Risk of contamination of germplasm during cryopreservation and cryobanking in IVF units

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Cryopreservation of sperm, embryos and, more recently, oocytes plays an important and increasing role in assisted reproduction, due to improvements of old, and introduction of new technologies. In parallel, concerns are increasing about the technical and biological safety of these procedures. However, published data regarding the confirmed or theoretical hazards of these procedures are sparse and sometimes contradictory. The purpose of this review will summarize data and opinions about one of the most disputed risks, the potential hazard of contamination and disease transmission through cryopreservation. Special attention is concentrated on the weak points of the technology including open vitrification systems, sterilization of liquid nitrogen and safety of commonly used storage tanks including straws and cryovials. Suggestions are also made for practical measures to avoid these dangers while preserving the benefits and perspectives of new cryopreservation technologies.

Key words: cryopreservation / vitrification / freezing / contamination / liquid nitrogen

Introduction

This article reviews the risk of pathogen transmission by cryopreserved semen and embryos and the sanitary aspect of cryopreservation and germplasm banking. For background on basic cryobiology and germplasm cryopreservation, readers are referred to the publication by Karrow and Critser (1997) and to the more recent articles by Mortimer (2004), Vajta and Nagy (2006), Yavin and Arav (2007) and Kuyawama (2007). The risk of disease transmission in humans and animals during assisted reproductive technology (ART) procedures was recently described in detail by Bielanski (2006).

Since semen, ooocytes and embryos can be infected with many micro-organisms, the use of cryopreserved germplasm for artificial insemination (AI) in vitro fertilization (IVF) and embryo transfer (ET), as well as in other related new reproductive technologies, is of concern to health authorities. During assisted reproductive procedures, cryostorage is the only situation where large quantities of biological materials of patients are kept together in a common liquid medium. Although the temperature of liquid nitrogen is −196 °C, it may transmit infective agents from one sample to the other if they are not sealed properly. Accordingly, apart from accidental mistakes and/or profound deviations from the safety manuals, cryopreservation and cryostorage of germplasm mean the potential for danger of disease transmission.

According to the latest available data, >70 000 cryopreserved embryos were thawed or warmed in Europe in 2004 and >12 000 babies were born as the result of cryopreserved embryo transfer in the same year (Andersen et al., 2008). The number of babies born after oocyte cryopreservation is growing rapidly, approaching 1000 worldwide, and the efficiency of some techniques is reported to be competitive with that of embryo cryopreservation. In domestic animals, according to the latest surveys, >260 million doses of bull semen were produced, out of which 95% were deep-frozen, 20 million were moved internationally and >220 000 cryopreserved bovine embryos were transferred (Thibier and Wagner, 2002; Thibier, 2006).

However, despite the enormous number of AI and ET procedures performed over the years in domestic animals, only a few cases of disease transmission by cryopreserved germplasm, e.g. infectious bovine rhinotracheitis virus (IBRV) by semen (Kupferschmied et al., 1986) and bovine viral diarrhoea virus (BVDV) by embryos (Lindberg et al., 2000), have been documented. This success can be credited, at least in part, to the advances in knowledge gained through research on...
the interaction of pathogenic micro-organisms with semen and embryos, and the consequent recommendations of the International Embryo Transfer Society (IETS) (Stringfellow and Seidel, 1998) and the Office International des Epizooties (OIE) on sanitary procedures for handling semen and embryos of livestock (Anon, 2005). A special annex (Anon, 2005, Appendix 3.3.3) refers to processing micromanipulated embryos. It clearly states that prior to micromanipulations (ICSI, biopsy etc.), embryos must be sequentially washed, zona pellucida (ZP) of each embryo should be examined over the entire surface area and certified to be intact and free of the adherent material.

In humans, evidence of contamination of patients through AI has been described for hepatitis B virus (HBV), human immunodeficiency virus (HIV), Neisseria gonorrhoeae, Ureaplasma urealyticum, Mycoplasma hominis, Chlamydia trachomatis, Trichomonas vaginalis and streptococcal species (Kleegman, 1967; Barwin et al., 1985; Berry et al., 1987; Mascola and Guinan, 1987; Araneta et al., 1995; Englert et al., 2004). Transmission of HBV by cryopreserved embryos to patients was also reported (van Os et al., 1991).

