

Symposium: Cryopreservation and assisted human conception

Current and future concepts and practices in human sperm cryobanking



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David Mortimer gained his PhD from Edinburgh University in 1977 and received post-doctoral training in Edinburgh, Paris and Birmingham before joining the faculty of the University of Calgary in 1983. There he continued his research on human sperm pathophysiology and was Scientific Director of the Infertility Programme. In 1991 he moved to Sydney IVF where he developed the novel sequential culture media and incubators that now constitute the Cook Culture System. He held positions at Sydney University and the Royal Prince Alfred Hospital, worked on the WHO's *Manual for the Standardized Investigation, Diagnosis and Management of the Infertile Male*, and was Programme Chairman for the 11th World Congress on IVF and Human Reproductive Genetics held in Sydney in 1999. By 2000 he had moved back to Canada and established an international consulting company based in Vancouver. Major consultancy projects since 1986 have included andrology labs, sperm banks and IVF units, and advice on accreditation, Total Quality Management and risk management. He has 120 publications to his name (including the book *Practical Laboratory Andrology*) and made over 200 conference presentations worldwide. In May 2000 he was appointed to the Advisory Board of the Wildlife Breeding Resource Centre (Pretoria, South Africa).

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Abstract

This article focuses on a range of issues that are of current concern to those working in human sperm cryobanking and considers them within a general framework of risk analysis and management, taking into account the available evidence and perceptions of 'best practice' from both the medical and legal perspectives. In particular, issues arising from concerns over the risk of cross-contamination between samples during storage immersed in liquid nitrogen have been analysed in detail. Even though such an occurrence has never been reported for spermatozoa or embryos, and the risk is generally accepted to be vanishingly small, it does represent a finite risk and all reasonable measures should be taken to reduce the chance of its occurring. It is concluded that all methods used to collect, cryopreserve, store, thaw and use cryobanked human spermatozoa must address the risk of contamination from any source throughout the entire process. To assist workers in this area, a series of recommendations as to current best practice are made, based upon conclusions from risk analyses using currently available information.

Keywords: banking, biosafety, cryostorage, freezing, human, spermatozoa

Introduction

Many reviews of human sperm preservation have been published. Therefore, rather than provide another comprehensive review of the literature on human sperm cryobanking and its application in human reproductive conservation and infertility treatment, this article will focus on a series of specific issues that are of current concern to those working in these areas. These issues will be considered within a general framework of risk analysis and management (Mortimer and Mortimer, 2005), taking into account the available evidence and perceptions of 'best practice' from both the medical and legal perspectives. Because many of the issues are inter-related, it has not been

possible to discuss each one fully in a strict logical sequence and cross-referencing has been provided to avoid repetition. Hopefully, this will not prove too confusing to readers.

For background on, and general reviews of, basic cryobiology and sperm cryopreservation, readers are referred to articles by Watson (1979), David and Price (1980), Mortimer (1994), Royere *et al.* (1996), Gao *et al.* (1997), Critser (1998), Leibo and Bradley (1999), Oehninger *et al.* (2000), Nijs and Ombelet (2001), Leibo *et al.* (2002) and Pegg (2002), as well as those by Sherman (1973, 1977, 1986, 1990). Ethical aspects of gamete banking or donor screening are not considered (see Barratt and Cooke, 1993; Daniels and Haines, 1998; Englert, 1998).

Although a few experimental studies on the vitrification of human spermatozoa have been reported very recently (for review, see Isachenko *et al.*, 2003), the approach remains unproven for clinical application. Furthermore, cells being vitrified are typically exposed directly to liquid nitrogen or other cryogenic agents, either in order to achieve sufficiently rapid cooling rates or as a consequence of the packaging system (e.g. using cryoloops or open pulled straws), a factor that greatly complicates, and in many situations precludes, effective biocontainment. This major problem must be resolved before such techniques can be applied clinically under safe working practices.

For ease of expression in the text, 'LN₂' is used to refer to liquid nitrogen, and 'LNV' to liquid nitrogen vapour, i.e. the gaseous phase above LN₂ inside a cryostorage Dewar flask.

Historical perspectives

The most significant, albeit accidental, discovery pertaining to sperm cryopreservation was the observation of the effectiveness of glycerol as a cryoprotectant for bovine spermatozoa by Polge and colleagues in 1949. At that time the emphasis in semen cryopreservation was focused upon farm animals (principally cattle), but shortly afterwards Sherman reported the successful cryopreservation and storage of human spermatozoa on dry ice (-78.5°C) with the production of pregnancies and apparently normal offspring. The use of LNV freezing was also described by Sherman in the early 1960s, along with the first normal births from this method. Detailed historical reviews of this early period have been published by Sherman (1973, 1977, 1980, 1990).

Despite unfounded and misleading concerns over the possible dangers of genetic and functional instability of cryopreserved spermatozoa, greater appreciation of the potential and applications of human semen cryobanking developed with wider use of cryopreserved donor semen during the early 1970s. The first commercial human semen cryobanks were opened in the USA during the 1970s, aimed at providing 'fertility insurance' for the anticipated millions of men who would have vasectomies. Poor growth in that market slowed the expansion of commercial semen banks until the second half of the 1980s when the absolute need for quarantined donor semen to reduce the risk of AIDS transmission was established. Some donor sperm banks now advertise availabilities of 200 or more donors (e.g. Cryos, Århus, Denmark; Fairfax Cryobank, Fairfax, VA, USA), and 30,000 births/year from frozen donor sperm insemination are estimated worldwide.

Human sperm cryobanking is practised worldwide but France remains the most organized country, with its national network of sperm banks. The original Centre d'Étude et de Conservation du Sperme Humain (CECOS) established by Professor Georges David at Le Kremlin-Bicêtre in 1973 became the headquarters of the Fédération Française des CECOS (Federation CECOS). As a national organization, CECOS had 14 centres by 1979, 20 by 1989, 22 by 1994 and now 23 centres (David, 1989; Federation CECOS *et al.*, 1989; Le Lannou *et al.*, 1998). Cumulative results from donor spermatozoa provided by CECOS now exceed 50,000 live births.

The longest period of cryopreservation that has resulted in a human birth is at least 15¼ years (Dr JH Olson, personal communication), although frozen bovine spermatozoa have been used to produce normal blastocysts by IVF after 37 years in cryostorage (Leibo *et al.*, 1994). This confirms that semen cryopreservation maintains the reproductive potential of human spermatozoa during effectively indefinite storage at -196°C.

Rigorous standards of operation are essential for all sperm banks and must be distinguished from the specific requirements for donor screening and quarantining. Licensing is required in some countries and sperm banks are inspected using specific lists of standards, guidelines or regulations (e.g. USA, UK) which have been developed, and usually updated regularly, either by professional organizations (e.g. British Andrology Society, 1999; Canadian Fertility and Andrology Society, 2000; American Association of Tissue Banks, 2002) or government agencies. The European Union is currently considering a directive on setting standards of quality and safety for the donation, procurement, testing, processing, storage, and distribution of human tissues and cells. The Directive will include human gametes and embryos, and is expected to be applied also to centres that only handle them for autologous use. It incorporates a requirement for the accreditation of all European tissue banks by an 'accredited, designated, authorized or licensed by a competent authority', and is expected to be passed in April 2004 with a subsequent 2-year compliance period.

Applications

Human semen cryobanking can be divided into two broad areas of autoconservation (i.e. semen banking for one's own future use, sometimes referred to 'client depositors') and donor banking, although there are many permutations in its clinical application.

Semen autoconservation

(i) Preservation of reproductive potential before chemical, radiological or surgical cancer therapy that could render an individual sterile, severely subfertile or impotent. (ii) 'Fertility insurance' for a man before he undergoes surgical sterilization (vasectomy). (iii) 'Convenience cryobanking' to ensure availability of a man's spermatozoa to treat his partner during his absence (e.g. military service) or as a reserve in case he is unable to provide a fresh ejaculate at the precise moment it is required for an IVF or IUI procedure. (iv) Storing and subsequently pooling ejaculates from oligozoospermic patients for their later combined use (although this is of limited benefit due to the poor cryosurvival of spermatozoa from such men).

Donor semen cryopreservation

Cryobanking confers a number of important advantages over the use of fresh semen in the provision of donor semen for therapeutic procedures. (i) Storage of donor semen pending results of microbiological tests on the ejaculate to prevent the transmission of infectious organisms by donor insemination. (ii) Quarantining donor semen to permit repeat testing of the donor for viral infections such as HIV or hepatitis, which can

be transmitted sexually. (iii) Ready availability of a wide selection of donor phenotypes and genotypes.

Fecundity of cryopreserved donor semen

The much-debated issue of poor fertility achieved using cryopreserved semen during the 1980s was largely related to comparisons between fresh semen and poor-quality cryobanked semen and concern caused early resistance to the use of cryobanked donor semen. The debate became academic subsequent to the AIDS epidemic and demands for adequate and responsible infection control.

It has also been demonstrated that comparable fecundity rates can be achieved using cryobanked donor semen. (Fecundity is defined as the chance of conceiving per cycle of trying, whereas fertility is the actual occurrence of conception after a defined period.) Fecundity rates of 10–12% per *unstimulated* insemination cycle can be achieved provided that proper recruitment and acceptance criteria are employed for semen donors, individual ejaculates and ‘doses’ (i.e. individual units of semen, e.g. a straw or a cryovial) so that at least 5×10^6 progressively motile spermatozoa can be inseminated into the lower cervical canal on 2–3 occasions during the peri-ovulatory period (Scott *et al.*, 1990). Ovarian stimulation provides a substantial increase in fecundity rates: odds ratio = 2.63 (95% confidence interval = 1.85–3.73), especially in cases where the results with previous cervical insemination had been poor (O’Brien and Vanderkerckhove, 2002). Intrauterine insemination (IUI) also obviates the need for multiple inseminations per cycle (Khalifa *et al.*, 1995).

