: Yanagimachi 1994) and the laser scanning confocal microscopy image of the bovine sperm head stained with FITC-conjugated anti-acrosome antibody.

1.1.4 Flagellum

The flagellum, also called the sperm tail, is the site of ATP production and provides propulsion for motility. It is a complex and highly organised structure. Lengthwise it is divided into four discrete sections: connecting piece, middle piece, principal piece and end piece. The connecting piece attaches the tail to the head of sperm. The axoneme of the middle piece of bull sperm is surrounded by a triple helix of about 72 mitochondria contained in the mitochondrial capsule (Bahr and Engler 1970). The centre of the cross-section of a sperm tail (Figure 1.3) is occupied by the axoneme, which has the same general organisation as cilia and flagella of other plant and animal cells. At the centre of the axoneme lies a pair of microtubules. These are surrounded by nine other pairs of microtubules lying around the perimeter of the axoneme. The outer microtubule pair is

made up of two types of microtubules, a full microtubule A and attached to it a C-shaped microtubule B. The microtubule A is connected to inner and outer dynein arms. Multiple copies of the two types of dynein arms extend from the microtubule A of one of the perimeter doublets towards the microtubule B of the adjacent perimeter doublet along the length of the axoneme. Nine radial spokes project from the central pair of microtubules towards the surrounding ones. The axoneme is essentially an ATP-powered molecular motor driving the flagellar motion. Around the axoneme lie cytoskeletal elements of the tail such as outer dense fibres, satellite fibres and the fibrous sheath. They provide mechanical strength to the flagellum and together with the axoneme produce an effective flagellar beat (Eddy and O'Brien 1994).

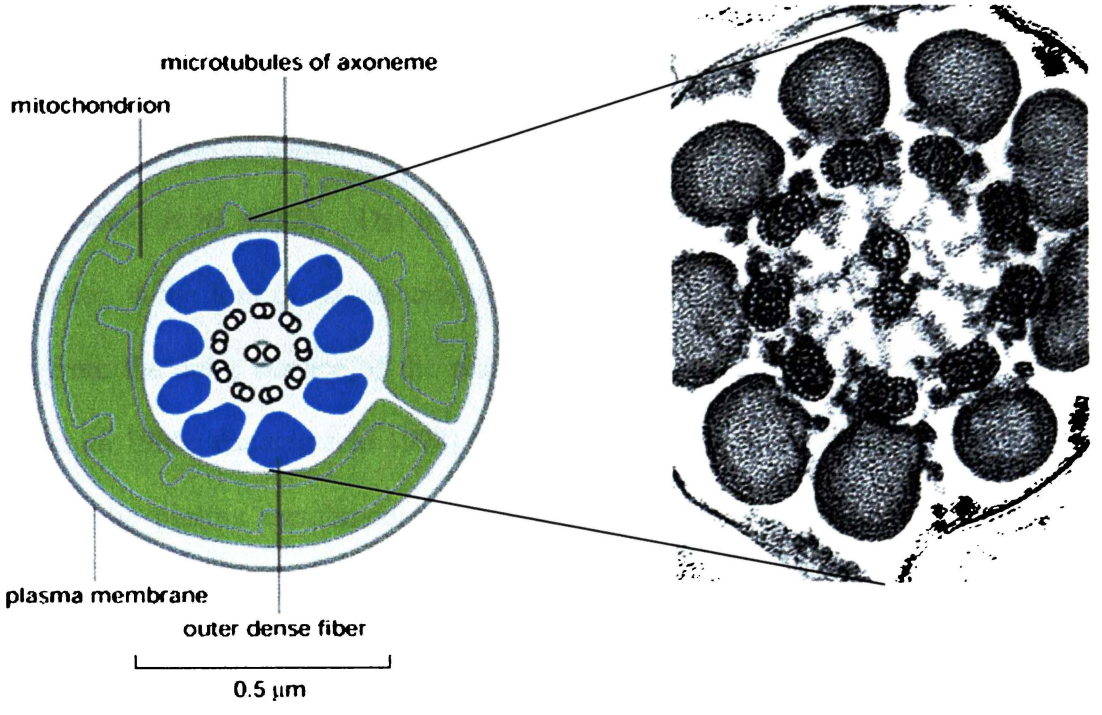


Figure 1.3 Schematic representation of the cross-section of the mammalian sperm through the midpiece region (from: Alberts *et al.* 1994) and the transmission electron micrograph of the cross-section of the axoneme of mammalian sperm (from: Cummins 2000).

1.1.5 Fertilisation - sperm's raison d'être

A succession of co-ordinated events constituting fertilisation is summarised in Figure 1.4. It begins *in vivo* with the deposition of sperm in the female reproductive tract (either by natural mating or artificial insemination). Immediately after deposition in the female tract, sperm are vigorously motile but are not able to fuse with the oocyte. To become able to unite with the oocyte, sperm have to undergo a process called capacitation either within the uterine environment or *in vitro*. In general, the term capacitation could be defined as the process of preparing ejaculated sperm to recognise the oocyte and respond to oocyte signals in an appropriate manner *i.e.* undergo a physiologically triggered acrosome reaction.

After deposition in the female tract the vast majority of sperm are eliminated and only a small number migrate successfully to the site of fertilisation (Drobnis and Overstreet 1992). The process of capacitation probably begins during migration through cervical mucus (Gould *et al.* 1985). The initial modification believed to be associated with capacitation is the loss of de-capacitating proteins of epididymal and seminal plasma origin from the outer surface of the plasma membrane (Florman and Babcock 1991; Gould *et al.* 1985; Yanagimachi 1994). Removal or lateral transfer of cholesterol from or within the plasma membrane causing changes in its architecture and fluidity is thought to follow the removal of de-capacitating factors (Parrish 1993). Changes in the concentration of the intracellular Ca^{2+} are also linked to capacitation but this aspect is controversial, perhaps reflecting differences between species or different responses of sperm cells to different *in vitro* treatments (Yanagimachi *et al.* 1992).

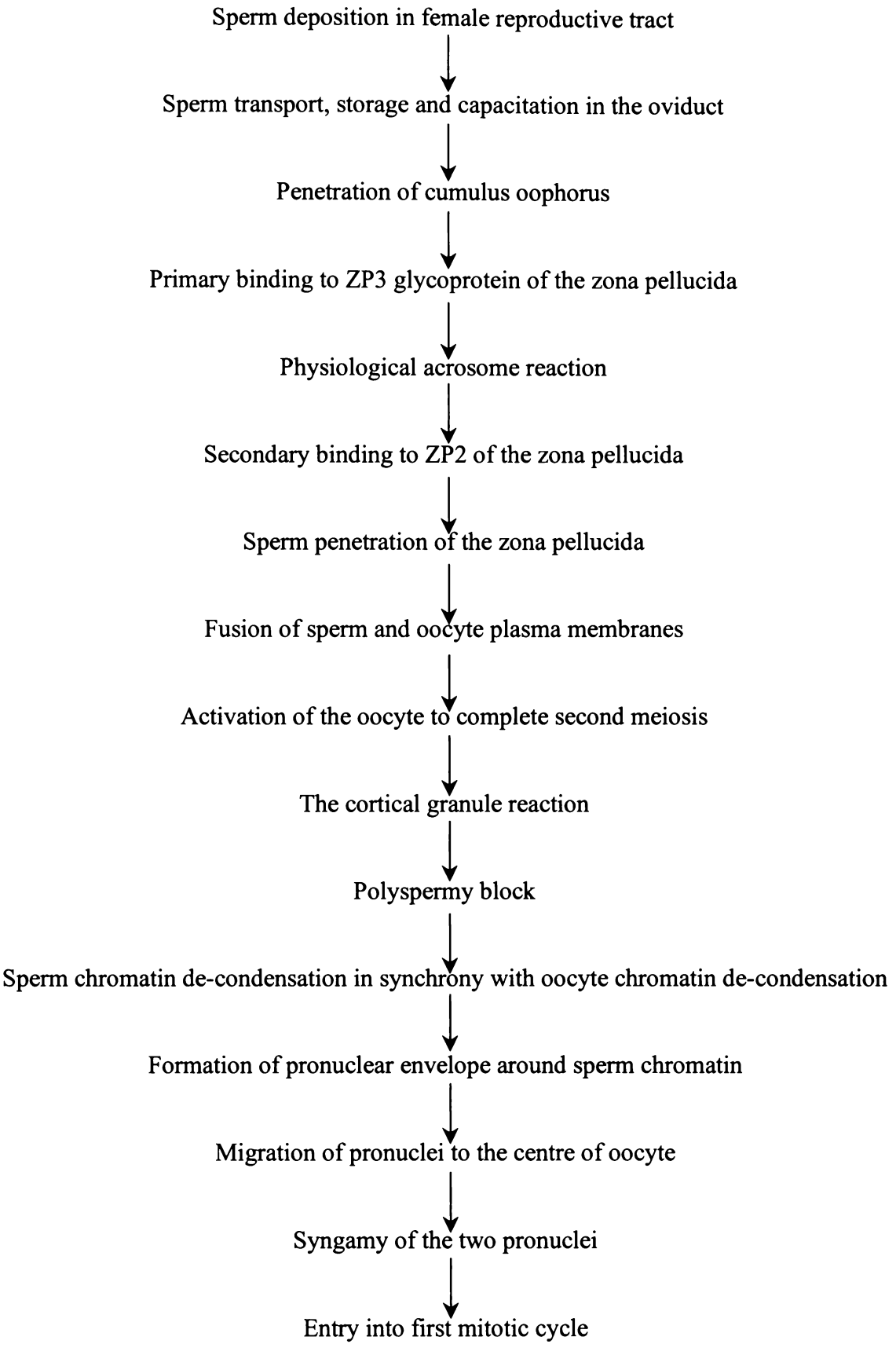


Figure 1.4 The succession of events comprising mammalian fertilisation.

In vitro studies indicate that cAMP may play an important role in regulating the pace of capacitation (Galantino-Homer *et al.* 1997; Leclerc *et al.* 1996). Numerous recent reports demonstrate an increase in tyrosine phosphorylation of sperm proteins during capacitation, suggesting that molecular events involved in capacitation are mediated by the tyrosine kinase/phosphatase signalling pathways (Duncan and Fraser 1993; Emiliozzi and Fenichel 1997; Galantino-Homer *et al.* 1997; Leclerc *et al.* 1996; Luconi *et al.* 1998; Visconti *et al.* 1995a; Visconti *et al.* 1995b). Both capacitation and tyrosine phosphorylation appear to be modulated by the redox potential of the cell (Aitken *et al.* 1996; Aitken *et al.* 1995) and in particular by the production of low levels of superoxide anion ($O_2^{-\bullet}$) (de Lamirande and Gagnon 1993c; de Lamirande *et al.* 1998a), hydrogen peroxide (H_2O_2) and nitric oxide (NO) (Zini *et al.* 1995). A dramatic change in sperm motility termed hyperactivation takes place during capacitation (Burkman 1990; Katz and Yanagimachi 1980; Mortimer and Maxwell 1999). Hyperactivated motility in low viscosity medium is characterised by high velocity and lateral head displacement together with low linearity and whip-lash like movement of the tail (de Lamirande and Gagnon 1993b). These motility characteristics may assist sperm to negotiate highly viscous cervical mucus or cumulus oophorus, and may also help in the mechanical penetration of zona pellucida (Parrish 1993). The site where capacitation is completed *in vivo* is uncertain (Parrish 1993). Capacitating sperm that survive the female immune response attach themselves to the epithelial cells of the oviduct to await their union with the oocyte (Ellington *et al.* 1998; Hunter *et al.* 1999; Hunter 1995). As the oocyte is transported from the ovary down the oviduct some sperm detach themselves from the oviductal epithelial cells and travel towards the oocyte. At this stage the ratio of number of sperm to the oocytes at the *in vivo* fertilisation site could be as low as 1:1 (Yanagimachi *et al.* 1992, and references therein).

The mammalian sperm-oocyte interactions during fertilisation are summarised in Figure 1.5.

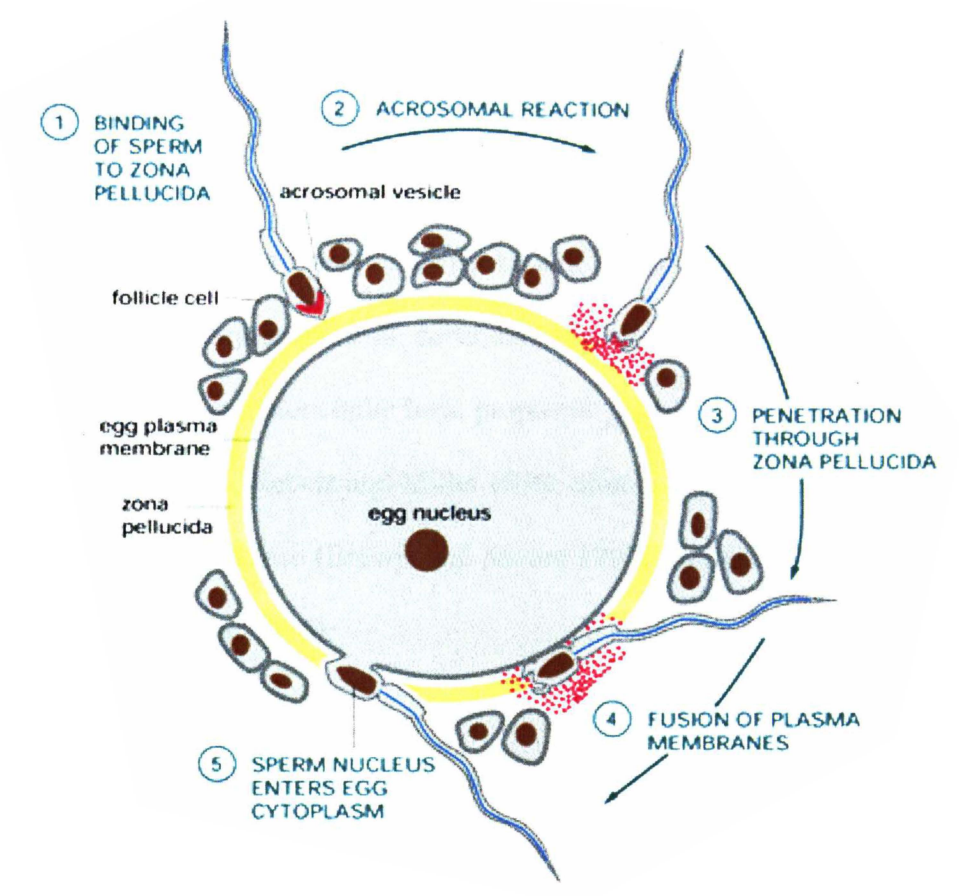


Figure 1.5 Schematic representation of the mammalian sperm-oocyte interaction during fertilisation (from: Alberts *et al.* 1994).

This subject is a very active field of research and the molecular details of gamete interactions have been comprehensively reviewed (Aitken 1997; Brewis and Moore 1997; Brewis and Wong 1999; McLeskey *et al.* 1998; Myles 1993; Myles and Primakoff 1997; Shalgi and Raz 1997; Snell and White 1996; Topfer-Petersen 1999; Wassarman *et al.* 1999; Wassarman 1987; Wassarman 1994; Wassarman 1995; Wassarman 1999a; Wassarman 1999b).

Capacitated sperm (probably with the aid of hyaluronidase activity of PH20 protein present on sperm plasma membrane) penetrate the cumulus oophorus. This is composed of cumulus cells enclosed within a matrix of polymerised hyaluronic acid that surrounds the oocyte. On reaching the surface of the zona pellucida of the oocyte, sperm recognise and bind to the carbohydrate residues of ZP3 glycoprotein of the zona. This primary binding to the zona is mediated by the receptors present in the sperm plasma membrane over the acrosomal cap (Burks and Saling 1992; Wassarman 1987). The identity of those receptors is still debatable (Brewis and Wong 1999) but the following candidate ZP3 receptors have been proposed: galactosyltransferase (Lopez *et al.* 1985; Lu and Shur 1997; Rebeiz and Miller 1999; Shur and Hall 1982), sp56 (Cheng *et al.* 1994) zona receptor kinase (Brewis and Moore 1997; McLeskey *et al.* 1998) and spermadhesin (Calvete *et al.* 1996).

The next event in the fertilisation sequence is the exocytotic release of the acrosome contents, a process called the acrosome reaction. This is an absolute prerequisite for subsequent sperm-oocyte fusion (Yanagimachi *et al.* 1992). During the acrosome reaction, the sperm plasma membrane fuses with the underlying outer acrosome membrane and eventually the whole acrosome cap is lost releasing the hydrolytic enzymes contained within. These enzymes perhaps take part in penetration of the zona by digesting the zona proteins, but this mechanism of zona penetration is still debatable (Bedford 1998).

Mutant females that produce oocytes without zona are infertile (Wassarman *et al.* 1999). After the acrosome reaction is complete, secondary binding of sperm to the zona takes place (Wassarman 1994). The ZP2 protein serves as a secondary sperm receptor.

Again, the identity of the zona-receptor responsible for secondary binding is hotly debated. PH20, proacrosin, sp38 and sp17 proteins have all been reported to participate in secondary binding of acrosome-reacted sperm to zona pellucida (McLeskey *et al.* 1998). The zona pellucida plays an active role during-oocyte interactions leading to fertilisation. Some of the carbohydrate residues of the zona undergo modifications that assist in the establishment of a block to polyspermy (Shalgi and Raz 1997).

A series of events are triggered once a single sperm cell traverses the zona pellucida thickness into the perivitelline space. The contact between the gametes takes place in the region of the oocyte that contains microvilli and the equatorial region of the sperm head (Parrish 1993). The fusion of the oocyte and sperm plasma membranes is thought to be facilitated by oocyte's integrin and the sperm's fertilin beta proteins (McLeskey *et al.* 1998; Myles and Primakoff 1997). After the fusion of the sperm and oocyte plasma membranes, the oocyte is activated to complete the second meiotic division, resulting in the formation of the female pronucleus. The content of the cortical granules is then extruded into the perivitelline space resulting in a zona block to polyspermy, presumably mediated by the enzymes contained in cortical granules. Another block to polyspermy takes place at the plasma membrane level (Parrish 1993).

After the sperm cell is engulfed by the oocyte cytoplasm the unpacking of the nuclear genetic message begins. Sperm do not contribute to the mitochondrial genome of the zygote, as the sperm mitochondria are actively destroyed by oocyte cytoplasm after fertilisation (Sutovsky *et al.* 1999; Sutovsky *et al.* 1996). Some cytoplasmic contribution to the zygote is made by the sperm in form of the centriole which plays an important role in formation of the zygote's centrosome in most mammals. Other sperm

structures are discarded from the oocyte in a tidy manner after fertilisation (Sutovsky and Schatten 2000). The sperm nucleus loses its nuclear envelope. Then a process reversing chromatin condensation, which had occurred during spermatogenesis, begins (Wright 1999). Glutathione, a tripeptide containing a free sulfhydryl group (γ-Glu-Cys-Gly), present in the oocyte cytoplasm, reduces disulphide links between protamine molecules of the sperm nucleus and protamines are replaced with histones (Crozet 1993) resulting in an increase in volume and acquisition of a more rounded shape (Storey 1995). Sperm nucleus decondensation proceeds correctly only if it is preceded by orderly condensation during spermatogenesis (Storey 1995) and if chromatin is not compromised in the time interval before fertilisation. Subsequently, the sperm nucleus acquires a new nuclear envelope and condenses again to form a male pronucleus (Parrish 1993). Formation of male pronucleus is co-ordinated with the formation of female pronucleus. Fertilisation is completed when both haploid pronuclei migrate to the centre of the oocyte, and their nuclear membranes interdigitate, the centrioles replicate, the nuclear envelopes break down, the chromosomes then re-aggregate into a single mitotic spindle and the cell enters the first mitotic cell cycle, as shown in Figure 1.6 (Parrish 1993). Because fertilisation is such a complex puzzle of co-ordinated molecular interactions, damage to any cellular element involved in this process can result in loss of fertility.

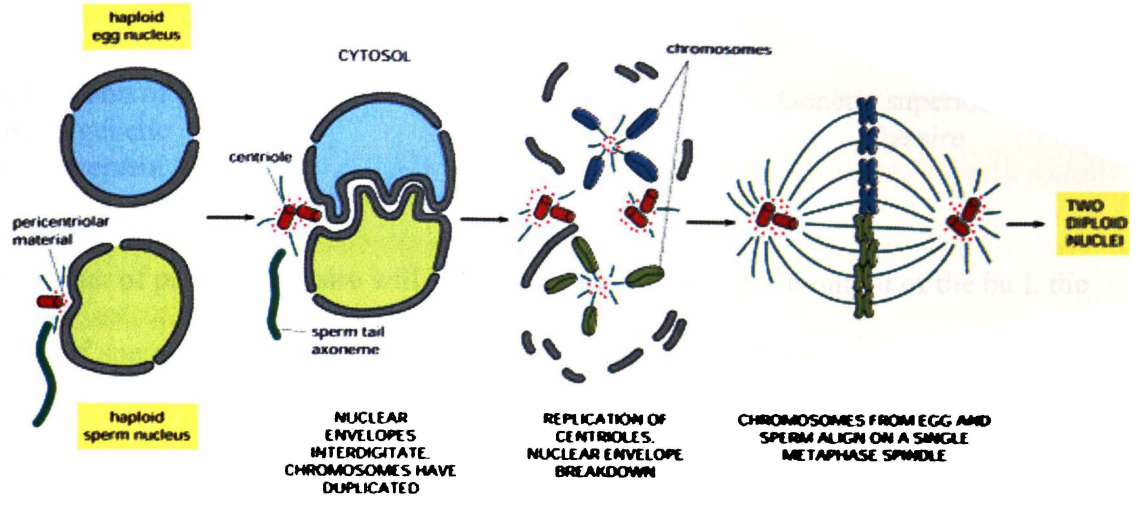


Figure 1.6 Syngamy of male and female pronuclei during mammalian fertilisation (from: Alberts *et al.* 1994).

1.2 ARTIFICIAL BREEDING

1.2.1 Benefits of artificial breeding

Artificial breeding can be defined as transfer of sperm into the female reproductive tract by means other than natural mating. The main advantage of this technology is the ability to disseminate genes, from selected sires that are superior in terms of the animal's economic performance, to as large number of dams as possible at minimal cost to the farmer (Hopkins and Evans 1989). The steady level of genetic progress in dairy cattle is for the most part due to advances in semen technology. Accurate identification of superior sires was made possible by establishment of precise measurements of production and other important traits through ancestry records and progeny testing programs (Rasbech 1993).

The potential genetic contribution of a sire is described by (Foote 1998) as:

Contribution of a sire to genetic improvement	=	Number of progeny per sire	x	Genetic superiority of the sire

The number of progeny per sire will be determined by total sperm output of the bull, the number of sperm used per insemination, and the percentage of cows calving to a single insemination. This can be represented as:

Number of progeny per sire	=	Number of sperm per sire / number of sperm inseminated per cow	x	Proportion of cows calving to a single insemination

These principles effectively determine the number of bulls required to service a dairy cow population. The equation becomes particularly attractive when very few bulls are needed to service a large population of dairy cows, thereby significantly raising the selection intensity (Shannon 1978).

The major spin-off of artificial breeding is a reduction in the incidence of sexually transmitted diseases in herds (Hopkins and Evans 1989). As insemination timing is crucial to the success of artificial breeding programs, better oestrus detection methods had to be developed (Rasbech 1993). In New Zealand all the elements of artificial breeding are highly integrated into a very efficient system. Diluted semen is distributed in single dose straws either frozen in liquid nitrogen (enabling almost indefinite storage) or in liquid form at ambient temperature as a branded product called Long Last Liquid™ semen.

1.2.2 Long Last Liquid™ semen technology

Any artificial breeding technology interrupts the natural process of direct deposition of sperm from the cauda epididymis (after admixture with accessory sex gland fluids) into a female tract. The purpose of the diluent is to support sperm fertility over the time of storage and dispensing of sperm in a large volume and much lower concentration than in raw ejaculate to allow large number of single insemination doses. To fulfil this purpose, the diluent has to maintain acceptable pH and osmolarity, provide an energy substrate for sperm metabolism, minimise cold shock and oxidative damage and eliminate microbial growth (Coulter 1992). The main advantage in using sperm stored *in vitro* at ambient temperature, rather than frozen is that the same non-return rate (rate of successful fertilisation) can be achieved with sperm numbers that are approximately fifteen times lower (Vishwanath *et al.* 1996). Liquid storage technology for bovine semen has been improved over the last 35 years and is now widely applied in New Zealand for intensive spring mating (Shannon 1964; Shannon 1968; Shannon 1978; Shannon and Curson 1972; Shannon and Curson 1983a). There are many diluents for storage of semen and their composition has been extensively reviewed (Maxwell and Salamon 1993; Salisbury *et al.* 1978; Vishwanath and Shannon 2000). Currently, in New Zealand, liquid semen is used for up to three days after collection because fertility declines below commercially acceptable levels when stored beyond three days even though sperm are still highly motile. A steady decline in the number of sperm cells per insemination dose has been achieved since 1959 (Figure 1.7). Liquid semen technology was in large part successfully developed on a trial-and-error basis, and at least in cattle it must now be regarded as a mature technology. The dose rate of liquid semen is probably close to the optimum in terms of genetic gain and future economic improvement will most likely be made by extending the "shelf life" of diluted semen.

The challenge for basic research now is to investigate what exactly happens to sperm during liquid storage. Once the nature of detrimental changes to sperm cells is identified, fine-tuning of current diluents and storage procedures can be attempted to extend the "shelf life" of semen doses.

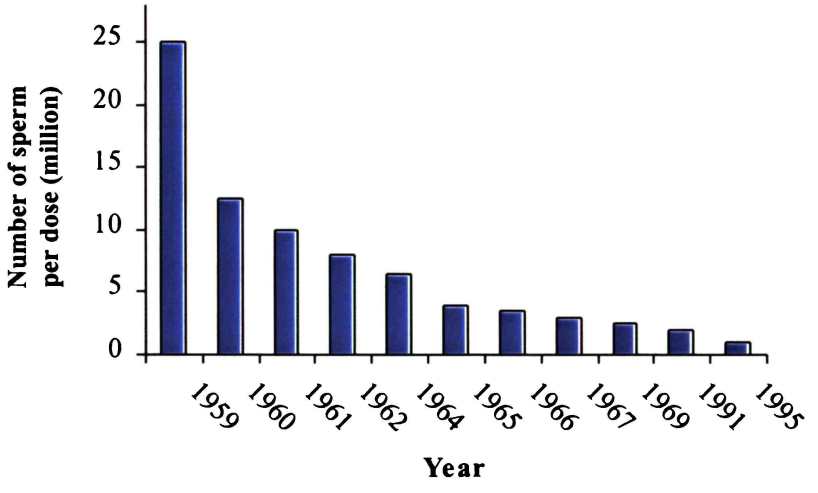


Figure 1.7 Changes in the number of sperm cells used in a standard insemination dose of Long Last Liquid semen stored at ambient temperature by Livestock Improvement Corporation NZ.

1.2.3 The effects of storage on sperm physiology

Various effects of liquid storage on sperm have been described to date. During *in vitro* incubation or storage, the enzymes contained in the acrosome diffuse out, increasing their concentration in the semen or the diluent (Salisbury *et al.* 1978). Sperm membranes become more permeable during storage (Mann and Lutwak-Mann 1973). A decline in the percentage of motile sperm and the intensity of the movement during *in vitro* storage has been reported (Vishwanath and Shannon 1997). But sperm motility and membrane integrity is retained longer than the ability to complete gamete fusion (Barros and Bustos-Obregon 1984). Increased instability of mouse sperm chromatin

during storage has been detected by means of the Sperm Chromatin Structure Assay (Ellington *et al.* 1999; Ellington *et al.* 1998; Estop *et al.* 1993).

An earlier report suggested an increase in the number of disulphide bonds within chromatin during sperm ageing (Beil and Graves 1977), this increase seems unlikely in the light of recent research on chromatin structure which shows that in mature sperm all the sulfhydryl groups in protamines are oxidised (Balhorn *et al.* 1991). When sperm are stored at ambient temperature for different periods, and are subsequently incubated at 37°C, the length of time they are able to survive upon incubation declines with the period of storage (Shannon 1978). All the changes in sperm observed at the biochemical and cellular level during storage may contribute to the widely reported decline in sperm fertilising capacity and in their ability to induce normal development of the conceptus (Coulter 1992; Dziuk and Henshaw 1958; First *et al.* 1963; Foote and Parks 1993; Mann and Lutwak-Mann 1973; Maxwell and Salamon 1993; Maxwell and Stojanow 1996; Roche *et al.* 1968; Salisbury *et al.* 1952; Salisbury and Flerchinger 1967; Salisbury and Hart 1970; Shannon and Vishwanath 1995; Shaver and Yanagimachi 1978; Smith and Lodge 1987; Vishwanath and Shannon 1997; Vliet and Hafez 1974).

1.3 SPERM AND REACTIVE OXYGEN SPECIES

1.3.1 Sources of reactive oxygen species in diluted semen

Sperm are produced in the testis and stored in the epididymis until ejaculation. The environment that sperm are stored in is virtually anaerobic (Setchell 1978). During semen collection, sperm pass into an aerobic environment. In raw bull semen straight

after ejaculation the cell density is very high and the dissolved O_2 is probably depleted very rapidly (Baas 1985). Consequently, up to the point of dilution, the exposure of sperm to reactive oxygen species is relatively low.

Interestingly, in boar semen, the sperm density is much lower than in bull semen. Also, the concentration of fructose in boar semen is about ten times lower than in bull semen (Mann 1964; Mann and Lutwak-Mann 1981). Boar sperm are immotile under anaerobic conditions (Nevo *et al.* 1970) indicating that, unlike bull sperm, they are adapted to relatively oxygenated seminal plasma that is poor in glycolysable substrates. This adaptation is achieved through strikingly different metabolic strategy of generating ATP almost exclusively via aerobic respiration.

Upon dilution of bull semen in an O_2-containing medium, there is a drastic increase in the potential for oxidative damage. The major sources of reactive oxygen species in diluted semen are sperm mitochondria (Holland and Storey 1981; Max 1992), seminal leukocytes (Kessopoulou *et al.* 1992), and the oxidation of amino acids catalysed by the aromatic amino acid oxidase present in sperm tail (Shannon and Curson 1981; Tosic and Walton 1946; Tosic and Walton 1950).

The production of reactive oxygen species by somatic cell mitochondria and the role of this process in cellular ageing are well documented (Boveris and Chance 1973; Gadaleta *et al.* 1998; Takasawa *et al.* 1993; Tanhauser and Laipis 1995). At the whole organism level, studies of transgenic *Drosophila melanogaster* called *methuselach* strongly support the link between ageing and reactive oxygen species. This genetically engineered strain expresses human superoxide dismutase specifically in adult motor

neurones, leading to increased capacity for the elimination of the superoxide radical. Their longevity is 40% higher than that of wild type *Drosophila* (Parkes *et al.* 1998) and they are more resistant to artificially induced oxidative stress (Lin *et al.* 1998).

In presence of O_2 each mitochondrion produces about 10^7 superoxide radicals per day (Max 1992). As a result, the life-span of the individual mitochondrion in somatic cells is on average considerably shorter than the life-span of the cell as a whole (Max 1992). Sperm cells, and in particular bovine sperm (Saaranen *et al.* 1989), are relatively rich in mitochondria and can potentially attain high rates of oxidative phosphorylation. In contrast to those in somatic cells, sperm mitochondria do not reproduce after cell differentiation during spermatogenesis (Cummins *et al.* 1994). In sperm cells about a third of the $O_2^{-\bullet}$ is apparently produced by the mitochondria, with the rest originating in the cytosol (Max 1992). Some of $O_2^{-\bullet}$ is subsequently dismutated to H_2O_2 as described in the reaction equation below (Boveris and Chance 1973), and exported to the diluent (Holland and Storey 1981).