It has to be stated that none of the reported infections after insemination or embryo transfer in humans and domestic animals can be clearly attributed to the applied cryopreservation and storage procedure. However, as proved under experimental conditions, such possibilities exist (Bielanski et al., 2000, 2003), concerns are justified and measures should be taken to prevent possible future disease transmissions via cryopreserved germplasm.

**Current cryopreservation technologies for oocytes and embryos**

The discovery by Polge et al. (1949) of the cryoprotective properties of glycerol for bovine spermatozoa outlined the route to exploit advantages of preserving the germplasm of farm animals at ultra low temperatures. Successful cryopreservation of mammalian embryos was first reported by Whittingham et al. (1972) and Wilmut (1972) and for human embryos by Trounson and Mohr (1983). Currently, most embryos have been frozen in sealed straws and vials by the slow cooling (0.5–1°C/min) equilibrium method in static vapours or using computerized, controlled rate freezers.

Using this methodology, embryos require ‘seeding’ at subzero temperatures to induce ice nucleation and to avoid cell injury by releasing a latent heat prior to further controlled cooling (−40 to −80°C) followed by plunging into liquid nitrogen (LN2). An alternative approach of cryopreservation is vitrification, applied first successfully for mouse embryos by Rall and Fahy (1985) and improved recently to the point of practical application for mammalian oocytes and embryos. Features of most successful vitrification techniques are the very rapid cooling rates (>20 000°C/min) and simplicity, with higher embryonic survival and pregnancy rates when compared with conventional, slow cooling. However, to achieve the maximal rates of cooling to reduce chilling injury and eliminate ice crystal formation without the need of high cryoprotectant (CP) concentrations, embryos have been exposed directly to LN2 in tiny droplets of a CP (<1 μl, in some methods <0.1 μl). Despite many unquestionable advantages, concerns have been raised by sanitary regulatory bodies regarding the safety of these procedures, as LN2 may mediate transfer of pathogenic agents between samples (discussed in detail later).

**Survival of micro-organisms in low temperatures and transmission to germplasm by LN2**

Most micro-organisms (either in the form of pure cultures or in association with germplasm) can survive storage at low temperatures, including in LN2 (−196°C). Although the preparation of embryo culture media and semen extenders from specific ingredients in human clinics is avoided, it continues to be a common practice in animal ART. Nevertheless, many ingredients of embryo culture media and semen extenders may act as stabilizers for micro-organisms at freezing temperatures (e.g. milk, serum or serum albumin, sucrose, sorbitol and other sugars). Unfortunately, the most common CPs applied in embryo and oocyte cryopreservation (glycerol, DMSO, ethylene glycol, propylene glycol, methanol etc.) also efficiently protect bacteria and viruses from cryoinjuries, e.g. concentrations of DMSO as low as 5% defend enveloped viruses against the trauma of freezing (Wallis and Melnik, 1968). On the other hand, non-enveloped viruses are not inactivated by freezing and thawing even in the absence of protective agents. The fact that micro-organisms survive in association with germplasm is not only important from the potential of disease transmission to recipients by embryo transfer, but also in approaches to the storage and testing of samples for health certification of embryos for international movement. The effect of methods of sample handling, testing, storage and preservation on survival of viruses was investigated by Van der Maaten (1987). Aliquots of embryo collection fluid were spiked with various isolates of IBRV, BVDV, bovine parainfluenza virus type 3 and bluetongue virus (BTV) and then placed directly in storage at 4, −20 and −70°C without using controlling cooling rates. In general, all viruses retained their titres for 6 days at 4°C. After storage at −20°C for up to 3 months, some decrease in viral titres was observed, but all of the viruses tested seemed well-preserved during storage at −70°C. Other viral agents such as herpes simplex-2 virus and cytomegalovirus also survive in association with sperm cells without loss during routine cryopreservation and storage (Sherman and Menna, 1986; Hammit et al., 1988).