Issues

Cryoprotectants

Cryoprotectant agents (CPA) are generally classified as ‘penetrating’ or ‘permeating’ cryoprotectants that actually cross the plasma membrane of the cells being frozen, and ‘non-permeating’ ones that do not (although those in the latter category are not, strictly speaking, actually cryoprotectant agents). The solution used to dilute the semen or sperm suspension (‘extend’ in the parlance of theriogenologists, for whom the dilution of the original semen sample is often quite substantial) is a buffered medium that contains a permeating CPA and, usually, one or more non-permeating CPA; such a solution is often referred to as a ‘cryoprotectant medium’ or ‘CPM’.

Glycerol is by far the most widely used and successful permeating CPA for human spermatozoa; a final concentration of 6.0–7.5% (v/v) seems to be optimum. Dimethylsulphoxide (DMSO) not only has direct deleterious effects on human spermatozoa but also requires their exposure to cold shock. Propanediol (PrOH), which is the most successful cryoprotectant for cleavage stage human embryos, has seen little application with spermatozoa while ethylene glycol has been proposed as a suitable CPA for human spermatozoa but has received little attention (Gao *et al.*, 1997).

Non-permeating CPA are often macromolecules or sugar molecules that increase the extracellular osmolality and aid in dehydrating the cells during slow freezing, e.g. sucrose,

trehalose. Hen’s egg yolk, a common component of CPM for human spermatozoa, is also a non-permeating cryoprotectant that maintains sperm plasma membrane fluidity. Indeed, some workers have reported that adequate cryosurvival can be obtained with egg yolk in the absence of glycerol (see review by Sherman, 1990).

Buffering the pH of the CPM during freezing is essential to avoid damaging the spermatozoa. Buffering is effected by glycine and citrate in Ackerman’s GEYC (glycerol–egg yolk–citrate) medium (Ackerman and Behrman, 1975), but more modern recipes employ a combination of the zwitterionic buffers TES and TRIS. This TES–TRIS combination (usually abbreviated to ‘TEST’) is most often used in conjunction with egg yolk and citrate with glycerol as the permeating cryoprotectant (Weidel and Prins, 1987; Mortimer, 1994; Jeyendran *et al.*, 1995; Stanic *et al.*, 2000). Media based on phosphate-buffered saline are not recommended, due to the poor pH buffering provided by such solutions at lower temperatures (van den Berg and Rose, 1959).

Is one cryoprotectant medium the best?

No one CPM formulation (or freezing regimen) has been proven to be better than others when considered over a population of individuals. Pertinent points include: (i) Great inter- and intra-individual variability exists in the cryosurvival of spermatozoa between different CPMs and cryopreservation methods (Friberg and Gemzell, 1977). (ii) Studies using pooled semen conceal such inter-individual variability and hence contribute little to establishing an optimized method with broad applicability. (iii) The ‘human sperm preservation medium’ (‘HSPM’) developed by Mahadevan and Trounson (1983) was formulated as a compromise medium based on Tyrode’s solution; in addition to 20 mmol/l HEPES it includes 31 mmol/l sodium bicarbonate. Therefore, basic chemistry dictates that if such a solution is kept under an air atmosphere, its pH buffering will be stressed. This might be at least a partial explanation of why TYG was found to be superior to HSPM (Stanic *et al.*, 2000). (iv) If one needs to cryopreserve semen from a specific man, it might be necessary to try several different CPMs to establish the one best suited for his spermatozoa. (v) There still remain some men who, in spite of apparently normal sperm quality pre-freeze and post-thaw, and even proven fertility of their fresh semen, fail to achieve pregnancies when their cryopreserved spermatozoa are used for insemination. (vi) For semen donors, the problem has been circumvented by only accepting those donors who show good cryosurvival by the specific method that is standard for the cryobank.

Notwithstanding the above, over the past 10 years or so there has been a substantial shift away from the traditional Ackerman’s GEYC medium to TEST–yolk–glycerol (TYG) medium, which has now become the standard CPM for most centres freezing human semen.

IUI-ready sperm samples

Perhaps as a result of dissatisfaction with the cryosurvival of donor semen frozen in cryovials, an increasing number of fertility centres began to perform sperm washing post-thaw so that it could be used for IUI. A method for processing semen (at least from normozoospermic men) through density gradients prior to cryopreservation so that the sperm sample could be inseminated directly into the uterine cavity post-thaw was reported by Larson *et al.* (1997) and extensive clinical results published by Wolf *et al.* (2001). Although this protocol for preparing 'IUI-ready' specimens employed Percoll (which was withdrawn from all clinical use on 1 January 1997 by its manufacturer: see Mortimer, 2000) alternative products such as PureSperm (Nidacon International AB, Göteborg, Sweden) can be substituted for it.

A further problem exists with the current protocols of some commercial sperm banks in that they wash the spermatozoa using simple centrifugation and resuspension (which can cause decreased sperm function, see Mortimer, 2000) and/or use a CPM containing hen's egg yolk. There have been anecdotal reports of severe uterine cramps in some women following IUI with washed donor spermatozoa suspended in TYG medium. Clearly hen's egg yolk does not normally enter the human uterine cavity and, from a medico-legal standpoint, it would seem unwise to allow it to do so. If a woman receiving such an inseminate suffered severe cramps and did not become pregnant in that treatment cycle, it would be unknown whether her failure to conceive might have been due to an adverse reaction to the inseminate.

Consequently, safe IUI-ready specimens should be prepared using a validated density gradient method to avoid iatrogenic damage to the spermatozoa and then cryopreserved using a CPM formulation that does not contain any xenoproteins.

Rate of addition/removal of cryoprotectant

From published protocols, some workers remain unclear about the rate of addition of CPM to semen/sperm suspensions and how to handle cryopreserved specimens post-thaw during CPA removal during washing on density gradients. Both processes must be performed slowly, using stepwise addition of CPM and dilution of thaw material, because mammalian spermatozoa are very sensitive to osmotic stress. For a detailed discussion of this topic, readers are referred to the work of Critser's laboratory (for review, see Gao *et al.*, 1997).

When a permeating CPA is added to cells, they undergo substantial dehydration as water leaves the cells due to the osmotic gradient, and hence they shrink. Then, as the permeating CPA enters the cells (which it does more slowly than water leaves them, due to higher membrane permeability coefficient for water compared with CPA), the cells return to their isotonic volume. Upon removal of the permeating CPA by dilution of the post-thaw specimen, water enters the cells quickly due to the osmotic gradient and the CPA leaves the cells more slowly; hence the cells swell before equilibrium is restored.

All cells have critical volume limits which, if exceeded during these volume excursions, result in irreversible damage to the

cell, presumably via the integrity of its cytoskeleton. Only with extreme swelling will cells burst, so cells can have been damaged but still be 'alive' (i.e. membrane intact) and, in the case of spermatozoa, will still probably be motile. Using a 5% loss of motility as the criterion for damage, the upper and lower critical volume limits for human spermatozoa are 110 and 75% of their isotonic volume (Gao *et al.*, 1995).

Consequently, CPM addition must be stepwise, and most protocols involve a drop-wise addition with constant mixing over several minutes, something known for bovine spermatozoa for half a century (Miller and VanDemark, 1954). Upon thawing, if insemination is to be intra-cervical (or into the uterine cavity with 'IUI-ready' spermatozoa), then CPA removal occurs as the spermatozoa migrate from the semen/sperm suspension + CPM mixture into the fluids of the female reproductive tract. However, if the spermatozoa are to be washed in any way, the thawed specimen must be diluted slowly using stepwise addition of culture medium (ideally a HEPES-buffered medium, or 'sperm buffer', to avoid pH shifts that could occur with a bicarbonate-buffered medium under a non-CO₂-enriched atmosphere: Mortimer, 2000). Again, this is not new information, but many laboratories seem unaware that too rapid dilution can damage cryopreserved spermatozoa. Adding a 10-times volume of sperm buffer slowly (drop-wise to begin with, and then in increasingly larger volumes as the sample is progressively diluted, and not exceeding 1/10 of the current volume of the diluted specimen) will enable optimum yields using PureSperm gradients (unpublished observations). For donor semen, the same effect seems to be achievable using only a five-times volume (T Ebbeson; Cryos, Århus, Denmark, personal communication).

Packaging

Four main types of packaging container have been used over the years for human spermatozoa: (i) Glass ampoules (which have been strongly discouraged for many years on safety grounds due to their fragility). (ii) Plastic screw-top vials or 'cryovials', primarily the NUNC™ CryoTube® range of products (Nunc A/S, Roskilde, Denmark and Nalge Nunc International, Naperville, IL, USA) made from polypropylene with either polypropylene or polyethylene screw caps. (iii) Plastic straws or 'paillettes' (Cassou, 1964) commercialized by Cassou's company, Instruments de Medicine Veterinaire (IMV: L'Aigle, France) made from either polyvinyl chloride (PVC) or polyethylene terephthalate glycol (PETG). PVC straws were withdrawn in 1998 because they could not be sterilized by irradiation without compromising their mechanical integrity. In recent years, plastic straws from other companies (e.g. Minitüb, Tiefenbach, Germany) have also been used. (iv) Straws made from an ionomeric resin (CBS High Security Straws, commonly referred to as 'CBS straws': CryoBioSystem, Paris, France).

