

Preservation and evaluation of semen for artificial insemination

Lindsay Gillan^{A,B}, W.M. Chis Maxwell^A and Gareth Evans^A

^ACentre for Advanced Technologies in Animal Genetics and Reproduction, The Faculty of Veterinary Science, The University of Sydney, NSW 2006, Australia. ^BTo whom correspondence should be addressed, email: lindsayg@vetsci.usyd.edu.au

Abstract. Many years of research have been devoted to improving the fertility of preserved semen of small ruminants. There have been few significant advances in preservation in recent times, but considerable knowledge has been gained on the effect of preservation on the structure and function of spermatozoa. It has become evident that preservation greatly affects many sperm attributes, such as motility, respiratory activity, membrane status and DNA quality. Consequently, viability is reduced, transport in the female reproductive tract is inhibited, the timing of fertilisation is altered and embryo development is affected following insemination of preserved, compared to fresh spermatozoa. A greater understanding of their functional condition may lead to the development of methods of preventing these alterations or to improved methods of using the preserved spermatozoa for artificial insemination in their altered state.

Extra keywords: frozen storage, liquid storage, small ruminant, spermatozoa.

Introduction

Breakthroughs in semen preservation were made with the discoveries of the protective agents in egg-yolk for cooling (Phillips 1939) and glycerol for freezing (Bernstein and Petropavlovsky 1937; brought into prominence by Polge *et al.* 1949). This was followed by a period of intensive empirical study, with numerous manuscripts published demonstrating the effects of different storage diluents, protective agents and other additives, cooling rates, storage temperature, warming rates and extending semen preservation methods across many livestock species. The aim of semen preservation is either to prolong the lifespan of the spermatozoa from ejaculation to insemination, or beyond to fertilisation, by depressing the metabolism of the spermatozoa at a reduced temperature or by some other means (liquid storage), or to completely arrest the metabolic processes and in essence suspend the spermatozoa in time (frozen storage).

The obvious disadvantage of liquid-stored spermatozoa is their limited lifespan, restricting transportation over large distances and preventing long-term banking. However, if semen utilisation is high within the shelf-life period after dilution, the economic advantages are significant; large numbers of females can be inseminated with a relatively low dose of spermatozoa, storage is inexpensive and the semen can easily be used in the field. There have been several extensive reviews on the preservation of small ruminant semen in a liquid state (see Maxwell and Salamon 1993; Leboeuf *et al.* 2000; Salamon and Maxwell 2000).

Frozen storage has the advantages of being able to preserve important genes for future use, provide insurance against loss

of a particular sire, permit the transport of semen over long distances and allow large numbers of females to be inseminated over extended periods of time or at different times of the year. However, it is relatively expensive to store spermatozoa in this state and, owing to the low numbers of spermatozoa surviving the process, high total numbers of spermatozoa are required for subsequent AI. A wealth of information has been accumulated on all aspects of frozen storage of small ruminant semen and this has been thoroughly reviewed elsewhere (Salamon and Maxwell 1995a, [1995b](#), 2000; Leboeuf *et al.* 2000). The only recent significant advancement in semen preservation technology that has arisen since the reviews were written is 'multi-thermal gradient' (MTG) freezing (Arav *et al.* 2002). Multi-thermal gradient is based on directional freezing where biological material is moved in a linear temperature gradient so that the cooling rate and ice front propagation are precisely monitored, facilitating full control over ice nucleation and ice crystal morphology. Although the post-thaw motility is acceptable when MTG is used (for example, ram: 57%, bull: 65%, boar: 45%) perhaps it will be most useful in the future for banking frozen semen in large volumes. Despite the thorough research, progress in the preservation of small ruminant semen has reached a plateau of achievement. As evidence of this, a recent study by Salamon *et al.* (2004) showed that ram semen cryopreserved 35 years ago yielded fertility results after intrauterine insemination that are within today's acceptable standards. Nevertheless, over the past 30 years there has been much knowledge gained on the functional condition of the spermatozoa after preservation. Recent studies have revealed that preservation techniques do

not merely suspend spermatozoa in time, but induce sublethal damage that affects their functional capacity that makes them behave very differently to fresh spermatozoa.

In the present manuscript, evidence of reduced sperm function following preservation will be reported, with particular reference to the ram. We will discuss the underlying causes of the altered state of spermatozoa after preservation identifying the problem, showing evidence of its effect on fertility and offer a discussion on how this can be prevented where appropriate.