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Similar defensive strategies are used in tissues lacking intracellular catalase such as brain, lung and heart that export H_2O_2 to the blood stream where it is destroyed by erythrocyte-bound catalase (Holland and Storey 1981). More recent reports indicate that only about a third of the reactive oxygen species produced inside sperm is released extracellularly (Plante *et al.* 1994). Semen of infertile males produce abnormally large amounts of reactive oxygen species, and the source of these is often intracellular (Aitken *et al.* 1992; Gomez *et al.* 1996).

The second potential source of reactive oxygen species in semen are seminal leukocytes (Aitken *et al.* 1998a; Cummins *et al.* 1994; de Lamirande and Gagnon 1994; Gagnon *et al.* 1991; Kessopoulou *et al.* 1992). Lymphocytes produce and release H_2O_2 via dismutation of $O_2^{-\bullet}$ during a sharp increase in glucose catabolism termed the "respiratory burst" that occurs after phagocytosis (Gabig and Babior 1979). The respiratory burst is initiated by NADPH oxidase that mediates the transfer of electrons from NADPH or NADH to oxygen (Yamashita *et al.* 1985). This enzyme, located on the surface of the leukocyte plasma membrane, is inactive in dormant cells but is activated during phagocytosis to become a potent anti-microbial agent (Cummins *et al.* 1994). Various types of leukocytes present in ejaculated semen have been demonstrated to be a significant source of reactive oxygen species (Aitken *et al.* 1994; Geva *et al.* 1998; Kessopoulou *et al.* 1992).

The third major source of reactive oxygen species in sperm is the generation of H_2O_2 under aerobic conditions through the reaction catalysed by aromatic amino acid oxidase, originally described by Tosic and Walton (1950).

$$R.CH_2CH(NH_2).COOH + O_2 + H_2O \xrightarrow{AAAO} R.CH_2COCOOH + H_2O_2 + NH_3$$

(R=phenyl, p-hydroxyphenyl or indolyl groups and AAAO=aromatic amino acid oxidase).

In bovine sperm, it was established that aromatic amino acid oxidase is inactive in live cells but the membrane-bound enzyme is activated and released from dead sperm in the presence of citrate (Shannon and Curson 1972). It is confined to the tail region of bull

sperm (Shannon and Curson 1981) and is specific for L-aromatic amino acids and in particular, L-phenylalanine (Macmillan *et al.* 1972). Egg yolk, a commonly used additive in semen diluents, is an abundant source of substrates for this enzyme (Vishwanath and Shannon 2000).

1.3.2 Physiological roles of reactive oxygen species in sperm

The current consensus of opinion is that while reactive oxygen species are harmful to sperm at high levels, precisely timed production of very low and controlled amounts of reactive oxygen species plays a part in the physiological reactions of normal sperm. In fact there appears to be a fine balance between beneficial and detrimental roles of reactive oxygen species in sperm (Aitken and Fisher 1994; de Lamirande and Gagnon 1994; de Lamirande and Gagnon 1995).

Sperm undergo a change from the virtually anaerobic environment to the relatively aerobic environment during ejaculation (Max 1992). This change can possibly act as a signal for sperm cells to change their metabolic rate and prepare for fertilisation. At a metabolic level, the presence of oxygen allows oxidative phosphorylation to occur but it does not necessarily impose it on sperm (Salisbury *et al.* 1978). A physiological signalling mechanism, which could indicate to the sperm cells that they are out of the testis and should start preparing for fertilisation, may involve reactive oxygen species. As reactive oxygen species are produced in semen under aerobic but not under anaerobic conditions, and as they are involved in the regulation of cellular functions of sperm (de Lamirande and Gagnon 1993c), a switch from aerobic to anaerobic conditions during ejaculation could trigger the cascade of molecular events leading to capacitation. De Lamirande and colleagues first noticed that $O_2^{-\bullet}$ triggers

hyperactivation in human sperm while studying toxic effects of reactive oxygen species (de Lamirande and Gagnon 1993c). They also demonstrated that addition of active but not denatured superoxide dismutase abolished hyperactivation under conditions conducive to the generation of $O_2^{-\bullet}$, confirming a role of this reactive oxygen species in capacitation of human sperm (de Lamirande and Gagnon 1993a; de Lamirande and Gagnon 1993c). The role of $O_2^{-\bullet}$ in capacitation may be species-specific, as in golden hamster sperm hydrogen peroxide rather than $O_2^{-\bullet}$ has been shown to promote capacitation and catalase to inhibit it (Bize *et al.* 1991).

The scavenging capacity of various biological fluids for reactive oxygen species was demonstrated to be inversely proportional to their ability to induce sperm capacitation and spontaneous acrosome reaction (de Lamirande *et al.* 1993). This may explain the decreased fertility of males with a low scavenging capacity for reactive oxygen species in seminal plasma, because sperm of such males may become prematurely capacitated (de Lamirande *et al.* 1998a). Reactive oxygen species have also been shown to regulate the acrosome reaction (de Lamirande *et al.* 1998a; de Lamirande *et al.* 1998b). The role of reactive oxygen species during sperm capacitation seems to be executed through the regulation of protein tyrosine phosphorylation (Aitken *et al.* 1996; Aitken *et al.* 1998b; Aitken *et al.* 1995; de Lamirande *et al.* 1998a; Leclerc *et al.* 1997). In relation to *in vitro* storage it is worth noting that capacitated and acrosome-reacted sperm do not survive as long as non-capacitated sperm, thus storage of sperm under the conditions inhibiting capacitation may extend their life span. Stopping generation of reactive oxygen species may be one of the strategies to inhibit capacitation.

Sperm capacitation and apoptosis seem to share some features. Both can be induced by reactive oxygen species and generation of nitric oxide (Zini *et al.* 1995), and both capacitation and *in vitro* induced apoptosis in some cell culture systems are inhibited by catalase (Aitken *et al.* 1996; Tilly and Tilly 1995). It can be speculated that early cell death of acrosome-reacted sperm that fail to fertilise is a result of an apoptosis-like process.

Apart from capacitation, ROS play some role in other processes that influence the fertility of sperm. Small amounts of H_2O_2 or lipid hydroperoxides are required to complete protamine cross-linking during the final stages of chromatin condensation (Aitken *et al.* 1998a). Also, a low level of oxidative stress enhances the ability of sperm to bind to zona pellucida (Aitken *et al.* 1989), reduces DNA fragmentation and increases rates of *in vitro* sperm oocyte fusion (Aitken *et al.* 1998a), indicating that ROS may play multiple roles during fertilisation.

1.3.3 Detrimental effects of reactive oxygen species on sperm

Early life and most of its basic molecular mechanisms are considered to have evolved under anaerobic conditions. However, because of photosynthesis oxygen gradually accumulated in the atmosphere and, as a later adaptation, aerobic organisms developed the capacity to use oxygen for energy generation via respiration. This brought the benefits of a more ample energy supply, but also the risk of long term accumulation of oxidative damage (Sohal and Weindruch 1996). The fact that oxygen is necessary for life of aerobic organisms and at the same time sub-lethally toxic is sometimes called the "oxygen paradox" (Gauduel and Duvelleroy 1984). Toxicity is inherent in the atomic structure of oxygen. O_2 is a bi-radical which, after accepting single electrons,

successively produces partially reduced molecules called reactive oxygen species. In the aerobic cell around 2 to 3% of consumed oxygen is converted into reactive oxygen species (Chance *et al.* 1979) including the superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide H_2O_2. Superoxide ion and hydrogen peroxide can react in presence of iron or copper and produce hydroxyl free radical ($^{\bullet}OH$).

$$(O_2^{-\bullet}) + H_2O_2. \xrightarrow{\text{iron or copper ions}} {}^{\bullet}OH + OH^-$$

The resulting hydroxyl free radical is very reactive and it can in turn generate an array of additional reactive metabolites and cause extensive damage to the delicate arrangement of biological molecules within a living cell (Sohal and Weindruch 1996). In various cells and tissues, oxidative damage had been reported to manifest itself as peroxidation of membrane unsaturated fatty acid chains, DNA alterations (including base substitutions, single strand breaks, sister chromatid exchanges, and cross-linking of DNA with proteins), and carbonylation and loss of thiol groups in proteins leading to enzymatic inactivation (Sohal and Weindruch 1996).

The detrimental effects of reactive oxygen species on sperm were identified much earlier than their positive physiological role. The possibility of oxidative damage to sperm was first reported by MacLeod (1943), when he observed that human sperm lost motility rapidly when incubated under high oxygen tension. Sperm are particularly vulnerable to the damage inflicted by reactive oxygen species for number of reasons. The high content of polyunsaturated fatty acid chains in their plasma membrane makes them vulnerable to oxidative attack as susceptibility of fatty acids to peroxidation rises with the increase in the number of double carbon-carbon bonds (Hughes *et al.* 1998;

Jones and Mann 1973; Jones *et al.* 1979; Sevanian 1988). Consequences of lipid peroxidation include disruption of membrane architecture, increase in cell permeability, ATP-depletion, loss of motility, un-controlled flux of ions, formation of cytotoxic products of lipid peroxidation that can spread to neighbouring cells, and ultimately cell death (Cummins *et al.* 1994). Some of these effects may be due to excessive hydrolysis of membrane lipids by phospholipase A_2. Phospholipase A_2 is known to preferentially hydrolyse peroxidised unsaturated lipids leading to increased membrane instability and enhanced phospholipase A_2 attack (Sevanian *et al.* 1988). Activation of phospholipase A_2 plays a normal physiological role in acrosome reaction but, if it is excessive or premature it can lead to a loss of membrane integrity (Dominguez *et al.* 1999; Dominguez *et al.* 1996; Roldan 1998).

During exposure of sperm to reactive oxygen species malondialdehyde is formed. It inhibits motility and apart from being an indicator of lipid peroxidation is cytotoxic in its own right (Alvarez and Storey 1983).

The extreme fragility of the sperm membrane due to its high content of polyunsaturated fatty acids may be an adaptive mechanism to eliminate those cells that have been exposed to oxidative attack from the pool of cells that are able to fertilise the oocyte, and thus avoid the possibility of passing damaged DNA to the offspring (Aitken *et al.* 1998a). The fragility of the messenger may be a safety valve ensuring that only the intact genetic message is delivered to the oocyte cytoplasm. If so, it is not entirely efficient, as demonstrated by the birth defects in animals resulting from fertilisations with aged sperm (Rowson 1973).

Unlike in somatic cells, DNA in mature sperm is not protected by repair enzymes (Hughes *et al.* 1998), but some protection from reactive oxygen species is assured by tight condensation of sperm chromatin. In raw semen, sperm are also protected from oxidative damage by various antioxidants present in seminal plasma (Dawra and Sharma 1985; de Lamirande and Gagnon 1993b; Hughes *et al.* 1998; Jeulin *et al.* 1989; Jones *et al.* 1979).

Further evidence of the detrimental role of reactive oxygen species on sperm comes from clinical research on male infertility (Geva *et al.* 1998). Several authors have reported that infertility in humans is correlated with the imbalance between production of reactive oxygen species and the antioxidant capacity of sperm and seminal plasma (Agarwal *et al.* 1994; Alkan *et al.* 1997; D'Agata *et al.* 1990; de Lamirande *et al.* 1995; Hendin *et al.* 1999; Iwasaki and Gagnon 1992; Lewis *et al.* 1995; Sharma *et al.* 1999; Smith *et al.* 1996).

Sperm do not possess effective mechanisms to counteract the generation of reactive oxygen species. They are also poorly protected by scavenging enzymes for reactive oxygen species in the cytoplasm, as most of the cytoplasm is discarded during spermatogenesis (Hughes *et al.* 1998). Exactly how ineffective are sperm in their ability to neutralise reactive oxygen species remains controversial. Some reports claim that sperm are completely devoid of catalase activity (Holland *et al.* 1982; Holland and Storey 1981). Other investigators have been able to detect catalase activity in human sperm (Jeulin *et al.* 1989), and noted that motility spontaneously recovered several hours after the arrest caused by reactive oxygen species, suggesting some form of intracellular protection against the effects of oxidative stress (Gagnon *et al.* 1991).

Although they posses glutathione peroxidase activity, the lack of glutathione makes this enzyme ineffective in removing H_2O_2 (Max 1992). A recent report has shown that when reactive oxygen species are generated by leukocytes, H_2O_2 is the most detrimental of all reactive oxygen species to the motility and energy metabolism of sperm, while $O_2^{-\bullet}$ and $^{\bullet}OH$ are not involved in oxidative injury (Armstrong *et al.* 1999). This is probably due to the fact that although $O_2^{-\bullet}$ and $^{\bullet}OH$ are violently reactive they are short lived and their damaging effects are limited to the site of their formation (in this case leukocytes). The effect of H_2O_2 on motility is probably due to the oxidation of sulfhydryl groups that have to be in a reduced form to maintain activity of enzymes crucial to motility (Armstrong *et al.* 1999). Catalase activity has been detected in bovine oviductal fluid and it is thought that its role is to protect sperm during transit, as it fluctuates during the oestrus cycle, reaching a maximum immediately prior to ovulation. Oviductal catalase binds to the acrosomal region of sperm (Lapointe *et al.* 1998). This binding is specific, as liver catalase does not bind to sperm (Lapointe *et al.* 1998).

1.3.4 Various approaches to limiting reactive oxygen species damage to sperm during *in vitro* storage

All cells living in aerobic environments are at risk of damage from the highly reactive oxygen species that are normal by-products of metabolism. In response to this risk, aerobic cells have evolved strategies to protect themselves from oxidation. These strategies include presence of intracellular catalase in liver and kidney cells, export of H_2O_2 to the blood stream by brain and lungs where it is neutralised by erythrocyte catalase, and the intracellular presence of glutathione and glutathione oxidase (Meister 1983). However, these protective mechanisms are either absent in semen as discussed

in the previous section or are insufficient in the contrived situation of artificial breeding because their concentration is reduced by dilution. It has been demonstrated in animal models that reducing oxidative stress by lowering metabolic rates through chronic food restrictions postpones the onset of senescence (Kohno *et al.* 1985; Sohal and Weindruch 1996). There are basically two possible strategies to reduce oxidative injury to cells preserved *in vitro*. The primary one is to eliminate the production of reactive oxygen species by arresting or reducing the rate of oxidative metabolism. The second strategy is to rapidly neutralise reactive oxygen species once they are produced to render them harmless. The first strategy can be pursued by cryopreservation, or in cells that are facultative anaerobes, by eliminating oxygen from the storage medium. Neutralisation of reactive oxygen species can be achieved by supplementing cell suspensions with various scavenging enzymes for reactive oxygen species, or with small molecular weight antioxidants. Both strategies have been applied to storage of sperm *in vitro*, either separately or in tandem.

In early diluents designed for liquid storage of bovine sperm, saturation with CO_2 was used to inhibit sperm metabolism (Salisbury *et al.* 1978). This inhibition may be due to lowering O_2 tension in the diluent and in part to reduction in pH (Foote 1964). Shannon (1965) showed that oxidative damage to stored sperm could be limited by reducing O_2 tension in the diluent with N_2 gassing.

Antioxidants are commonly added to sperm diluents. Ascorbic acid, alpha tocopherol and urate have been shown to protect human sperm DNA during handling procedures used during sperm preparation for IVF (Hughes *et al.* 1998). However, in diluent containing egg yolk, ascorbic acid was not beneficial to bovine sperm survival during *in*

vitro storage (Vishwanath *et al.* 1992). A commercial antioxidant solution (Sperm-Fit®) has been demonstrated to enhance human sperm survival during *in vitro* processing (Parinaud *et al.* 1997).

The positive effect of the addition of H_2O_2-destroying catalase to the storage diluent on sperm survival is well documented (MacLeod 1943; Macmillan *et al.* 1972; Shannon and Curson 1972; Tosic and Walton 1950) and in New Zealand it is routinely used in commercial liquid semen extender. It breaks down H_2O_2 present in the diluent outside the sperm cells regardless of its origin.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Macromolecular, enzymatic reactive oxygen species scavengers such as catalase and superoxide dismutase are relatively ineffective in preventing oxidative injury to sperm by the free radicals retained inside the cell (Sharma *et al.* 1999). Lopes and co-workers found that under conditions of *in vitro* exposure of sperm to exogenously generated reactive oxygen species, reduced glutathione and hypotaurine were protective against DNA fragmentation while catalase was ineffective (Lopes *et al.* 1998). They concluded that while H_2O_2 may be an important cause of lipid peroxidation, it has limited effect on chromatin (Lopes *et al.* 1998). However it is more likely that catalase does not decrease DNA fragmentation because it is not membrane-permeable and thus can not act inside the cell in close proximity to the chromatin where some H_2O_2 may still be present. The protective effect of hypotaurine is exerted through its ability to directly react with cytotoxic products of lipid peroxidation and thus protect thiol groups of sperm proteins (Lopes *et al.* 1998). Reduced glutathione has the ability to neutralise $O_2^{-\bullet}$, and is also a

substrate for glutathione peroxidase which neutralises H_2O_2 and $^{\bullet}OH$ (Lopes *et al.* 1998). Kodama and colleagues demonstrated a significant decrease of *in vivo* oxidative damage to the DNA of sperm from some sub-fertile patients after treatment with the antioxidants vitamin C, vitamin E and glutathione (Kodama *et al.* 1997). The antibiotic penicillin widely used in semen diluents to control bacterial growth also has antioxidative side effect (Al-Somai 1994).

1.4 AIMS OF THIS STUDY

The main aim of this study was to investigate the biochemical and physiological changes that take place in sperm during long term storage at ambient temperature and which may contribute to the drop in sperm fertility. Better understanding of these processes would assist in modifying storage conditions *in vitro* in such a way that the time span during which insemination doses retain fertility at a commercially acceptable level will be extended. In this study effort was concentrated on comparing parameters of sperm stored under aerobic and truly anaerobic conditions. Whereas in previous work nitrogen-gassed storage conditions were used to create "anaerobic" environment, which allowed some oxygen to be present, the aim in the present work was to incubate sperm under strictly anaerobic conditions thus eliminating all potential sources of reactive oxygen species. The biochemical and physiological parameters of sperm that were investigated in this study included: motility, plasma membrane permeability, chromatin structure, protein tyrosine phosphorylation and *in vitro* fertilising ability.

As a gradual decline in sperm motility over the time of storage under nitrogen-gassed conditions has been observed, measurements of changes in sperm swimming velocities

during storage at ambient temperature were performed to quantify this decline. A possible cause of this decline could be deterioration in the ability of sperm to generate ATP through mitochondrial respiration, caused by the oxidative damage due to incomplete elimination of oxygen from nitrogen-gassed medium. To test if the decline in motility upon storage could be ascribed to mitochondrial damage, a study of the motility of freshly ejaculated sperm under fully anaerobic and aerobic conditions was undertaken. If a fully functional respiratory system is necessary to maintain a high level of motility then the intensity of motility (sperm velocities) of freshly ejaculated sperm would be substantially higher under aerobic than under anaerobic conditions. Although there have been some investigations of bovine sperm motility under conditions that either allowed or prohibit aerobic respiration, the results reported were contradictory to each other (Hammerstedt *et al.* 1988; Prendergast 1994; Wilson 1985), so this study was also necessary to clarify those contradictions.

Protein tyrosine phosphorylation is involved in regulation of various cellular processes crucial to the fertility of sperm, therefore any changes in tyrosine phosphorylation of sperm proteins during storage could, potentially, be related to the decline in fertility. As the tyrosine phosphorylation state of a single cytosolic protein has been previously demonstrated to correlate with changes in motility of epididymal bovine sperm (Vijayaraghavan *et al.* 1997). In the present study the attempt was made to determine whether the decline in sperm motility that occurs during storage of ejaculated sperm correlates with the changes of tyrosine phosphorylation of this protein. During capacitation, changes in tyrosine phosphorylation of numerous sperm proteins are known to be regulated by the oxidative state of the medium (Leclerc *et al.* 1997). The

aim of the present study was to investigate whether a similar regulation of protein tyrosine phosphorylation occurs during storage at ambient temperature.

Damage to sperm DNA, including oxidative damage, has been demonstrated to result in diminished fertility (Lopes *et al.* 1998b). Therefore the present study attempted to determine if the diminishing fertility of stored semen is correlated with increased damage to sperm DNA. The exposure to different levels of reactive oxygen species in currently used diluent could potentially result in damage to sperm DNA. Thus studies of changes in sperm chromatin stability utilising the Sperm Chromatin Structure Assay (SCSA) were undertaken to determine whether the DNA damage induced by reactive oxygen species occurs in stored sperm. It was also attempted to distinguish between the direct effects of reactive oxygen species and the effects of nucleases on the integrity of sperm DNA during storage by incubating sperm with general nuclease inhibitor aurintricarboxylic acid under both aerobic and anaerobic conditions.

After three days of storage there is a considerable decline in the fertility of sperm used for artificial breeding. *In vitro* fertilisation can be used to investigate changes in fertilising ability of sperm. An *in vitro* fertilisation study was undertaken to compare the *in vitro* fertility of sperm stored at ambient temperature under aerobic, nitrogen-gassed and fully anaerobic conditions in order to establish if the elimination of oxidative damage would improve the maintenance of fertility of stored sperm. The second objective was to determine if the decline in *in vitro* fertility of stored sperm could be compensated by increasing the number of motile sperm per oocyte used in fertilisation drops.

Chapter 2

INVESTIGATION OF THE IMPORTANCE OF AEROBIC RESPIRATION AND OF MITOCHONDRIAL DAMAGE DURING STORAGE TO THE MOTILITY OF BOVINE SPERM

2.1 INTRODUCTION

It is widely accepted that sperm must be sufficiently motile to penetrate cumulus cells and the zona pellucida to allow merging with the oocyte membrane during fertilisation. It has been observed previously that during extended storage at ambient temperature (15°C to 21°C) the motility of bovine sperm gradually decreases (Vishwanath and Shannon 1997). A possible explanation for this decrease in motility of stored sperm is a gradual decline in the ability to generate ATP through mitochondrial respiration as a consequence of mitochondrial ageing (Cummins *et al.* 1994; Vishwanath and Shannon 1997). If that explanation were true then the aerobic rate of generation of ATP by sperm with undamaged mitochondria would have to be substantially higher than the anaerobic rate. Thus the swimming velocities of sperm freshly collected and diluted in a medium permitting both glycolysis and respiration would be significantly lower under fully anaerobic conditions than that under aerobic conditions.

There are marked differences between sperm of various species in their energy metabolism necessary for motility. Boar and guinea pig sperm can maintain motility only in the presence of O_2 regardless of the availability of glycolysable substrates (Frenkel *et al.* 1975; Nevo *et al.* 1970). Human sperm have been shown, by multiple exposure photography, to maintain similar velocities under aerobic and anaerobic

conditions if glycolysis is possible (Makler *et al.* 1992). Bovine sperm are motile under both aerobic and anaerobic conditions if glycolysable substrates are present in the diluent (Ford and Rees 1990). There are however contradictory reports on the ability of sperm to maintain the same intensity of motility under conditions that permit or prohibit aerobic respiration. A sharp decline in translational and rotational velocities of ejaculated bovine sperm after 15-40 minutes incubation at 37°C in an air-tight chamber was observed by Wilson (1985), Prendergast (1994), and Vishwanath and Shannon (1997). In the work of these authors the incubation medium contained 16.7 mmol/l D-glucose and velocities were measured using a Transit Time Laser Velocimeter. The length of time before the onset of decline in velocity was proportional to the initial O_2 concentration in the diluent. When sperm were removed from the airtight chamber and re-aerated velocities returned to the initial higher values. Wilson (1985) and Prendergast (1994) attributed the decrease in sperm velocities to the transition of metabolism from aerobic respiration to anaerobic glycolysis.

Contrary to the above results, Hammerstedt *et al.* (1988) reported that ejaculated bull sperm maintained the same progressive velocity and percentage of motile cells in both the presence and absence of aerobic respiration. In their experiments, respiration was prevented by the electron transport chain inhibitors rotenone and antimycin A. In their work velocities were measured by analysing microscopic video images of swimming sperm on a Cell Soft Motility Computer System (CRYO Resources Ltd., NY, USA).

The first objective of the present study was to quantify the known decline of sperm swimming velocities during storage at ambient temperature under standard nitrogen-gassed conditions.

The second objective was to determine if the decline seen during storage could be due to oxidative damage to mitochondria limiting ATP production for motility. Crucial to this was to determine whether the motility of sperm is lower when aerobic metabolism is prevented from occurring.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Analytical grade chemicals were used in this study unless otherwise stated. Citric acid and D-glucose were obtained from Riedel-deHaën AG, Seelze, Germany. O_2-free N_2 and an O_2-free pre-mixed gas blend of 5% H_2 and 95% N_2, were purchased from British Oxygen Co., Hamilton, New Zealand. Streptomycin sulphate, penicillin, bovine serum albumin (fatty acid free), n-[p-aminobenzenesulfonyl]acetamide (sulfacetamide), 2-deoxy-D-glucose, antimycin A, and rotenone, were purchased from Sigma Chemical Co., St. Louis, MO, USA. Tris-hydroxymethylamine (Tris) was purchased from United States Biochemical Corporation, Cleveland, OH, USA. Catalase was extracted, from livers of eighteen-month-old, disease-free bulls owned by Livestock Improvement Corporation, Hamilton, New Zealand using a method described by Summer and Mybrack (1951), and egg yolks were taken from hen eggs obtained from a local poultry farm. Sodium chloride was purchased from Scientific Supplies Ltd., Auckland, New Zealand. Absolute ethanol was purchased from Ajax Chemicals, Auburn, Australia. All other chemicals were purchased from BDH Chemicals Ltd., Poole, England.

2.2.2 Preparation of diluents

Compositions of semen diluents were based on 14G buffer (Shannon 1965), which consisted of tri-sodium citrate 68 mmol/l, glycine 133.2 mmol/l, D-glucose 16.7 mmol/l, di-potassium hydrogen orthophosphate 3-hydrate 35 mmol/l, glycerol 162 mmol/l, n-hexanoic acid 2.15 mmol/l, citric acid 0.66 mmol/l, magnesium chloride 2.2 mmol/l and calcium chloride 2.5 mmol/l, penicillin and streptomycin sulphate each 1.25 mega-units per litre, sodium sulfacetamide 0.1 μmol/l and catalase 800-1000 IU/ ml.

Modifications of the diluent for particular experiments are shown in Table 2.1. Diluent A was used for assessment of motility parameters in a medium that allowed both glycolysis and respiration. For measurements of motility parameters in a medium that allowed respiration only, Diluent B was used. In this diluent egg yolk was replaced with bovine serum albumin as a protective agent to avoid introducing any glycolysable substrate and 2-deoxy-D-glucose was included as a competitive inhibitor of glycolysis. Diluent C was used to assess the influence of mitochondrial electron transport chain inhibitors (rotenone and antimycin A) on sperm motility. These inhibitors were first dissolved in absolute ethanol and then added to 14G buffer containing 5% egg yolk. The final concentration of ethanol in Diluent C was 0.1%, and an equivalent amount was added to Diluent A for these experiments. In the diluents containing egg yolk, insoluble components were removed from suspension by centrifugation at 1000 *g* for 30 minutes in a bench-top centrifuge (Sorvall® TC6® Sorvall Products, L.P., Newton, CT, USA). All diluents were sterilised by filtration through sterile 0.2 μm filters (Whatman International Ltd. Maidstone, England).

Table 2.1 Variations of normal 14G buffer used for sperm incubations.

Component	Diluent A	Diluent B	Diluent C
D-Glucose	16.7 mmol/l	Nil	16.0 mmol/l
2-Deoxy-D-Glucose	Nil	3.0 mmol/l	Nil
Pyruvic Acid	Nil	9.00 mmol/l	Nil
Rotenone	Nil	Nil	4.00 μmol/l
Antimycin A	Nil	Nil	4.00 μmol/l
Bovine serum albumin	Nil	3.0%	Nil
Egg Yolk	5.0%	Nil	5.0%

2.2.3 Semen collection, evaluation and preparation

Semen was collected from mature dairy bulls using an artificial vagina, and ejaculates were assessed for concentration and percentage motile sperm. The concentration of sperm in the raw ejaculate was measured using a Semen Concentration Photometer (Instruments Medecine Veterinaire, L'Aigle-Cedex, France) according to manufacturer's instructions. The initial evaluation of motility was done immediately after collection by placing a drop of the semen into 3 ml of Diluent A. A drop of suspended semen was transferred to a pre-warmed slide and observed under a magnification of x 125 using an Olympus BH2 microscope equipped with phase contrast optics. Each ejaculate was given score for percentage motile and the quality of movement. The subjective motility score (SMS) reflected the speed and normality of motion and was marked out of 10 as follows:

SUBJECTIVE MOTILITY SCORE (SMS)	MOTILITY	IRREGULARITIES OF MOTILITY (eg. returned tails or shocked)
10	very fast	none
9	fast	few
8	moderately fast	few
7	moderate	noticeable
6		increasing
5		
4		
3	slow	
2	slight	irregular
1	very slight	very irregular
0	none	extremely irregular

Only ejaculates containing 80% or more motile sperm with SMS of at least 8 were used in the experiments.

After evaluation, semen was initially diluted into the appropriate diluents at 29°C, to a final concentration of 100 x 10^6 sperm/ml and allowed to slowly cool down to room temperature (18-20°C). For measurements of sperm motility parameters in a medium that does not support glycolysis, 15 ml of semen diluted in Diluent B was centrifuged at 400 *g* for 5 minutes in a Sorvall® TC6® centrifuge using conical bottom tubes. The sperm pellet was re-suspended again in same volume of Diluent B. This washing process was repeated three times to remove any glycolysable substrates originating from seminal plasma. Semen was then diluted at room temperature to a final concentration of 7 x 10^6 sperm/ml.

To measure sperm motility parameters under anaerobic conditions, dilutions were performed with the diluents that had been made anaerobic by purging with the gas mixture contained inside the anaerobic chamber (Section 2.3.2) for sixteen hours, and the secondary dilution was completed inside the anaerobic chamber.

2.2.4 Measurements of the swimming velocities of sperm with computer aided sperm analysis (CASA)

Precise measurements of sperm motility parameters became possible with the advent of Computer-Assisted Sperm Analysis (CASA) technology. In this study sperm swimming velocities were measured with the Hobson Sperm Tracker (Hobson Tracking Systems Ltd., Sheffield, U.K.), one of the most advanced CASA machines. This system continuously tracks up to 250 sperm in real time, analysing movement parameters as they pass through the field of view of the microscope.

To obtain objective measurements of sperm swimming velocities with the CASA system, microscopic video recordings of swimming sperm were made using a video camera Model KP-C200/201/205 (Hitachi Ltd., Tokyo, Japan) connected to an Olympus BH2 microscope (Olympus, Optical Co., Ltd., Tokyo, Japan). The video camera was attached to a Camera Control Unit Model KP-C200/201/205 (Hitachi Ltd., Tokyo, Japan) and a video cassette recorder (Sharp Corporation of New Zealand Ltd., Auckland, New Zealand). The microscope was equipped with a warm stage, which maintained a temperature of 37°C ± 1°C. Video recordings of 1.5 minute duration were made using a 20 x negative phase objective. Disposable Semen Analysis Chambers of 20 μm depth (Fertility Technologies Inc., Natick, MA, USA) were used for sperm

motility analysis. The volume of the analysed drop was 7 µl. The analyser was calibrated for linear distances using a haemocytometer grid (Neubauer, Germany).

Two types of swimming velocities were analysed, *i.e.* curvilinear velocity (VCL) and straight line velocity (VSL), from the video recordings. Velocities of all the sperm tracks from each 1 minute time window of 1.5 minute recording were measured using the following analysis settings: minimum tracking time 0.3 s, maximum tracking time 40 s, search radius 18.50 µm, framing rate 50 Hz. Median values of VCL and VSL were obtained from all measured individual sperm tracks.