Straws are widely used for packaging human semen, especially in Europe, although plastic cryovials are also used in many laboratories, especially in the USA. Reasons quoted to the author for the early preference of plastic cryovials by US sperm banks include their perceived easier use compared with straws, and physicians' perceived need for higher volume insemination aliquots. So is one system better than the other? What are the pros and cons of each system? Technical

arguments in the 'straws versus cryovials' debate involve issues concerning the cooling and warming rates as well as biocontainment, issues that are tightly interconnected because both are governed by the physical characteristics of the packaging systems. Matters pertaining primarily to biocontainment will be discussed later in the section on 'Storage'.

Straws versus cryovials: fecundity rates post-thaw

There have been no reliable prospective trials comparing the relative fertility of human spermatozoa frozen in straws versus cryovials. However, lower fertility of bull spermatozoa frozen in ampoules compared with straws has been known for almost 40 years (see Watson, 1979) and Whittingham reported that 8-cell mouse embryos showed significantly lower cryosurvival and post-thaw blastocyst development rates when frozen in plastic cryotubes compared with straws (see McLaughlin *et al.*, 1999 or Wood, 1999).

In general, US sperm banks and physicians expect at least 30×10^6 motile spermatozoa per dose post-thaw, whereas users of straws achieve comparable success rates using far fewer ($5\text{--}15 \times 10^6$) motile spermatozoa per insemination (Le Lannou, 1990; Moghissi, 1990; Mortimer, 1990). For example, Byrd *et al.* (1990) reported that for semen frozen in cryovials, doses with an average of 30×10^6 motile spermatozoa post-thaw achieved a fecundity rate of 3.9% when used for intra-cervical insemination; and Patton *et al.* (1992) achieved an average fecundity rate of 5.1% for cryovial-frozen doses containing a mean of 44×10^6 motile spermatozoa post-thaw when inseminated intracervically. Using inseminates with at least 40×10^6 spermatozoa with good progression per insemination, Bordson *et al.* (1986) achieved an average fecundity rate of 10.3%. However, for donor semen frozen in straws, Johnston *et al.* (1994) reported an average fecundity rate of 8.9% when using intra-cervical insemination with approximately 12×10^6 motile spermatozoa, and David *et al.* (1980) reported fecundity rates of 4, 10 and 13% for single inseminations containing <5 , $5\text{--}10$ and $>10 \times 10^6$ motile spermatozoa.

The difference in post-thaw fecundity could be as much as 6- to 8-fold better for straws, but this argument can only be answered definitively by a properly designed and executed study. However, the question may now be moot (see Conclusions, below).

Straws versus cryovials: cooling/warming rates

Simple physics tells us that the larger radius of cryovials will impede heat transfer (**Figure 1**). Consequently, not only will there be uneven heat exchange throughout the sample, but the cooling rate achieved inside a cryovial will also lag behind the programmed rate in controlled rate freezers (Morris, 2002), especially those operating via the temperature of the vapour inside the cooling chamber (e.g. Planer Kryo-10), since vapour provides less effective heat exchange than does contact with a cooled mass of metal. Similarly, when a cryovial is removed from cryostorage its contents will thaw more slowly and less uniformly than those of a straw, even if immersed in a 30 or 37°C water bath. Rapid thawing is required for optimum cryosurvival (transferring a 0.25 ml straw to 22°C will achieve

a warming rate of about 400°C/min; see Henry *et al.*, 1993), so cryovials are again sub-optimal.

Measuring the temperature experienced by specimens during cooling or warming is actually rather more difficult than anticipated because the thermocouple wire, being a better thermal conductor than water, will increase the effective rate of change of temperature of the specimen, especially in a small cylindrical object such as a straw. This effect will be seen both at the point of measurement and elsewhere, since the wire must pass through half the length of the sample. Consequently, more accurate values for cooling curves inside straws would be expected if calculated mathematically. A mathematical model of cooling in cylindrical objects has been developed by Diarmaid Douglas-Hamilton (Hamilton Thorne Biosciences, Beverly, MA, USA: personal communication) and **Figure 2** shows the relative cooling rates that would be experienced at different positions within cylindrical objects of 3 and 1 mm radius (upper and lower panels respectively). The hypothetical objects were taken from 37°C and placed in a +5°C environment before cooling at a rate of -40°C/min. The material was modelled as being aqueous (i.e. having a thermal conductivity equivalent to water, 6 mW/cm/K) with a freezing point of -10°C, at which point latent heat of fusion was released. For the 3 mm radius specimen, there was significant retardation and alterations of the cooling experienced within the cylinder, especially closer to its centre. For reference, a 1 mm radius corresponds to a traditional IMV 0.25 ml straw, while a CBS 0.3/0.5 ml High Security Straw has a radius of 1.55 mm (see **Figure 1**). A 1.0 ml NUNC™ CryoTube® has a radius of just over 6 mm, so the perturbations to, and variations in, the cooling rate through a cryovial's contents will be greater than the 3 mm radius model.

Practical measurements have also been made of the cooling curves experienced in the centre of various packaging systems (**Figure 3**) and these have revealed that while materials packaged in all sizes of straws (IMV 0.5 and 0.25 ml and CBS 0.5 ml High Security Straws) experience very similar cooling curves, material packaged in Nunc 1.8 ml cryovials will experience cooling that has a considerable delay behind the programmed curve. This will be very important in determining the correct time to perform seeding, and could require a substantial increase in the 'soak' or holding time to ensure that a cryovial's contents have actually reached the intended seeding temperature. The recorded cooling curves closely match those predicted by the mathematical model, verifying the model's validity and validating its use for investigating the temperatures experienced at different positions within the various packaging vessels.

Effective warming rate is the other side of this issue and impacts not only the effective thawing of specimens but also their warming during handling for brief periods outside the cryogenic storage tank (e.g. while checking the identity of a specimen before thawing or during cryobank audits). In this regard, the ability to achieve rapid warming rates in straws is a double-edged sword, as it will lead to an increased risk of damage. A 0.25 ml straw will warm to -80°C within 15 s in air at ambient temperature (Tyler *et al.*, 1996; **Figure 4**), a very important issue in relation to frozen water undergoing recrystallization that can disrupt cells even in the frozen state (see 'What temperature must spermatozoa be stored at?')

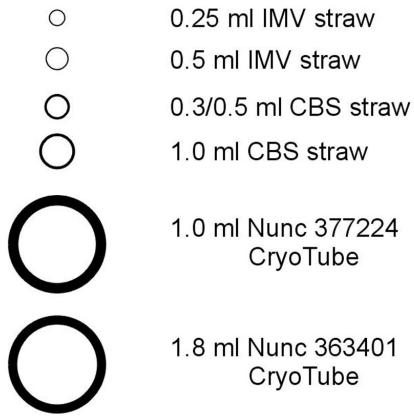


Figure 1. Relative dimensions of various products used for packaging human semen.

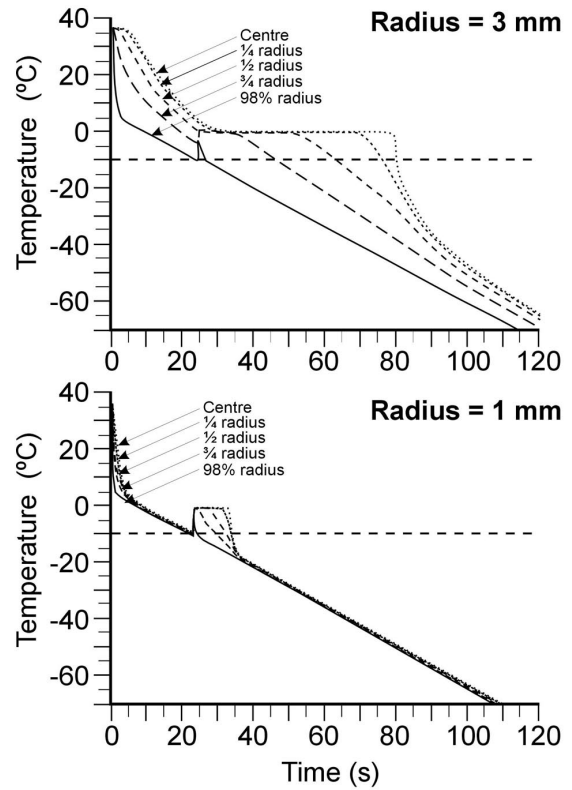


Figure 2. Cooling rates at various locations inside aqueous objects of radius 3 and 1 mm (upper and lower panels, respectively) calculated using a mathematical model. The horizontal broken lines indicate the projected freezing point of -10°C . Curves have been re-drawn from data supplied by Diarmaid Douglas-Hamilton (Hamilton Thorne Biosciences, Beverly, MA, USA: personal communication).