Evidence of reduced sperm function *in vivo* after preservation

Irrespective of the type of diluent or method of storage used, damage occurs to a significant portion of spermatozoa resulting in a reduction in motility, a decline in viability and decreased fertility with increased length of storage of liquid semen and as a consequence of frozen storage. Maxwell and Salamon (1993) summarised critical studies on the use of liquid-stored ram semen for cervical insemination and reported that fertility decreased at a rate of 10-35% per day of storage. Thus, although 68-75% of ewes lambed from insemination with fresh semen in a single cycle, the lambing rates for semen stored for 24, 48 and 72 h were 45-50%, 25-30% and 15-20% respectively. Similarly, after frozen storage the fertility of small ruminant semen is greatly reduced and fertility results similar to those achieved for fresh semen have only rarely been obtained following cervical insemination (reviewed by Salamon and Maxwell 1995a, 1995b?).

The viability of spermatozoa in the female reproductive tract is also affected by semen preservation. Lopyrin (1971) flushed the tract of the ewe after cervical AI and reported that fresh spermatozoa survived ~27 h, spermatozoa liquid-stored for 24 h survived 22-23 h and spermatozoa liquid-stored for 48 h survived 19-22 h in the cervical canal. Spermatozoa stored at ambient temperatures (16-18°C) survived longer than those at 0°C. Gillan *et al.* (1999) examined the rate of loss of fresh compared to frozen-thawed spermatozoa from the cervix of ewes in synchronised oestrus after intrauterine insemination. At the time of insemination, a balloon catheter was inserted into the vagina to collect spermatozoa lost from the tract. Similar numbers of fresh and frozen-thawed spermatozoa were lost from the tract, but those frozen-thawed were lost at a faster rate than fresh.

There is also an increased incidence of embryonic mortality after insemination with preserved semen. Rabocev (1965) and Lopyrin and Rabocev (1968) observed that after AI with liquid-stored semen a high proportion of the zygotes showed signs of degeneration. When semen stored at 5°C was used for cervical AI there was a steeper decline with the age of semen in embryonic survival after single than double inseminations suggesting that further ageing of the preserved spermatozoa in the female reproductive tract was detrimental

(Salamon *et al.* 1979). Langford *et al.* (1979) reported 33% embryonic mortality with frozen-thawed spermatozoa and only 6% for fresh spermatozoa after intrauterine AI.

Several investigators (reviewed by Salamon and Maxwell 1995b) have also reported inadequate transport of preserved spermatozoa through the reproductive tract of small ruminants after cervical AI (cervix: Lopyrin and Loginova 1958; utero-rubal junction or both structures: Mattner *et al.* 1969; Platov 1983).

However, fertility of preserved semen can be increased if the depth of cervical insemination is increased. Achieving sufficient penetration of the cervix is problematic and obtaining acceptable conception rates is variable, leading to the requirement for the cervix to be bypassed surgically. Satisfactory fertility has been achieved after intrauterine insemination with chilled semen stored for 8 days, and some spermatozoa retained their fertilising capacity for up to 10 days (Salamon *et al.* 1979). When frozen-thawed spermatozoa are deposited into the uterus or oviduct, high (85-95%) fertilisation rates have been obtained (Salamon and Maxwell 1995b).

The observations from these studies show that preserved spermatozoa are fertile, but behave very differently *in vivo* than fresh spermatozoa. They have prompted the initiation of efforts to identify the causes of the decreased fertility.

Identification of the underlying causes of decreased fertility of preserved spermatozoa

Many studies aiming to improve the fertility of preserved spermatozoa have addressed the issue by increasing the number of motile spermatozoa inseminated. Although there is an improvement in fertility after cervical insemination with a greater dose of preserved spermatozoa, this does not fully explain their low fertility. For example, a minimum of 100 million motile fresh ram spermatozoa is required to be deposited in the cervix for adequate fertility. In order to achieve comparable fertility rates with a frozen-thawed sample published estimates range from 160 (Lightfoot and Salamon 1970) to 800 million motile frozen-thawed spermatozoa (Colas 1979). This suggests that the spermatozoa, although motile, are functionally compromised in some manner that prevents them from either reaching the site of fertilisation or participating in fertilisation. The use of laboratory tests of sperm quality and function has led to improvements in our understanding of the underlying mechanism responsible for their altered state. In this section we will discuss the effects of liquid and frozen storage on various sperm attributes and suggest reasons for decreased fertility.