On the bacterial side, many micro-organisms tolerate very high DMSO concentrations without visible toxic effects and some (e.g. Acinetobacter spp., Corynebacterium spp., Bacillus spp. and Streptomyces spp.) are even capable of multiplication in a growth medium containing 2–45% DMSO (Hirayama, 2007). Garcia et al. (1981) reported that bacterial agents (β-hemolytic Streptococcus, Staphylococcus aureus, Pseudomonas, Escherichia coli) isolated from fresh ejaculated semen were also recovered from a portion of frozen in LN without appreciable reduction. Also the parasitic agent, T. vaginalis, survives (90%) cryopreservation at −196°C in their growth medium, trypsinase yeast maltose with DMSO (Sherman et al., 1991).

However, cryopreservation may reduce the concentration of common bacterial contaminants of bull semen (Mazurova et al., 1975).
It has also been shown, for example, that with a concentrated suspension of *Brucella* bovis, 64% of the organisms failed to survive a simple freezing and thawing cycle in an antibiotic-free embryo culture medium (Stringfellow et al., 1986). Nevertheless, bacteria possess a relatively high tolerance, contrary to fungi which proved very sensitive to the freezing procedure and to the toxicity of higher concentrations of CPs (e.g. DMSO). For example, cryopreservation reduced the concentration of fungi in human semen by >90% (Hubalek, 2003). However, it should be noticed that the minimum infectivity titres for most of the pathogenic agents associated with cryopreserved semen and embryos transferred in utero to recipients remain unknown. Therefore, the potential for disease transmission by even a residual amount of the agents associated with the ZP or spermatozoa may still exist. This view can be supported by few reports on suspected transmission of BVDV, bovine herpesvirus-1 (BHV-1) and HBV to recipients by cryopreserved embryos and semen (Kupferschmied et al., 1986; Van Os et al., 1991; Lindberg et al., 2000; Drew et al., 2002).

The clinical risk of transmission of viral agents such as HBV, herpes simplex, adenovirus and papillomavirus to patients throughout the dermatologic practices of direct exposure to LN2 has been discussed in several publications (Charles and Sire, 1971; Schaffer et al., 1976; Burke et al., 1986; Jones and Darville, 1989). Jones et al., (1995) demonstrated that it is possible to transfer herpes simplex and adenovirus type 2 into and out of a Dewar flask by means of a cotton wool swab. It was also concluded that it seems quite possible that virus particles could be transferred from patient to patient by repeated dipping of cotton swabs into the same Dewar container. However, the safety of cryopreserved germplasm has received a great degree of attention after the discovery of a case of transmission of human hepatitis B via bone marrow transplants cryopreserved in LN2 (Tedder et al., 1995). Experimentally, Pasecka-Serafin (1972) was first to demonstrate the possibility of translocation of bacteria from infected semen pellets to sterile ones in LN2. Of the sterile samples, 94% became infected with *E. coli* and *S. aureus* within 2 h after placing them in a container holding contaminated LN2. More recently, Bielanski et al. (2000) have demonstrated the possibility of infection of embryos through LN2 contaminated with BVDV and BHV-1 when the samples were not sealed. Also, retrospective studies in which commercial LN2 cryotanks were examined after 35 continuous years of service revealed various bacterial and fungal contaminations in the LN2 detritus. Many of the identified bacteria isolated in these studies were ubiquitous environmental micro-organisms and were rare opportunistic pathogens of low significance in producing disease in humans or animals (Table I). It should be acknowledged that some of the isolates may have been derived from laboratory contamination during semen and embryo processing for cryopreservation rather than genuinely being present within the sample.