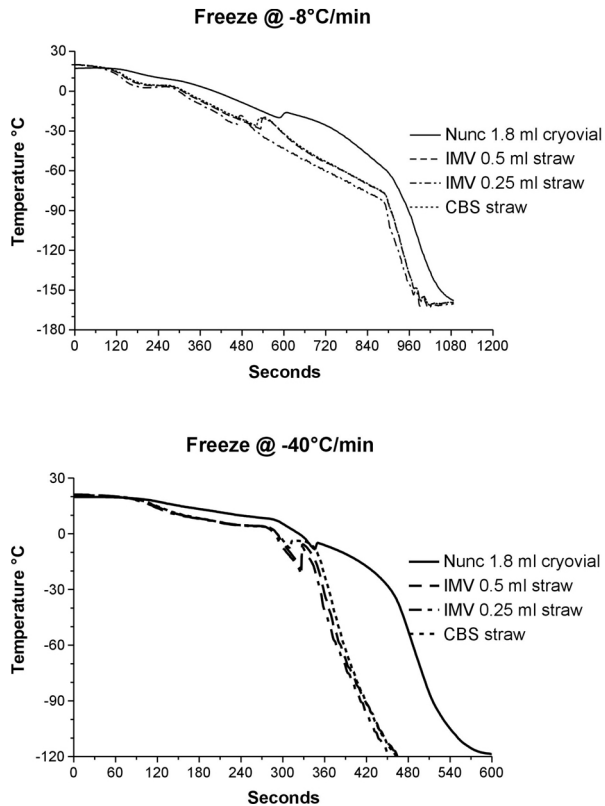


Figure 3. Actual cooling curves measured by thermocouple inside various packaging systems. Data generously supplied by Agnès Camus (CryoBioSystem, Paris, France).

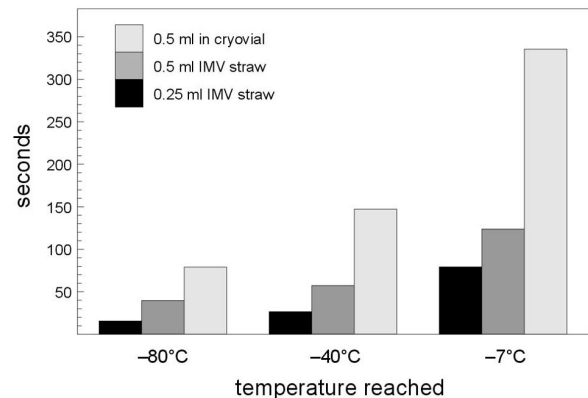


Figure 4. Relative warming times for various products used for packaging human semen (data from Tyler *et al.*, 1996).

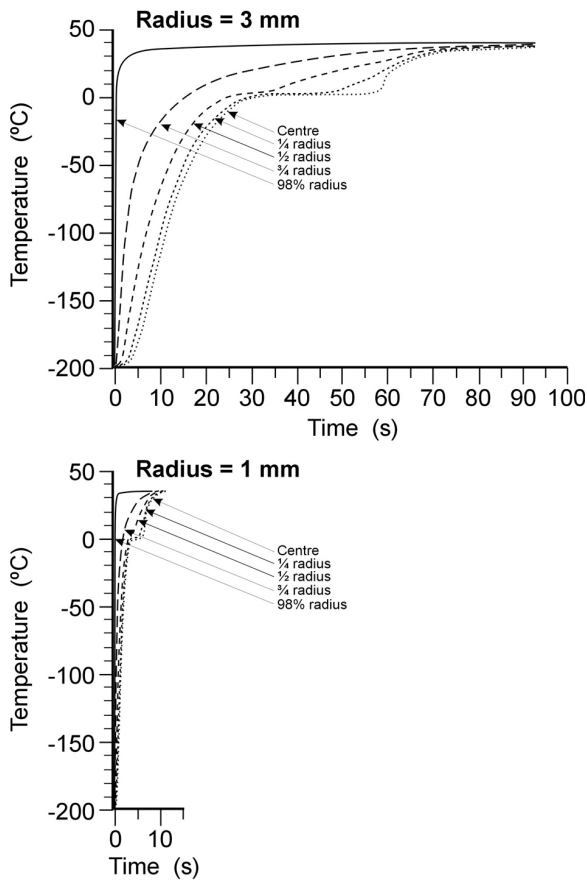


Figure 5. Warming rates at various locations inside aqueous objects of radius 3 and 1 mm (upper and lower panels, respectively) calculated using a mathematical model. Curves have been re-drawn from data supplied by Diarmaid Douglas-Hamilton (Hamilton Thorne Biosciences, Beverly, MA, USA: personal communication).

below). A further application of the mathematical models of Diarmaid Douglas-Hamilton is the warming rate at various locations within the frozen objects: **Figure 5** shows these curves for the same objects as in **Figure 2** after they were taken from -200 to 37°C . In this case, the effective warming experienced throughout an object the same size as an IMV 0.25 ml straw (lower panel) would be expected to be even more rapid than measured by Tyler using a thermocouple: much, if not all of the specimen will be above -80°C within 2–3 s. The massive variation in warming throughout the 3 mm radius object (upper panel) eloquently illustrates the concerns over the intra-sample post-thaw variability of material frozen in cryovials.

Direct thermocouple recordings of the warming curves experienced in the centre of various packaging systems are shown in **Figure 6**. Again, the ‘real world’ data closely match the curves predicted by the mathematical model, with warming at 37°C revealing magnified differences between straws and cryovials. Overall, the curve for straws warmed at 37°C is very close to that predicted for specimens of 1 mm diameter, while that for the 1.8 ml Nunc cryovial (6 mm diameter) shows even greater lags compared with the curve modelled for a 3 mm

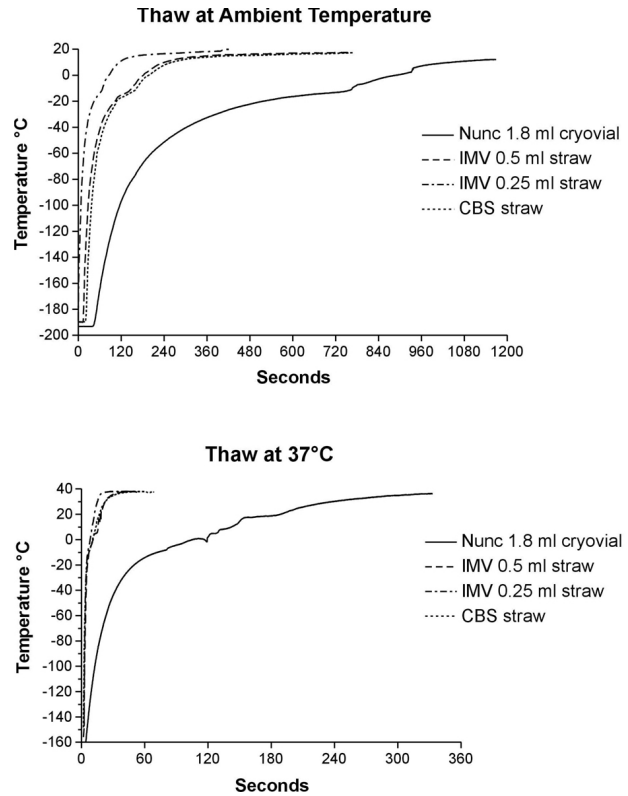


Figure 6. Actual warming curves measured by thermocouple inside various packaging systems. Data generously supplied by Agnès Camus (CryoBioSystem, Paris, France).

diameter specimen. The ‘wobbles’ in the warming curve for the cryovial might reflect convection currents within the cryovial’s contents as they thaw.

Straws versus cryovials: effective sealing

Serious concerns regarding the use of cryovials have been expressed (Byers, 1998). Most sperm banks use cryovials with internal threads which, in conjunction with the silicone gasket, provides the best possible seal. Nonetheless, it is clearly stated in both the Nunc catalogues and the *Cryopreservation Manual* (Nalge Nunc International, 1998) that storage of cryovials immersed in LN_2 is not advised, and that for storage under such ‘extreme’ conditions CryoTube[®] vials must be correctly sealed in NUNC[™] CryoFlex[™] tubing. However, because the use of CryoFlex tubing as a secondary seal hinders the secure attachment of cryovials to canes, very few banks use it routinely. A recent paper reported an alternative secondary sealing method using a product called Nescofilm (Bahadur and Tedder, 1997a), but it does not seem to have become widely used. Indeed, in a survey conducted in the UK it appeared that no sperm banks (or IVF centres) were using secondary containment while over 60%

of sperm banks were using cryovials (see Wood, 1999). A small study reported by Clarke (1999) found that, in spite of strict laboratory technique in filling and sealing cryovials, 45% of Nunc cryovials without an O-ring (Nalge Nunc product no. 340711) and 85% of Iwaki cryovials with an O-ring (Asahi Techno Glass Corporation Scitech Division, Tokyo, Japan) absorbed up to 1 ml of LN₂ during a 3 h immersion.

However, poor sealing is not a problem restricted to cryovials. After being filled, PVC or PETG straws are sealed either by filling the open end with polyvinyl alcohol (PVA) powder which polymerizes upon contact with moisture, using either solid plastic (nylon) plugs, plastic spheres, steel balls, haematocrit tube sealant, ultrasonic welding, or by heating. Thermal soldering is used for the CBS ionomeric resin straws. In all cases, there should be an air space left inside the straw to allow for the expansion of the semen + CPM mixture as it cools (since water has maximum volume at +4°C). Early IMV documentation stated that 'the air bubble or space at the open end of the paillette is essential so that the column of semen in the paillette can extend [expand] during the freezing process'. Without the air space, the plug can be ejected as the column of semen + CPM expands, but the air space also represents an opportunity for LN₂ to enter the straw if the seal is faulty.

Another concern here is the use of crude heat sealing devices such as forceps heated in a Bunsen burner flame with the traditional IMV straws because flattening of the cylinder causes stress fractures at the corners, making them more liable to crack during freezing, leak during storage, and explode upon thawing. The sealing machine sold by IMV for these straws was actually an ultrasonic welder.

Finally, the PVA powder that is tamped into the open end of the filled straw and then cured by moisture lining the straw will form a poor seal unless the PVA powder is fully cured. Again, the IMV documentation indicated that, after tamping in the PVA powder, straws should be immersed in water to 'allow the plugs to become firmer'.