Altered motility and respiratory activity

There is a significant reduction in motility with length of time of liquid storage and as a result of freezing and thawing. It has been reported that there is a disruption to the axonemal elements of the tail (Watson 1995) and this may not only reduce

the number of motile spermatozoa but also affect the type of motility. Using computer-assisted semen analysis (CASA), Moses *et al.* (1995) determined that all kinetic parameters changed significantly after freezing and thawing of ram spermatozoa. There have been several studies, particularly in dairy cattle, where tests of sperm function were carried out in the laboratory and correlations drawn with fertility after AI, in which some sperm motility parameters have shown a correlation. For example, one to five sperm motility parameters (Farrell *et al.* 1998), linear motility (Zhang *et al.* 1998) and total number of motile frozen-thawed bull spermatozoa assessed by CASA (Januskauskas *et al.* 2003) have all been correlated with fertility. Therefore, the effect of preservation on motility may explain the reduced ability of preserved spermatozoa to penetrate the cervix following cervical AI and may also explain the increase in fertility that occurs when preserved spermatozoa are inseminated directly into the uterus in closer apposition to the oocyte (Maxwell 1986).

Treatment with gonadotrophin-releasing hormone (GnRH) in combination with progestagen sponges and pregnant mare serum gonadotrophin (PMSG) has been used to reduce the period of time over which ovulation occurs in ewes synchronised for AI with frozen semen (Eppleston *et al.* 1991). Although reports of the use of GnRH with sheep has been variable when a commercial dose of spermatozoa is used for AI (Salamon and Maxwell 1995), when Smith *et al.* (1986) used a low dose of spermatozoa for intrauterine insemination there were increased pregnancy rates when GnRH was administered. With the elucidation of the altered state of preserved spermatozoa perhaps further optimisation of the use of GnRH in small ruminants would have a beneficial effect on fertility.

Motility may also decrease following preservation owing to a disruption in the availability of energy produced in the midpiece to the axoneme, although it is unknown whether this affects the pattern of motility. The architecture of the mitochondria in the midpiece is altered during freezing and thawing, resulting in a loss of structure (see Watson 1979; Courtens and Paquignon 1985; Courtens *et al.* 1989) and this damage has been shown to decrease the respiratory rate of spermatozoa (Windsor and White 1995; Windsor 1997). Using the fluorochrome rhodamine 123 (R123), which accumulates in the mitochondria of actively respiring spermatozoa, Gillan and Maxwell (1999) reported that the mitochondrial respiration of frozen-thawed spermatozoa decreases more rapidly than that of fresh spermatozoa over a 6-h incubation period (37°C). Also using R123, Windsor (1997) observed the effect of cryoinjury on mitochondrial respiration and demonstrated that mitochondrial respiration is important for ram spermatozoa to be able to successfully penetrate the cervix. Windsor (1997) also reported that mitochondrial inhibition of ram spermatozoa reduced fertility after cervical, but not laparoscopic intrauterine insemination.

Ruminant spermatozoa are characterised by a high capacity for oxidative respiration (Mann and Lutwak-Mann 1981) and the cervix of the ewe contains adequate quantities of lactic acid and oxygen, but is low in glucose and other glycolysable sugars emphasising the role of oxidative respiration to transit through this region of the tract. Furthermore, several glycolytic enzymes (hexokinase, glucose phosphate isomerase and lactate dehydrogenase) leak from the spermatozoa during freezing and thawing, possibly a consequence of membrane damage (Harrison and White 1972). Thus, mitochondrial injury may be a significant contributor to the poor fertility observed after cervical insemination with spermatozoa and may explain the improvement in fertility when the cervix is bypassed and the spermatozoa are placed directly into the uterus. Although at present it is not known how damage to the mitochondria can be prevented, it would be expected that methods of avoiding direct damage to spermatozoa owing to ice crystal formation might be beneficial.

Taken together the altered sperm kinetics and mitochondrial injury occurring during semen preservation may explain the impaired sperm transport, resulting in the premature death of large numbers of preserved spermatozoa in the lower parts of the female tract after cervical or vaginal deposition.