2.2.5 Statistical analysis of results

The results from the Hobson Sperm Tracker were analysed by a least squares analysis of variance (SAS Institute, Cary, NJ, USA), fitting the effects of treatment (anaerobic and aerobic) using VSL and VCL as the dependent variables. The bull effects were treated as a random variable (n=3) and time as a covariate. The interactions between time and treatment were also tested and regression estimates obtained to determine parallelism. Trend lines (linear or third order polynomial) were fitted to obtain the best fit for the data. The differences in means for all other results were subjected to a 't' test.

2.3 EXPERIMENTS

2.3.1 Measurements of changes in sperm motility during extended storage at ambient temperature under standard (partially anaerobic) nitrogen-gassed conditions

In the first experiment, sperm diluted in Diluent A were stored under standard storage conditions used for artificial insemination at ambient temperature. Diluted semen was stored in tightly sealed 100 ml sterile Schott bottles (Schott Glaswerke, Mainz, Germany) wrapped in aluminium foil with the headspace of the bottle filled with nitrogen. To assess changes in swimming velocities of sperm over a 14-day period of storage, aliquots were taken from the bottles on designated days for measurements of swimming velocities by CASA under aerobic conditions. The headspace of the bottles was refilled with N_2 each time an aliquot was taken.

2.3.2 Measurements of the swimming velocities of sperm under fully anaerobic and fully aerobic conditions in diluent allowing glycolysis

In the second experiment, sperm collected from three bulls were separately incubated at 37°C, and their swimming velocities measured, under anaerobic and aerobic conditions for over 2 h in Diluent A. During each incubation, aliquots were drawn every 2-4 minutes for measurements of the swimming velocities of sperm to determine if the velocities are lowered by the elimination of the oxygen from the diluent forcing sperm to rely solely on glycolysis for energy generation.

Microscopic video recordings of swimming sperm for measurements of velocities under anaerobic conditions were made inside a microbiological anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA) filled with a gas mixture of 5% H_2 and

95% N_2. Absence of oxygen in the atmosphere in the anaerobic chamber was confirmed by use of Anaerobic Indicator BR55 (Unipath Ltd., Basingstoke, England). The chamber was equipped with an air lock to allow transfer of equipment and solutions without contaminating the internal atmosphere with air. A positive pressure was maintained inside the chamber limiting the possibility of air access to the interior. Any residual O_2 inside the chamber was reduced to water by H_2 in the gas mixture with the help of a palladium catalyst. The oxygen concentration in the diluents was measured with a Dissolved Oxygen Meter Model 57 (Yellow Springs Instruments Inc., Yellow Springs, OH, USA). Measurements of swimming velocities of sperm under aerobic conditions were made from video recordings of swimming sperm performed under conditions of exposure to air.

2.3.3 Measurements of the swimming velocities of sperm under fully anaerobic and fully aerobic conditions in the diluent prohibiting glycolysis

In the third experiment, sperm motility was recorded under both anaerobic and aerobic conditions, as in the second experiment, but in Diluent B that does not support glycolysis. This was to confirm that the anaerobic conditions under which velocities measurements were taking place were not allowing any respiration to take place. Therefore, under truly anaerobic conditions sperm with inhibited glycolysis would have no means to generate ATP and would lose motility. Recording continued until all motility in the anaerobic medium ceased. This took approximately 45 min. Immediately after cessation of motility, the semen was removed from the anaerobic chamber and re-aerated by moderate shaking with the contents of the test tube exposed to air. Swimming velocities of sperm from re-aerated samples were then recorded as described in Section 2.2.4 for five aliquots of sperm from each of three bulls.

2.3.4 Measurements of swimming velocities of sperm in the presence and absence of electron transport chain inhibitors

In the fourth experiment velocities were measured under aerobic conditions as described in Section 2.2.4 for twelve aliquots of sperm from each of three bulls. For this experiment semen was diluted in the diluent containing electron transport chain inhibitors (Diluent C) and in control medium (Diluent A) containing 0.1% ethanol.

This was to confirm, independently from the experiments using exclusion of oxygen from the medium to inhibit respiration, that the sperm could maintain the same level of motility with and without respiration.

2.3.5 Assessment of mitochondrial functionality

After the experiments described in Sections 2.3.2 - 2.2.4 had been carried out the mitochondrial function of the sperm used in those experiments was assayed at the end of each incubation in diluents A, B and A containing 0.1% ethanol. After the end of incubations, sperm were washed from their incubation medium into Diluent B by centrifuging for 5 minutes in a Sorvall® TC6® centrifuge at 400 *g* and re-suspending the sperm pellet in Diluent B. This process was repeated three times to ensure that there was no residual sugar in the final suspension. The washing procedure was unnecessary for sperm that were already incubated in diluent B. Sperm were then incubated at 37°C ± 1°C for 10 minutes and the motility was assessed visually. In this medium, motility could be supported only by mitochondrial respiration. To confirm that no glycolysis was taking place, motility was evaluated again after antimycin A and rotenone was added to aliquots to the final concentration of 4 μmol/l each.

2.4 RESULTS

2.4.1 Motility of sperm during extended storage at ambient temperature under standard nitrogen-gassed conditions

During storage both Curvilinear Velocity (VCL) and Straight Line Velocity (VSL) declined over first four days and then remained quite stable until day eleven of storage (Figure 2.1).

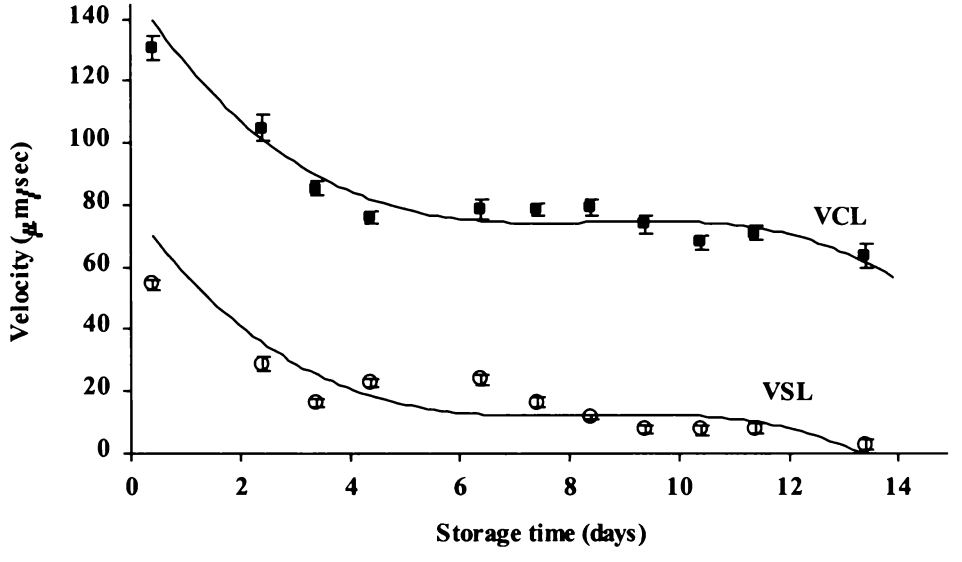


Figure 2.1 Changes in velocities during storage under nitrogen-gassed conditions of sperm from three bulls (VCL ■, VSL ○).

2.4.2 Motility of sperm under fully aerobic and fully anaerobic conditions in the diluent allowing glycolysis (Diluent A)

The concentration of O_2 in the diluent at 21°C under aerobic conditions was 7.9 mg/l (89% saturation) while at the same temperature, under anaerobic conditions the O_2 levels were below the detection limits of the Dissolved Oxygen Meter (<0.1 mg/l, 1.1% saturation).

Values of mean curvilinear velocities (VCL) and mean straight line velocities (VSL) were significantly higher ($p<0.05$) for sperm cells recorded under anaerobic conditions compared with those under aerobic conditions (Figure 2.2).

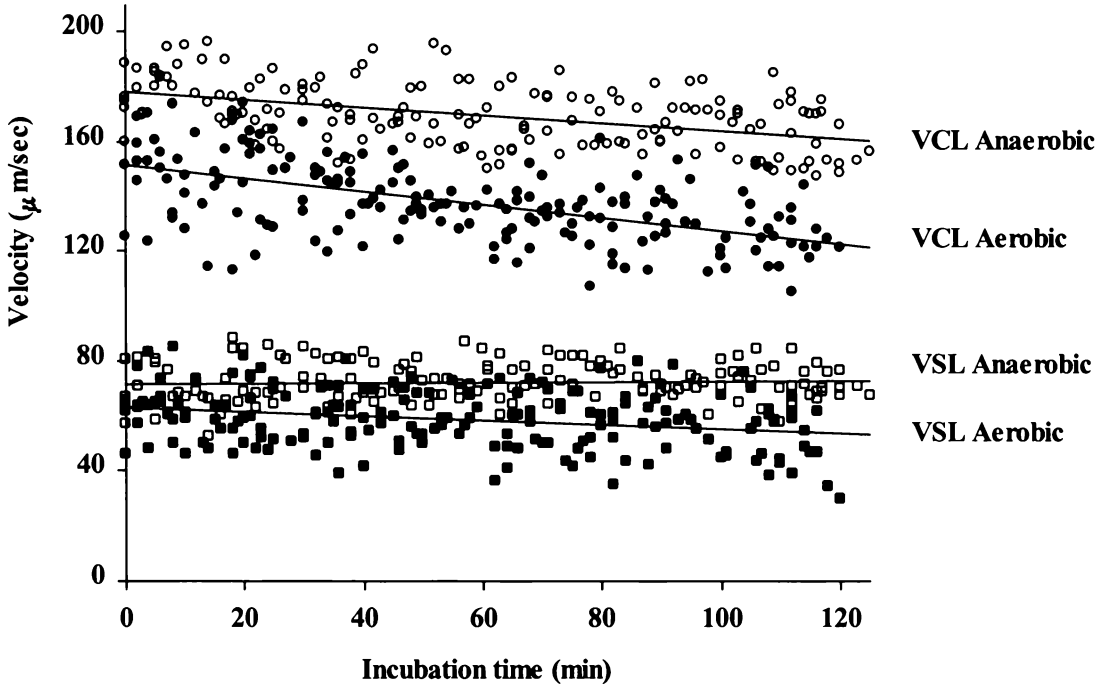


Figure 2.2 VCL and VSL of sperm tracks from three bulls. Semen was diluted and analysed in a medium that allows glycolysis (Diluent A). Each point represents the mean of velocities of all the sperm tracks analysed in each 1 minute time window under anaerobic (VCL ○, VSL □) and aerobic (VCL ●, VSL ■) conditions.

The regression estimates for VCL were not significantly different between aerobic and anaerobic incubations (Table 2.2) but the rate of decline of VSL was significantly higher for sperm incubated under aerobic than anaerobic conditions over the incubation period ($p<0.01$).

Table 2.2 Regression estimates of changes in sperm velocities with time under aerobic and anaerobic conditions in the medium that allows glycolysis (Diluent A) (n=3). * Significant difference between aerobic and anaerobic treatments (P<0.01).

	VCL (μm/sec/min)	VSL (μm/sec/min)
Anaerobic	-0.1635	-0.0116
Aerobic	-0.2157	-0.0600*

2.4.3 Motility of sperm under fully aerobic and fully anaerobic conditions in the diluent prohibiting glycolysis (Diluent B)

The concentration of oxygen in the diluent at 21°C under aerobic conditions was 8.0 mg/l (89% saturation) and at the same temperature, under anaerobic conditions the dissolved O_2 concentration was below the sensitivity of the Dissolved Oxygen Meter (<0.1 mg/l, 1.1% saturation). Sperm were washed in the diluent prepared under anaerobic conditions, however the washing procedure involving centrifugation and re-suspension was performed under aerobic conditions. After washing into Diluent B sperm were transferred into the anaerobic chamber and diluted 1:14 in O_2 free Diluent B. During this final dilution of semen into the anaerobic diluent inside the anaerobic chamber, a small amount of dissolved oxygen would have been carried into the final suspension from the washing procedure. Initially values of VCL and VSL of sperm measured under aerobic and anaerobic conditions were about the same (Figure 2.3). Under aerobic conditions the velocities did not change during incubation. In contrast, velocities declined sharply after 25 to 30 minutes of incubation in the anaerobic diluent. Sperm forward motility (VSL) ceased after 35 to 45 minutes.

VCL and VSL values of sperm measured in Diluent B on re-aeration after anaerobic incubation were not significantly different (P>0.05) from the initial aerobic velocities of

sperm from the same ejaculate measured at the beginning of the incubations (Table 2.3). Only the initial VSL of sperm from bull II was significantly higher than that of re-aerated.

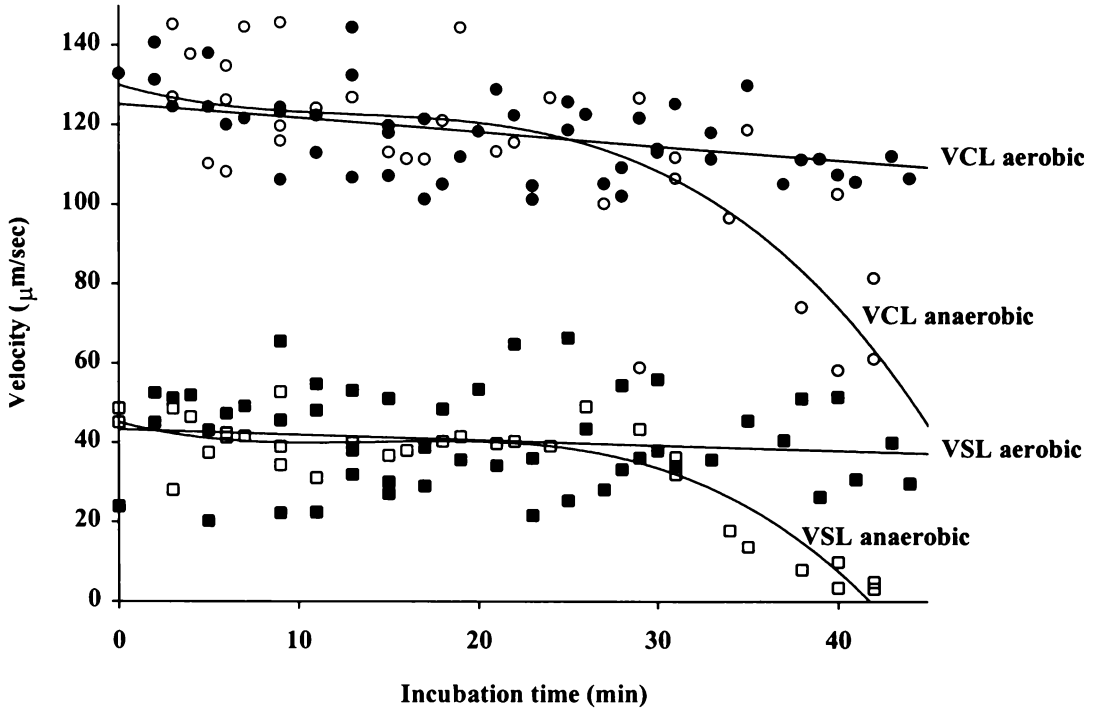


Figure 2.3 Changes in velocities of sperm from three bulls incubated in the medium that prevents glycolysis (Diluent B) under anaerobic (VCL ○, VSL □) and aerobic (VCL ●, VSL ■) conditions. Data was fitted to a linear or a cubic order polynomial trend line to obtain best fit.

Table 2.3 Curvilinear Velocity (VCL) and Straight Line Velocity (VSL) values (n=5, mean ± s.d.) of sperm from three bulls incubated in Diluent B that did not support glycolysis, under anaerobic conditions and reactivated after cessation of motility by introducing O_2 to the medium. Values marked with * are significantly different from each other ($P<0.05$).

	Initial aerobic velocities (μm/sec)		Velocities after re-aeration (μm/sec)	
	VCL	VSL	VCL	VSL
Bull I	118.9±10.19	27.7±6.24	113.0±10.01	26.8±5.59
Bull II	119.6±6.24	56.2±6.14*	121.2±16.12	38.1±2.69*
Bull III	124.4±12.11	38.5±5.12	120.3±13.14	39.6±3.08

2.4.4 Motility of sperm under fully aerobic conditions in the medium containing respiratory inhibitors (Diluent C)

There was no significant difference (P>0.05) between VCL and VSL values of sperm measured in Diluent C (containing 4 μmol/l antimycin A and 4 μmol/l rotenone) and those of sperm from same ejaculates incubated in control Diluent A (Table 2.4). Both diluents contained 16.7 mmol/l glucose, 0.1% ethanol and were 89% saturated with O_2.

Table 2.4 Mean curvilinear velocity (VCL) and straight line Velocity (VSL) values (n=12, mean ± s.d.) of sperm from three bulls measured in the presence and absence of electron transport chain inhibitors, in diluent A containing glucose. There is no significant difference (P>0.05) between values of VCL and VSL of sperm from the same bull in the presence or absence of inhibitors.

	VCL (μm/sec)		VSL (μm/sec)	
	Antimycin and Rotenone (4 mol/l) each	Control	Antimycin and Rotenone (4 mol/l) each	Control
Bull I	114.9±15.8	113.8±21	49.6±7.3	43.3±7.3
Bull II	143.5±16	133.6±12.7	39.7±12.7	44.7±12
Bull III	122.7±10.5	116.7±6.9	35.5±5.6	35.7±5.4

2.4.5 Mitochondrial functionality

When, at the end of each experiment, sperm were washed and re-suspended in the medium that does not support glycolysis (Diluent B) at least 70% of the sperm were motile (Table 2.5). Their motility was terminated immediately after addition of rotenone and antimycin A to the diluent, demonstrating that no ATP was generated through the glycolytic pathway after sperm were washed and re-suspended into Diluent B. This demonstrated that sperm were capable of mitochondrial respiration during the experiments.

Table 2.5 Mitochondrial functionality (percentage of motile sperm) after incubation and motility assessment under aerobic and anaerobic conditions in Diluents A and B and in Diluent A containing 0.1% ethanol. Sperm were subsequently washed into Diluent B to inhibit glycolysis (as described in Section 2.2.7) and motility estimated visually in the presence and absence of electron transport chain inhibitors (4 µmol/l antimycin A and 4 µmol/ rotenone).

	A		B		A + 0.1% ethanol	
	No inhibitors	With inhibitors	No inhibitors	With inhibitors	No inhibitors	With inhibitors
Bull I anaerobic	70	0	75	0		
Bull I aerobic	73	0	73	0	70	0
Bull II anaerobic	73	0	70	0		
Bull II aerobic	73	0	75	0	75	0
Bull III anaerobic	70	0	70	0		
Bull III aerobic	73	0	70	0	75	0

2.5 DISCUSSION

The gradual decline in the percentage of sperm motile during extended *in vitro* storage at ambient temperature has been well documented in the past (Vishwanath and Shannon 1997). Subjectively estimated decline in sperm velocities during storage has also been previously reported but not quantified (Shannon and Pitt; personal communications). Unlike the decline in the percentage motile, the loss of swimming velocities observed in the present study occurred mostly during the first three days of storage and velocities remained fairly constant for about ten days afterwards (Figure 2.1). But an important factor to consider is that velocity values derived from CASA measurements represent the average of the velocities of the sperm that retain motility, and not that of whole

population, *i.e.* they do not include immotile sperm with velocities equal to zero. That may explain why, in the present study, after the initial drop, the velocities remained almost constant, albeit at the lower, level as the sperm that became immotile were excluded from the population measured.

The initial hypothesis to explain the decline in motility of stored sperm was that during storage sperm mitochondria are damaged causing a motility-limiting deterioration in ability to produce ATP. Sperm with damaged mitochondria would rely solely on the energetically less efficient process of glycolysis for their supply of ATP. If that were true, inhibiting respiration through exclusion of oxygen or inhibition of the electron transport chain in fresh sperm would cause a significant drop in sperm velocities. If, however, bovine sperm could produce enough ATP through anaerobic glycolysis to ensure that the intracellular ATP concentration does not limit motility, one would expect velocities to be the same under anaerobic and aerobic conditions.

The present study demonstrated that the initial hypothesis explaining decline in sperm motility during storage by the loss of mitochondrial function was incorrect, because in the medium containing glucose, sperm velocities were slightly higher under anaerobic than under aerobic conditions (Figure 2.2). This being higher is probably due to experimental conditions rather than a physiological phenomenon. The precision of temperature control of both microscope warm stages was ± 1°C. This could possibly mean a maximum difference in analysis temperature of 2°C between anaerobic and aerobic measurements. Sperm velocities are highly sensitive to temperature (Hammerstedt and Hay 1980; Inskeep and Hammerstedt 1985). However, O_2 is known to have a detrimental effect on sperm (Aitken and Clarkson 1988; Alvarez *et al.* 1987).

Sperm motility could have been compromised by exposure to O_2 during the time between collection and velocity measurements. Furthermore, the rate of decline in VSL during incubation is higher under aerobic conditions, suggesting that O_2 was indeed damaging the sperm.

In Diluent A, which contained glucose, there was no significant difference in VCL and VSL values when respiration was stopped by electron transport chain inhibitors. In Diluent B where glycolysis was completely inhibited by excess 2-deoxy-D-glucose, motility stopped immediately after addition of rotenone and antimycin A. These results agree with a report which showed no difference in the motility of bovine sperm when incubated in presence of glycolysable substrate, in the absence and presence of rotenone and antimycin A (Hammerstedt *et al.* 1988) but contradict the conclusion of Wilson (1985) and Prendergast (1994) that bovine sperm require O_2 to maintain a high level of motility. The possibility that the diluent used in the anaerobic incubations in the present study contained O_2 was excluded by the evidence that when sperm were incubated under the same experimental conditions in a medium devoid of glucose and containing excess of 2-deoxy-D-glucose, motility was not sustained. The cessation of motility could not have been an effect of 2-deoxy-D-glucose on anything other than glycolysis as motility was restored by re-aeration.

The high initial motility of sperm in the diluent that prohibits glycolysis (experiment three) under anaerobic conditions was probably the result of a small amount of O_2 being introduced when the diluent was exposed to air during centrifugation and re-suspension of the pellet as part of the washing procedure. When human sperm have been incubated under similar conditions, motility has declined rapidly after 30 minutes (Makler *et al.*

1992). However, the time-course of decline is unclear because motility was assessed in 30 minutes time windows. The authors concluded that the initial motility was supported by intracellular stores of ATP, which were rapidly exhausted. The authors did not attempt to eliminate O_2 from the diluent prior to the incubation under 95% N_2 and 5% CO_2 thus the initial motility might have been supplied with energy by respiration using residual O_2 until it was consumed (Makler *et al.* 1992).

When glycolysis was prevented in anaerobic incubations, sperm became immotile after 35 to 45 minutes (Figure 2.3). Similar results for bovine sperm were reported by Lardy and Phillips (1942). In this study motility was restored, in two bulls, to the same level as the initial motility of sperm incubated under aerobic conditions (Table 2.5) by removing them from the anaerobic chamber and re-aerating, thus confirming that the loss of motility was due to lack of O_2 and glycolysable substrate and therefore an inability to generate ATP. VSL values of sperm from the third bull after re-aeration were significantly lower than the initial aerobic VSL. Nevertheless, vigorous motility was restored from the state of complete immotility. Lowering of VSL may be attributed to the damage incurred by the sperm during short periods of anoxia in the medium that does not support glycolysis, the sperm being unable to generate energy through either respiration or glycolysis. Alternatively, motility might have been slightly compromised by handling between removing from the anaerobic chamber and motility analysis after re-aeration. Sperm incubated in the medium containing glucose did not have to be washed before incubations, therefore no O_2 was introduced to the anaerobic incubations, and they maintained stable velocities for over 120 minutes. This reiterates the point that they have been incubated under truly anaerobic conditions in the presence of glycolysable substrate (Figure 2.2).

Values of VCL and VSL of sperm incubated under aerobic conditions in Diluent B which prevents glycolysis remained almost constant for 60 minutes (Figure 2.3). It is clear that bovine sperm can sustain motility through either glycolysis alone, respiration alone or combination of the two processes provided an appropriate substrate is supplied. It is possible to argue that if sperm mitochondria were rendered dysfunctional during handling, sperm velocities would be the same under aerobic and anaerobic conditions because, irrespective of O_2 concentration in the medium all the ATP available to the sperm would need to be generated by glycolysis. Sperm with damaged mitochondria however, would be immotile in the medium that does not permit glycolysis. In experiments presented here sperm were shown to have functional mitochondria by demonstrating motility in aerated Diluent B at the end of each incubation (Table 2.5).

Most of the ATP produced by sperm is converted to mechanical force by the dynein ATPase of the flagellum. Estimations of actual proportion of the total ATP used for motility range from 70 to 80% (Ford and Rees 1990). Under aerobic conditions at least 10% of the total O_2 uptake is used for processes other than motility (Bohnensack and Halangk 1986).

In theory, respiration is far more efficient than glycolysis in generating ATP. When glucose is a substrate, a theoretical yield from glycolysis is 2 molecules of ATP for each molecule of glucose degraded to lactate while, oxidation to CO_2 theoretically yields 38 molecules of ATP for each molecule of glucose oxidised. In addition, in the presence of O_2, other substrates such as glycerol and phospholipids can be oxidised to generate energy. Under aerobic conditions bovine sperm convert 85% of available glucose to lactate and excrete it to the surrounding diluent: the balance is oxidised to CO_2

(Hammerstedt and Lardy 1983). Other estimations of partition of glucose catabolism in ejaculated bovine sperm suggest that 16% of glucose is oxidised to CO_2, 18% is unaccounted for and 66% is catabolised to lactate (Voglmayr and Amman 1973). Despite the overwhelming proportion of glucose being metabolised to lactate, the ATP yield from this pathway under aerobic conditions is very low in bull sperm. Calculations of the proportion of ATP derived from glycolysis under aerobic conditions by bull sperm range from 18% (Voglmayr and Amman 1973) to less than 5% (Inskeep and Hammerstedt 1985) to none (Hammerstedt and Lardy 1983). Under aerobic conditions the bulk of ATP is generated by mitochondrial respiration. In contrast, human sperm obtain 80% of ATP from the glycolytic pathway under aerobic conditions (Ford and Rees 1990).

The detailed study of bovine sperm metabolism by Hammerstedt *et al.* (1988) revealed that under aerobic conditions at 37°C, there is no net ATP gain from degradation of glucose to lactate, and all ATP available to the cell is produced by mitochondrial respiration. The reason for the nil net ATP yield from glycolysis is an extensive substrate cycling in ejaculated bull sperm. They have also demonstrated that the actual net ATP yield from mitochondrial oxidation of 1 molecule of glucose to CO_2 is only 2.9 molecules of ATP. This is far less than the theoretical yield of 38 ATP molecules gained by oxidation of 1 molecule of glucose. Under anaerobic conditions or when mitochondrial respiration is eliminated by inhibitors, all the glucose is catabolised to lactate, which is excreted to the medium. When mitochondrial respiration is inhibited by rotenone and antimycin A, ATP-consuming substrate cycling of the glycolytic pathway is eliminated and the net ATP yield of glycolysis increases to 1.83 molecules of ATP for each molecule of glucose catabolised to lactate, which is very close to

theoretical maximal yield of 2 ATP molecules. The ATP yield from glucose obtained by glycolysis under anaerobic conditions is thus only slightly lower than the actual ATP yield of respiration. It is clear that aerobic respiration in sperm is only slightly more efficient than anaerobic glycolysis. Moreover, the flux through the Embden-Meyerhof pathway is considerably stimulated by inhibition of respiration (Hammerstedt and Lardy 1983; Hammerstedt *et al.* 1988; Lardy and Phillips 1943).

Sperm motility is independent of concentration of ATP or ATP/ADP ratio above a given threshold. This suggests that supply of energy does not normally limit sperm motility (Ford and Rees 1990). Increased flux through the glycolytic pathway together with increased efficiency due to reduced substrate cycling under anaerobic conditions could provide sperm with sufficient energy to sustain motility with velocities similar to those of sperm utilising aerobic metabolism.

The ability of bovine sperm to meet all their energy needs from anaerobic metabolism is interesting in the light of the fact that once sperm leave seminal plasma, which occurs soon after ejaculation, they are in an environment where concentrations of potential energy substrates are low (Gibbons *et al.* 1974). Exogenous substrates for respiration are practically absent in cervical mucus. This indicates that bovine sperm probably depend on endogenous reserves of phospholipids to maintain *in vivo* motility after leaving seminal plasma and before fertilisation. The anaerobic mode of energy generation for sustaining motility and viability may be utilised by sperm in fructose-rich seminal plasma where they are present at high concentrations and where O_2 depletion is very rapid. It appears that conditions in the female genital tract are sufficiently aerobic to support oxidation of sperm intracellular stores of phospholipids (Hartree and Mann

1961). The available data on O_2 concentration in the female reproductive tract of rhesus monkeys, hamsters and rabbits indicate that sperm respiration is possible in this environment (Fischer and Bavister 1993).

The damage to the sperm from reactive oxygen species produced via mitochondrial respiration may well be a factor in the decline of fertility during storage but it seems that it is not likely to be the cause of a motility limiting loss of ability to produce ATP.

2.6 CONCLUSIONS

Bovine sperm can maintain a similar level of motility aerobically and anaerobically if a glycolysable substrate is available. Published data on bovine sperm energetics support this view. The complete elimination of respiration, by the exclusion of oxygen from the medium, does not seem to harm the ability of sperm to sustain adequate energy production to support its most energy-demanding function that is, motility.

Chapter 3

INVESTIGATING IF PROTEIN TYROSINE PHOSPHORYLATION DURING *IN VITRO* STORAGE OF EJACULATED BOVINE SPERM IS REGULATED BY THE OXIDATIVE STATE OF THE MEDIUM

3.1 INTRODUCTION

Sperm motility declines during storage at ambient temperature (18-20°C) due to *in vitro* ageing (Vishwanath and Shannon 1997) and also during short-term incubation at 37°C (Brandt and Hoskins 1980). This decline can be reversed to an extent by incubation with phosphodiesterase inhibitors such as theophylline (Brandt and Hoskins 1980), which increases intracellular concentration of cAMP. The exact mechanism of action of cAMP in sperm is still unknown (Vijayaraghavan *et al.* 1997). A complex network involving cAMP, calcium, protein tyrosine kinase/phosphatase and PKA pathways probably controls sperm motility in various species. There is evidence that bovine caudal and caput epididymal sperm motility is closely related to the cAMP-dependent tyrosine phosphorylation state of a soluble protein with M_r=55 000 (Vijayaraghavan *et al.* 1997). This protein was recently identified as glycogen synthase kinase-3 α (Vijayaraghavan *et al.* 2000). In trout sperm, tyrosine phosphorylation of an axonemal protein with M_r=15 000 activates motility of quiescent cells (Hayashi *et al.* 1987). Similarly, Ciona sperm motility has been shown to be dependent on protein tyrosine phosphorylation (Dey and Brokaw 1991), and this process is also involved in maintenance of flagellar movement of fowl sperm (Ashizawa *et al.* 1989).

A number of proteins from whole sperm extracts display a time-dependent increase in tyrosine phosphorylation under capacitating conditions. It has been demonstrated that inhibition of phosphodiesterase induces an increase in tyrosine phosphorylation in ejaculated bovine sperm during capacitation (Galantino-Homer *et al.* 1997).

There is growing evidence that reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical and superoxide, apart from being detrimental to sperm, also play a positive physiological role in capacitation (de Lamirande *et al.* 1993; de Lamirande and Gagnon 1993a; de Lamirande and Gagnon 1993b; de Lamirande and Gagnon 1993c; de Lamirande *et al.* 1998a). The increase in protein phosphorylation during capacitation and the role of ROS in capacitation are probably interrelated. Under some conditions capacitation is mediated by ROS-regulated protein tyrosine phosphorylation (Aitken *et al.* 1995; Aitken *et al.* 1996). Generally, oxidising conditions were found to promote tyrosine phosphorylation and stimulate sperm function whereas an opposite effect was observed under reducing conditions when sperm were incubated in a medium promoting capacitation (Aitken *et al.* 1995). ROS also regulate protein tyrosine phosphorylation in other types of cells such as H-35 rat hepatoma cells (Koshio *et al.* 1988).