The arguments offered in favour of and against secondary sealing or secondary containment, or 'double bagging' as it is sometimes referred to, can be summarized as follows: *Pro (i)*: When cryovials are immersed in LN₂, the air space inside them is under reduced pressure due to contraction of the cooled air. It has been calculated that the air pressure inside a vessel at -196°C will be only 26% of that before cooling (Rall, 2003: although this does not allow for shrinkage of the vessel or the expansion of the water in the specimen) and LN₂ will be drawn into the air space if there is a faulty seal. Upon rapid warming, e.g. when specimens are removed for thawing, this LN₂ rapidly turns into nitrogen gas with a 700-fold volume expansion that can cause the cryovial to explode, representing a significant physical risk to the operator. *Pro (ii)*: A non-hermetic seal will constitute a breach of biocontainment between the specimen and the LN₂ inside the cryotank (see Storage, below). *Con (i)*: The extra layer of plastic will affect the cooling and warming rates of the specimen. This will be exacerbated if air is trapped inside the secondary sealing envelope. *Con (ii)*: The extra layer of plastic, with or without a layer of trapped air, will create difficulties when seeding (see Wood, 1999). *Con (iii)*: A loose fitting secondary containment sheath makes handling specimens more cumbersome and also creates difficulties attaching cryovials to canes.

Straws versus cryovials: fragility at -196°C

Another concern is that straws are more fragile than cryovials at -196°C. However, it has been the author's experience that if an appropriate inventory system is used, i.e. one that does not expose straws to any bending stress upon transfer into, during, or removal from, storage (and if straws are not over-filled), then broken straws are extremely rare. For example, when the traditional IMV straws are stored in narrow tubes (9.2 mm diameter 'visotubes') attached to canes, as is usual with embryos, many straws will be broken when trying to extract them from the visotube: this is because at -196°C (and even at -140°C in super-cold vapour/air storage) straws become extremely rigid with essentially no flexibility, a problem that is worse with the 0.25 ml straws compared with the 0.5 ml straws.

However, in this regard the CBS straws have a major improvement in that they cannot be broken, even at cryogenic temperatures. The author has verified this personally by bending CBS straws immediately upon their removal from LN₂.

Should everyone use 'High Security Straws'?

In the early 1990s, the veterinary products corporation IMV introduced a new type of straw called the 'High Security Straw', which was launched for human assisted reproduction applications by IMV's medical subsidiary CryoBioSystem (Paris, France) in 1995. The French Federation CECOS switched to CBS High Security Straws en masse (22/23 centres) in 2000. These straws (often nowadays referred to as the 'CBS straw' by people working in the human assisted conception field) are made from an ionomeric resin, and have the following features: (i) Heat sealable using a special thermal welding device (the SYMS sealer). Properly sealed CBS straws are guaranteed to be absolutely leakproof at pressures of up to 150 kg.cm⁻². (ii) Mechanically resistant, i.e. shatterproof even at -196°C. (iii) Bacteria and virus proof (see below). (iv) A special filling nozzle so that none of the material being loaded into the straw ever comes into contact with the outside of the straw (see Russell *et al.*, 1997). (v) Extensive biocompatibility testing of every batch to verify that the straws are non-toxic (low endotoxin) and free of bacterial and viral contamination. The CBS company guarantees that the straws will cause no specific damage to human or bovine spermatozoa or mammalian embryos. (vi) Sterile (after irradiation at 25 kGy in accordance with European Pharmacopoeia standards). (vii) Two-compartment design using a hydrophobic filter (for embryo straws) that excludes all medium and cells from the plug and hence no aqueous material can enter the second, identification, compartment. CBS sperm straws with a three-part plug (two hydrophilic parts enclosing PVA powder) are also available, either as a 0.3 ml two-compartment straw or a single-compartment 0.5 ml straw. Two-compartment straws allow identifying information to be sealed inside the straw itself, making identification tamper-proof. (A larger straw of maximum 1 ml volume is also available.) (viii) Secure external identification labelling option using sleeves that cannot slide off the straw once sealed (due to the flat 'tabs' formed at each end when the straw is welded shut). (ix) Approved for human applications by the US Food and Drugs Administration with a 510(k) pre-market notification clearance K002595 and CE marked as a medical device according to CE 93/42.

Studies on the microbial impermeability of the CBS straws were performed using a variety of bacteria and bovine hepatitis virus

I conducted or supervised by Dr B Guérin (Laboratoire pour le Contrôle des Reproducteurs, Ministère de l'Agriculture, Maisons-Alfort, France). His report concluded that there was an absence of release of contaminating bacterial agents from CBS straws and that the contents of CBS straws were not contaminated even under highly contaminated environmental conditions. A study by Benifla *et al.* (2000) reported some leakage of HIV-1, but they did not test the CBS straws under correct conditions of use. As illustration of the overall confidence in biosafety of these straws, a containment level 4 laboratory in France is using them to cryopreserve organisms of the infectiousness of *Ebola*.

Since the advent of the CBS High Security Straws, several workers, including the author, have contemplated the question of what the legal position might be if it was decided *not* to use them and a pathogen was transmitted that would presumably have been prevented had the CBS straws been used (see also commentary by J. Tyler in McLaughlin *et al.*, 1999, p. 15): certainly, ignorance is no defence! As a consequence of this, the author always advises centres that operate cryobanks about the CBS straws, and recommends that they should be used on the grounds of best practice. Most of the laboratories that have chosen not to use them did so because of the cost of the SYMS sealer (about US\$2500) and the higher cost of the straws. Yet CBS sperm straws have a list price of just 56¢ in the USA and about €0.35, before bulk discounts. Therefore, for an average ejaculate of 3.3 ml, which would require perhaps 22 × 0.3 ml straws, the cost of straws would be a maximum of about \$12 or €8. While this is certainly several times higher than the cost of bulk veterinary straws, such products are not approved for medical use (and the use of which for medical applications, in itself, contravenes some countries' government regulations). All things considered, rational risk assessment would identify this as a classic example of 'penny wise but pound foolish'.

In conclusion, it would seem that, given all the benefits of avoiding contamination of the LN₂ because there is no material on the outside of these straws, because of their mechanical resistance, guaranteed leakproof seals and microbial biocontainment, CBS straws should be used by everyone. Furthermore, the issue of vapour storage (see below) would also become unimportant.

Cooling procedures

Although some studies have reported specific optimum cooling rates (e.g. Henry *et al.*, 1993) human spermatozoa are generally considered to be relatively insensitive to cooling rates within the range of 1–25°C/min. However, this might be due to the generalized use of sub-optimal CPM formulations and the masking of damage by rapid thawing (Gao *et al.*, 1997). Several technical methods have been used to achieve controlled rate cooling for human spermatozoa:

Static vapour phase. (i) In trays suspended horizontally in a large Dewar (e.g. 10 min at 25 cm above the LN₂ for 0.25 ml IMV straws, or 15 min each at 36 and 15 cm above the LN₂ for IMV 0.5 ml straws, before plunging); (ii) in the necks of storage-type Dewars (rather less well-controlled).

Computer-controlled freezers. (i) Liquid nitrogen vapour-filled chambers (e.g. Kryo-10, Planer, Sunbury, UK and Nicool

models, Air Liquide, Bussy-Saint-Georges, France); (ii) cooled metal blocks (e.g. Freeze Control models, CryoLogic, Mulgrave, Vic, Australia and model DB1, Biotronics, Leominster, UK)

A generic cooling programme for human spermatozoa relying on spontaneous ice nucleation (i.e. no 'seeding') is shown in **Table 1**. If ice nucleation is to be induced ('seeding') then the alternative programme shown in **Table 2** can be used. See also To seed or not to seed? (below).

Provided that the desired cooling curve is achieved, there is little to choose between the various approaches so long as the following important points are observed.

Because a temperature gradient is established through a static vapour phase, straws frozen by placing them in a static vapour phase above LN₂ must be placed horizontally in the vapour so that they will experience the same cooling effect along their length. Moreover, it is vital that straws are arranged in a monolayer, not in bundles or multiple layers, so that all the straws will experience the same cooling effect. Straws in bundles will experience different cooling rates, and this will contribute to poor inter-dose reproducibility in post-thaw survival and quality. A particularly poor technique is to place straws in a bundle, usually inside a 'visotube', in the neck of an open storage Dewar vessel for a certain period of time. This method results in very unreliable cryopreservation with differences between straws on the outside and inside of the bundle, and perhaps even in variations in cryosurvival along the length of each straw. Again, IMV documentation instructed users to freeze straws horizontally in racks in LNV and stated 'we do not recommend freezing paillettes in goblets'. (Modern parlance differentiates between 'goblets', which are the larger plastic cups that fit inside canisters, and 'visotubes', which are the small plastic holders that are placed inside goblets, usually holding straws from a single specimen.)

To seed or not to seed?

The vast majority of sperm cryobanks do not induce ice nucleation during cooling, typically because it has not been shown to confer any significant improvement in cryosurvival. Ice nucleation will occur somewhere between –5 and –8°C depending on the CPM formulation being used, but it is also affected by the very nature of its being 'spontaneous'. For example, spontaneous seeding might happen in one straw at –7°C and in another at, say, –16°C, quite arbitrarily. Certainly this could contribute to between-straw variability in cryosurvival, and perhaps especially so when frozen in bundles.