Accelerated capacitation

Ejaculated spermatozoa cannot fertilise until they undergo capacitation, which is the first stage of membrane destabilisation events involving an efflux of cholesterol and redistribution of intrinsic membrane proteins and lipids (Harrison 1996). This destabilisation continues with the acrosome reaction and ultimately results in a loss of viability. Thus, it is essential that these events take place at the appropriate time and place within the female reproductive tract if spermatozoa are to participate in fertilisation. Early scientists were largely unaware of this intricate timeline of events that begins in the epididymis and ends in fertilisation or cell death when they initiated studies into semen preservation. It is only in the last few years that the relevance of the timeline of events in the lifespan of the spermatozoon to semen preservation has become fully appreciated.

Frozen-thawed spermatozoa are immediately capable of penetrating oocytes, whereas fresh spermatozoa require preincubation before attaining similar penetration rates (boar: Clarke and Johnson 1987; Wang *et al.* 1991; bovine: Wheeler and Seidel 1986; gorilla: Lambert *et al.* 1991; human: Critser *et al.* 1987; ram: Garde *et al.* 1993; Siberian tiger: Byers *et al.* 1989). Gillan and Maxwell (1999) also reported that *in vitro* matured ovine oocytes were penetrated earlier by frozen-thawed than by fresh spermatozoa, as evidenced by the presence of more advanced zygotes 24 h after insemination. In addition, frozen-thawed bovine spermatozoa have been shown to have the capability of fertilising in the absence of heparin (Cormier *et al.* 1997), which is necessary to induce

capacitation in this species (Parrish *et al.* 1988). Preservation suspends the continuum of changes in spermatozoa that begins in the epididymis and ends at fertilisation or cell death and it was traditionally thought that the process merely interrupts most biological processes, with those cells surviving possessing the same characteristics as in their original pre-storage state. However, the ability of preserved spermatozoa to immediately penetrate oocytes in an IVF system is further evidence of their altered functional state, suggesting that they are in a more advanced state than fresh spermatozoa. This led Watson (1995) to suggest that the spermatozoa emerge from frozen storage in a state resembling that of capacitation.

To test Watson's hypothesis in sheep, Gillan *et al.* (1997) investigated the proposal that freezing and thawing may cause ram spermatozoa to undergo membrane changes similar to capacitation. Using chlortetracycline (CTC), to measure calcium-mediated changes thought to be associated with capacitation, these authors found that a high proportion of the spermatozoa were capacitated after freezing and thawing compared to fresh spermatozoa. This has also been observed by several others (bull: Cormier *et al.* 1997; mouse: Fuller and Whittingham 1997; ram: Perez *et al.* 1996). It is thought that it is the process of cooling and re-warming that induces capacitation-like changes, rather than events associated with ice formation, and thus cells merely cooled to 0–4°C also display this effect (boar: Maxwell and Johnson 1997; bull: Cormier *et al.* 1997; mouse: Fuller and Whittingham 1997). Green and Watson (2001) reported that the changes between cooled and re-warmed spermatozoa and those of spermatozoa capacitated *in vitro* ('true' capacitation) were similar when analysed using CTC. However, using Merocyanine 540 (Molecular Probes, Eugene, OR, USA) to assess changes to the sperm membrane lipid bilayer fluidity, and protein tyrosine phosphorylation to assess intracellular signalling pathways, the events occurring in cooled and re-warmed spermatozoa do not completely mirror those of true capacitation. The authors suggested that alternate routes of activation occur during both processes, with the spermatozoa arriving at a similar end-point, namely destabilised membranes that will readily undergo an acrosome reaction. A similar observation was made by Cormier and Bailey (2003) who found that capacitation induced by either heparin or the freezing and thawing of bull spermatozoa was associated with a different profile of phosphotyrosine-containing proteins.