The raising of cAMP levels has been demonstrated to restore declining motility and increase protein tyrosine phosphorylation (Brandt and Hoskins 1980; Vijayaraghavan *et al.* 1997). There is a significant decline in sperm motility during extended storage *in vitro*. Part of this investigation was to determine the regulatory role of protein tyrosine phosphorylation in the storage-related decline of motility.

If tyrosine phosphorylation of soluble proteins declined in parallel with the decline in motility seen during *in vitro* storage of sperm at ambient temperature, the hypothesis was that this decline should at least to some extent be reversed by increasing intracellular concentration of cAMP through phosphodiesterase inhibition.

The other hypothesis was that there will be changes in tyrosine phosphorylation of proteins from whole sperm extracts and that the pattern of these changes would be significantly affected by both phosphodiesterase inhibition and oxygen tension in the storage medium. To test this, sperm were stored with and without a phosphodiesterase inhibitor under standard nitrogen-gassed storage conditions to determine if the tyrosine phosphorylation of sperm proteins alters upon storage. In a separate experiment, the impact of complete elimination of oxygen from the storage medium on the tyrosine phosphorylation of proteins extracted from sperm was investigated. The motility and viability of sperm during storage at ambient temperature under aerobic and fully anaerobic conditions was also monitored in this experiment to determine if the completely anaerobic storage conditions were not harmful to sperm survival. Changes in the pattern of protein tyrosine phosphorylation in whole cell extracts from sperm resulting from storage under these conditions were determined.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Analytical grade chemicals were used in this study, purchased from suppliers as specified in Section 2.2.1. Additional ones used here, EGTA, theophylline, sodium orthovanadate, O-phospho-DL-tyrosine, n-[p-Aminobenzenesulfonyl], were purchased

from Sigma Chemical Co., St. Louis, MO, USA. Bovine serum albumin was purchased from Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany. Tris was purchased from United States Biochemical Corporation, Cleveland, OH, USA. Anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology Inc., Lake Placid, NY, USA. Complete™ protease inhibitor cocktail tablets were purchased from Boehringer Mannheim, GmbH, Germany. Pre-cast continuous gradient (10%-20%) gels for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), supported nitrocellulose membrane, Laemmli sample buffer, 2-mercaptoethanol, Silver Stain Plus Kit, and polyoxyethylene sorbitan monolaurate (Tween® 20) were purchased from Bio-Rad Laboratories, Hercules, CA, USA. Horseradish peroxidase conjugated anti-mouse sheep antibody was purchased from AMRAD-Silenus, Melbourne, Australia. Enhanced chemiluminescence (ECL) Western blot detection reagents were purchased from Amersham International Plc., Buckinghamshire, England. X-OMAT™ AR Scientific Imaging Film was purchased from Eastman Kodak Company, Rochester, NY, USA. Propidium iodide (PI) and SYBR-14 were purchased as *Ferti*Light™ Sperm Viability Kit from Molecular Probes, Inc., Eugene, OR, USA.

3.2.2 Preparation of diluents

The semen diluent used was 14G buffer (Shannon 1965). Its preparation is described in Section 2.2.2. When standard storage conditions were used, the diluent was purged with oxygen-free nitrogen for 30 minutes to reduce oxygen concentration. For experiments requiring anaerobic storage conditions, the diluent was purged with oxygen-free nitrogen for 30 minutes and placed inside an anaerobic chamber as

described in Section 2.2.3. When sperm were stored under aerobic conditions, the diluent was not gassed at all.

For motility analysis, bovine serum albumin was added to the diluent to a final concentration of 1% to prevent sperm from adhering to glass slides.

3.2.3 Semen collection, evaluation and preparation

Semen collection and evaluation procedures described were as in Section 2.2.3. For all treatments, semen was initially diluted at 32°C in an anaerobic diluent, to a concentration of 240 x 10^6 sperm cells/ml, and allowed to cool to ambient temperature (18-20°C). It was then diluted again to a final concentration of 20 x 10^6 sperm cells/ml, in either aerobic, nitrogen-gassed or anaerobic diluent at ambient temperature. Diluted semen were stored at ambient temperature in sealed 100 ml sterile Schott bottles wrapped in aluminium foil, under aerobic, anaerobic or nitrogen-gassed conditions, depending on the experiment.

For storage under aerobic conditions the bottles in which semen was stored were open daily and aerated by gentle shaking. Under nitrogen-gassed conditions, the headspace of the bottles was filled with oxygen-free nitrogen. The anaerobic and nitrogen-gassed storage bottles were shaken with the caps sealed. For storage of sperm under anaerobic conditions, they were suspended in an anaerobic medium and stored inside an anaerobic chamber.

3.2.4 Assessing percentage of sperm with intact plasma membrane by flow cytometry

Flow cytometry is an instrumentation technology that allows examination of cell properties in fluid suspension. During flow cytometric examination, cells are carried at high speed by a fluidics system through the pathway of a laser beam. Scattered and fluorescent light signals generated by cells are collected by a system of mirrors and prisms (optical system) and converted to proportional electronic signals by an electronics system. Digitised electronic signals are analysed by a computer in real time. The advantage of this technology over microscopy for analysis of cells in suspension lies in its capacity to quantify multiple characteristics of a large number of individual cells in a short time. One can only analyse 100 to 200 cells per sample using microscopy, while flow cytometers can typically measure properties of more than 5000 cells per second. Statistical accuracy of measurements is greatly increased by having a large number of cells analysed. Flow cytometry was first developed in the late 1960's for applications in haematology and began to be applied to investigation of sperm in late 1970's.

In this study the proportion of sperm with intact plasma membranes was quantified by flow cytometry using a Becton Dickinson FACS analytical flow cytometer with a built-in air-cooled argon ion laser (Becton Dickinson, San Jose, CA). Green fluorescence events were collected through a 515 nm band pass filter (FL1) and red fluorescence events were collected through a 630 nm band pass filter (FL3). The fluorochromes were excited with a 488 nm laser light. The results were analysed on a Macintosh Quadra 650 computer with CellQuest™ Becton Dickinson Software.

Aliquots of sperm were stained with *Ferti*Light™ Sperm Viability Kit according to the manufacturer instructions. Sperm with intact plasma membranes ("live") fluoresce green (515 nm) while sperm with damaged plasma membrane ("dead") fluoresce red (630 nm) (Figure 3.1). Distributions of 10,000 green- and red-stained sperm sub-populations were displayed as two-parameter dot plot cytograms for log FL1 and log FL3 (Johnson *et al.* 1996) (Figure 3.2). The proportion of sperm with intact plasma membrane was quantified using quadrant analysis (Johnson *et al.* 1996) to delineate green only, red only, double green and red fluorescence sperm populations. Percentage of sperm live was calculated by dividing the number of cells in the green fluorescence quadrant by the sum of sperm cells in all of the quadrants. Each aliquot was run in triplicate.

3.2.5 Assessment of sperm motility

The percentage of motile sperm and the Subjective Motility Score (SMS) were assessed visually in six observation fields, as described in Section 2.2.3.

For CASA, aliquots were drawn from incubations and six video recordings of 1.5 minute duration each were made. Curvilinear Velocities (VCL) and Straight Line Velocities (VSL) were analysed from the video recordings as described in Section 2.2.4.

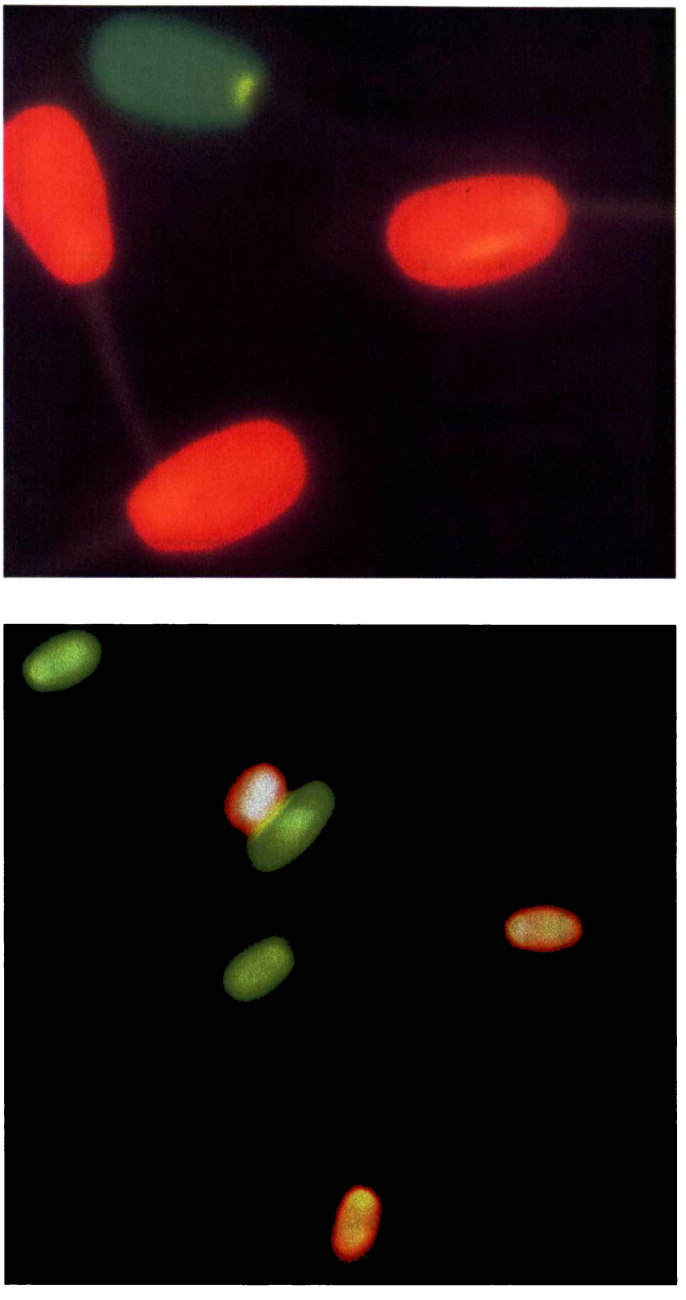


Figure 3.1 Fluorescent microscope images of sperm stained with *Ferti*Light™ Sperm Viability Kit (Molecular Probes, Inc., Eugene, OR, USA). Live cells (stained with membrane-permeable SYBR-14 stain) are fluorescing green. Dead cells with ruptured membranes (stained with membrane-impermeable PI) are fluorescing red.

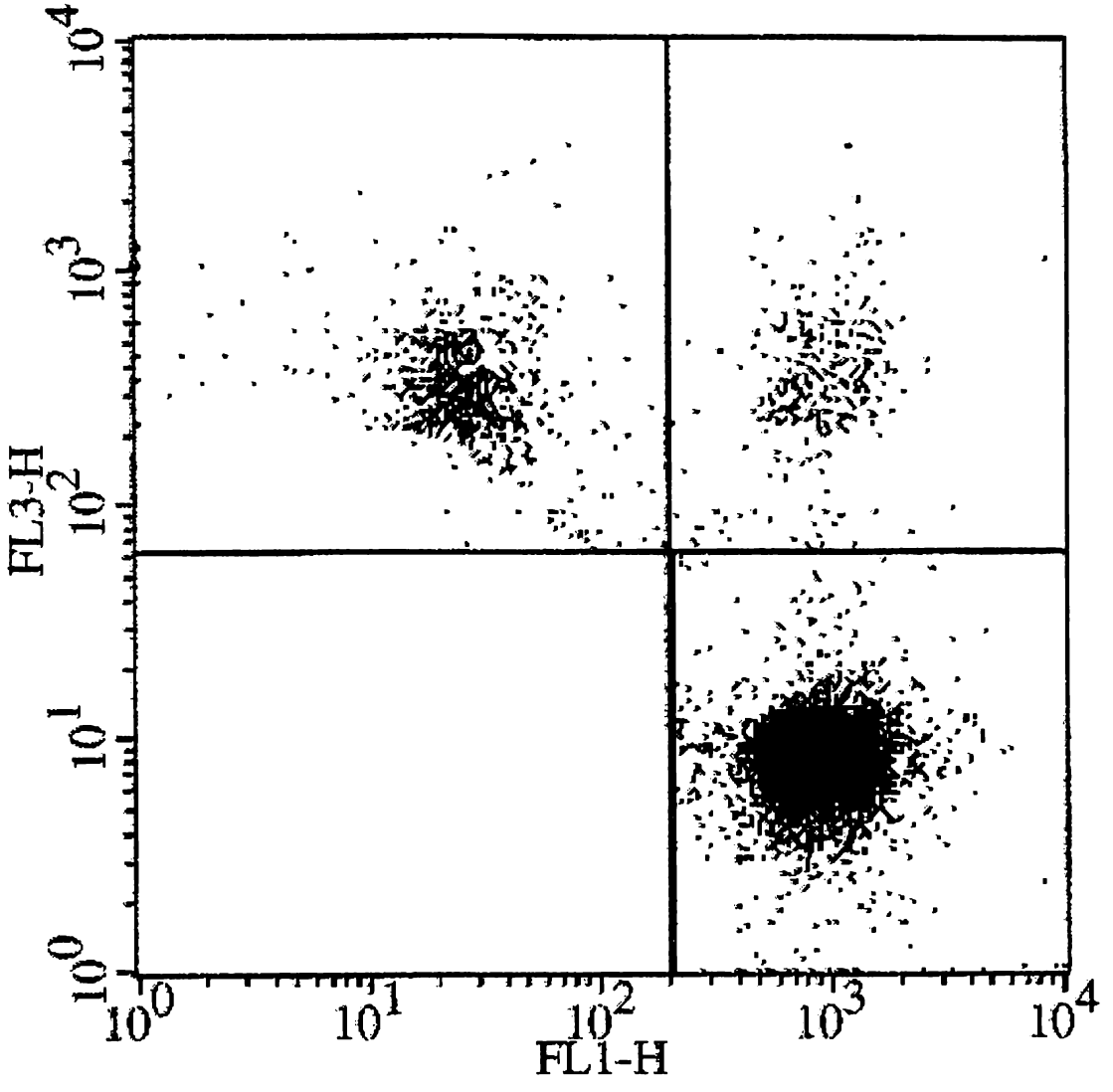


Figure 3.2 Representative two-parameter dot plot cytogram for log FL1-height (green fluorescence) and log FL3-height (red fluorescence) of sperm population stained with *Ferti*Light™ Sperm Viability Kit (Molecular Probes, Inc., Eugene, OR, USA). Dots represent individual cells. The position of the sperm cell dot along the X-axis is the value of its green fluorescence intensity (proportional to the amount of SYBR-14 bound to sperm DNA), while its Y-axis position is the measure of its red fluorescence intensity (proportional to the amount of PI bound to sperm DNA). Cells in the lower right quadrant (green fluorescence only) were designated as live. Cells in the upper right quadrant (double green and red fluorescence) and the upper left (red only fluorescence) were designated as dead.

3.2.6 Statistical analysis of results

The results from the Hobson Sperm Tracker were analysed by a least squares analysis of variance (SAS Institute, Cary, NJ, USA) fitting the effects of treatment (anaerobic and aerobic, theophylline-stimulated and non-stimulated) using median VSL and VCL as the dependent variables. The bull effects were treated as a random variable (n=3) and time as a covariate. Trend lines (linear or third order polynomial) were fitted to obtain the best fit for the data shown in Figures 3.6, 3.11 and 3.12. The differences in means for all other results were subjected to a 't' test.

3.2.7 Washing of sperm to remove seminal plasma proteins prior to protein extraction

To 35 ml of stored suspension containing 20 x 10^6 sperm cells/ml, 5 ml of milliQ water and 10 ml of Tris-buffered saline (TBS; consisting of 150 mmol/l NaCl, 25 mmol/l Tris-HCl pH 7.4) supplemented with 1 mmol/l sodium orthovanadate was added. This suspension was centrifuged at 3 000 *g* for 15 min. The supernatant was carefully aspirated, avoiding removal of any sperm, and leaving 5 ml of the buffer above the pellet. Sperm were subsequently re-suspended in 50 ml of TBS supplemented with 1 mmol/l sodium orthovanadate and centrifuged as before. The pellet, after aspiration of the supernatant, was re-suspended with 1 ml TBS and carefully transferred to a 1.5 ml Eppendorf tube, and centrifuged again at 16 000 *g* for 5 min. The supernatant was carefully aspirated and the pellet was frozen and stored in liquid nitrogen until extraction of the proteins.

3.2.8 Extraction of soluble proteins from sperm pellets

Soluble protein extracts were obtained using a method described by Vijayaraghavan *et al.* (1997) with minor modifications. Frozen sperm pellets were thawed and re-suspended in 600 µl of ice-cold homogenisation buffer (10 mmol/l Tris-HCl, 1 mmol/l EGTA, 1 mmol/l sodium orthovanadate and 3.2 mg/ml of Complete™ protease inhibitors; pH 6.7) and sonicated using a microprobe (Sonicator W380 Heat Systems-Ultrasonic Inc., Farmingdale, New York, NY, USA), with four 5-second bursts, with power output set at 4 and percentage duty cycle at 90%. The sperm sonicate was centrifuged at 16 000 *g* for 15 minutes at 4°C and the collected supernatant centrifuged again at 100 000 *g* for one hour. Immediately before SDS-PAGE, a volume of 400 µl of the 100 000 *g* supernatant, called the soluble fraction in the rest of this thesis, was mixed and boiled for five minutes with 800 µl of Laemmli sample buffer containing 5% 2-mercaptoethanol.

3.2.9 Whole cell protein extracts

Whole cell protein extracts were obtained as described by Galantino-Homer *et al.* (1997). The frozen sperm pellet obtained from the washing procedure to remove seminal plasma (described above) was thawed and re-suspended in 800 µl of Laemmli sample buffer without 2-mercaptoethanol. It was then boiled for 5 min, and centrifuged at 16 000 *g* for 10 min. The supernatant was removed and 2-mercaptoethanol was added to this to reach a final concentration of 5%. This mixture was then boiled for 5 minutes immediately before SDS-PAGE.

3.2.10 SDS-PAGE and Western blotting

Proteins of whole sperm extracts (5 µl) or of the soluble fraction (35 µl) were separated by SDS-PAGE, using Modular Mini-PROTEAN Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA) under constant voltage conditions (150 V) until the bromophenol blue tracer front ran to the edge of the gel. The proteins were then electrophoretically transferred to supported nitrocellulose membrane (0.2 µm pore size) using a wet transfer system (100 V, 1 h). The non-specific protein binding sites on the membrane were then blocked with 5% bovine serum albumin in TBS for 2 h at room temperature. The membrane was then incubated overnight at 4°C with anti-phosphotyrosine monoclonal antibody 4G10 at 1:3000 dilution (0.3 µg/ ml) in TBS containing 0.1% Tween-20 (TTBS), and 1% bovine serum albumin.

After three 15 minutes washes with TTBS containing 1% bovine serum albumin the blots were incubated at room temperature for 2 h with sheep anti-mouse immunoglobulin antibody conjugated with horseradish peroxidase at 1:3000 dilution (0.3 µg/ ml) in TTBS. The blots were then subjected to three 15 minutes long washes in 40 ml of TTBS and the protein bands containing phospho-tyrosine residues were detected with an ECL detection system.

3.2.11 Quantification of tyrosine phosphorylation

The ECL contact photographs of tyrosine-phosphorylated protein bands on Western blot membranes were scanned with a Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) using reflectance mode and 600 dpi resolution. Scanned images were analysed using a Power Macintosh computer equipped with Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA, USA).

To quantify changes in protein tyrosine phosphorylation of different protein bands, boxes were drawn around bands on scanned digital images of ECL contact photographs of Western blots. The optical density of all the pixels within the box was integrated and adjusted for background (optical density of the region on the contact photograph image outside protein lines) for each band. These values are proportional to the degree of tyrosine phosphorylation of the protein bands. Resulting values for phosphotyrosine signal (PY signal) in arbitrary units are displayed in figures under the blot images.

3.2.12 Estimation of the variations in the amount of total protein extracted from different samples for Western blotting

To ensure that the differences observed in protein tyrosine phosphorylation were not an artefact caused by variations in the total amount of protein loaded on the particular line of electrophoresis gel, the uniformity of protein extraction and loading of each sample were determined. To do this, an identically prepared gel for every one used for Western blotting was run simultaneously in the same two-gel electrophoresis chamber (Luconi *et al.* 1996). These second gels were silver-stained, according to the manufacturer's instructions, and were subsequently scanned with a Model GS-700 Imaging Densitometer using reflectance mode and 600 dpi resolution. Scanned images of the silver-stained gels were analysed using a Power Macintosh computer equipped with Molecular Analyst software.

To estimate uniformity between samples of total protein loaded into a gel, boxes were drawn around five major bands in each loading lane, corresponding to the different experimental sample, on the scanned digital images of silver-stained gels as shown in Figure 3.3. The optical density of all the pixels within the box was integrated and

adjusted for background (optical density of the region on the gel image outside protein lines) for each band. The sum of adjusted optical densities of all measured bands within a loading lane was regarded as a representation (estimation) of total protein concentration in this particular sample. This procedure is justified by the fact that gels were run under reducing conditions ensuring that no protein aggregation was taking place and because proteins were separated on the basis of size, changes in charge of protein did not affect band migration. Protein loading did not vary by more than 25% between samples in any gel, and variations were random without a time-dependent trend, thus the larger and time-dependent variations in protein tyrosine signal on Western blots could not have been due to the amount of protein loaded on the gels.

Integration results **90** **100** **83** **94** **92** **100** **94** **89**

Figure 3.3 An example of the gel used for estimation of variation in total protein concentration between different experimental samples. Representations (estimations) of total protein loading (arbitrary units) to each line are displayed under the image of a silver-stained gel.

3.3 EXPERIMENTS

3.3.1 *In vitro* storage of sperm

Three *in vitro* storage experiments were carried out. In experiment one, semen was collected and diluted as described in Section 3.2.3, and stored under standard nitrogen-gassed conditions for eight days. The percentage of sperm with intact plasma membrane was determined by flow cytometry on days 0, 2, 5, and 8 after ejaculation. On the same days, theophylline was added to aliquots of sperm to a concentration of 5.5 mmol/l and sperm were incubated at 37°C for 30 min. SMS and percentage of motile sperm were assessed for both theophylline-stimulated and non-stimulated sperm. The cells were then harvested by centrifugation from both treatments, washed and rapidly frozen in liquid nitrogen. The degree of tyrosine phosphorylation of soluble and whole cell proteins extracted from the frozen sperm was determined by Western blotting. Velocities of theophylline-stimulated and non-stimulated sperm were measured using CASA on days 0, 1, 3, 5, and 7 of storage.

In the second experiment semen was divided, after collection and dilution, and one half was stored under fully aerobic and the other under fully anaerobic conditions. Subjective motility score, percentage motile and percentage of sperm with intact plasma membrane were assessed on days 0, 2, 4, 6, 8, 10, and 12 after ejaculation for sperm stored under aerobic and anaerobic conditions. On the same days sperm were washed and frozen as before for assessment of the state of protein-tyrosine phosphorylation by Western blotting.

In the third experiment, semen was divided after collection and dilution and stored under fully anaerobic and fully aerobic conditions. On day five of *in vitro* storage one

half of the semen stored under anaerobic conditions was transferred from an anaerobic chamber to an aerobic environment. Similarly, one half of the semen stored under aerobic conditions was transferred to the anaerobic chamber. On days 6, 9, and 12, sperm cells were harvested, washed and frozen as before, from the four treatments (*i.e.* semen stored all the time under anaerobic conditions, semen transferred on day five from an anaerobic to an aerobic environment, semen stored all the time under aerobic conditions, semen transferred on day five from an aerobic to an anaerobic environment). Proteins were then extracted from these samples and protein-tyrosine phosphorylation was assessed by Western blotting.

3.4 RESULTS

3.4.1 The effect of storage under standard nitrogen-gassed conditions on sperm motility and plasma membrane integrity

The percentage of motile sperm declined from around 85% on day of collection (day zero) to 15% on day eight (Figure 3.4). The percentage of motile sperm increased significantly ($p<0.05$) when samples were stimulated with theophylline. The difference between the motility of sperm in theophylline stimulated and non-stimulated samples increased steadily over the time of storage and by day eight the percentage of motile sperm in theophylline-stimulated samples was more than four-fold higher than that in non-stimulated samples. The SMS declined during storage in both stimulated and non-stimulated sperm, but scores were significantly higher in samples stimulated with theophylline on all sampling days ($p< 0.05$).

The VSL and VCL values of sperm stored for more than three days were significantly higher ($p<0.05$) after stimulation with theophylline compared with non-stimulated sperm (Figure 3.5). The percentage of sperm with intact plasma membranes ("live") decreased only slightly during storage (Figure 3.6). Incubation with theophylline seemed to accelerate disintegration of the plasma membrane in stored sperm, as the percentage of "live" sperm is lower in sperm stimulated with theophylline than in non-stimulated sperm on later days of storage.

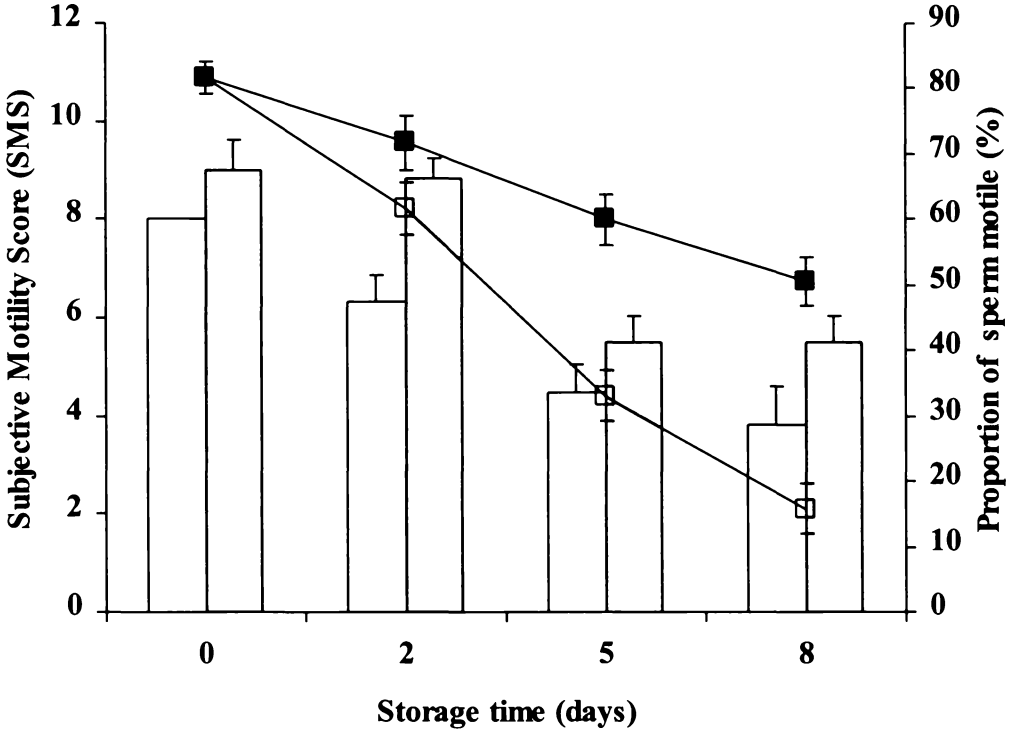


Figure 3.4 Changes in SMS and percentage sperm motile during *in vitro* storage at ambient temperature (18-20°C) under standard nitrogen-gassed conditions. The open columns depict the SMS of non-stimulated sperm; shaded columns represent the SMS of sperm stimulated with theophylline. (□) represent percentage of motile sperm in non-stimulated samples, and (■) represent percentage of motile sperm in samples stimulated with theophylline.

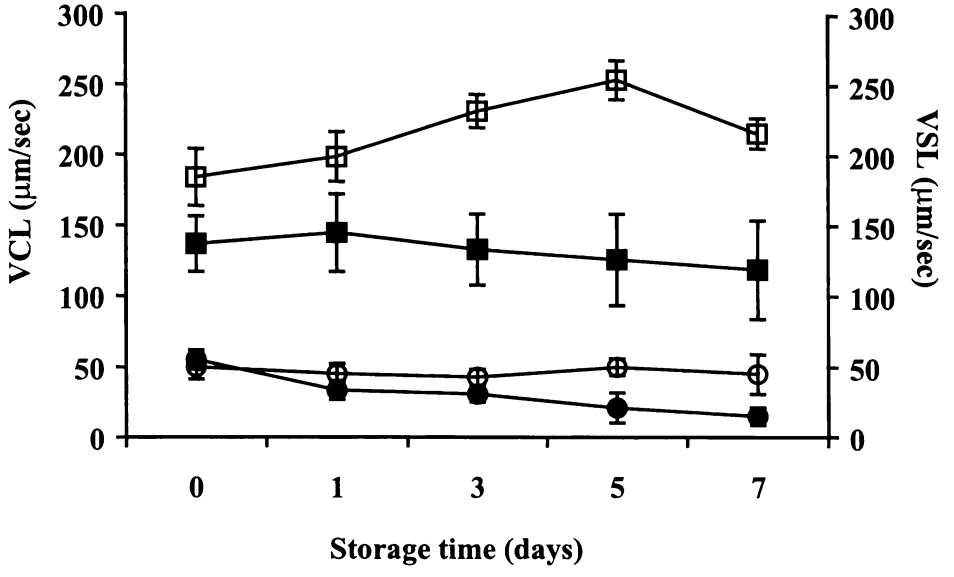


Figure 3.5 Changes in VCL and VSL during *in vitro* storage of sperm at ambient temperature (18-20°C) under standard, nitrogen-gassed conditions. (□) represents VCL of sperm stimulated with theophylline, (■) represents VCL of non-stimulated sperm, (O) represents VSL of sperm stimulated with theophylline, (●) represents VSL of non-stimulated sperm.

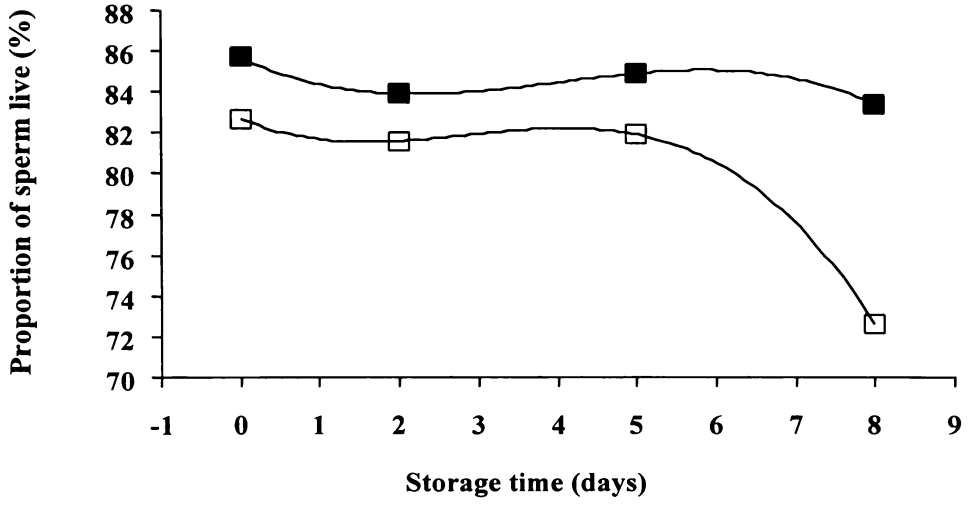


Figure 3.6 Changes in percentage of sperm with plasma membrane intact (live sperm) during storage *in vitro* at ambient temperature (18-20°C) under standard nitrogen-gassed conditions. (□) represents percentage of live sperm after stimulation with theophylline (■) represents percentage of live sperm when not stimulated with theophylline.

3.4.2 The effects of storage of sperm under standard nitrogen-gassed conditions on protein tyrosine phosphorylation of the soluble protein extracts

In all the samples two major bands of M_r=67 000 and M_r=36 000 were immuno-detected with the anti-phospho-tyrosine 4G10 antibody (Figure 3.7). The results between bulls differ substantially, hence, Western blots of all three bulls are presented. Although changes in tyrosine phosphorylation of the proteins from the soluble fraction were not consistent between bulls, a general downward trend was observed, most noticeably in bull three. In all samples, the degree of protein tyrosine phosphorylation declined in one of the bands within the treatment, corresponding to declining sperm motility.