Morris (2002) has provided an interesting discussion of the effects of the latent heat of fusion that is released at ice nucleation and its effects on the temperature inside the straw as well as on the extent of rapid cooling that occurs as this heat is lost from the straw. He comments that for men with normal sperm quality the consequent loss in viability might not be important, but for oligozoospermic or asthenozoospermic samples these losses might be more significant. Findings from animal studies are difficult to translate to human sperm cryopreservation due to the high inter-individual variability of human spermatozoa. Critser *et al.* (1987) reported that incorporating a hold for 10 min at –5°C and seeding at that temperature when using HSPM as the CPM and packaged in 0.25 ml IMV straws improved human sperm

Table 1. Generic cooling curve for human sperm relying on spontaneous ice nucleation (no 'seeding'). 'Free fall' denotes uncontrolled cooling, i.e. a cooling rate as fast as the system being used can achieve.

Ramp	Description	Comments
Start at 20°C		i.e. ambient temperature, after addition of CPM to the semen
Ramp 1	-5°C/min to +4°C	
Ramp 2	Hold for 1 min	Optional
Ramp 3	-10°C/min to -80°C	See Henry <i>et al.</i> (1993)
Ramp 4	Free fall to -120°C	Or plunge into LN ₂ , but this must be done very rapidly
Plunge into LN ₂		

Table 2. Generic cooling curve for human sperm including ice nucleation ('seeding').

Ramp	Description	Comments
Start at 20°C		i.e. ambient temperature, after addition of CPM to the semen
Ramp 1	3°C/min to -5°C	
Ramp 2	Hold for 10 min	After 5 min seed the straws using cold forceps or an LN ₂ -soaked Q-Tip
Ramp 3	-10°C/min to -80°C	See Henry <i>et al.</i> (1993)
Ramp 4	Free fall to -120°C	Or plunge into LN ₂ , but this must be done very rapidly
Plunge into LN ₂		

motility post-thaw. Anecdotally, no improvement in cryosurvival has been found when seeding either 0.5 ml IMV straws or CBS straws using TYG or glycerol-in-PBS (data not available), although the cooling rate from ambient temperature was faster than the -0.5°C/min employed by Critser *et al.* (1987).

Storage

What temperature must spermatozoa be stored at?

Although this question keeps being asked by workers in the fertility field, it is simple to answer. All biological material must be stored below the glass transition temperature of water (about -132°C) in order to stop all biological activity (Mazur, 1984). Even at -80°C in an 'ultracold' mechanical refrigerator or -79°C on dry ice some biological activity might continue, and degradation certainly accumulates over time. Ackerman (1968) reported that human spermatozoa stored at -79°C showed a progressive decline in post-thaw motility, an effect that was accelerated at storage temperatures above -75°C. More recently, Trummer *et al.* (1998) reported that human spermatozoa stored in a mechanical freezer at -70°C showed lower cryosurvival than did parallel samples stored at -196°C; furthermore, the detrimental effect was greater after 3 months of storage compared with 7 days. As an aside, bovine spermatozoa might be more hardy, since functional survival

has been demonstrated after 4 years storage at -79°C, as well as after 33 years at -196°C (Leibo, 1999).

Of particular relevance to the storage and handling of cryopreserved materials is what happens as water warms from cryogenic storage temperatures. The glass transition temperature of an already frozen aqueous solution is not a sudden event at exactly -132°C, glass transition will occur progressively between this temperature and about -90°C, so that by, say, -80°C there is a great risk of substantial change having occurred. Damage occurs primarily because during warming energy is returned to the system, energy that permits molecules to resume their natural orientation. Very small ice crystals have a large surface area:volume ratio, and hence they are unstable and will fuse together to reduce that ratio. Because cryogenic storage temperatures are so low, it is likely that water molecules will have sufficient time to crystallize during warming before reaching their actual melting point.

Consequently, cryopreserved material must be kept below -132°C, and hence storage temperatures of -150°C (the often reported temperature of LNV) or -196°C (the temperature of LN₂) are employed. Clearly, the lower the temperature the greater the margin of safety when a specimen is removed briefly to check its identity. This is also why it is better to run a controlled rate freezer to as low a temperature as possible before removing the specimens and transferring them into

cryogenic storage; it will reduce the extent of glass transition-induced damage during the brief exposure to warmer temperatures during the transfer.

Risk of cross-contamination

In 1995 a cluster of six cases of acute hepatitis B virus (HBV) infection occurred among multiply transfused patients undergoing cytotoxic treatment (Tedder *et al.*, 1995). All had been HBV negative at the start of treatment and subsequent investigations revealed that the only common factor linking the patients, who had all been treated at different times and/or locations, was that the harvested bone marrow or peripheral blood stem cells had been stored in the same cryogenic tank (Hawkins *et al.*, 1996). Moreover, PCR analysis of an aqueous sample taken from the decommissioned cryogenic tank revealed HBV sequences identical to those in the patients. The source of this contamination was found to be due to splitting of the heat seals of the blood bags in which the cells had been frozen and stored in LN₂ allowing entry of LN₂ through the imperfect seals. This particular type of blood bag was recalled, and professional opinion is that the product was of low quality (Mericka *et al.*, 2003).

Therefore, this problem, the only reported case of cross-infection via LN₂ storage, and which gave rise to enormous concern resulting in substantial expense by many organizations, was apparently due to a poor quality product combined with imperfect technique. In one experimental study, LN₂ contaminated with three pathogenic viruses did not contaminate specimens stored in properly sealed cryovials or straws immersed in it (Bielanski *et al.*, 2000), while in another the micro-organisms contained in properly sealed straws neither leaked out in the LN₂ nor contaminated 'clean' samples in the same tanks (Bielanski *et al.*, 2003). Also of relevance is the observation that in the case in which semen from an HIV-positive donor was unknowingly used to inseminate several women who became infected (Stewart *et al.*, 1985), no other infections occurred following many inseminations with donor semen that had been stored contemporaneously in the same cryotank (JPP Tyler, personal communication).

Several authors have reported that there is no direct evidence of any cross-contamination in a cryobank within a fertility clinic or sperm bank setting (Kuleshova and Shaw, 2000; Tomlinson and Sakkas, 2000; Centola, 2002) and a senior cryobank inspector in the United States once described to the author that the risk was 'vanishingly small'. Worldwide enquiries by the author using Internet list servers in 1997 (EmbryoMail, Androlog and ARTLog), and again in 2003 (EmbryoMail and Androlog) all returned no reports of any occurrence of cross-contamination in a reproductive cryobank using straws or cryovials (although the long-established problem with animal semen frozen in pellets and stored 'naked' in LN₂ was well known, e.g. Piasecka-Serafin, 1972). While the risk of cross-contamination cannot be ignored, it is certainly unquantifiable (Tomlinson and Sakkas, 2000), rendering impossible robust risk analysis to ascertain its real likelihood. However, the risk cannot be presumed to be theoretical, and all workers involved in human gamete and embryo cryobanking must understand its origins and be able to take every available practical step to minimize the risk in their banks.

The current standards of the American Association of Tissue Banks (AATB) require that 'cells and/or tissue shall be processed by methods known to be validated to prevent contamination and cross-contamination', that 'reproductive cells and/or tissues shall be stored either in the liquid phase of liquid nitrogen, or provided that the storage method has been validated, in the vapour phase of liquid nitrogen', and that 'oocytes and embryos shall be stored in the liquid phase of liquid nitrogen'.

Of particular importance for the storage of spermatozoa (and also oocytes and embryos) is the relative viral load and consequent risk of infection: even a straw or cryovial of spermatozoa (i.e. 5–20 × 10⁶ cells) must carry several orders of magnitude fewer viral particles than a bone marrow sample, and a single oocyte or embryo at least 10⁻³ lower again. Of course, for donor spermatozoa the risk is minimized further by the extensive screening to which men are subjected before and during their time as donors. Finally, for cross-contamination to occur the infectious organisms must not only be released into the LN₂ but also gain access to the 'clean' unit in order to contaminate its content. This is where the leakiness of cryovials without a secondary seal, or incorrectly sealed straws, is of further concern; the issue goes beyond physical safety of the operator at the time of thawing to the breach in biocontainment that it represents.

This issue of adequate biocontainment has given rise to such statements that '[straws] are microbiologically hazardous' and the recommendation of screw-capped cryotubes for storage of semen and embryos (UK Royal College of Pathologists; see McLaughlin *et al.*, 1999 or Wood, 1999), plus recommendations that both secondary seals and vapour phase storage should be used (e.g. Rall, 2003). However, some experts (e.g. Rall, 2003), including the author, consider that properly sealed straws are the more secure packaging system. Other authorities, however, have recommended caution and, in the absence of proof of the safety and efficacy of secondary containment and gaseous phase storage systems, awaiting evaluation of new packaging and storage systems (i.e. the CBS High Security Straws) before implementing such changes (British Andrology Society, 1999).

In order to address this issue properly a risk assessment must be undertaken and the individual component risks addressed. So what are the various mechanisms by which LN₂ can become contaminated?