After insemination, spermatozoa must survive long enough to reach and colonise the oviduct in order to fertilise the oocyte. If the life of the spermatozoa is reduced by premature capacitation as a consequence of storage at reduced temperatures, the spermatozoa will only have a short time to achieve fertilisation compared to fresh spermatozoa. Thus, the advanced membrane status of preserved spermatozoa would explain their premature death in the lower regions of the female reproductive tract following cervical insemination, the loss of preserved spermatozoa from the tract

quicker than fresh spermatozoa and the decreased fertility of preserved spermatozoa. Although it is not possible to draw definitive conclusions about the importance of sperm membrane changes associated with preservation and fertility when the semen is used for AI, we can gain some insight from fertility studies. Thundathil *et al.* (1999) used CTC staining and reported that the fertility of frozen-thawed bull spermatozoa increased with an increased proportion of viable, non-capacitated (F-pattern) spermatozoa in an inseminating dose. Collin *et al.* (2000) also used CTC to show the proportion of acrosome-reacted (AR-pattern) frozen—thawed bull sperm was related to fertility after AI. Furthermore, the increase in fertility observed when frozen-thawed spermatozoa are inseminated close to the time of ovulation supports the limited lifespan of the spermatozoa and their readiness to fertilise (Maxwell 1986).

An attempt to improve fertility by altering the membrane state was made by Maxwell *et al.* (1999) after finding that the post-thaw addition of seminal plasma to ram spermatozoa resulted in less capacitated and AR cells when assessed by CTC. The frozen-thawed spermatozoa in the presence of seminal plasma had increased motility, an increased ability to penetrate cervical mucus *in vitro* and increased fertility after cervical AI. Interestingly, the seminal plasma had no effect on the fertility of fresh spermatozoa or when frozen-thawed spermatozoa were used for laparoscopic intrauterine AI. The addition of seminal plasma brought the fertility of frozen-thawed spermatozoa after cervical AI within the normal ranges of fertility of spermatozoa inseminated in the uterus. This suggests that the seminal plasma stabilised the sperm membranes and may prove useful as a thawing solution. An attempt was also made by Cormier and Bailey (2003) to inhibit preservation induced capacitation by adding glucose to a bull semen extender to inhibit heparin-induced capacitation. Unfortunately, this attempt was unsuccessful in preventing capacitation following preservation.

Altered interaction with oviduct epithelial cells

The accelerated capacitation-like state of spermatozoa after preservation would also be expected to affect the ability of these cells to colonise the oviduct. In all animal models studied so far, the isthmus of the oviduct acts as a reservoir and conserves the function of spermatozoa if mating or insemination takes place before ovulation. A minimum of 6–8 h is required for a functional population of spermatozoa to be established in the oviducts of sheep and cows mated at oestrus (Hunter and Wilmut 1984; Hunter and Nichol 1986). Immediately before ovulation the oviduct activates a portion of these spermatozoa to progress to the site of fertilisation in the ampulla (Hunter and Nichol 1983). It is clear that spermatozoa undergo necessary membrane changes during intimate contact with the epithelial cells of the oviduct. Suzuki *et al.* (1997) found that frozen-thawed bull spermatozoa

reacted in a different way from fresh spermatozoa when co-incubated with bovine oviduct epithelial cells, but they did not observe membrane status. When fresh and frozen-thawed ram spermatozoa were incubated with an oviduct epithelial cell monolayer (OECM) (Gillan *et al.* 2000), frozen-thawed spermatozoa immediately bound to the OECM whereas fresh spermatozoa bound more slowly. The frozen-thawed spermatozoa were released quickly and the fresh spermatozoa more gradually. These observations may explain the limited lifespan of preserved spermatozoa in the female reproductive tract and the importance of the correct timing of insemination to ovulation. In fertility studies, De Pauw *et al.* (2002) found a correlation between the log_e transformed number of frozen-thawed spermatozoa bound to 0.1mm² oviduct epithelium and fertility after AI, provided the membrane integrity of the spermatozoa was greater than 60%. This suggests that a decreased ability of preserved spermatozoa to interact with oviduct epithelial cells would also adversely affect their fertility. Although it is not known how to prevent the altered interaction of frozen-thawed spermatozoa with the epithelial cells of the oviduct, perhaps this may be alleviated by the post-thaw addition of seminal plasma to spermatozoa.

Altered capacity for normal embryo development

There is increased embryonic loss after insemination with preserved semen, which decreases the fertility. It is possible that ageing already functionally capacitated spermatozoa in the female reproductive tract may not prevent fertilisation but reduce subsequent embryo development. There is some evidence that embryonic loss may be increased when stored spermatozoa are further aged in the female reproductive tract, resulting in asynchrony between the age of the spermatozoa and the oocyte (Salamon *et al.* 1979). Furthermore, the advanced stage of development of embryos fertilised by spermatozoa that have bypassed 'normal' capacitation may result in abnormal embryo development. It has been suggested that ewes inseminated with frozen-thawed spermatozoa timed closely with ovulation to prevent further ageing of the spermatozoa in the tract will produce more viable embryos (Gillan *et al.* 1997).