In Bull 1 the tyrosine phosphorylation of the soluble protein with M_r=67 000 declined by almost half in non-stimulated samples, but did not show any consistent trend in samples stimulated with theophylline. Tyrosine phosphorylation of the soluble protein with M_r=36 000 does not show a clear trend in intensity over the time of storage in a non-stimulated sample, but in the presence of theophylline, phosphorylation declined to less than one seventh over the time of storage.

In Bull 2 the tyrosine phosphorylation state of the protein with M_r=67 000 remained relatively unchanged when not treated with theophylline, but it declined to just over one third over eight days of storage when stimulated with theophylline. In contrast, tyrosine phosphorylation of the protein with M_r=36 000 in samples not stimulated with theophylline declined to approximately one fifth. The bands on the Western blot of the protein with M_r=36 000 stimulated with theophylline was too faint to quantify by densitometry.

In Bull 3, the protein band with M_r=67 000 on Western blot was too faint to quantify by densitometry, in both theophylline-treated and untreated samples. Tyrosine phosphorylation of the protein with M_r=36 000 declined to approximately one third over eight days in both theophylline-treated and untreated samples.

3.4.3 The effect of storage of sperm under standard nitrogen-gassed conditions on protein tyrosine phosphorylation in the whole cell protein extract

There was a time-dependent increase in tyrosine phosphorylation of a number of proteins from whole cell extracts, during storage of sperm in nitrogen-gassed diluent (Figure 3.8). Two major bands of M_r=220 000 and M_r=80 000 showed the most obvious increase in tyrosine phosphorylation.

There was no substantial difference in the pattern of protein tyrosine phosphorylation changes between extracts from sperm stimulated with theophylline and those from non-stimulated sperm cells. The increase in tyrosine phosphorylation with storage ranged from approximately sixty-fold for the protein with M_r=220 000 to eight-fold for the protein with M_r=80 000.

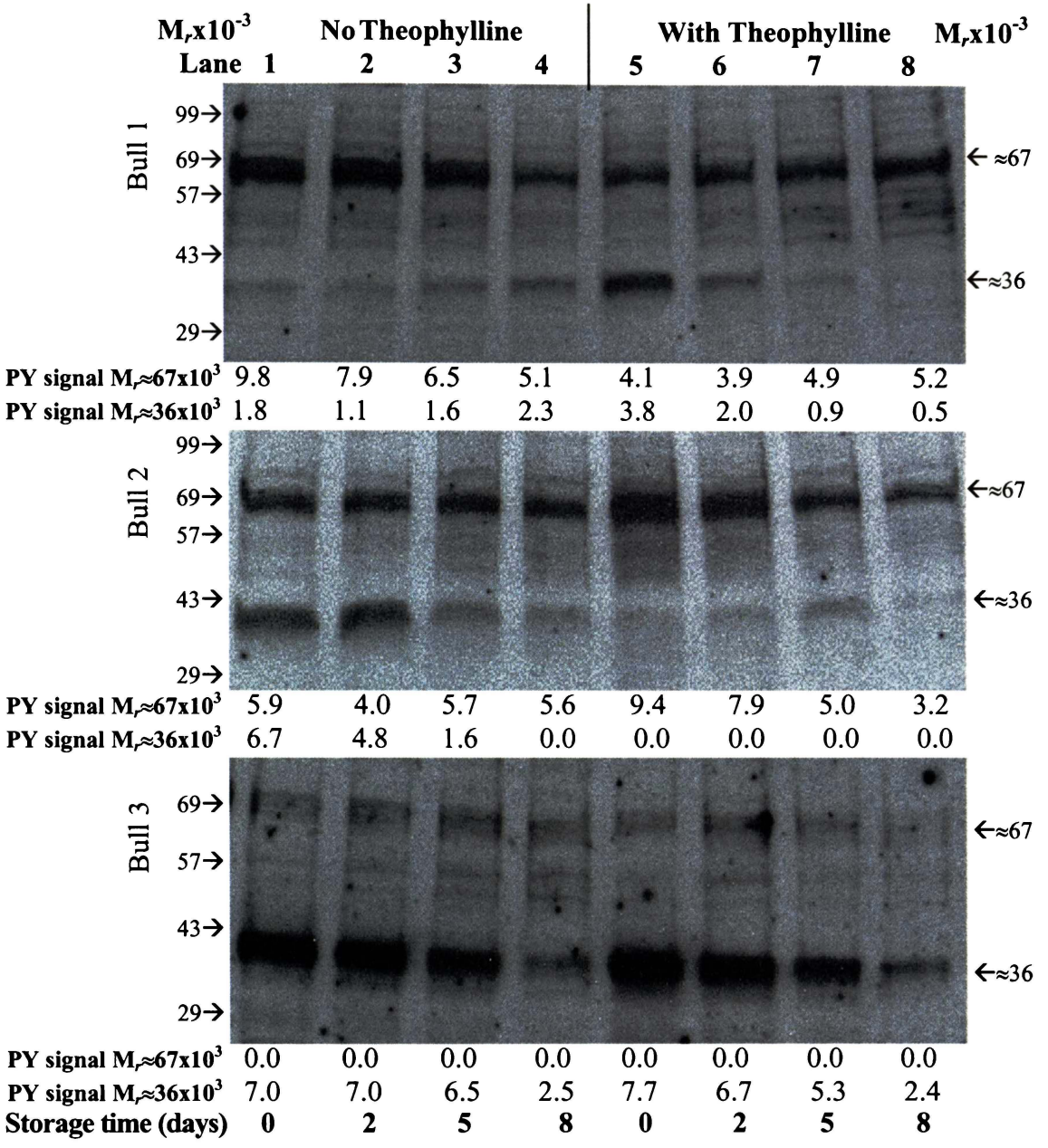


Figure 3.7 Effects of storage of ejaculated bovine sperm *in vitro* at ambient temperature (18-20°C) under standard nitrogen-gassed conditions, for the length of time indicated at the bottom of the figure, on tyrosine phosphorylation of soluble proteins extracted from the sperm. Sperm of three bulls were either washed and harvested directly from the storage diluent (Lanes 1-4) or were pre-incubated in the presence of 5.5 mmol/l theophylline at 37°C for 30 minutes prior to washing and harvesting (Lanes 5-8). The photographs show the results of Western blot analysis, using 4G10 anti-phospho-tyrosine antibody, performed on soluble fraction as described in Section 3.2.9. Phosphotyrosine (PY) signal values (arbitrary units) for the $M_r \approx 36 \times 10^3$ and $M_r \approx 67 \times 10^3$ bands are shown under each photograph.

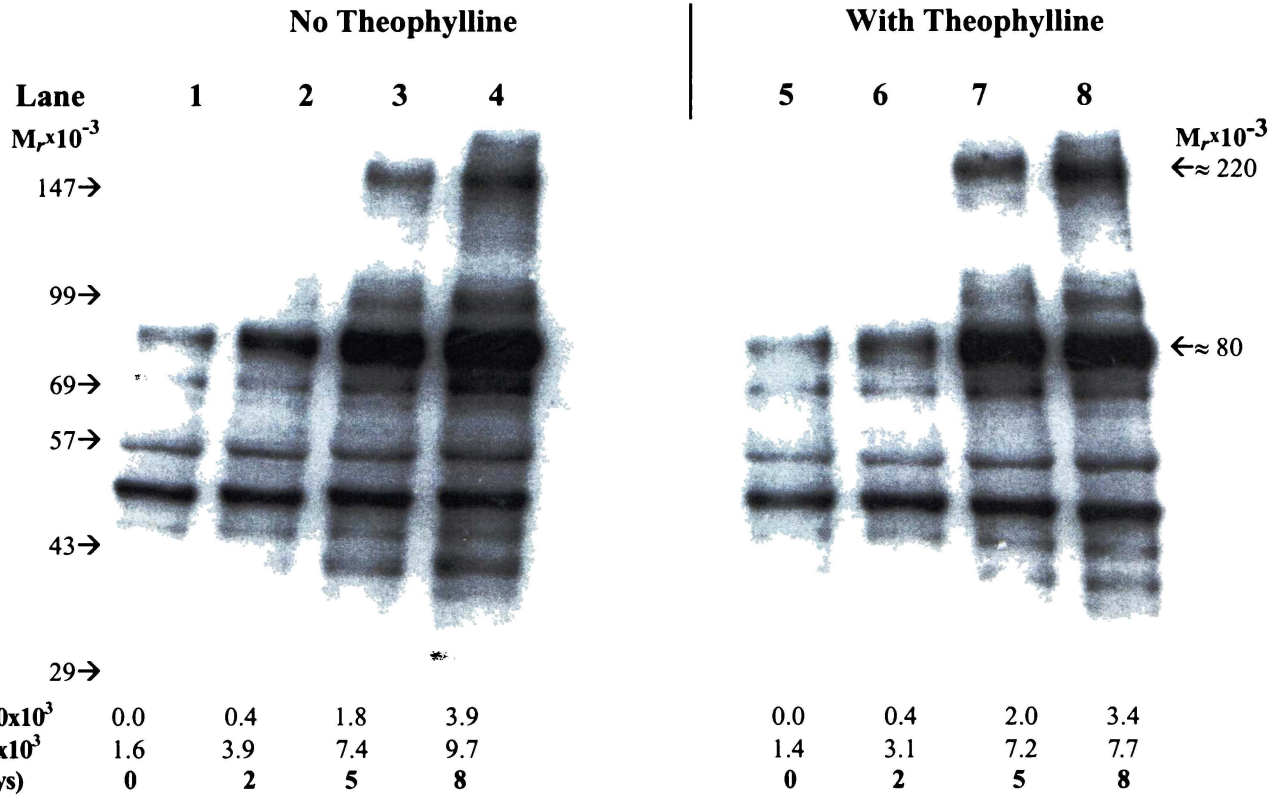
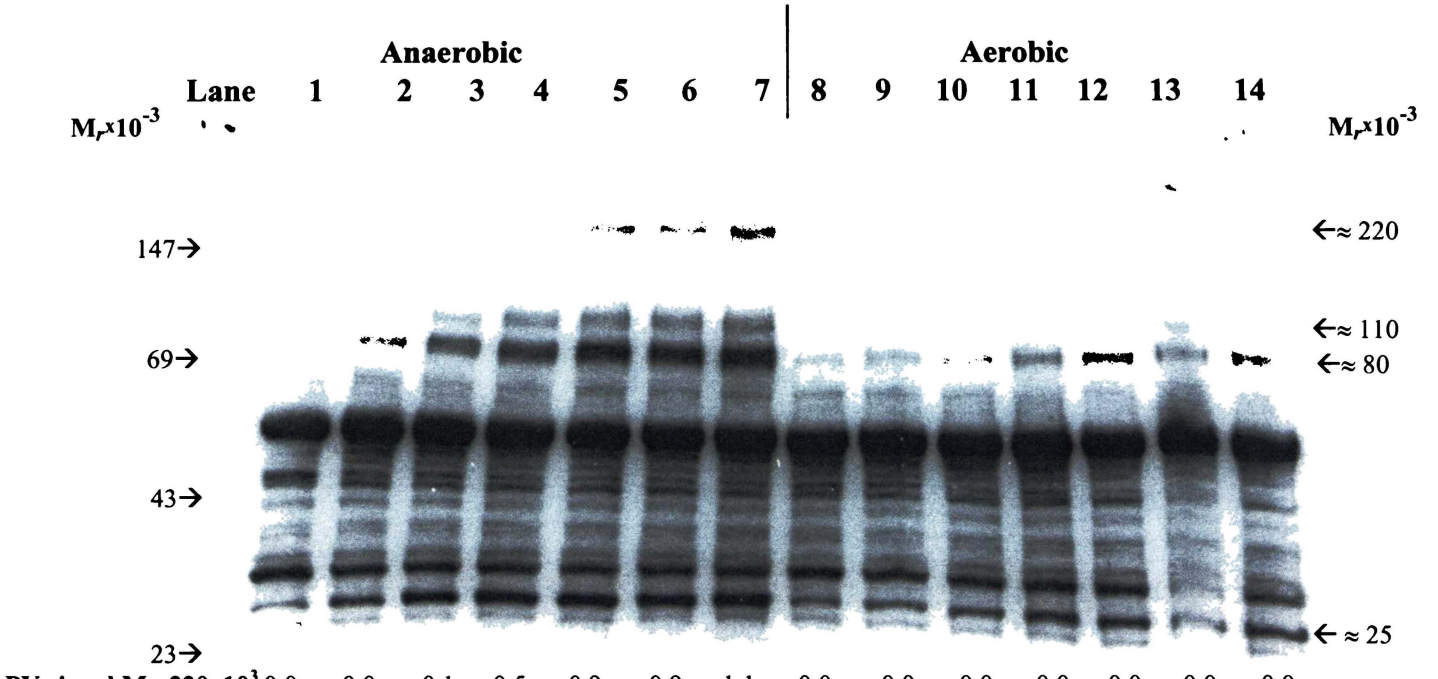


Figure 3.8 Effects of storage *in vitro* on tyrosine phosphorylation of proteins in whole sperm extracts. Ejaculated bovine sperm were stored *in vitro* at ambient temperature (18-20°C) under standard nitrogen-gassed conditions, for the length of time indicated at the bottom of the figure. Sperm cells of three bulls were either washed and harvested directly from the storage diluent (Lanes 1-4) or were pre-incubated in presence of 5.5 mmol/l theophylline at 37°C for 30 minutes prior to washing and harvesting (Lanes 5-8). The photograph shows the representative Western blot of proteins extracted from the sperm of one bull. Western blot analysis was as described in Section 3.2.9. Phosphotyrosine (PY) signal values (arbitrary units) for the M_r≈80x10^3 and M_r≈220x10^3 bands are shown under the photograph.

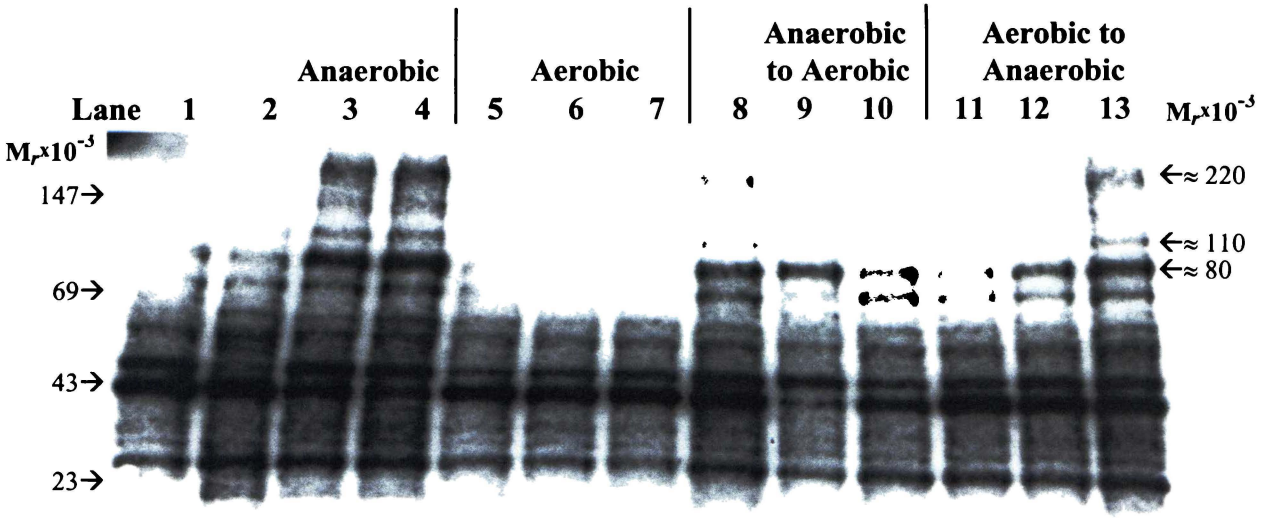
3.4.4 The effect of storage of sperm under anaerobic and aerobic condition on tyrosine phosphorylation of the proteins in whole cell extracts

Tyrosine phosphorylation of a number of proteins from sperm stored under anaerobic conditions increased in a time-dependent manner between day zero and day twelve of storage (Figure 3.9). In the case of the protein with M_r=220 000 the increase was more than twenty five-fold, for the protein with M_r=110 000 the increase was six-fold, for the protein with M_r=80 000 the increase was seventeen-fold and for the protein with M_r=25 000 it was more than four-fold. No increase in protein tyrosine phosphorylation was observed in sperm stored under aerobic conditions (Figure 3.9). The same trends were observed in sperm stored under aerobic and anaerobic conditions in a subsequent experiment (Figure 3.10). When sperm were transferred from anaerobic to aerobic conditions after five days of storage, the same proteins that were phosphorylated during anaerobic storage were de-phosphorylated between days six and twelve of aerobic storage (Figure 3.10). Conversely when sperm initially stored under aerobic conditions were transferred to an anaerobic environment, proteins which did not display increased protein tyrosine phosphorylation until this point became progressively phosphorylated on tyrosine residues between days six and twelve (Figure 3.10).



PY signal $M_r \approx 220x10^3$	0.0	0.0	0.1	0.5	0.9	0.9	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PY signal $M_r \approx 110x10^3$	0.3	0.4	0.5	0.9	1.5	1.6	1.8	0.6	0.5	0.4	0.6	0.5	0.7	0.5
PY signal $M_r \approx 80x10^3$	0.3	0.7	2.2	3.2	4.3	5.2	5.5	0.6	0.8	0.9	1.3	1.2	1.4	1.4
PY signal $M_r \approx 25x10^3$	1.0	1.6	2.3	2.9	3.7	4.0	4.4	2.1	2.0	2.1	2.3	2.4	1.8	2.7
Storage time (days)	**0**	**2**	**4**	**6**	**8**	**10**	**12**	**0**	**2**	**4**	**6**	**8**	**10**	**12**

Figure 3.9 Changes in tyrosine phosphorylation of proteins in whole cell extracts of sperm stored *in vitro* at ambient temperature under anaerobic (Lanes 1-7) and aerobic conditions (Lanes 8-14) for the length of time indicated at the bottom of the figure. The photograph shows the results of Western blot analysis, using 4G10 anti-phospho-tyrosine antibody, performed on whole cell extracts as described in Section 3.2.9. Experiments were performed with sperm collected from three bulls. The results of all experiments were similar. Shown is a representative Western blot of proteins extracted from the sperm of one bull. Phosphotyrosine (PY) signal values (arbitrary units) for the bands indicated by the arrows on the right are shown under the photograph.



PY signal M_r≈220x10^3	1.5	1.3	3.6	4.6	0.0	0.0	0.0	2.3	1.5	1.1	1.0	1.4	3.2
PY signal M_r≈110x10^3	1.3	1.4	2.6	3.0	1.0	1.0	0.9	2.0	1.4	1.1	1.0	1.5	2.5
PY signal M_r≈80x10^3	3.0	3.1	7.7	9.2	2.1	1.9	1.5	7.8	6.0	3.5	2.6	5.2	8.1
Storage time (days)	**0**	**6**	**9**	**12**	**6**	**9**	**12**	**6**	**9**	**12**	**6**	**9**	**12**

Figure 3.10 Changes in tyrosine phosphorylation of proteins in whole cell extracts of sperm stored *in vitro* at ambient temperature under anaerobic and aerobic conditions, also changed after five days of storage from anaerobic to aerobic and vice versa. Lane 1 represents tyrosine-phosphorylated proteins from freshly collected sperm. Ejaculated bovine sperm were stored in 14G diluent under anaerobic (Lanes 2-4) or aerobic (Lanes 5-7) conditions for five days, then sperm from anaerobic storage were transferred to aerobic conditions (Lanes 8-10) and those from aerobic storage were transferred to anaerobic conditions (Lanes 11-13), and stored for a further seven days. The photograph shows the result of Western blots analysis, using 4G10 anti-phospho-tyrosine antibody, performed on whole cell extracts from samples taken on days six, nine and twelve of storage as described in Section 3.2.9. Three experiments were performed with sperm collected from three bulls. The results of all experiments were similar. Shown is a representative Western blot of proteins extracted from the sperm of one bull. Phosphotyrosine (PY) signal values (arbitrary units) for the bands indicated by the arrows on the right are shown under photograph.

3.4.5 The effect of storage of sperm under anaerobic and aerobic conditions on motility and viability

The SMS and the percentage of motile sperm (Figure 3.11), and the percentage of sperm with intact plasma membrane (Figure 3.12) declined under both anaerobic and aerobic conditions. For the first five days of *in vitro* storage the rate of decline of those parameters was similar under anaerobic and aerobic conditions, after which sperm stored under aerobic conditions deteriorated more rapidly ($p<0.005$).

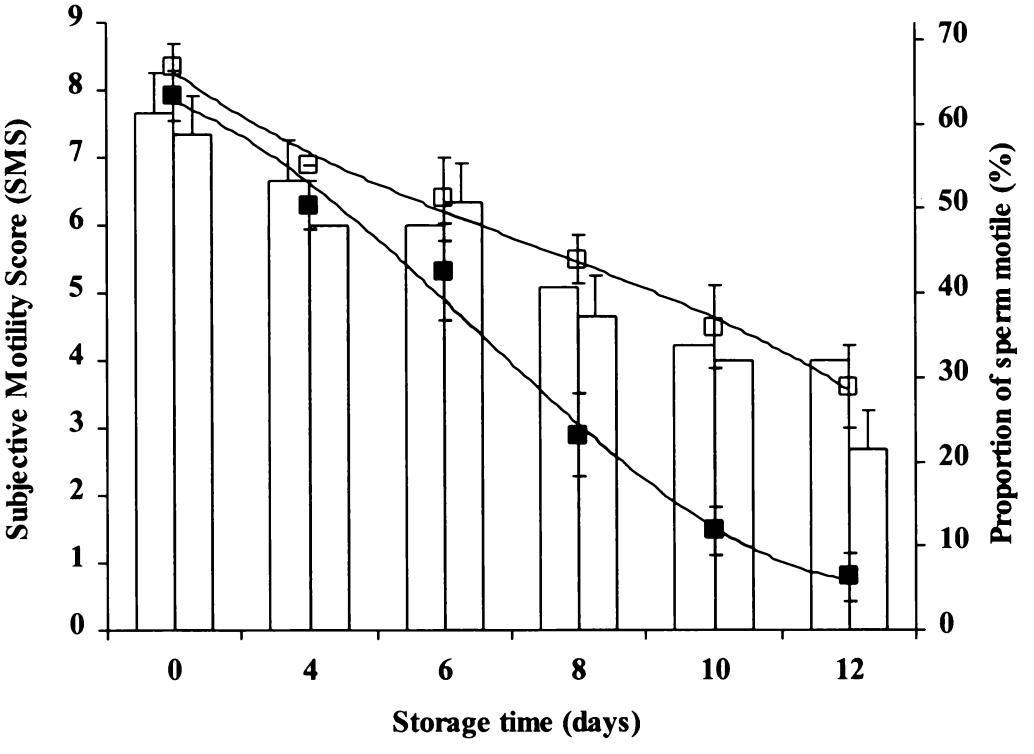


Figure 3.11 Changes in SMS and percentage of sperm motile during storage of sperm *in vitro* at ambient temperature (18-20°C) under aerobic and anaerobic conditions. The open columns depict SMS of sperm stored under anaerobic conditions; shaded columns represent SMS of sperm stored under aerobic conditions. (□) represents percentage motile of sperm stored under anaerobic conditions, and (■) represents percentage motile of sperm stored under aerobic conditions.

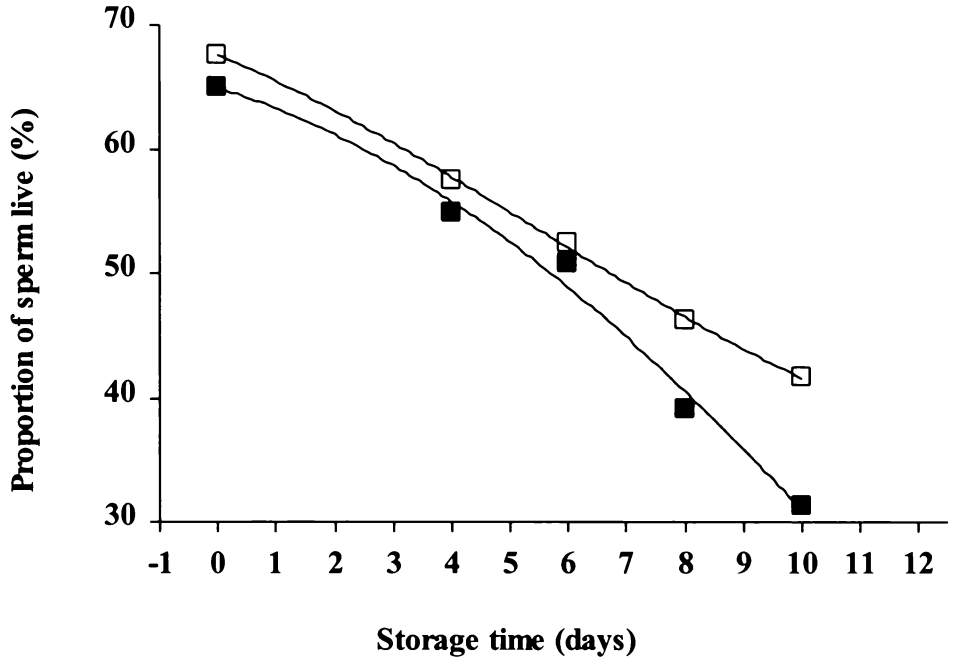


Figure 3.12 Changes in percentage live during *in vitro* storage of sperm at ambient temperature (18-20°C) under aerobic and anaerobic conditions, as determined by flow cytometric assay of plasma membrane integrity. (□) represents percentage of live sperm stored under anaerobic conditions, (■) represents percentage of live sperm stored under aerobic conditions.

3.5 DISCUSSION

It is well known that sperm immobilised by removing motility-stimulating factors contained in seminal plasma by repetitive washing (Baas *et al.* 1983) or short incubation at 37°C (Brandt and Hoskins 1980) can be reactivated by inhibition of cyclic nucleotide phosphodiesterase. It was demonstrated in the present work that the decline in motility observed during prolonged *in vitro* storage at room temperature can also, to a great extent, be reversed in the same way. This indicates that immobilised sperm are not dead, and at least part of the motility loss could be attributed to defective regulation rather than structural damage to the axoneme or loss of ability to generate ATP.

Many cellular regulatory processes are mediated via tyrosine phosphorylation and de-phosphorylation. Western blots of tyrosine-phosphorylated proteins from soluble fraction of ejaculated and stored sperm presented here are quite different from those presented by Vijayaraghavan *et al.* (1997). The soluble tyrosine-phosphorylated protein with M_r=55 000 described by Vijayaraghavan *et al.* (1997) was not detected in any of the samples from three different bulls. This may be due to the fact that ejaculated sperm was used here while they used caudal and epididymal sperm. In addition, some components of diluent used in this study, which is different from that used by Vijayaraghavan *et al.* (1997), may have possibly altered the pattern of tyrosine phosphorylation of soluble sperm proteins.

Proteins detected on all Western blots were specifically tyrosine phosphorylated, since immunoabsorption of primary antibody with O-phospho-DL-tyrosine almost entirely abolished immunoreactivity (Figure 3.13). When the binding site of 4G10 antibody is blocked by excess of free phosphotyrosine, binding to phosphorylated proteins on the blot is greatly reduced.

Since variations in total protein loading in different experimental samples estimated by the densitometry of silver-stained gels were small compared with the differences in the phospho-tyrosine signal on Western blots, and were random rather than time dependent, the detected changes in the degree of tyrosine phosphorylation of sperm proteins could not be attributed to an artefact of increased total protein concentration in the experimental samples. Moreover, if increased total protein loading were a cause of the increased protein tyrosine signal, then there would have been uniform increase in all tyrosine-phosphorylated proteins, but this clearly did not occur (Figure 3.9).

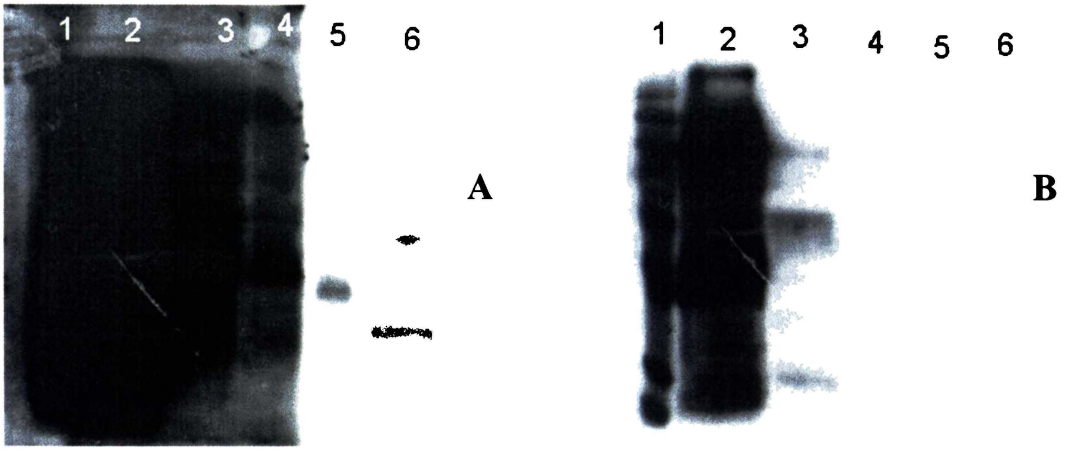


Figure 3.13 To determine the specificity of 4G10 antibody binding site, Western blots were incubated with 4G10 antibody (Lanes 1,2,3,4) or 4G10 antibody that has been pre-incubated overnight at 4°C with excess of free phosphotyrosine to block the binding site (Lanes 5 and 6). Subsequently those blots were then incubated with secondary anti-mouse antibody conjugated to HRP and developed using the Enhanced Chemiluminescence method. During detection X-ray film was exposed for 5 minutes (A) and 5 sec (B). Lane 1: pre-stained molecular weight standards. Lane 2: EGF stimulated A431 protein preparation (positive control). Lanes 3 and 5: whole cell sperm extracts. Lanes 4 and 6: soluble sperm protein extract.

The difference between bulls in the pattern of immuno-detected protein bands on Western blots could possibly be due to proteolysis. In spite of protease inhibitors being present in the extraction buffers throughout the process, it is possible that the smaller protein is a product of proteolytic fragmentation of the larger one. The sum of the phosphotyrosine signal from larger and smaller soluble proteins in the same loading lane declines with the time of storage within a treatment in all bulls. This is what one would expect if the smaller protein was a proteolytic fragment of the larger one and if the tyrosine phosphorylation of these proteins declines during *in vitro* storage, in parallel with the decline in motility. An alternative explanation for this is that different proteins are tyrosine-phosphorylated in ejaculated sperm from those in epididymal

sperm. Upon ejaculation, sperm cells are mixed with seminal plasma which contains several motility activators (Hoskins *et al.* 1975). It is conceivable that these activators initiate tyrosine phosphorylation on a different set of proteins from those phosphorylated in the epididymis. Another possibility is that tyrosine-phosphorylated proteins from seminal plasma form complexes with sperm proteins and are not removed by washing. This study did not attempt to determine if the phosphorylated proteins from soluble extracts of ejaculated sperm are related to the soluble tyrosine-phosphorylated protein with M_r=55 000 from epididymal sperm described by Vijayaraghavan *et al.* (1997).