Contamination of the LN₂ with pathogenic organisms (in decreasing order of their perceived likelihood)

(i) By semen contaminating the outside of the packaging unit, particularly straws. This is a well-known problem (Russell *et al.*, 1997), and requires that the straws not just be wiped but also disinfected prior to freezing. (ii) By split or broken straws. (iii) By removing LN₂ from a contaminated cryotank to handle units being frozen (e.g. for seeding) or while being transferred from the freezing machine to the cryobank, between cryotanks within the bank, or to fill a dry shipper. This represents very poor practice and has been eschewed by cryobanking experts, especially since all cryotanks must be considered to be contaminated by pathogens (e.g. Rall, 2003). (iv) From room

air or the exhaled breath of operators. This is what causes the 'fogging' when a cryotank is opened, and hence constitutes the major source of the material that forms the 'sludge' at the bottom of cryotanks. (v) Skin commensals from operators while leaning over an opened cryotank. (vi) From the LN₂ supply at point of manufacture. Suggestions that 'sterile LN₂' can be created either by ultrafiltration or UV irradiation (Vajta *et al.*, 1998; Kuleshova and Shaw, 2000) have not been demonstrated experimentally. Therefore it is best to assume that LN₂ might have been contaminated during manufacture (Rall, 2003). (vii) From frozen material out of imperfectly sealed cryovials or straws. (viii) Directly through the wall of intact, properly sealed plastic straws, although there is no evidence for this mechanism. (ix) By LN₂ that escapes from an imperfectly sealed cryovial or straw that had been contaminated by the material stored in that cryovial or straw. There is no physical reason why this would happen and available evidence does not support such an event.

Risk status of various cryobanked materials

(i) Semen from unscreened men. These should only be 'rush' freezes for either oncology patients or a few patients undergoing assisted conception treatment. (ii) Semen from screened men. All other men, including pre-vasectomy and other 'client depositors', should be screened prior to storage. N.B. With the discovery of new infectious organisms (e.g. new strains of the hepatitis virus) even the most rigorous screening such as that applied to sperm donors cannot be considered perfect and must leave some small future risk that such sperm samples might be discovered to carry a presently unknown pathogen (Bahadur and Tedder, 1997b; Clarke, 1999; Tomlinson and Sakkas, 2000).

Use of 'quarantine' tanks

Many banks use separate tanks to hold specimens, even from screened donors, while waiting for follow-up infectious disease testing. Only 'cleared' samples are then moved into the long-term storage tanks. One bank even uses one tank per 3-month period and waits for all donor samples in a tank to be 'cleared' before allowing any samples to be transferred, and if even one sample tests positive for a pathogen the entire tank contents are discarded and the tank emptied and sterilized (Janssens, 1997). While this is extremely cautious and secure, it could result in great wastage of valuable donor semen and still not cover the problem that some samples 'cleared' from quarantine might be infected with an as yet unknown pathogen.

While quarantine and repeat screening is an essential part of providing safe donor semen, and is a standard requirement of regulatory authorities worldwide, it does not guarantee totally pathogen-free semen for insemination. Donor screening requirements (the most strict being those required by Health Canada, 2000) evolve with time, especially as new pathogens are identified, so it is impossible to state whether some new organism will be discovered tomorrow that will be included in the minimum required screening for donors. The Special Access Programme operated by Health Canada (2000) is an illustration of how such evolution affects regulation. Health Canada also expects that donor semen and patient semen will be stored in separate tanks as a further measure to reduce the

risk of cross-infection.

For 'client depositors' who have the luxury of time, e.g. men considering a vasectomy, a bank can insist upon screening for pathogens as a safety precaution for the security of other men's semen stored in the same cryotanks. However, men with cancer often need to bank semen at such short notice as to preclude precautionary screening. These men's semen could be placed in a quarantine tank until the requisite screening had been completed. But what is the true status of a cryotank, and all the specimens stored within it, if a single sample is found to be contaminated? Remember also that it has already been established that cryotanks are not sterile repositories (see above). A further complication is that some Australian IVF centres no longer screen patients for HIV before treatment because: (a) no consequence counselling is typically provided before performing an AIDS test, as is required by law; and (b) it is unlawful to refuse treatment solely on the grounds of a patient being HIV positive, so the clinical value, and financial costs, of the HIV tests have no impact upon whether the couple will receive treatment. At Sydney IVF this led to the development of the concept of 'universal contamination' for all cryostorage tanks, a concept that has now been adopted by other banks in Australia (e.g. commentary by J Tyler in McLaughlin *et al.*, 1999, p. 15).

The other situation is, of course, where samples are cryopreserved for patients who are known to carry an infection, e.g. HIV- or hepatitis-positive men. These can be stored in separate 'dirty' tanks (Tomlinson and Sakkas, 2000), but of course there must be a separate tank for each combination of recognized pathogenic organisms.

Therefore, while the use of a quarantine system and the separate storage of patient and donor samples will reduce the risk of storing samples that are infected by a known pathogen alongside samples from uninfected individuals, the practical application of the scheme to achieve optimum risk minimization can be very expensive. Consequently, while there are reasonable arguments in favour of separate storage vessels for the four categories of donor, pre-screened, unscreened and 'known positive' cases, this does not eliminate the external (and possible internal) contamination of straws or cryovials by organisms present in the LN₂. Therefore, beyond this degree of separate storage, it seems only logical that the concept of 'universal contamination' must become standard.

Liquid or vapour phase storage?

Subsequent to the report that cross-infection of bone marrow and blood stem cells had occurred via the LN₂ in which they were being stored (Tedder *et al.*, 1995) there were several calls for storage in LN₂ to be abandoned in favour of storage in the vapour or gaseous phase either above a layer of LN₂ (LNV storage) or in newer design freezers that enclosed the cryogenic LN₂ inside a sealed vessel so that cold was transferred through the walls of the vessel, sometimes with the aid of heat shunt devices ('cold fingers') to minimize temperature gradients, and material is therefore stored in what is, in reality, super-cold air [e.g. Isothermal Vapor Storage ('IVS') models from Custom BioGenic Systems, Shelby Township, MI, USA]. Another option is to use mechanical cryogenic freezers (e.g. Ultima

II Series from Revco, Asheville, NC, USA: Burden, 1999), although these are very expensive to run (typically in excess of 4 kW) and, unlike systems employing LN₂ as the cryogenic refrigerant, provide little security if the mains power fails without a substantial emergency generator system (many models also require three-phase power).

While LNV storage does go a long way to reducing the risks of cross-contamination via LN₂, especially with leaky specimen packaging, it is not an absolute solution because pathogens have been isolated from LNV (Fountain *et al.*, 1997) and presumably the same will be true of IVS systems for organisms that are frozen out of the air that enters the storage chamber every time the lid is opened. Although environmental organisms and skin commensals appear to be common, low level, contaminants of LN₂ and LNV, along with occasional enteric contaminants, high concentrations of a potential pathogen (*Aspergillus* spp.) have been reported in LNV (Fountain *et al.*, 1997).

A major concern is that LNV and super-cold air have poor heat transfer rates and very low thermal capacity (see McLaughlin *et al.*, 1999; Wood, 1999), as a consequence of which they cool poorly and heat up all too quickly in the presence of a 'warm' object, even ambient air. It is vital for long-term storage that specimens be maintained below the glass transition temperature of water, and every second spent above that temperature, and especially above -80°C (see above), will cause the irreversible accumulation of damage to the frozen cells. Consequently, extreme care must be taken to ensure that specimens are kept below -132°C as they are manipulated during transfer into the cryobank from the freezing system, during storage, and when they are being retrieved (Simione, 1999).

How safe is auditing?

Extreme care must be taken whenever a cryopreserved specimen is handled, not just from the perspective of the safety of the operator, but to protect the specimen from damage that will accumulate, irreversibly, whenever the temperature of even part of it exceeds -132°C. This problem is greatest for material frozen in straws, especially the older 0.25 ml IMV straws.

The British HFEA (Human Fertilisation and Embryology Authority) Code of Practice requires that all centres carry out a periodic review (at least annually) of the status of all stored gametes and embryos in order to ensure that the centre's records reconcile with the material actually in storage (HFEA, 2001). While this annual audit has been perceived as necessary in order to ensure that a cryobank's contents match the patients' records, and *vice versa*, it must also be considered that it might put patients' specimens (and the quality of frozen donor semen) at risk due to the accumulation of latent cryodamage.

Efficient and safe auditing of a cryobank relies upon the following factors: (i) An inventory system that allows for easy and quick access to specimens within the cryogenic storage tanks. (ii) Unambiguous (and secure) labelling systems that facilitate the rapid and accurate identification of each specimen. (iii) Skilled staff who can handle material at cryogenic temperatures quickly, safely and securely. This is probably the biggest single area of

weakness in the performance of any audit. (iv) Proper maintenance of records, either in paper or electronic form, so that the records *do* reconcile with the material actually in the cryobank.

As to the true value and importance of the HFEA's requirement for annual cryobank audits, a risk assessment must be undertaken to balance the potential deterioration of cryopreserved material during handling against the likelihood of identifying a discrepancy between the centre's records and the cryobank's actual contents. If a centre can demonstrate that it has accurate records and that its standard operating procedures minimize all opportunities for discrepancies to occur, then the negative risk of potential cryodamage will outweigh any positive 'verification' benefit and the 'need' for annual audits should be rejected. Obviously each cryobank must undertake its own risk assessment on this question and establish its own level of confidence.

Further reducing risk

Disinfecting the outside of straws

Clearly when cryovials or CBS straws are filled, there should be no contamination of their outsides with the biological material, but when filling the old-style IMV straws the open end of the straw is immersed in the semen + CPM mixture as it is aspirated into the straw. After sealing it is essential that residual material on the outside of the straw be removed, usually by wiping with a paper tissue (otherwise adjacent straws during freezing become 'cemented' together at LN₂ temperatures and cannot be separated without grave risk of breaking them. Many laboratories also disinfect the outside of the straws with an alcohol wipe before cooling them although CBS recommends that straws be disinfected using a less volatile disinfecting agent (e.g. hypochlorite), followed by rinsing with sterile water.