A direct effect of the preservation process on the sperm nucleus may also be responsible for increased embryonic loss after insemination with preserved semen. There are reports that the proportion of double-stranded DNA decreases after freezing and thawing (Royere *et al.* 1988; Gillan and Maxwell 1999) and that the surface area of the sperm head also decreases significantly, possibly owing to the 'overcondensation' of the chromatin in the nucleus (Thompson *et al.* 1994; Gravance *et al.* 1998). From fertility studies it has been reported that the quality of the DNA in the spermatozoa in an insemination dose is correlated with fertility and may be helpful in understanding the increased embryonic mortality observed with preserved semen. Using the

terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay, which identifies DNA strand breaks, Duran *et al.* (2002) found that if greater than 12% of human spermatozoa contained fragmentation there was no pregnancy and Benchaib *et al.* (2003) observed no pregnancies if this value was greater than 20%. Furthermore, DNA fragmentation was higher in the spermatozoa from couples experiencing unexplained, recurrent pregnancy loss when compared with control samples with proven fertility (Carrell *et al.* 2003). The sperm chromatin structure assay (SCSA®) detects abnormal chromatin structure. This assay has been used on small ruminants (Sailer *et al.* 1995), and good correlation with fertility has been reported in several species (boar: Evenson *et al.* 1994; bull: Ballachey *et al.* 1988; Karabinus *et al.* 1990; Januskauskas *et al.* 2001; human: Evenson *et al.* 1999; mouse: Evenson *et al.* 1980; stallion: Love and Kenney 1998) where spermatozoa from males with low fertility exhibit more chromatin denaturation than spermatozoa from high fertility males.

Variation between individual animals in their ability to withstand the preservation processes ('freezability') may be owing to the susceptibility of their DNA to the preservation-induced damage. Using computer-aided sperm head morphometry analysis (Moruzzi *et al.* 1988), Gravance *et al.* (1997, goat; 1998, bull) assessed the effect of frozen storage on sperm head structure. No overall significant freezing effect was found in goats but there was some individual buck variation. Bull sperm heads were significantly smaller following freezing and thawing, which might be expected to be a result of condensation of the DNA. The authors suggested that this variability in sperm head morphology might be a reason for variations in the ability of individuals to tolerate freezing.

Conclusions

Many years of research have been devoted to improving the fertility of preserved semen (reviewed by Salamon and Maxwell 2000). Most have been empirical studies on diluent components, increasing the number of spermatozoa in a dose, hormonal treatment of the females or semen and double or deep inseminations. Although there have been few significant advances in small ruminant semen preservation in recent times, considerable knowledge has been gained on the effect of the preservation processes on the structure and function of spermatozoa. It has become apparent that preservation greatly affects many sperm attributes, such as motility, respiratory activity, membrane state and DNA quality. Consequently, the viability is reduced, transport in the female reproductive tract is inhibited, the timing of fertilisation is altered and embryo development is affected following insemination of preserved, compared to fresh spermatozoa. The next significant improvements in fertility of preserved spermatozoa may result from the identification of the mechanisms behind their altered state and this may lead to the development of

methods of preventing these alterations or to better methods of using the preserved spermatozoa in their altered state.

By thorough evaluation of preserved spermatozoa it may be possible in the future to identify molecular markers for resistance to the detrimental effects of preservation and reduced fertility after preservation. Progress in this direction has been provided by Thurston *et al.* (2002) who identified DNA markers associated with successful 'freezability' of individual boar ejaculates using a novel molecular technique, amplified fragment length polymorphism. The authors used this procedure to visualise differences in DNA sequences between individual boars classified as having poor, average and good post thaw recovery as assessed by the percentage motile cells, motility characteristics assessed by CASA, plasma membrane integrity and acrosome integrity. Sixteen candidate genetic markers for semen freezability in the boar were identified. A molecular explanation for poor sperm quality after preservation may lead to more successful methods of preservation and identify methods to protect the spermatozoa to prevent them from undergoing these changes altogether.

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