Incubation with theophylline does not seem to increase tyrosine phosphorylation of soluble proteins (Figure 3.7). This is in contrast to the observation of Vijayaraghavan *et al.* (1997) that the increase in cAMP concentration led to increased tyrosine phosphorylation of a cytosolic protein with M_r=55 000. It is not known if the decline in the degree of tyrosine phosphorylation of soluble proteins shown in this study has a causal link to time-dependent decline of sperm motility or if the two processes are simply occurring at the same time without any cause-effect relationship. The M_r=55 000 protein described by Vijayaraghavan *et al.* (1997) has recently been identified as glycogen synthase kinase-3 α (GSK-3α) (Vijayaraghavan *et al.* 2000) and it has been demonstrated that its inactivation via tyrosine phosphorylation (a mechanism opposite to that observed in somatic cells) is associated with development of motility (Vijayaraghavan *et al.* 2000).

As antibodies against tyrosine-phosphorylated and non-phosphorylated forms of GSK-3α are commercially available it would be relatively easy to determine with Western blotting if one of the cytosolic tyrosine-phosphorylated proteins detected in this study is also GSK-3α.

Where proteins from whole cell extracts are concerned, changes in tyrosine phosphorylation are very slow when compared with typical receptor-mediated tyrosine phosphorylation, or those changes observed in ejaculated bovine (Galantino-Homer *et al.* 1997) and human (Luconi *et al.* 1995) sperm incubated under capacitating conditions. The changes that occur during storage span across days rather than seconds or hours. For example, insulin receptor kinase requires only fifteen seconds to reach maximum tyrosine autophosphorylation after insulin binding or ten minutes when stimulated with hydrogen peroxide (Koshio *et al.* 1988). Capacitation-associated protein tyrosine phosphorylation reaches a maximum after 4 h of incubation (Emiliozzi and Fenichel 1997; Galantino-Homer *et al.* 1997). With the *in vitro* storage the present study demonstrated that tyrosine phosphorylation was still increasing even after ten days of storage (Figures 3.9 and 3.10).

There was no major difference in the pattern of protein tyrosine phosphorylation of whole sperm cell extracts between theophylline-stimulated and non-stimulated sperm (Figure 3.8). In contrast, inhibition of phosphodiesterase in bovine sperm incubated under capacitating conditions strongly promotes protein tyrosine phosphorylation and is even able to overcome glucose inhibition of tyrosine phosphorylation (Galantino-Homer *et al.* 1997).

In order to determine if the exclusion of oxygen, and thus prevention of respiration, during storage would affect protein tyrosine phosphorylation, sperm were stored *in vitro* under aerobic and anaerobic conditions. Contrary to the initial expectations, a time-dependent increase in tyrosine phosphorylation was observed under anaerobic but not under aerobic conditions (Figure 3.9). One possible reason for this may be the composition of the diluent. It contains glucose, which is known to inhibit bovine sperm capacitation and is a mild reducing agent. Other strong, cell permeable-reducing agents, such as 2-mercaptoethanol, suppress tyrosine phosphorylation under capacitating conditions (Aitken *et al.* 1995). Catalase, which also inhibits capacitation-related tyrosine phosphorylation in bovine sperm, acts via the rapid removal of hydrogen peroxide from the medium, thus inhibiting tyrosine phosphorylation. It can be speculated that the capacitation-related increase in protein tyrosine phosphorylation would be inhibited in this medium under both aerobic and anaerobic conditions, and that the observed time-dependent increase in protein tyrosine phosphorylation during storage is distinct from the capacitation-related increase in tyrosine phosphorylation.

When sperm stored under anaerobic conditions were transferred to aerobic conditions, tyrosine phosphorylation of proteins that were phosphorylated during storage was reversed. When sperm stored for five days under aerobic conditions were transferred to an anaerobic environment, protein tyrosine phosphorylation of the same set of proteins which were phosphorylated during storage under anaerobic conditions began to proceed in a time-dependent manner (Figure 3.10). This indicates that both protein tyrosine kinase and phosphatase activities are regulated by the redox status of the cell, probably through the generation of ROS, but this modulation of protein tyrosine

phosphorylation occurs in a direction opposite to that which is documented for sperm undergoing capacitation.

Sperm probably did not undergo capacitation during storage in medium used here as no hyperactivated motility was observed in stored sperm either with or without theophylline stimulation. Sperm were stored during the experimental period in a commercial diluent empirically optimised over the years to maintain sperm viability at ambient temperature rather than promote capacitation (Vishwanath and Shannon 1997). This diluent does not contain heparin, bovine serum albumin or sodium carbonate which are known to be required for capacitation and tyrosine phosphorylation associated with capacitation (Emiliozzi and Fenichel 1997; Visconti *et al.* 1995a). On the contrary, it contains glucose which has been shown to inhibit bovine sperm capacitation (Parrish *et al.* 1989) and tyrosine phosphorylation (Galantino-Homer *et al.* 1997). The medium also contains Ca^{2+}, which has been shown to promote capacitation in murine sperm (Visconti *et al.* 1995a), but has been reported to negatively modulate capacitation-associated tyrosine kinase activity in human sperm (Luconi *et al.* 1996). Catalase, which is an ingredient of the medium, has also been demonstrated to inhibit capacitation (Aitken *et al.* 1996).

3.6 CONCLUSIONS

The increase in protein tyrosine phosphorylation during storage is probably different from the increase in phosphorylation of tyrosine observed during capacitation since unlike the latter it is not responsive to phosphodiesterase inhibition nor is it enhanced by oxidative conditions. On the contrary, it is inhibited in the presence of oxygen and is reversed upon transfer of sperm cells from an anaerobic to aerobic environment. Furthermore, it takes place across a much longer time span than capacitation. The role of protein tyrosine phosphorylation in sperm storage and its relation to other tyrosine phosphorylation mediated processes will only be understood when the proteins that are phosphorylated during storage are isolated and identified.

Chapter 4

CHANGES IN SUSCEPTIBILITY OF BOVINE SPERM TO *IN SITU* DNA DENATURATION DURING STORAGE AT AMBIENT TEMPERATURE UNDER CONDITIONS OF EXPOSURE TO REACTIVE OXYGEN SPECIES AND NUCLEASE INHIBITOR

4.1 INTRODUCTION

During *in vitro* storage at ambient temperature sperm undergo detrimental changes to both their cellular machinery and the haploid genome; consequently, their oocyte penetrating ability and the potential to initiate and sustain the development of the embryo may be impaired (Salisbury and Hart 1970; Vishwanath and Shannon 1997). Reduced fertility may result from sperm DNA fragmentation (Sun *et al.* 1997). Sperm nuclear chromatin integrity can be investigated using the DNA-binding properties of acridine orange ~~acridine orange~~ which intercalates into double-stranded (ds) DNA as a green-fluorescing monomer and binds to single-stranded (ss) DNA as a red-fluorescing aggregate. The Sperm Chromatin Structure Assay (SCSA) was developed to measure sperm DNA susceptibility to *in situ* acid denaturation by quantifying the metachromatic shift from green fluorescence of acridine orange bound to dsDNA to red fluorescence emitted by acridine orange bound to ssDNA (Evenson *et al.* 1980). Reduced sperm chromatin stability as measured by SCSA has been demonstrated to correlate strongly with DNA strand breaks (Aravindan *et al.* 1997) and sub-fertility in bull, human and boar (Ballachey *et al.* 1988; Ballachey *et al.* 1987; Evenson *et al.* 1999; Evenson *et al.* 1994). A decline in chromatin integrity during storage at ambient temperature has been detected by SCSA in mouse sperm (Estop *et al.* 1993) and human sperm (Ellington *et*

al. 1998). Bull sperm stored in milk or egg yolk extender have also shown increased SCSA values over time (Karabinus *et al.* 1991) indicating decreased chromatin stability.

Reactive oxygen species (ROS) are harmful to sperm at elevated levels (Aitken and Fisher 1994; Aitken *et al.* 1989; Alvarez *et al.* 1987; Armstrong *et al.* 1999; Cummins *et al.* 1994; D'Agata *et al.* 1990; Jones and Mann 1973) and are a major source of damage to sperm DNA (Gagnon *et al.* 1991). The major sources of ROS in diluted semen stored at ambient temperature are aromatic L-amino acid oxidase (Shannon and Curson 1981) released from dead and damaged sperm (Shannon and Curson 1972), mitochondrial respiration (Aitken and Clarkson 1987) and seminal leukocytes (Aitken *et al.* 1992; Kessopoulou *et al.* 1992). Because sperm are almost devoid of cytoplasm they possess only very low amounts of ROS-scavenging enzymes that protect somatic cells from oxidative damage. Moreover, DNA repair enzymes are apparently absent in mature sperm, making these cells uniquely susceptible to oxidative damage (Hughes *et al.* 1998). Functional sperm rely on tight packing of their DNA around protamines, which reduces exposure to free radicals, and on antioxidants present in the seminal plasma for protection from oxidative damage (Hughes *et al.* 1998). During *in vitro* manipulation of sperm samples, oxidative damage to sperm DNA can be alleviated by supplementing the diluent with antioxidants (Hughes *et al.* 1998), ROS-degrading enzymes (Shannon and Curson 1982) and elimination of oxygen from the diluent (Shannon and Curson 1982).

Exchange of nuclear histones during spermatogenesis, first by transition proteins and subsequently by protamines, is characterised by scission and religation of DNA by topoisomerases in order to accommodate the torsional stresses involved in these protein

exchanges (McPherson and Longo 1993a; McPherson and Longo 1993b; Roca and Mezquita 1989). Some activity of those DNA-hydrolysing enzymes may persist in mature sperm. DNA strand breaks in sperm may also occur due to endogenous deoxyribonucleases activated by external stimuli, which cleave both exogenous and genomic DNA. This leads in some cases to cell death that in some respects resembles apoptosis (Blanc-Layrac *et al.* 2000; Spadafora 1998). This type of DNA damage in mouse sperm can be prevented by pre-incubation with the deoxyribonuclease inhibitor aurintricarboxylic acid (ATA) prior to *in vitro* fertilisation, leading to increased efficiency of embryo production (Zaccagnini *et al.* 1998). It has been proposed that the ATA acts by protecting sperm nuclei from induced or spontaneous DNA damage and/or by priming sperm chromatin for the events of early embryogenesis (Zaccagnini *et al.* 1998).

The present study was performed to investigate whether the stability of bull sperm chromatin deteriorates as a result of deoxyribonuclease activity during extended storage at ambient temperature under fully aerobic and fully anaerobic conditions in commercial 14G diluent developed for *in vitro* storage of sperm. Also investigated was whether the stability of the chromatin is affected by exposure to ROS under these storage conditions. Instability of the chromatin was assessed as an increase in DNA susceptibility to *in situ* denaturation by acid. The influence of endogenous deoxyribonucleases on chromatin stability was investigated by the inclusion of the general deoxyribonuclease inhibitor aurintricarboxylic acid (ATA) in the storage medium. Increased levels of exposure to ROS were achieved by storing sperm in the presence of H_2O_2 added directly to the diluent.

In addition, the effect of the inclusion in the diluent of L-phenylalanine, a substrate in an oxidation reaction catalysed by aromatic L-amino acid oxidase, which results in generation of H_2O_2 as a by-product was also investigated (Shannon and Curson 1972). Reduced levels of ROS were achieved by including catalase in the diluent, and eliminating O_2 from the diluent.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Analytical grade chemicals were used in this study purchased from the same suppliers as specified in Sections 2.2.1 and 3.2.1. In addition, 2.0 N Standardised HCl, Triton X-100, phenylalanine, and aurintricarboxylic acid (ATA; practical grade) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Chromatographically purified acridine orange acridine orange was purchased from Polysciences, Warrington, PA, USA.

4.2.2 Semen collection, evaluation and preparation

Diluents for anaerobic and aerobic storage were prepared as described in section 3.2.2.

For semen collection, evaluation and preparation for anaerobic and aerobic storage, the procedures described in Section 3.2.3 were followed. Sperm viability as determined by plasma membrane integrity was measured as described in Section 3.2.4. The percentage of motile sperm in storage samples was visually assessed as described in Section 2.2.3.

4.2.3 Sperm Chromatin Structure Assay (SCSA)

A Becton Dickinson fluorescence-activated cell sorter (FACScan) with built-in air-cooled argon ion laser operating at 488 nm emission spectra was used in this study. The FACScan was interfaced to a Macintosh Quadra 650 computer with CellQuest™ Becton Dickinson Software (Becton Dickinson, San Jose, CA). A 515 nm band pass filter was used to collect green fluorescence (FL1) and a 630 nm band pass filter was used to collect red fluorescence (FL3).

SCSA procedures as described in detail by Evenson and Jost (1994) were used in this study. Samples taken from storage bottles at designated time intervals were snap-frozen and stored in liquid nitrogen. SCSA of all samples from different treatments of the sperm from the same ejaculate were performed on the same day. The whole SCSA procedure was performed in triplicates for each thawed sample. To confirm the stability of the assay and reliability of the instrument the reference bull samples were assayed after each fifteen experimental samples. Reference samples were diluted aliquots of the same ejaculate frozen under identical conditions and used throughout the trial to set the photomultiplying tubes of the flow-cytometer to the values that ensure that the mean red and green fluorescence of the same samples fall within ±5 channels of each repeated measurement of the same sample.

For SCSA, sample tubes were removed from liquid nitrogen and immediately thawed by immersion in a 37°C water bath. Immediately after thawing, each sample was diluted with ice cold TNE buffer (0.01 mol/l Tris-HCl, 0.15 mol/l NaCl and 1 mmol/l EDTA, pH 7.4) to a concentration of 8 x 10^6 sperm cells/ml and the tubes were placed on wet ice. Using a Falcon tube (Becton Dickinson), 200 µl of diluted sample was

mixed with 400 µl of ice-cold acid detergent solution (0.1% v/v Triton X-100, 0.08 mol/l HCl, 0.15 mol/l NaCl). Exactly 30 second later 1.2 ml of staining solution (0.1 mol/l citric acid monohydrate, 0.2 mol/l di-sodium hydrogen orthophosphate, 1 mmol/l di-sodium EDTA, 0.15 mol/l NaCl, 6 µg/ml acridine orange, pH 6.0) was added. The sample tube was placed into the flow cytometer and sample flow started. Signal acquisition was started after two minutes equilibration.

Flow cytometry was performed using the slow flow rate setting (12 µl/min of sample) with 15 mW laser output. Prior to SCSA measurements the instrument was calibrated using CaliBRITE™ calibration beads (Becton Dickinson), then a mixture of ¾ acridine orange staining solution and ¼ of acid detergent solution was passed through the instrument's sample lines for 20 minutes to equilibrate acridine orange with the sample tubing. After the last sample was analysed, a 0.4% (w/w) solution of sodium hypochlorite was passed through the sample lines, followed by a water rinse, to remove all traces of acridine orange. Green (515 nm) and red (≥630 nm) fluorescence of 5000 cells was acquired to listmode at about 75 events/sec. Listmode data files were then analysed using WinList™ Software (Verity House Inc., Topsham, ME, USA).

The level of DNA denaturation in each individual cell was quantified by alpha t (α_t) where α_t=red/(red+green) fluorescence. The following SCSA parameters were obtained from α_t distribution histograms: cells outside main population (COMPα_t) reflecting number of cells with increased DNA denaturability, and the mean and standard deviation of α_t reflecting the degree of chromatin instability in the gated cell population.

4.2.4 Statistical analysis of results

Estimates of motility, viability and SCSA parameters were analysed using General Linear Model (GLM) procedures of SYSTAT® 8.0 Statistics (SPSS Inc., Chicago, IL, USA). At each time interval, matrixes of *P* values for Tukey's pair-wise comparisons were obtained. Differences with the *P* value <0.05 were regarded as statistically significant for all analyses.

4.3 EXPERIMENTS

Semen samples from three bulls were used throughout this study, the samples being treated separately.

Two storage experiments were performed. In both experiments diluted semen from the same ejaculate was divided into two halves. One half was stored under fully anaerobic conditions while the other was stored under fully aerobic conditions. All the samples were stored at ambient temperature in sealed 100 ml sterile Schott bottles (Schott Glaswerke, Mainz, Germany) wrapped in aluminium foil. For storage of sperm under anaerobic conditions, the sperm were suspended in an anaerobic medium and stored inside an anaerobic chamber. For aerobic storage the bottles were left on the bench top. Bottles in which sperm were stored were opened daily and the diluted semen was equilibrated with the gas storage phase by gentle shaking for 10 seconds in order to maintain the presence of oxygen during the aerobic storage.

In the first experiment, sperm were diluted in the following media: normal 14G buffer (contains catalase), 14G buffer without catalase, 14G buffer supplemented with 100

μmol/l H_2O_2, 14G buffer without catalase supplemented with 100 μmol/l hydrogen peroxide, 14G buffer supplemented with 1 mmol/l phenylalanine, and 14G buffer without catalase supplemented with 1 mmol/l phenylalanine. Diluted semen was stored for 9 days in each of the diluents under aerobic and anaerobic conditions at ambient temperature. Samples of the stored semen were removed, on days 0, 3, 6, and 9 of storage for assessment of motility and viability. Samples for SCSA were removed and frozen in liquid nitrogen at the same time.

In the second experiment sperm were diluted in normal 14G buffer, and in 14G buffer supplemented with 25 μmol/l ATA. These samples were then stored under fully aerobic or fully anaerobic conditions for 8 days. Motility and viability of stored sperm were assessed and samples for SCSA were removed and frozen on days 0, 2, 4, 6, and 8 of storage. All motility assessments were performed under aerobic conditions.

4.4 RESULTS

4.4.1 The effects of exposure to ROS on sperm viability and motility

Sperm motility and viability declined over the period of storage under all treatments but not at the same rate (the results are presented in Table 4.1). Motility was affected most severely by inclusion of exogenous H_2O_2 in the medium under anaerobic storage conditions. Under aerobic storage conditions there was no significant difference between the detrimental effect on motility of H_2O_2 directly added to the diluent and that of H_2O_2 generated via oxidation of phenylalanine under aerobic conditions. Apart from the treatment where H_2O_2 was included in the diluent, motility was maintained better under anaerobic than under aerobic storage conditions. Whether H_2O_2 was added

directly to the medium or generated in the course of the oxidation of phenylalanine, catalase protected stored sperm from the detrimental effects of H_2O_2 on motility.

H_2O_2, from whatever source, had a far less marked effect on viability than it had on motility. Viability in the presence of exogenous H_2O_2 declined more sharply under aerobic conditions. Where exogenous H_2O_2 was added to the diluent under anaerobic conditions motility ceased on day three of storage without as marked loss of plasma membrane integrity as observed under aerobic conditions in the presence of H_2O_2 or phenylalanine. Sperm exposed to exogenous H_2O_2, either directly added to the diluent or generated by aromatic amino acid oxidase, lost membrane integrity more rapidly under aerobic storage conditions than under anaerobic conditions. There was no significant difference in the viability of sperm between those stored in the presence or the absence of catalase if no exogenous H_2O_2 was included or generated in the diluent on any of the storage days within aerobic and anaerobic treatments.

H_2O_2 caused accelerated loss of membrane integrity, in the absence of catalase, under both aerobic and anaerobic storage conditions, although the effect was stronger under aerobic conditions on days six and nine. Phenylalanine did not have a significant effect on viability during storage under anaerobic conditions (where H_2O_2 would not be produced from its oxidation) but caused membrane disintegration during storage under aerobic conditions in the absence of catalase. Sperm viability was maintained better under anaerobic than under aerobic conditions.

Table 4.1 Percentages of motile sperm and sperm with intact plasma membrane (n=9; mean ±SE) during storage at ambient temperature in diluents that allow exposure to different levels of ROS. Values within rows that do not share a letter in superscript are significantly different (P<0.05).

	Aerobic						Anaerobic					
	Diluent alone		Diluent + H_2O_2		Diluent + phenylalanine		Diluent alone		Diluent + H_2O_2		Diluent + phenylalanine	
	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase
% motile												
Day 0	75±1.8^{d}	75±1.8^{d}	73±2.1^{cd}	67±2.8^{b}	73±2.1^{cd}	75±1.8^{d}	72±1.1^{cd}	70±2.0^{bc}	68±2.1^{b}	60±2.8^{a}	67±4.2^{b}	73±1.1^{cd}
Day 3	48±3.8^{e}	48±2.1^{e}	38±2.8^{cd}	15±1.8^{b}	47±4.2^{e}	13±4.6^{b}	40±6.3^{d}	37±4.6^{c}	33±6.4^{c}	0±0.0^{a}	45±4.8^{de}	35±1.8^{c}
Day 6	15±4.8^{bc}	12±2.8^{b}	17±2.8^{c}	0±0.0^{a}	22±7.4^{cd}	0±0.0^{a}	35±6.3^{d}	32±4.6^{d}	30±6.6^{d}	0±0.0^{a}	33±6.9^{cd}	32±2.8^{d}
Day 9	0±0.0^{a}	0±0.0^{a}	0±0.0^{a}	0±0.0^{a}	0±0.0^{a}	0±0.0^{a}	25±4.8^{b}	18±5.6^{b}	18±5.6^{b}	0±0.0^{a}	20±6.6^{b}	18±4.6^{b}
All days	35±6.7^{bc}	35±6.7^{bc}	32±6.5^{b}	20±6.5^{ab}	35±6.5^{bc}	22±7.3^{ab}	43±4.1^{c}	39±4.5^{bc}	38±4.4^{bc}	15±6.1^{a}	41±4.0^{c}	40±4.8^{c}
% live												
Day 0	79±4.3^{ab}	82±3.2^{b}	82±3.4^{b}	75±2.6^{ab}	78±3.9^{ab}	78±1.5^{ab}	81±1.8^{b}	79±2.8^{ab}	78±6.1^{ab}	71±2.1^{a}	75±1.7^{ab}	82±4.0^{b}
Day 3	68±5.9^{bc}	67±6.4^{bc}	67±6.5^{bc}	43±4.0^{a}	66±7.6^{b}	58±7.5^{ab}	67±6.7^{bc}	71±5.6^{c}	71±6.2^{c}	46±9.9^{a}	68±6.6^{bc}	68±6.6^{bc}
Day 6	58±7.8^{c}	57±7.6^{c}	59±7.4^{c}	17±4.7^{a}	53±8.2^{c}	25±5.3^{ab}	65±6.7^{c}	63±7.1^{c}	66±7.5^{c}	35±9.7^{b}	65±6.5^{c}	65±6.7^{c}
Day 9	45±9.1^{c}	29±5.8^{bc}	45±9.2^{c}	3±1.8^{a}	34±9.9^{bc}	3±1.4^{a}	58±7.1^{d}	58±7.4^{d}	59±7.7^{d}	24±5.9^{b}	57±7.3^{d}	57±7.0^{d}
All days	63±2.4^{c}	59±3.7^{c}	63±2.6^{c}	35±5.2^{a}	58±3.1^{c}	41±5.6^{ab}	68±1.6^{d}	68±1.6^{d}	68±1.4^{d}	44±3.4^{b}	66±1.2^{cd}	68±1.7^{d}

4.4.2 The effects of exposure of sperm to H_2O_2 on the susceptibility of their DNA to *in situ* acid denaturation

The SCSA parameters of sperm stored under conditions that exposed them to different levels of ROS are presented in Table 4.2. There was virtually no change in the SCSA parameters of sperm stored without the exposure to exogenous reactive oxygen species under both aerobic and anaerobic conditions (Figure 4.1A and Table 4.2). Sperm exposed to exogenous H_2O_2 that was either directly added to the diluent or generated by aromatic amino acid oxidase in the presence of phenylalanine under aerobic conditions suffered significant decline in the resistance of their DNA to *in situ* acid denaturation. From day 6 onwards, catalase did not protect sperm chromatin from destabilisation by H_2O_2 under aerobic storage conditions and both the mean α_t and the mean of standard deviation of α_t were elevated. The effect of H_2O_2 generated via aromatic amino acid oxidase on sperm chromatin stability was most severe and was observed at the earliest time point during aerobic storage. As expected, the presence of phenylalanine in the medium under anaerobic conditions did not affect chromatin stability because the H_2O_2-yielding reaction of aromatic L-amino acid oxidation could not take place in the absence of dissolved oxygen. Somewhat unexpectedly, direct addition of H_2O_2 into the anaerobic diluent in the absence of catalase had no significant effect on sperm chromatin stability parameters apart from a slightly higher proportion of COMPα_t on day 9 of storage.

There are some differences in the pattern of changes to chromatin stability of sperm that are not well reflected in the numerical parameters shown in Table 4.2. The DNA of a sub-population of sperm exposed to H_2O_2 directly added to the diluent underwent a sudden and dramatic shift from high stability to almost complete denaturation and

possible nuclear fragmentation while the remaining sub-population was unchanged (Figure 4.1 B). Additionally, sperm that had been stored in presence of phenylalanine under aerobic conditions and thus exposed to H_2O_2 generated by aromatic amino acid oxidase exhibited a gradual shift in the chromatin stability of the entire population without a clear resolution of a sub-population of cells with highly unstable chromatin (Figure 4.1 C).

4.4.3 The effects of ATA on motility and viability of sperm stored under aerobic and anaerobic conditions

The results of assessment of percentages of motile sperm and sperm with intact plasma membrane during storage in the presence and absence of ATA under aerobic and anaerobic conditions are presented in Table 4.3. There was no significant difference in the percentage of motile sperm between any of the treatments on day 8 of storage. Unlike the results presented in Table 4.1 where the motility of sperm stored in normal 14G buffer was generally preserved better under anaerobic storage conditions, the percentage of motile sperm was significantly lower under anaerobic than aerobic conditions on days 2, 4, and 6 of storage and was not significantly affected by the presence of ATA in the diluent.

Sperm viability in all treatments was not significantly different on day 2 of storage. On days 4 to 8 the viability of sperm stored in aerobic diluent containing ATA was significantly ($P<0.05$) lower than in all other treatments.

Mean viability on day 8 and across all days of storage was slightly but significantly higher under anaerobic than under aerobic storage conditions.

Table 4.2 SCSA parameters (n=9, mean ±SE) during storage at ambient temperature in the diluents that allow exposure to different levels of ROS. Values within rows that do not share a letter in superscript are significantly different (P<0.05).

	Aerobic						Anaerobic					
	Diluent alone		Diluent + H_2O_2		Diluent + phenylalanine		Diluent alone		Diluent + H_2O_2		Diluent + phenylalanine	
	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase
Meanα_t												
Day 0	223±1.9^{b}	220±2.9^{ab}	222±2.3^{b}	219±0.9^{ab}	221±1.2^{ab}	219±0.7^{ab}	216±0.6^{a}	222±1.9^{ab}	221±2.1^{ab}	222±1.7^{ab}	222±2.4^{b}	219±1.5^{ab}
Day 3	221±2.2^{a}	219±0.8^{a}	222±2.9^{a}	221±0.9^{a}	222±1.8^{a}	229±3.3^{b}	220±0.7^{a}	221±0.9^{a}	218±1.6^{a}	220±2.7^{a}	220±1.7^{a}	219±1.5^{a}
Day 6	224±1.4^{ab}	217±0.4^{a}	229±1.1^{b}	240±2.2^{c}	220±1.4^{a}	240±3.5^{c}	220±1.1^{a}	222±1.8^{a}	220±1.4^{a}	221±2.4^{a}	221±1.5^{a}	219±1.7^{a}
Day 9	218±2.3^{a}	217±0.2^{a}	258±7.2^{b}	286±4.7^{c}	223±1.4^{a}	259±4.0^{b}	225±1.2^{a}	224±1.5^{a}	223±1.6^{a}	226±3.1^{a}	224±0.8^{a}	222±0.8^{a}
All days	221±1.0^{a}	218±0.8^{a}	233±3.2^{b}	242±4.7^{b}	222±0.7^{a}	237±2.9^{b}	220±0.7^{a}	222±0.8^{a}	220±0.9^{a}	222±1.3^{a}	222±0.9^{a}	220±0.7^{a}
SDα_t												
Day 0	27±1.3^{a}	25±1.5^{a}	22±1.8^{a}	26±1.9^{a}	26±1.9^{a}	27±1.7^{a}	23±1.7^{a}	28±3.0^{a}	22±1.6^{a}	23±1.3^{a}	24±1.9^{a}	22±1.6^{a}
Day 3	25±1.7^{ab}	25±1.8^{ab}	23±1.9^{ab}	24±1.7^{ab}	26±1.8^{ab}	30±2.1^{b}	27±1.8^{ab}	25±1.7^{ab}	22±1.7^{a}	20±1.5^{a}	23±1.4^{ab}	21±1.8^{a}
Day 6	25±1.5^{a}	25±2.0^{a}	60±11.0^{b}	116±5.3^{c}	26±2.9^{a}	62±10.9^{b}	23±1.6^{a}	26±1.9^{a}	24±0.9^{a}	24±1.4^{a}	24±1.7^{a}	23±1.7^{a}
Day 9	24±1.7^{a}	28±2.3^{a}	122±25.9^{c}	190±8.0^{d}	41±6.5^{b}	104±13.2^{c}	25±1.8^{a}	27±1.0^{a}	23±1.9^{a}	24±2.2^{a}	26±2.6^{a}	25±1.8^{a}
All days	25±0.8^{a}	26±0.9^{a}	57±9.7^{b}	89±11.9^{c}	30±2.5^{a}	56±6.7^{b}	25±0.9^{a}	26±1.0^{a}	23±0.8^{a}	23±0.8^{a}	24±0.9^{a}	23±0.8^{a}
COMPα_t												
Day 0	8±0.4^{b}	5±0.8^{a}	9±0.6^{b}	7±0.7^{ab}	7±0.8^{ab}	6±0.8^{ab}	5±0.7^{a}	8±0.4^{b}	7±0.6^{ab}	6±0.9^{ab}	9±1.2^{b}	6±0.4^{ab}
Day 3	8±0.5^{a}	7±1.0^{a}	8±0.6^{a}	8±0.9^{a}	8±0.3^{a}	22±3.8^{b}	7±0.8^{a}	7±0.9^{a}	6±0.8^{a}	6±1.5^{a}	7±0.7^{a}	7±0.4^{a}
Day 6	9±1.8^{a}	6±0.8^{a}	9±0.4^{a}	10±0.8^{a}	7±0.5^{a}	27±6.7^{b}	7±0.7^{a}	9±0.3^{a}	7±0.6^{a}	7±1.5^{a}	8±1.0^{a}	7±0.8^{a}
Day 9	11±0.7^{ab}	6±0.7^{a}	14±1.3^{b}	18±1.2^{b}	8±0.6^{ab}	33±6.8^{c}	11±1.5^{ab}	10±0.4^{ab}	10±1.8^{ab}	14±5.1^{b}	10±1.2^{ab}	9±0.9^{ab}
All days	8±0.6^{ab}	6±0.4^{a}	10±0.5^{ab}	11±0.8^{b}	7±0.3^{ab}	22±3.0^{c}	8±0.6^{ab}	8±0.3^{ab}	8±0.6^{ab}	8±1.4^{ab}	8±0.6^{ab}	7±0.4^{ab}

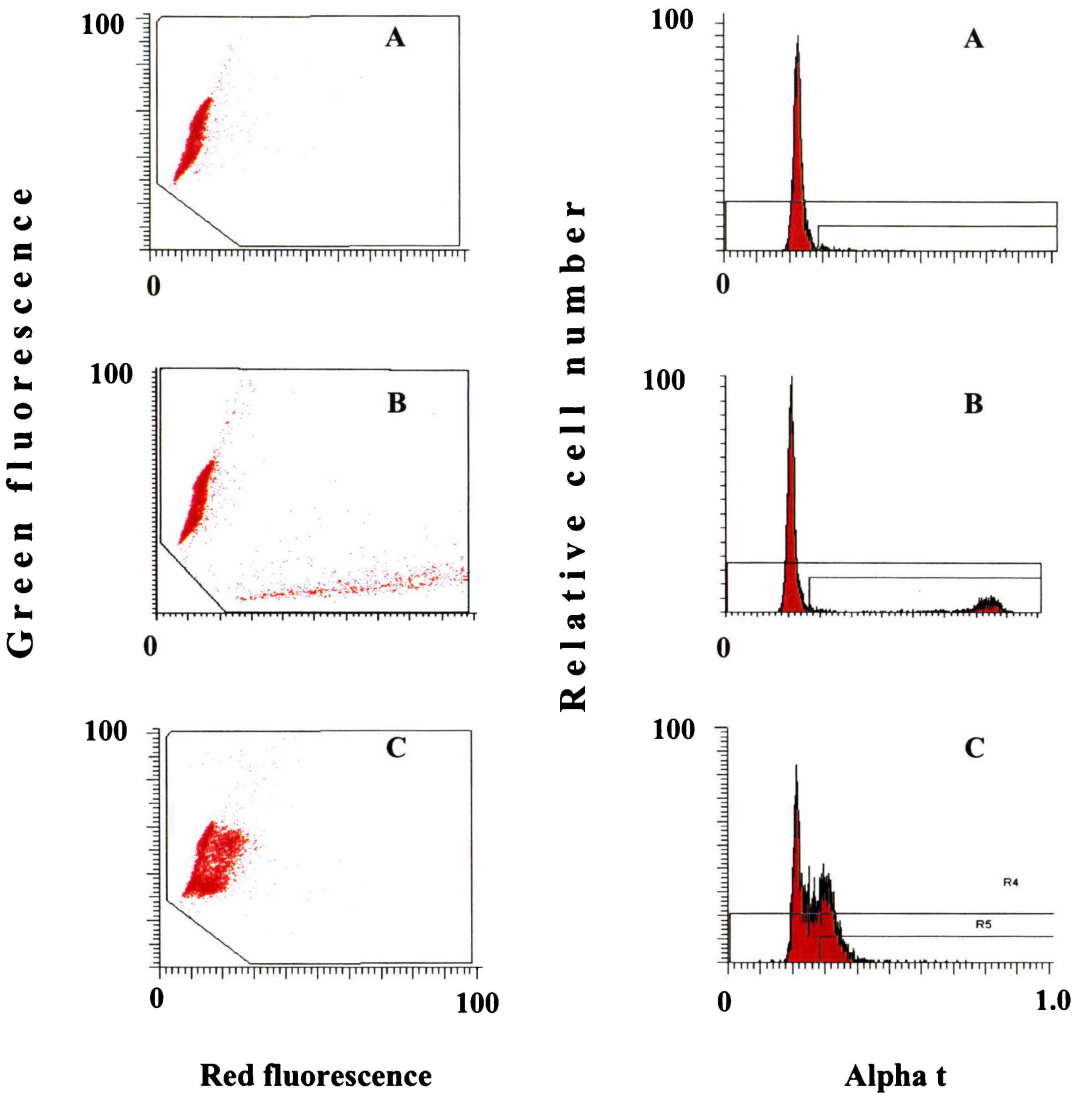


Figure 4.1 Representative SCSA cytograms and corresponding histograms of bovine sperm stored for 9 days at ambient temperature under the following conditions: A) sperm diluted in buffer containing catalase, stored under anaerobic conditions; B) sperm diluted in buffer devoid of catalase and containing 100 μmol/l H_2O_2 stored under aerobic conditions C) sperm diluted in buffer devoid of catalase and containing 1 mmol/l phenylalanine stored under aerobic conditions. Dots represent individual cells. The position of the dot along the Y-axis is the value of its green fluorescence (dsDNA), while its X-axis position is the measure of its red fluorescence (ssDNA). The heterogeneity of the green fluorescence of DNA associated chromophores in sperm cells results in an elongated spread of dots and is due to an optical artefact caused by their highly asymmetrical shape of sperm (Evenson *et al.* 1994). This does not present a problem for measuring SCSA parameters since they are derived from the ratio of green and total fluorescence of individual cells (Evenson *et al.* 1994).