Upon thawing the outside of all containers will be contaminated with whatever organisms were present in the LN₂, even if vapour storage was used (Fountain *et al.*, 1997). Disinfecting the outside of specimens after thawing is a common feature of cryobank SOP and Clarke (1999) recommended that all straws should be disinfected (e.g. using hypochlorite solution) before cutting with a sterile scalpel blade or scissors. Safe laboratory practice should avoid all risks of 'finger stick' and similar injuries, therefore scalpel blades should not be used. Sterile disposable suture scissors (e.g. model R50.000 from Rocket Medical, Watford, UK) have been used for this purpose for several years. CBS have recently announced a custom-designed, easily sterilized device, the 'StrawCut', based on the hot-wire principle for opening High Security straws.

The biocide 'Expel' is an aqueous solution of stabilized chlorine dioxide that is highly effective as a bactericide, viricide and fungicide. It is widely used in many industries around the world and was chosen by the governments of Singapore, China and Hong Kong as the most effective

agent with which to ‘fog’ their airports and destroy the SARS virus. This product is undergoing evaluation for the disinfection of the outside of straws and cryovials, including confirmation that it does not pass through the walls of the various products and/or does not have any adverse effects upon spermatozoa inside them (Biotronics Ltd, Leominster, UK).

Finally in this regard, because they cannot be disinfected without damaging their contents, any straws that are broken, obviously cracked, or have lost their seal at either end, should be discarded (Clarke, 1999).

Sterilizing the controlled rate freezer

Certainly, if there is any risk of contaminating the inside of the controlled rate freezing machine with biological material that might contain pathogens, the cryobank must have a protocol in place to sterilize the contaminated surfaces; not only the freezer, but of all units that are frozen using it. When processing samples from pathogen-infected individuals, even using packaging that ensures biocontainment, it would be good practice to sterilize the cooling chamber, or at least the contact surfaces, afterwards.

Monitoring cryogenic storage tanks, auto-fill systems and alarms

All modern accreditation systems require that critical equipment be monitored for its correct performance on a continual basis and that a programme of preventive maintenance is in place, e.g. ISO 15189:2003 (International Standards Organization, 2003). As a consequence of such requirements, and in the interest of best practice (Tomlinson and Sakkas, 2000), the following measures are considered normal by various accreditation authorities around the world: (i) The levels of LN₂ in cryogenic storage tanks that are filled manually should be monitored on a regular basis, e.g. at the time of their regular filling. (ii) Large cryogenic storage tanks that use a lot of LN₂ should have an ‘auto-fill’ controller and be attached to a plentiful supply of LN₂, preferably via vacuum-insulated manifold. (iii) Low-level sensors should be installed in all cryogenic storage tanks and connected to an alarm that will alert laboratory personnel to problems that occur outside normal working hours.

While the regular documenting of LN₂ levels in smaller cryogenic storage tanks might appear redundant if low-level alarms are fitted, it remains important because it can provide early warning of tank failure. In the author’s personal 26-year experience, three tank failures were all identified early due to their increased consumption of LN₂ and replaced before their contents were compromised. Other workers have reported similar experiences on the EmbryoMail list server, one even describing a problem with a recently purchased tank.

Do cryogenic storage tanks have a useful life?

Another frequent question is how long a cryogenic storage tank should last. Manufacturers of cryogenic storage tanks rarely give a stated working life of n years, but some do give a vacuum warranty of 3 or 5 years. Of course, this does not preclude failure at any time before or after the warranty period, and manufacturers clearly exclude any liability for the use of their products. Whether cryogenic storage tanks should be replaced on a continuous contingency basis every n years remains a matter of discussion, but maintaining a cold spare tank of at least equal size to the largest tank in use is a very sensible precaution. Carefully monitoring the LN₂ consumption of tanks will provide advance warning of gradual vacuum loss, but will not alert one to a catastrophic vacuum loss, an event that is, fortunately, very rare except as a result of physical damage to a tank.

Divided storage

Some cryobanks that have undertaken a thorough risk assessment of the long-term security of their cryostorage services divide a man’s sperm specimens (or a couple’s embryos) between two or more separate storage tanks. There is a further refinement where part is sent to an entirely different site, as a precaution against events such as fire and earthquake. To many this might seem excessive, but for those who live in high risk areas (e.g. adjacent to the San Andreas fault or, perhaps areas with a high risk of terrorist activities) it would seem a wise precaution. Again, these are questions that each cryobank should answer for itself through a formal risk analysis process. However, the Jackson Laboratory (Bar Harbor, USA), whose cryobank holds almost 2 million mouse embryos from over 2400 strains, has taken this approach (SP Leibo, personal communication).

Post-thaw processing

Although cryopreserved semen has been employed widely, and very successfully, with cervical insemination (e.g. Mortimer, 1990; Lansac *et al.*, 1997) there is an increasing trend to wash seminal spermatozoa post-thaw using density gradients. This practice should be encouraged, especially for spermatozoa frozen in any packaging other than CBS straws, since it will further reduce the load of micro-organisms in the final inseminate (Tomlinson and Sakkas, 2000). Indeed, such processing methods are used very successfully when treating HIV or hepatitis discordant couples (Levy *et al.*, 2000, 2001; Meseguer *et al.*, 2002). Only products that are approved by the relevant regulatory authorities, e.g. USA FDA 510(k) clearance or CE marking as a medical device, should be used for such purposes and, regardless of their infectious status, sperm samples should be processed under conditions where both the specimen and the operator are protected, i.e. class II biohazard cabinets.

Conclusions

Regardless of whether the risk of cross-contamination between samples stored in LN₂ is 'vanishingly small', and has never been reported for spermatozoa or embryos, it must be accepted as a finite risk and all reasonable measures taken to reduce the likelihood of its occurrence. Moreover, all methods used to collect, cryopreserve, store, thaw and use cryobanked human spermatozoa must address the risk of contamination from any source throughout the entire process (Rall, 2003). In developing a best practice approach to human sperm cryobanking, the following recommendations would seem valid conclusions from literature reviews and risk analyses using currently available information.

1. With regard to cryoprotectant media and cooling rates for human spermatozoa: (i) no one medium is demonstrably better than others; (ii) media should be manufactured under proper conditions and certified for human use by the appropriate regulatory authorities; (iii) freezing rates of -8°C to -10°C per minute between $+4^{\circ}\text{C}$ and -80°C appear to provide the best cryosurvival; (iv) no benefit of seeding has yet been established.

2. All semen and washed sperm samples should be packaged in CBS High Security Straws in order to: (i) prevent contamination of the outside of the packaging with the specimen during loading; (ii) achieve a secure hermetic seal; (iii) achieve the most uniform cooling rates throughout the specimen; (iv) have maximum mechanical resilience at cryogenic temperatures; (v) employ secure, tamper-proof labelling of the specimen; and (vi) achieve maximum biocontainment.

3. Straws should be disinfected after filling and sealing, and before cooling.

4. Storage must be below -132°C but either liquid nitrogen (LN₂) immersion, liquid nitrogen vapour (LNV) or super-cold air (IVS) can be employed. However, there is no advantage of vapour phase storage using CBS straws and the risks of thermal instability of the specimens must be considered if choosing to use a system other than immersion in LN₂.

5. The inventory system should allow easy and quick access to specimens within the cryogenic storage tanks.

6. A robust records system must be implemented and SOPs developed that ensure documentation and the cryobank's actual contents reconcile. This system should be designed so as to eliminate the risk (so far as is humanly possible) the opportunities for samples to be placed into, or removed from, the cryobank without the correct records being kept.

7. Audits of the contents of a cryobank should be undertaken only after considering the perceived value of the information that might be obtained against the risk of accumulating irreversible damage to the specimens due to repeated transient warming above the glass transition temperature of water (i.e. -132°C). In this regard, it is essential that recommendations 5 and 6 be taken into account and the results of previous audits can be used to ascertain the likelihood of future audits revealing any discrepancies.

8. After thawing, the exterior of the straw must be disinfected before breaching the wall of the straw to remove its contents.

9. Open straws using either the CBS StrawCut device or disposable sterile suture scissors. Do not use scalpel blades or hypodermic needles.

10. Large storage tanks should have auto-fill controllers and be connected to a LN₂ supply manifold. Small tanks must be topped-up and have their levels monitored regularly (advance warning of tank deterioration).

11. All cryostorage tanks should have low-level and/or temperature alarms fitted that are connected to a dial-out alarm system.

12. All cryobanks should maintain a spare tank with a capacity at least equal to the largest tank in use at that site partially filled with LN₂.

13. Consideration should be given to separating specimens between two cryotanks as protection in case of tank failure and, in high-risk areas, to storing specimens between two geographically distant sites.

14. Consideration should be given to the separate storage of specimens from donors, patients and known 'high-risk' patients carrying pathogens, but only on public relations grounds since CBS straws will ensure effective biocontainment. The use of 'quarantine' tanks with CBS straws seems pointless provided that straws are disinfected post-thaw prior to breaching the straw.

15. Samples that were stored in cryovials or older types of straws must be treated according to the following additional rules: (i) Discard all broken or cracked units as well as straws that have lost a seal at either end. If a specimen is irreplaceable, or of great intrinsic value, then the patient(s) must be fully advised of the estimated risks to which the specimen has been exposed (noting that the problems were not previously recognized) and additional consent obtained before allowing use of the specimen; (ii) Disinfect the outside of the unit after thawing and before opening it to remove its contents; (iii) Process spermatozoa through density gradients to further reduce the risk of transmitting any pathogens that might have gained access to specimens, especially those where LN₂ was identified inside the unit upon removal from the cryotank; (iv) Separate tanks should be used for specimens from donors, screened patients, unscreened patients and various groups of patients known to be contaminated by pathogens.

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