Table 4.3 Percentages of motile sperm, sperm with intact plasma membrane and SCSA parameters of sperm (n=9, mean ±SE) during storage at ambient temperature in diluents containing no ATA or 25 µmol/l of ATA under aerobic and anaerobic conditions. Values within rows that do not share a letter in superscript are significantly different (P<0.05).

	Aerobic		Anaerobic	
	With ATA	Without ATA	With ATA	Without ATA
% motile				
Day 0	66±1.3^{a}	65±1.2^{a}	66±1.1^{a}	66±1.3^{a}
Day 2	54±1.0^{b}	53±1.2^{b}	46±1.1^{a}	48±1.5^{a}
Day 4	48±1.7^{b}	51±1.3^{b}	37±1.9^{a}	44±3.5^{ab}
Day 6	33±1.2^{b}	35±1.2^{b}	25±1.2^{a}	27±1.9^{a}
Day 8	16±1.0^{a}	13±1.5^{a}	13±1.2^{a}	15±1.9^{a}
All days	43±0.6^{a}	43±0.6^{a}	37±0.6^{a}	40±0.9^{a}
% live				
Day 0	78±2.3^{a}	78±2.8^{a}	78±2.2^{a}	80±2.1^{a}
Day 2	69±2.4^{a}	72±2.8^{a}	74±1.4^{a}	73±2.1^{a}
Day 4	64±3.1^{a}	69±2.9^{b}	69±1.4^{b}	69±1.8^{b}
Day 6	57±3.1^{a}	64±3.0^{b}	66±1.7^{b}	67±2.3^{b}
Day 8	45±3.2^{a}	54±3.6^{b}	63±1.1^{c}	65±2.4^{c}
All days	63±1.3^{a}	67±1.3^{b}	70±0.7^{c}	71±1.0^{c}
Meanα_t				
Day 0	219.0±0.84^{a}	217.2±0.71^{a}	357.6±6.50^{b}	391.6±4.11^{c}
Day 2	221.1±0.77^{a}	220.5±1.06^{a}	426.5±4.96^{b}	423.1±4.95^{b}
Day 4	221.1±0.77^{a}	218.8±0.66^{a}	438.1±7.99^{b}	459.0±7.71^{b}
Day 6	216.0±0.67^{a}	218.6±0.69^{a}	456.3±4.08^{b}	460.7±6.56^{b}
Day 8	216.6±0.76^{a}	219.2±0.63^{a}	458.0±2.25^{b}	492.1±6.26^{c}
All days	218.8±0.15^{a}	218.9±0.15^{a}	427.3±1.03^{b}	445.3±1.18^{c}
SDα_t				
Day 0	21.7±0.37^{a}	23.4±0.45^{a}	114.0±3.86^{b}	110.7±4.01^{b}
Day 2	22.9±0.42^{a}	22.6±0.43^{a}	168.6±1.58^{b}	178.5±3.77^{c}
Day 4	28.0±1.19^{a}	25.3±0.77^{a}	186.2±1.84^{b}	182.5±1.91^{b}
Day 6	23.1±0.74^{a}	23.4±0.35^{a}	196.1±2.98^{b}	214.5±3.15^{c}
Day 8	22.8±0.48^{a}	21.7±0.70^{a}	217.6±2.61^{b}	212.3±1.95^{b}
All days	23.7±0.13^{a}	23.3±0.11^{a}	176.5±0.51^{b}	179.7±0.59^{b}
COMPα_t				
Day 0	0.53±0.27^{a}	1.16±0.47^{a}	19.68±2.65^{b}	19.80±2.42^{b}
Day 2	0.44±0.23^{a}	1.82±1.01^{a}	27.53±2.24^{b}	29.63±2.72^{b}
Day 4	0.72±0.11^{a}	0.73±0.08^{a}	37.94±4.51^{b}	42.33±5.39^{b}
Day 6	0.47±0.27^{a}	0.94±0.21^{a}	41.72±4.92^{b}	39.58±3.81^{b}
Day 8	0.88±0.19^{a}	0.93±0.19^{a}	42.00±4.78^{b}	50.81±3.99^{b}
All days	0.61±0.14^{a}	1.12±0.18^{a}	33.78±1.71^{b}	36.43±1.64^{b}

4.4.4 The effect of ATA on the susceptibility of DNA of sperm stored under aerobic and anaerobic conditions to *in situ* acid denaturation

Inclusion of ATA in the sperm storage medium had a strong destabilising effect on sperm chromatin (Figure 4.2) as measured by all parameters derived from SCSA (Table 4.3). This effect was observed almost immediately after dilution of the sperm in the medium containing ATA, and increased with the time of storage. However, there was no change in viability and motility of sperm caused by ATA. Mean α_t on days 0 and 8 of storage as well as on average across all days of storage, and the SD of α_t on days 2 and 6 were slightly but significantly higher ($P < 0.05$) for sperm stored under aerobic than anaerobic conditions in the medium containing ATA. All SCSA parameters of sperm stored under aerobic and anaerobic conditions in the diluent that did not contain ATA were much lower than those of sperm stored in presence of ATA. All SCSA parameters of samples stored in the medium without ATA remained stable during the storage period. There was no significant difference ($P < 0.05$) between sperm stored under aerobic and anaerobic conditions in the absence of ATA on any of the storage days.

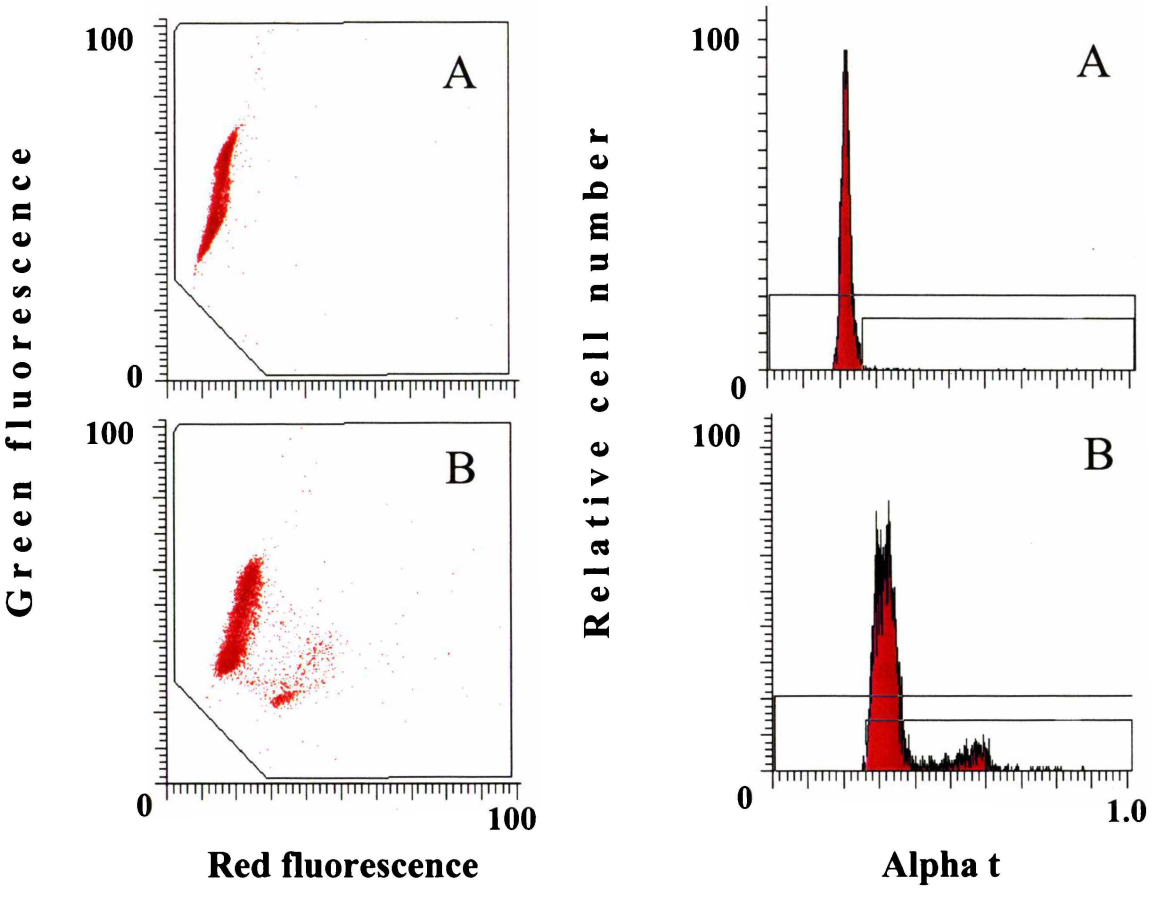


Figure 4.2 Representative SCSA cytograms and corresponding histograms of bovine sperm incubated at ambient temperature for 15 minutes under the following conditions: A) sperm diluted in 14G buffer; B) sperm diluted in 14G buffer containing 25 µmol/l ATA.

4.5 DISCUSSION

The effects of exposure to H_2O_2, on the motility and viability of sperm during storage at ambient temperature were similar to those observed in previous reports where exogenous H_2O_2 was either directly added to the diluent (Aitken *et al.* 1998a) or generated by aromatic amino acid oxidase (Shannon and Curson 1972; Shannon and Curson 1981; Shannon and Curson 1982; Shannon and Curson 1983b). The present study also confirmed the beneficial effect of catalase on sperm survival observed by Shannon and Curson (1982), although total exclusion of oxygen from the medium was more effective in maintaining sperm motility and viability during storage than addition of catalase alone (Table 4.1). Plasma membrane integrity was shown to be damaged by ROS more easily under aerobic than anaerobic conditions. This is likely to occur because lipid peroxidation chain reaction initiated by ROS is sustained by oxygen and thus can proceed under aerobic but not anaerobic conditions.

The finding that exposure to H_2O_2 during storage caused an increase in susceptibility of sperm DNA to *in situ* acid denaturation supports previous observations. DNA damage in sperm exposed to ROS *in vitro* has been demonstrated using single cell electrophoresis (COMET) assay (Aitken *et al.* 1998a) and deoxynucleotidyl transferase mediated end labelling (TUNEL) assay (Lopes *et al.* 1998). Detection of DNA breaks by single cell electrophoresis (Aravindan *et al.* 1997) and TUNEL assay (Evenson 1999) has previously been shown to correlate well with the SCSA. Interestingly, results obtained in this study show remarkable stability of bull chromatin stored in 14G diluent in the absence of exogenous ROS. In contrast a significant decline in chromatin stability was detected after only one day of storage in mouse sperm stored in un-supplemented T6 diluent (Estop *et al.* 1993), or human sperm stored in human tubal

fluid supplemented with human serum albumin (Ellington *et al.* 1998). The high stability of bull sperm chromatin observed in this study may in part be an intrinsic property of the sperm of this species which, unlike sperm of numerous primates and rodents (Balhorn *et al.* 1991), including that of humans (Domenjoud *et al.* 1988) and mice (Maleszewski *et al.* 1998), contain only one type of protamine. However, since other studies have demonstrated SCSA alterations during storage of bull sperm in commercial semen extender at a higher temperature (39°C) (Karabinus *et al.* 1991), it may be the function of the 14G diluent used here that provided protection to the genome during storage. The 14G extender has been experimentally developed over a number of years for maintenance of high viability and fertility of bovine sperm during *in vitro* storage at ambient temperature (18-22°C).

In contrast to the deleterious effects of ROS on sperm chromatin, any endogenous nuclease activity in the sperm was seen to be of little effect under present storage regime. Sperm cells possess endogenous nucleases that, according to some reports, can under stress conditions be activated to cleave both exogenous and genomic DNA, leading as a consequence to apoptosis-like cell death (Spadafora 1998).

ATA is a general nuclease inhibitor that has been reported to suppress endonuclease activity and promote long-term survival of some types of somatic cells under conditions which would otherwise lead to apoptotic cell death (Batistatou and Greene 1991; Okada and Koizumi 1995). Incubation of sperm with ATA prior to IVF has been shown to improve the yield of two-cell embryos (Zaccagnini *et al.* 1998). Since the susceptibility of sperm DNA to *in situ* acid denaturation as determined by SCSA has in limited studies been shown to be strongly correlated with DNA strand breaks (Aravindan *et al.* 1997),

it was expected that a potential protecting effect of ATA exerted via nuclease inhibition on sperm DNA stability would be detected.

A surprisingly high level of DNA instability that increased with the time of storage was detected in all samples stored in the presence of ATA, while virtually no change occurred in all SCSA parameters in samples stored in absence of ATA (Figure 4.2). The destabilising effect of ATA on chromatin was very rapid as it was detected in all samples frozen approximately 15 minutes after dilution (Figure 4.2). In this experiment, chromatin was destabilised by ATA before any change in motility or viability was observed (Table 4.3), thus showing that SCSA parameters were independent of classical semen measures. This observation indicates that increased SCSA values were not due to cell death as defined by the loss of motility and plasma membrane integrity. The mechanism of chromatin destabilisation by ATA may well be different from the mechanism of chromatin destabilisation caused by exposure to H_2O_2, as the latter occurs in parallel with the loss of motility and viability (Tables 4.1 and 4.2).

Zaccagnini *et al.* (1998) did not offer a clear explanation of the fact that ATA was most effective in increasing IVF yields at concentrations below those required for complete inhibition of sperm nucleases. They speculated that at low concentrations (5 μmol/l), ATA has an anti-apoptotic effect exerted through partial inhibition of nucleases, while higher concentrations (25-50 μmol/l) ensure complete endonuclease inhibition but also lower two-cell IVF yields. The results of the present study are contradictory to the hypothesis that ATA stabilises the sperm nucleus. It is possible that ATA, while inhibiting sperm nucleases, causes changes to protamines and DNA packing within the nucleus leading to higher DNA susceptibility to acid denaturation. In turn, such partial

de-condensation of chromatin could have a positive effect on IVF yields by making sperm chromatin easier to fully de-condense inside the oocyte during fertilisation. An alternative explanation for the decreased chromatin stability in the presence of ATA observed in our study is that ATA somehow caused DNA damage.

If ATA caused limited DNA strand breaks it would not necessarily decrease *in vitro* fertility because, as demonstrated by Ahmadi and Ng (1999), the oocyte can repair defective DNA contributed by sperm if the damage does not exceed 8%. In addition, sperm with extensive DNA damage can fertilise an oocyte with the same efficiency as an undamaged sperm. Furthermore, cleavage can proceed after fertilisation with sperm with damaged DNA up to the eight-cell stage, at which time paternal genomes are activated. Studies are needed that go up to and beyond the eight-cell stage. However, it is difficult to imagine that the DNA damage would actually increase IVF yields. It may be that ATA is acting in another way as it has been reported to inhibit numerous enzymes other than nucleases (Zaccagnini *et al.* 1998).

The fact that sperm stored for 4 to 8 days in the presence of ATA under aerobic conditions have lower viability than in all other treatments suggests that ATA is cytotoxic under aerobic but not anaerobic conditions.

In human sperm various markers of apoptosis have been reported. Fas molecule and DNA fragmentation are present in some cells of sub-fertile males (Sakkas *et al.* 1999) and are thought to be the result of uncompleted apoptosis that was initiated in the testis rather than induced following ejaculation. Externalisation of phosphatidylserine, detected with annexin-V and DNA fragmentation have also been observed in ejaculated

human sperm (Blanc-Layrac *et al.* 2000). However, those markers are not necessarily specific to apoptosis (Charriaut-Marlangue and Ben-Ari 1995; Glander and Schaller 1999). Since apoptosis is an active process requiring protein synthesis for its execution (Walker *et al.* 1988) and since sperm are devoid of ribosomes and thus can not synthesise proteins, classical apoptosis is unlikely in these cells. Also apoptosis-specific morphological changes such as budding of the whole cell and production of membrane-enclosed apoptotic bodies have not been reported in sperm. Even so, the possibility of a programmed or induced cell death mechanism in sperm different from apoptosis in somatic cells could not be excluded.

4.6 CONCLUSIONS

ATA makes sperm DNA more susceptible to *in situ* acid denaturation. It is yet not known, whether those changes are due to alteration of protein and/or DNA or to DNA strand breaks. Taking into account that ATA is a general nuclease inhibitor, the first explanation seems more likely. Further experiments involving SCSA together with estimation of DNA strand breaks through single cell electrophoresis (COMET assay) or *in situ* labelling of DNA breaks (TUNEL assay) of bovine sperm exposed to various concentrations of ATA would help clarify this question. An IVF trial using bovine sperm pre-stored with ATA and assayed for chromatin stability would demonstrate if partial destabilisation of sperm chromatin prior to IVF could be beneficial to the outcomes of IVF.

Chapter 5

EFFECT OF SPERM NUMBER AND OXYGENATION STATE OF THE STORAGE MEDIA ON *IN VITRO* FERTILITY OF BOVINE SPERM STORED AT AMBIENT TEMPERATURE

5.1 INTRODUCTION

The physiological function of sperm is to deliver paternal genetic material into the oocyte in such a condition that it would sustain normal development of the embryo and foetus. During *in vitro* storage at room temperature in artificial insemination procedures, both the ability of the sperm cell to fuse with the oocyte and the genetic material itself undergoes gradual detrimental changes (Salisbury *et al.* 1976). Damage to the messenger (cellular components involved in oocyte penetration) results in decreased percentage of fertilised oocytes, while damage to the message (haploid genome) results in increased embryonic mortality.

It is generally accepted that during storage, both *in vivo* and *in vitro*, sperm undergo detrimental genome alterations sooner than they lose their capacity for fertilisation and motility (Salisbury and Flerchinger 1967; Salisbury *et al.* 1976; Vishwanath and Shannon 1997). The ultimate measure of sperm quality is its ability to fertilise the oocyte *in vivo*. The closest approximation possible for the study of this in laboratory conditions is *in vitro* fertilisation.

The ageing of the male gamete during *in vitro* storage plays a major role in the practice of artificial insemination with semen stored at ambient temperature. The detrimental

effects of sperm ageing, both *in vitro* and *in vivo*, has been demonstrated in many species. Storage of human sperm in the female reproductive organs is linked to increased incidence of failed pregnancies (Guerrero and Rojas 1975). In the rabbit, concurrent insemination with both fresh and stored semen demonstrated great competitive advantage of fresh sperm (Roche *et al.* 1968). Shaver and Yanagimachi (1978) found with artificial insemination that hamster females inseminated with sperm aged *in vitro* for up to 5 days resulted in decreased fertility and increased frequency of abnormal development. The effect of *in vitro* sperm storage on fertility is best documented for the bull, and data from a number of publications shows explicitly that the non-return rate declines and embryonic mortality rises with duration of liquid storage (Salisbury 1965; Salisbury and Hart 1970; Vishwanath and Shannon 1997; Willett and Ohms 1955).

The objective of the study presented in this chapter was to assess the *in vitro* fertilising potential of sperm that retained motility after storage at ambient temperature for extended periods of time. The study was extended to determine whether the decline in fertility of stored sperm could be reduced by altering the oxygenation state of the storage medium.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Analytical grade chemicals were used in this study. The CO_2, and the gas mixture of 88% N_2, 7% O_2, and 5% CO_2 were purchased from British Oxygen Co., Hamilton, New Zealand. Lactate, HEPES, hypotaurine and heparin (from porcine intestinal mucosa) were purchased from Sigma Chemical Co., St. Louis, MO, USA. TCM 199 medium with Earle's salts, MEM essential and non-essential amino acids solutions, and foetal calf serum, were purchased from Life Technologies, New Zealand. Affinity purified bovine serum albumin "ABRD" and ovine luteinising hormone were purchased from Immuno-Chemical Products, New Zealand. Mineral oil was purchased from E.R. Squibb & Sons Inc., Princeton, NJ, USA. H33342 Hoechst fluorochrome was purchased from Calbiochem-Novabiochem Corporation La Jolla, CA, USA. All other materials were purchased from the same suppliers as described in previous chapters.

5.2.2 Semen collection and evaluation

Semen was collected and evaluated as described in Section 2.2.3.

Sperm viability throughout experiments described in this chapter was measured as described in Section 3.2.4.

The percentage of motile sperm in samples was assessed visually as described in Section 3.2.5.

5.2.3 Processing and storage of liquid semen

Semen was initially diluted at 32°C, to the concentration of 100 x 10^6 sperm/ml into CAPROGEN® (Livestock Improvement Corporation, Hamilton, New Zealand) commercial extender (Shannon 1965) containing 20% egg yolk, and allowed to cool slowly to ambient temperature (18-20°C) to avoid cold-shock. It was then diluted to the final concentration of 10 x 10^6 with the same extender but with 5% egg yolk instead of 20% egg yolk. When standard storage conditions were used, the diluent was purged with nitrogen for 30 minutes to reduce the concentration of oxygen in it. For experiments requiring aerobic and anaerobic conditions the diluent was further processed as described in Section 3.2.2. When sperm were stored under aerobic conditions, the diluent was not gassed at all.

Diluted semen was stored at ambient temperature in sealed 100 ml sterile Schott bottles wrapped in aluminium foil, under aerobic, anaerobic or nitrogen-gassed conditions, depending on the experiment. Under nitrogen-gassed conditions, the headspace of bottles was filled with nitrogen. For storage of sperm under anaerobic conditions, the sperm were suspended in an anaerobic medium and stored inside an anaerobic chamber as described in Section 2.3.2. For storage under aerobic conditions, the bottles in which sperm were stored were opened daily and aerated by gentle shaking. The anaerobic and nitrogen-gassed storage bottles were shaken with the caps sealed to ensure equilibration with the gas phase of storage.

5.2.4 Recovery and *in vitro* maturation (IVM) of oocytes

Bovine ovaries obtained from a local abattoir were transported to the laboratory within 3 h of slaughter in a thermos flask containing 0.9% NaCl, at approximately 30°C.

Follicles 2 to 6 mm in diameter were gently aspirated with an 18g needle into a tube connected to a vacuum-pump system. Cumulus-oocytes complexes and follicular fluid were collected into HEPES-buffered TCM 199 medium supplemented with 10 μg/ml heparin and 0.4% (w/v) bovine serum albumin. The cumulus-oocyte complexes were recovered and evaluated under a stereomicroscope. Only oocytes surrounded by a compact, multi-layered cumulus investment and homogenous cytoplasm were selected for maturation. Selected oocytes were washed twice in 5 ml of HEPES-buffered TCM 199 medium supplemented with 10% (v/v) foetal calf serum. Then oocytes were cultured in 50 μl droplets of maturation medium (TCM 199 supplemented with 10% foetal calf serum, 10 μg/ml ovine follicle stimulating hormone, 1 μg/ml ovine luteinising hormone and 1 μg/ml estradiol) under mineral oil, in a 35 mm diameter Falcon Petri (Becton Dickinson) at 39°C under an atmosphere of 5% CO_2 in humidified air for 24 h. Each droplet contained about 10 oocytes.

5.2.5 Preparation of stored sperm for *in vitro* fertilisation (IVF)

On the day of fertilisation a 15 ml aliquot of diluted semen of each bull was drawn out of the storage Schott bottles and centrifuged at 500 *g* for 5 min, the pellet was re-suspended in 1 ml of HEPES-Tyrode's Albumin Lactate Pyruvate medium (HEPES-TALP) (Bavister and Yanagimachi 1977) and divided into 4 equal parts. Aliquots were also taken from storage bottles for motility assessment and flow cytometry. Each 0.25 ml aliquot of suspension of sperm was carefully layered at the bottom of a 5 ml plastic test tube containing 1 ml of HEPES-TALP medium, and then incubated for 1 h at 39°C. Thereafter, the upper 3/4 of the medium, which contained motile sperm that had swum up into it, was collected by careful aspiration. The motility of the sperm selected by swim-up procedure was assessed under a microscope equipped with phase contrast

optics, while the concentration of sperm was determined by haemocytometry. The final concentration of motile sperm was adjusted to 2 x 10^6 motile sperm cells/ml by dilution with the fertilisation medium described by Lu *et al.* (1987).

5.2.6 *In vitro* fertilisation and culture (IVF-IVC)

After *in vitro* maturation as described in Section 5.2.4, oocytes with expanded cumulus investments were selected and washed twice by transferring them in a minimal volume of the medium to 5 ml of HEPES-TALP buffer in a new Petri dish, and then transferred, in groups of 5, into 40 µl droplets of fertilisation medium, under mineral oil. Sperm selected with swim-up procedure (as described in Section 5.2.5) were further diluted in the fertilisation medium as necessary, and a 10 µl portion was added to the droplets to achieve an in-droplet concentration of 1 000, 2 000 or 4 000 motile sperm per oocyte depending on the experiment. Sperm were co-cultured with oocytes in fertilisation droplets contained in a 35 mm diameter Petri dish under mineral oil at 39°C under an atmosphere of 5% CO_2 in humidified air. After 24 h of sperm-oocyte co-culture, the cumulus cells around the zygotes/oocytes were removed by repeated pipeting with an automatic pipette. Then, zygotes/oocytes were washed twice placing by them in a 35 mm diameter Petri dish containing 5 ml of the HEPES-buffered version of synthetic oviduct fluid medium described by Tervit *et al.* (1972) and pipeting them twice up and down the automatic pipette tip. Zygotes/oocytes were then transferred using the minimal possible volume of HEPES-buffered version of synthetic oviduct fluid to a 50 µl droplet of culture medium comprising of synthetic oviduct fluid supplemented with MEM essential and non-essential amino acids and 8 mg/ml fatty acid free bovine serum albumin (Gardner *et al.* 1994) positioned in the centre of the 35 mm diameter Petri dish under mineral oil. From there zygotes/oocytes were distributed into 20 µl droplets

placed in the same Petri dish under mineral oil. Each culture droplet contained about 10 zygotes/oocytes. The numbers of cleaved oocytes (at least 2 blastomeres of approximately the same size) were recorded at 24 h. Un-cleaved oocytes were stained with H33342 Hoechst fluorochrome to detect pronuclei. Cleaved oocytes were cultured for 7 days at 39°C under humidified gas mixture of 5% CO_2, 7% O_2, 88% N_2 with a transfer of zygotes on day 5 to droplets of fresh culture medium (synthetic oviduct fluid supplemented with MEM essential and non-essential amino acids and 8 mg/ml fatty acid free bovine serum albumin). The number of zygotes developing to blastocyst stage was determined under a stereo microscope.

5.2.7 Statistical analysis of results

Data for fertilisation and development to blastocyst were analysed by analysis of variance with days as independent variable, percentage of oocytes fertilised or developed to blastocyst stage as dependent variable and bulls as random effect.

5.3 EXPERIMENTS

Ejaculates collected from 3 bulls were processed as described above, and diluted semen was stored at ambient temperature. Three experiments were conducted. In all experiments the motility of stored sperm was estimated by microscopic observation in the storage medium before processing for IVF and plotted against the time of storage. The percentage of sperm with intact plasma membrane in the storage medium before processing for IVF was determined by flow cytometry. The motility of swim-up-selected sperm was also assessed during sperm preparation for IVF to ensure that there was uniform number of motile sperm in fertilisation droplets.

In all experiments oocytes that were to be used for IVF were aspirated from the ovaries one day prior to fertilisation and matured as described in Section 5.2.4. In the first experiment, oocytes were fertilised *in vitro* with sperm (from each of 3 bulls) stored for 0, 2, 4, 7, 9 and 11 days at ambient temperature in nitrogen-gassed medium. Between 25 and 41 oocytes per semen sample from each bull were fertilised on each fertilisation day. The number of motile sperm used in the fertilisation droplets was 1000 per oocyte.

In the second experiment, oocytes were fertilised *in vitro* with sperm (from each of 3 bulls) stored for 0, 3 and 7 days at ambient temperature in nitrogen-gassed medium using ratios of 1000, 2000 and 4000 motile sperm per oocyte in the fertilisation droplets. Between 31 and 39 per sample of the semen from each bull used at one of three different sperm to oocyte ratios were fertilised on each fertilisation day.

In the third experiment, oocytes were fertilised with sperm (from each of 3 bulls) stored for 0, 3 and 7 days under anaerobic, aerobic and nitrogen-gassed conditions using the ratio of 1000 motile sperm per oocyte in the fertilisation droplets. Between 47 and 56 oocytes per sample of the semen from each bull stored at each of three different storage conditions were fertilised on each fertilisation day.

5.4 RESULTS

5.4.1 Changes in viability and motility of sperm during storage at ambient temperature

In the first and second experiments the percentage of motile sperm and the percentage of sperm with intact plasma membrane in diluted semen stored at ambient temperature were similar for up to 2 days of storage (Figures 5.1 and 5.2), following which the percentage of motile sperm declined more rapidly than the percentage of sperm with intact plasma membrane. When all sperm became immotile, about 45% of them still maintained membrane integrity (Figure 5.1).

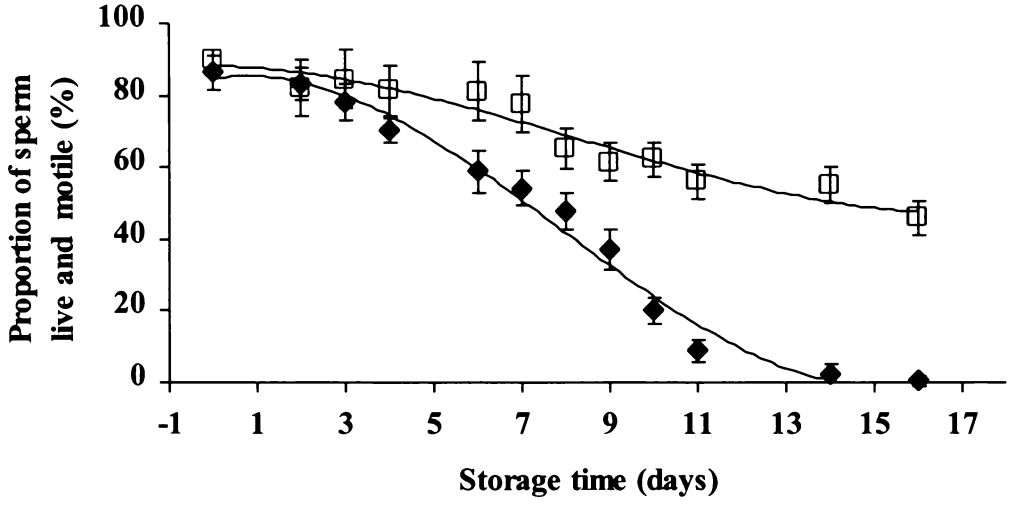


Figure 5.1 Changes in the percentage of sperm (from 3 bulls) with intact plasma membrane (□) (n=9, mean ± SD) and percentage of motile sperm (◆) (n=18, mean ± SD) during storage at ambient temperature under nitrogen-gassed conditions (first IVF experiment).

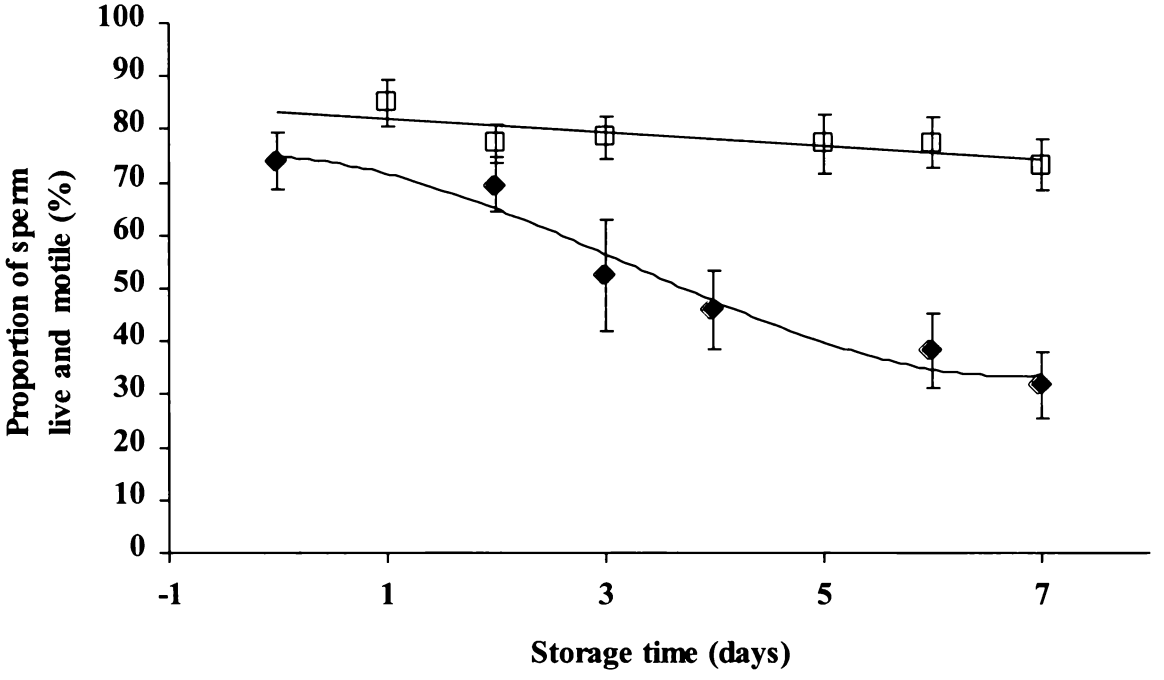


Figure 5.2 Changes in the percentage of sperm (from 3 bulls) with intact plasma membrane (□) (n=9, mean ± SD) and percentage of motile sperm (◆) (n=18, mean ± SD) during storage at ambient temperature under nitrogen-gassed conditions (second IVF experiment).

In the third experiment the percentages of sperm that were motile under aerobic, nitrogen-gassed and anaerobic storage conditions were similar on day 0 and on day 3 of storage, but on day 7 the motility of sperm was significantly lower ($p<0.01$) after storage under anaerobic than under aerobic and nitrogen-gassed storage conditions (Figure 5.3). The percentage of sperm with intact plasma membrane was lower in sperm stored under aerobic conditions ($p<0.01$) than in those stored under anaerobic and nitrogen-gassed conditions on days 3 and 7 of storage (Figure 5.4).

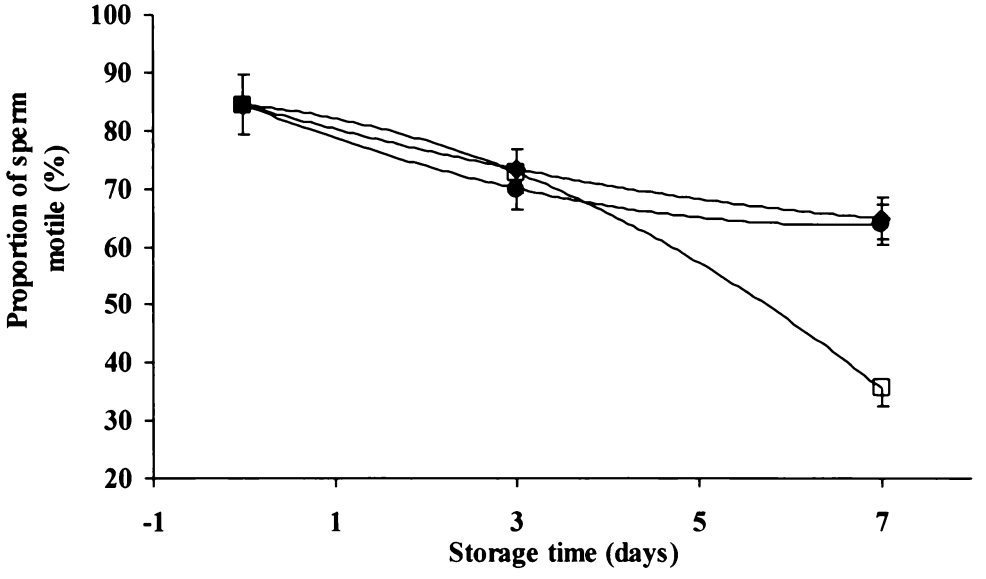


Figure 5.3 Changes in the percentage of motile sperm (from 3 bulls) during storage at ambient temperature under anaerobic (□), nitrogen-gassed (•) and aerobic conditions (◆) (n=18, mean ± SD).

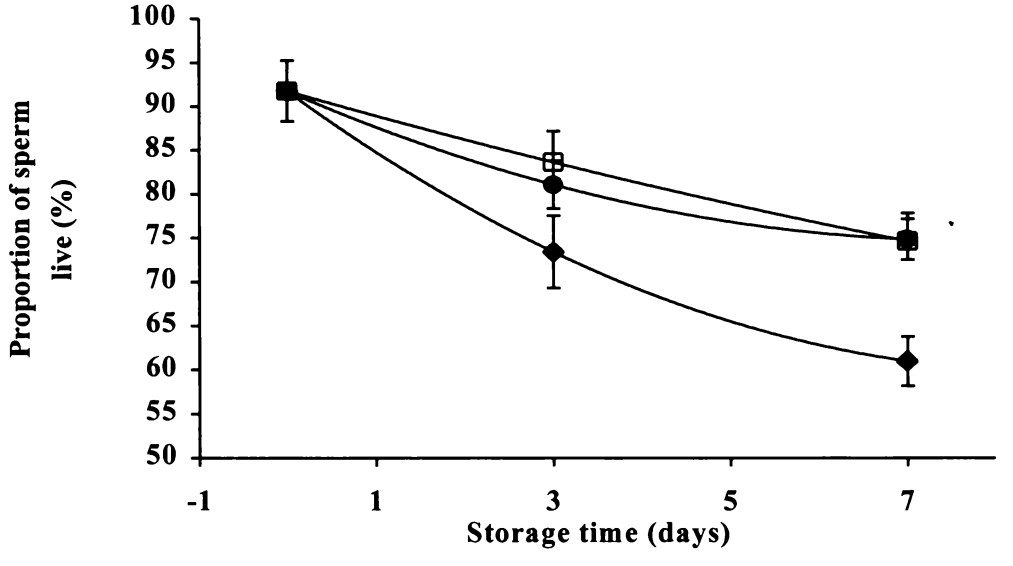


Figure 5.4 Changes in the percentage of sperm (from 3 bulls) with intact plasma membrane during storage at ambient temperature under anaerobic (□), nitrogen-gassed (●) and aerobic conditions (◆) (n=9, mean ± SD).

5.4.2 *In vitro* fertility of sperm stored at ambient temperature in nitrogen-gassed medium

The percentage of oocytes that were fertilised using the ratio of 1000 motile sperm per oocyte declined from 45.4% on day 0 of sperm storage to nil by day 11 (Table 5.1).

The percentage of fertilised oocytes that developed to blastocyst stage declined from 71.1% on day 0 to nil on day 7 of sperm storage, *i.e.* the early "embryonic" death increased from 8.9% of fertilised oocytes on day 0 to 100% on day 7 of sperm storage. In other words, some fertilisation was still taking place on day 7 and 9 without inducing subsequent development.

Table 5.1 Percentages of oocytes fertilised and developed to blastocyst stage when sperm stored at ambient temperature under standard conditions were used at the ratio of 1 000 of motile sperm per oocyte. Mean values (±SD) are shown for the percentages fertilised and developed to blastocyst stage after *in vitro* fertilisation by sperm from each of 3 bulls.

Day of storage	The total number of oocytes fertilised with sperm from 3 bulls	% of oocytes that were fertilised	% of fertilised oocytes that developed to blastocyst
0	101	45±4.9	71±6.7
2	85	32±5.1	49±9.6
4	96	28±4.6	36±9.1
7	98	10±3.0	0±0.0
9	119	5±2.0	0±0.0
11	75	0±0.0	0±0.0

In the second experiment the percentage of all the oocytes that were fertilised using ratios of 1000, 2000 and 4000 motile sperm per oocyte were overall much higher than in the first experiment, but again declined between day 0 and 7 of sperm storage for all the sperm to oocyte ratios used (Table 5.2).

The differences between percentages of oocytes fertilised achieved with different sperm to oocyte ratios were not significant but the percentage of fertilised oocytes that developed to blastocyst stage was significantly higher ($p<0.05$) when a higher sperm to oocyte ratio was used on days 0 and 3 of sperm storage. On day 7 of sperm storage there was no significant difference between the percentages of fertilised oocytes that developed to blastocyst stage achieved with different sperm to oocyte ratios.

At day 7 of storage only between 0% and 5% of fertilised oocytes were able to develop to blastocyst stage regardless of the sperm to oocyte ratio, in spite the fact that at all the ratios they were able to fertilise more than 40% oocytes.

5.4.3 The effect of storage atmosphere on the *in vitro* fertility of sperm stored at ambient temperature

The percentages of oocytes fertilised with sperm stored under aerobic, nitrogen-gassed and anaerobic conditions were not significantly different and declined from around 90% on day 0 to about 45% on day 7 (Table 5.3). Accidental loss of oocytes fertilised on day 0 of sperm storage meant that there were no data on the subsequent development to blastocyst stage. There is no significant difference in the percentage of fertilised oocytes developing to blastocyst stage with sperm stored under anaerobic, nitrogen-gassed or aerobic conditions and used for IVF on either day 3 or day 7 of sperm storage. For sperm stored under all conditions, the percentages of fertilised oocytes developing to blastocyst stage were much higher than those seen in first and second experiments on days 3 and 7 of sperm storage.

Table 5.2 Percentages of oocytes fertilised and developed to blastocyst stage when sperm stored at ambient temperature under standard conditions were used at various ratios of motile sperm per oocyte. Mean values (±SD) are shown for the percentages of oocytes fertilised and developed to blastocyst stage after *in vitro* fertilisation with sperm from each of 3 bulls (n is the total number of oocytes fertilised with semen from all bulls). Values marked with * are significantly higher ($p<0.05$) than those achieved with 1000 motile sperm per oocyte on the same day of storage.

	1000 sperm per oocyte			2000 sperm per oocyte			4000 sperm per oocyte		
Day of storage	n	% of oocytes that were fertilised	% of fertilised oocytes that developed to blastocyst	n	% of oocytes that were fertilised	% of fertilised oocytes that developed to blastocyst	n	% of oocytes that were fertilised	% of fertilised oocytes that developed to blastocyst
0	104	78±4.0	32±5.3	109	89±3.0	45±5.1	99	92±2.7*	54±5.2*
3	95	54±5.1	13±4.6	114	56±4.7	30±5.7	115	63±4.5	35±5.6*
7	100	48±5.0	2±2.2	95	44±5.1	0±1.0	98	48±5.0	5±3.2

Table 5.3 Percentages of oocytes fertilised and developed to blastocyst stage when sperm stored at ambient temperature under anaerobic, aerobic and nitrogen-gassed (standard) conditions were used at the ratio of 1 000 of motile sperm per oocyte. Mean values (±SD) are shown for the percentages of oocytes fertilised and developed to blastocyst stage *in vitro* fertilisation with sperm from each of 3 bulls. (n is the total number of oocytes fertilised with semen from all bulls).

	Anaerobic			Aerobic			Nitrogen-gassed		
Day of storage	n	% of oocytes that were fertilised	% of fertilised oocytes that developed to blastocyst	n	% of oocytes that were fertilised	% of fertilised oocytes that developed to blastocyst	n	% of oocytes that were fertilised	% of fertilised oocytes that developed to blastocyst
0	142	94±1.5		142	93±2.16		146	91±2.36	
3	155	93±2.6	64±4.0	152	88±2.68	67±4.09	151	95±1.82	64±4.01
7	168	40±3.9	56±6.2	165	52±3.89	63±5.21	169	44±3.82	61±5.64

5.5 DISCUSSION

A sharp decline in the percentage of motile sperm, without a corresponding decrease in the percentage of sperm with intact plasma membrane, suggests damage to regulatory mechanisms rather than cellular structures responsible for motility. This is also supported by the similar results obtained in the experiments described in Chapter 3 and discussed in Section 3.4 showing that a large proportion of sperm that lost motility as a result of storage could regain it when exposed to the phosphodiesterase inhibitor in order to raise their intracellular level of cAMP. When sperm were stored under aerobic conditions, plasma membrane integrity deteriorated faster than under anaerobic or nitrogen-gassed conditions, however the same is not true for sperm motility. On day 7 of storage, the motility of sperm stored in the anaerobic medium was lower than that of sperm stored under either aerobic or nitrogen-gassed conditions. This is not consistent with other experiments conducted in this laboratory, where the motility was better maintained under anaerobic than aerobic storage conditions (Bullen 1999).

In the experiments presented here, it was a sperm sub-population that retained motility that was tested for fertility, as the dead and immotile sperm were excluded using swim-up selection.

Because the decrease in fertilising ability was seen with the same concentration of motile sperm on different days of the storage trials it is clear that loss of *in vitro* fertility in experiments presented here was not caused by the loss of motility or plasma membrane perforation. However, more subtle changes, potentially occurring before the total loss of motility and plasma membrane integrity, might have been a cause of decline in fertility. This could include damage to nuclear material.

If sperm were loosing fertilising ability without concurrent damage to the genomic material, the proportion of fertilised oocytes developing to blastocyst would remain constant while the proportion of all oocytes which became fertilised would decline over time. If, on the other hand, only the genetic material were negatively affected, the percentage of oocytes being penetrated would remain constant with the percentage of normally developing zygotes declining. The present study showed that during storage sperm lose both the oocyte-penetrating ability and the ability to sustain early development of the zygote. The ability to sustain development declined more rapidly than the oocyte-penetrating capability in two experiments (Tables 5.1-5.2). In the third experiment the results are incomplete due to accidental loss of the samples during culture, but the percentage of fertilised oocytes that developed to blastocyst stage are higher than in other two experiments and do not change significantly between day 3 and 7 of storage. The results of first two experiments point to the relatively more rapid decline in the quality of the genomic material delivered to oocyte then the decline of the efficiency of cellular mechanisms involved in oocyte penetration. The results of the third experiment indicate that the sperm ability to penetrate the oocyte and to sustain early development of the zygote declined at very similar rates over the time of storage.

The percentage of oocytes developing to blastocyst stage is a better measure of fertility than just the percentage of oocytes penetrated by sperm because the oocytes that are fertilised but fail to develop would not result in a pregnancy. Some sperm retain the ability to penetrate the oocyte after they are damaged to the extent that they are unable to induce correct development of fertilised oocytes. Salamon *et al.* (1979) showed that the oocyte-penetrating capacity of ram sperm is preserved for up to 10 days at 5°C, but rates of lambing declined drastically after 3 days of semen storage.

In artificial insemination experiments, the effective motile sperm concentration at the fertilisation site would perhaps decline in parallel with the time-dependent decline in the percentage of motile sperm if the total insemination dose were kept constant throughout the storage trial. The other point of difference between current IVF results and the non-return data from artificial insemination with stored sperm is that only very early stages of embryo development (up to 7 days) were investigated here as opposed to longer-term effects observed when fertility is estimated from non-return rates. The small volume of the droplets used for fertilisation in this study, and the absence of cumulus vestments, means that motile sperm were essentially delivered almost to the surface of the oocytes, and thus did not need, as sperm in artificial insemination do, to cover the distance from the deposition site at the top of the cervix to the fertilisation site in the oviduct.

In artificial insemination the semen dose is correlated, up to an optimal value, with fertility, and the increased fertility is attributed to the increased probability of sperm reaching the oocyte surface (Shannon and Vishwanath 1995). The decline in fertility is more pronounced during storage when lower sperm doses are used for artificial insemination (Willett 1950; Willett 1953). The present study showed that, apart from increasing the probability of fertilisation, increased sperm numbers at the fertilisation site correlate with higher percentages of oocytes developing to blastocyst stage, particularly during the early stages of storage. This suggest that some form of sperm competition or oocyte selection occurs and is based on the ability of sperm to induce development rather than just on the ability of sperm to penetrate the oocyte.

A recent report (Van Dyk *et al.* 2000) suggests that human zona pellucida has the capacity to select against motile sperm with chromosomal abnormalities. The

mechanism of this selection remains unknown but it may also select against sperm with potential chromatin defects accumulated during *in vitro* storage. An alternative explanation that may be put forward is that higher numbers of sperm on the oocyte surface assist the development of the embryo resulting from fertilisation by a single sperm.

As seen in artificial insemination (Vishwanath and Shannon 1997), current results show that increasing the sperm to oocyte ratio does not increase the percentages of oocytes fertilised or developed to blastocyst stage achieved with semen stored for more than 3 days.

Maxwell and Stojanow (1996) investigated *in vitro* and *in vivo* fertility and viability of ram sperm stored in the presence and absence of antioxidants. They used a constant number of motile sperm in IVF droplets, and in artificial insemination each ewe was inseminated with the same total number of sperm throughout the storage trial. They found that the percentage of *in vitro* and *in vivo* fertilised ovine oocytes declined with the time of semen storage. The percentage of *in vitro* fertilised oocytes after 7 but not after 14 days of semen storage was higher in diluents containing superoxide dismutase and catalase than in control diluents. Pregnancy rates achieved with artificial insemination using fresh sperm were higher than those achieved with stored sperm, and overall the pregnancy rates were better with sperm stored in the diluent containing catalase and superoxide dismutase than in the medium without antioxidants. However on any single day of storage there was no significant difference between pregnancy rates achieved with semen stored in diluents containing antioxidants and those achieved with semen stored in the control diluent. Their results suggest that oxygen is probably

an important factor in reducing sperm viability and fertility during liquid storage because of lipid peroxidation caused by reactive oxygen species generated during respiration (Maxwell and Stojanow 1996). The reduction in the fertility of stored sperm may reflect the changes in the intensity of sperm motility or/and changes in their plasma membranes (Maxwell and Stojanow 1996). Maxwell and Stojanow speculated that the concentration of scavengers of reactive oxygen species was not sufficient to give protection from reactive oxygen species after 14 days of storage. After an extended storage period sperm may lose fertility regardless of the presence or absence of reactive oxygen species. The decline seen in artificial insemination results may be due to there being a lower probability of fertilisation with the lower number of motile sperm in the insemination dose, as a constant total number of sperm for each insemination was used. Probably, the overall decline in fertility is due to energy substrate exhaustion and general cellular deterioration. Another possibility is that sperm may possess a mechanism of programmed cell death similar to apoptosis in somatic cells.

The rationale behind the trial involving sperm storage in diluents with different oxygen content was the expectation to see a sharper decrease in fertility of sperm stored under aerobic conditions due to increased exposure to reactive oxygen species when compared with sperm stored under anaerobic and nitrogen-gassed media, because reactive oxygen species have long been implicated in deterioration of sperm quality during storage (Aitken 1989; Aitken 1994; Aitken and Clarkson 1987; Aitken *et al.* 1998a; Aitken *et al.* 1993; Cummins *et al.* 1994; Vishwanath and Shannon 1997). However, no differences in the *in vitro* fertility of sperm stored under aerobic, nitrogen-gassed and anaerobic conditions were detected. There might be several explanations for this result. The diluent in all storage treatments contains catalase that offers protection from

hydrogen peroxide, perhaps the most detrimental of all reactive oxygen species. Also, damaged sperm from all the treatments have been eliminated from the population used for fertilisation by the swim-up procedure. Therefore, the quality of sub-populations selected by swim-up from sperm stored under different conditions was higher and more uniform than that of the all the sperm cells in the storage bottle.

5.6 CONCLUSIONS

It was demonstrated here that the *in vitro* fertility of the motile sub-population of sperm declines over the time of storage. From the experiments presented here it seems that this decline is not caused by the oxidative damage to sperm due to the presence of oxygen in the diluent. This is possibly because sufficient protection is afforded by catalase present in the diluent. This decrease in fertility is not a result of diminishing probability of sperm oocyte fusion due to decline in the number of motile sperm available, but is the result of a decline in the quality of sperm that retained motility. Apart from the loss of sperm ability to penetrate the oocyte, even more rapid loss of the ability of stored sperm to induce and sustain early development of the embryo was observed in two experiments. The loss of sperm quality could be partially compensated by the increased quantity of sperm at the fertilisation site, which may be the result of either more sperm competition for the oocyte selection, or due to the effect of greater number of sperm on maturation of the oocytes.

Chapter 6

GENERAL DISCUSSION

In artificial breeding systems that use unfrozen semen, sperm cells are diluted to facilitate an extended use of superior sires and stored only for the length of time necessary to distribute insemination doses from the collection centre (where the bulls are located) to the dams dispersed over a wide area. Initially this was done with sperm diluted in aerobic diluent using relatively high sperm numbers per insemination dose. However the adverse effect of O_2 was realised fairly early in the history of artificial insemination and the cause of damage was identified as reactive oxygen species generated under aerobic incubation conditions (Aitken and Clarkson 1987; Dawra and Sharma 1985; de Lamirande and Gagnon 1992; de Lamirande and Gagnon 1994; de Lamirande and Gagnon 1995; Gagnon *et al.* 1991; Kessopoulou *et al.* 1992; Potts *et al.* 1999; Shannon 1965; Shannon 1968; Shannon and Curson 1972; Vishwanath and Shannon 1997). The success of Long Last Liquid™ semen technology in New Zealand is to a great extent based on research into the ways of minimising the effects of reactive oxygen species on sperm (Shannon 1965; Shannon 1968; Shannon 1978; Shannon and Curson 1972; Shannon and Curson 1981; Shannon and Curson 1983c). That research was taken further in the present study by comparing changes in various parameters of sperm occurring during storage under fully aerobic and fully anaerobic conditions. This has previously not been attempted. Motility was investigated in Chapter 2 and to the lesser degree in other chapters. In Chapter 3 the influence of the presence of O_2 in the medium on the tyrosine phosphorylation of sperm proteins was examined.

In Chapter 4 the impact of exposure to reactive oxygen species on sperm chromatin stability was evaluated, and in Chapter 5 an attempt was made to demonstrate the effect of the presence of O_2 in the diluent on *in vitro* fertility of stored sperm.

The work presented in Chapter 7 was undertaken to reconcile two initial observations. First, it was assumed, based on previous findings that bovine sperm require active aerobic respiration to maintain a high level of motility (Prendergast 1994; Wilson 1985). However, it was demonstrated in the present study that, contrary to the initial hypothesis, ejaculated bovine sperm can maintain a high level of motility under fully anaerobic conditions as long as a glycolysable substrate is available in the diluent. Thus, the complete exclusion of O_2 from the storage diluent provides a potential means of elimination of oxidative damage without depriving sperm of a necessary metabolic pathway for the generation of ATP. Also it is now known that sperm are stored anaerobically in the epididymis for extended periods and are only exposed to oxygen upon ejaculation when motility is activated (Max 1992). This biological storage system could inspire investigations to imitate it *in vitro*. Some interesting questions, however, were not answered in the study described in Chapter 7. What is the effect of presumably increased flux through the Embden-Meyerhof pathway on the changes in the pH of the storage medium? This has subsequently been investigated and in 14G diluent the pH drop during storage is significantly sharper under anaerobic than under aerobic conditions (Pitt; personal communication). Is the higher motility observed under anaerobic conditions an experimental artefact? For how long can the viability and fertility of sperm be maintained if they were to be stored in a medium devoid of glycolysable substrate under anaerobic conditions where their energy-generating metabolism would be eliminated? This question could be answered by storing sperm

under such conditions and then assessing motility, viability and fertility after re-supplying them with glycolysable substrate, or with pyruvate and oxygen.

As the motility of bovine sperm seems to be independent of aerobic respiration, its decline must be caused by factors other than damage to the mitochondria. It was demonstrated in Chapter 3 that swimming velocities of sperm stored for as long as 7 days could be restored to values at least equal to or even higher than the velocities of freshly ejaculated sperm (Figure 3.2) by raising the intracellular concentration of cAMP via phosphodiesterase inhibition. This result indicates that the decline in motility during storage is not caused by the exhaustion of energy substrate in the diluent or irreversible damage to sperm components involved in motility. Rather, it seems the regulation of sperm motility is adversely affected by storage. Changes in the percentage of motile sperm during storage present a slightly different picture. It can be seen from Figure 3.1 that a proportion of the sperm became immotile during storage and could not be stimulated to regain motility by the inhibition of phosphodiesterase. Those cells can be regarded as permanently damaged. However even on day 8 of storage the percentage of motile sperm was increased three-fold by stimulation with theophylline. Sperm that regain motility have clearly dysfunctional regulation but are mechanically able to be motile.

When the changes in tyrosine phosphorylation of proteins in stored sperm were studied (Chapter 3) the results were the opposite of what was expected. The expectation was to see increased protein tyrosine phosphorylation under aerobic conditions as it has been described for sperm undergoing capacitation. Instead, the tyrosine phosphorylation of several proteins increased during storage under anaerobic conditions, with little change

under aerobic conditions. This demonstrated a strong effect of the oxygenation state of the diluent on an important mechanism of cellular regulation. The regulation of tyrosine phosphorylation during sperm storage by the oxygenation state of the non-capacitating diluent seems to act in the opposite direction to the regulation of the same process during capacitation.

Overall, the effect of complete exclusion of oxygen from the storage medium on the percentage of motile sperm appears to be complex. In two experiments (Figure 3.8 and Table 4.1) the percentage of motile sperm declined more rapidly under aerobic storage conditions, but in one experiment there was no significant difference (Table 4.3) and in one experiment the decline in the percentage of motile sperm was more rapid under anaerobic conditions. This should be further investigated in a randomised study focused solely on the percentage of motile sperm using an increased number of bulls. Changes in the pH of the diluent and availability of glucose should be measured simultaneously as they are known to affect motility. Also, the gas mixture of the anaerobic chamber should be checked for contaminants that could affect motility.

Although motility is necessary for fertility, it is not all that is required. Motile sperm could be infertile because of the defects in the chromatin. A remarkable stability of bull sperm chromatin in normal 14G diluent was demonstrated under both aerobic and anaerobic storage conditions (Chapter 4). However, exclusion of catalase from this diluent together with addition of the aromatic amino acid oxidase substrate phenylalanine to this diluent caused a significant destabilisation of chromatin under aerobic conditions. This is relevant to the practice of artificial insemination where egg yolk, which contains aromatic amino acid oxidase substrates, is normally added to the

diluent and where fully anaerobic conditions during storage are not maintained. In this situation the integrity of the sperm chromatin depends solely on the effectiveness of catalase to remove the H_2O_2 generated by sperm during storage. This may not always be adequate and even subtle damage to the sperm chromatin that may not necessarily be detected by SCSA could be detrimental to the fertilising potential of sperm.

No difference in *in vitro* fertility of the motile sub-population of sperm stored under aerobic and anaerobic conditions was detected (Chapter 5). An interesting question arises from this, whether the *in vitro* fertility of the total sperm population would be the same for sperm stored under aerobic and anaerobic conditions. This could be investigated by conducting an *in vitro* fertilisation trial where the same total number of sperm stored under different conditions would be used on different days of storage without selection of motile sperm.

Recommendations for further research

In relation to changes in sperm motility during storage it would be interesting to investigate the following problems. The rate of glucose loss from the storage medium and the change in lactate concentration and pH should be measured under anaerobic and aerobic storage conditions to perhaps improve the buffering capacity of the current diluent. The change in viability and the possibility of restoring motility to sperm stored under conditions of arrested metabolism achieved by the absence of glycolysable substrate and O_2 in the diluent should also be investigated. Since reduced metabolic rate may improve sperm survival and maintenance of fertility during storage at ambient temperature.

As shown in Chapter 3, the tyrosine phosphorylation of several cytosolic and whole cell proteins changes during storage, and those changes are modulated by the oxygenation state of the diluent. The isolation and identification of proteins with storage-related changes in tyrosine phosphorylation would shed some light on their possible role in sperm ageing. Isolation of those proteins could be achieved by immuno-chromatography of cell extracts using anti-phosphotyrosine antibodies immobilised on agarose gel. Proteins separated by SDS PAGE could be blotted to PVDF membrane and the amino acid sequences of internal or terminal peptides obtained. Based on those amino acid sequences proteins could be identified.

In Chapter 4, ATA was shown to have a dramatic effect on sperm chromatin stability. It would be interesting to see if incubation of bovine sperm with ATA improves IVF efficiency as it does with mouse sperm. Whether the destabilisation of chromatin by ATA is the result of increased occurrence of DNA breaks or destabilisation of protamines in the sperm head should also be investigated. DNA breaks could be detected by COMET or TUNEL assays. The effects of ATA on sperm protein tyrosine phosphorylation could also be investigated as it has been reported to stimulate tyrosine phosphorylation in other cells (Okada and Koizumi 1995).

The multitude of effects due to incubation of sperm under fully anaerobic and fully aerobic conditions has been demonstrated in this thesis. Further studies are certainly required to further explore the impact of chronic anaerobiosis on sperm. This will provide a new impetus to the studies on the methods of sperm storage at ambient temperature.

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