It is interesting to observe that although *Pseudomonas aeruginosa* is a frequently isolated contaminant of bull semen, *Stenotrophomonas maltophilia* was the most prevalent bacterial strain detected in this study in association with cryopreserved germplasm and LN2 samples. Implications of the introduction of antibiotic-resistant strains of *Pseudomonas* spp., *Enterobacter cloacae*, *Staphylococcus sciuri*, *Actinobacter cacaoeticus* and *Flavobacterium* spp. by contaminated cryopreserved semen into IVF systems has been reported. Particularly relevant is the demonstration that *S. maltophilia* can affect sperm motility and severely suppress embryonic development (Stringfellow et al., 1997; Bielanski et al., 2003).

### Factors facilitating contamination of germplasm, and preventive measures

There are many factors which could lead to or influence the contamination of embryos with pathogens prior to and during cryopreservation and some of them are also applicable to semen processing (Bielanski, 2006). These include the integrity of the embryonic ZP, the cryopreservation method, loading and sealing of the container and the sterility of the LN2, as well as the storage container. Maintenance of the intact ZP throughout cooling and warming as well as post-warming manipulation is a powerful approach for prevention of embryonic cell contamination and infection. In domestic animals, the importance of inspection of the ZP for continuity as well as rigorous washing of the embryos prior to cryopreservation using the methodology recommended by the IETS (Stringfellow and Seidel, 1998) have been stressed in OIE Animal Health Code Appendices (Anon, 2005). However, under certain freezing and thawing conditions, >50% of mammalian embryos may have ZP fractures (Bielanski et al., 1986; Rall and Meyer, 1989; Van den Abbeel and Van Steirteghem, 2000), although the proper application of the latest vitrification methods seems to eliminate this problem.

Unfortunately, commonly applied techniques in human embryology (oocyte, polar body and embryo biopsy, ICSI assisted hatching or cryopreservation of hatched embryos) result in irreversible damage or lack of ZP; therefore embryonic cells of such embryos are more susceptible for contamination, compared with ZP-intact embryos. Accordingly, screening oocyte donors for blood-borne diseases, including at least HIV, hepatitis B and C and herpesviruses before embryos are considered for cryopreservation and storage in LN2 appears to be basic. On the other hand, the first step of the defence at the IVF laboratory level should be an application of efficient washing to oocytes and embryos prior to cryopreservation. As has been demonstrated in numerous animal models, multiple washing procedures is very effective in decreasing infectivity and in rendering oocytes or embryos free from many viral and bacterial pathogens (Stringfellow and Seidel, 1998). Additional washing of embryos after thawing or warming will likely facilitate a further decrease in their infectivity and reduce risk of cross-contamination between samples during cryobanking. The last indispensable step in prevention of the risk of disease transmission to recipients is storage of germplasm in a quarantine cryotank until the donors have been tested for seroconversion and/or the samples have been tested for the presence of infectious agent(s). From the sanitary point of view, at all times, it appears safest to store gametes from a patient known or suspected as infected in a separate LN2 tanks. Such procedures would eliminate the possibility of cross-contamination between gametes of different patients in case of leaking seals or broken straws. Alternatively, gametes from potentially infected patients with the same pathogenic agent could be quarantined together. Recognizing that semen (especially raw) may harbour a high microbial load, storing it together in the same tank with oocytes/embryos specimens should be avoided. Nevertheless, at any time, the storage of germplasm derived from...
<table>
<thead>
<tr>
<th>Sample tank</th>
<th>Identified microbial contamination</th>
<th>Years of storage</th>
<th>Total no. of stored samples</th>
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<tr>
<td><strong>Research</strong></td>
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<tr>
<td>Laboratory tanks</td>
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<tr>
<td>1</td>
<td>Staphylococcus auricularis</td>
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<tr>
<td>2</td>
<td>Bacillus licheniformis, Bacillus spp.</td>
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<td>3</td>
<td>CDC group IVc-2, Alcaligenes faecalis</td>
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<tr>
<td>4</td>
<td>Brevundimonas vesiculans</td>
<td>Stenotrophomonas maltophilia</td>
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<td>5</td>
<td>Stenotrophomonas maltophilia</td>
<td>Proteus vulgaris</td>
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<td>6</td>
<td>Staphylococcus capitis, unidentified</td>
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<td>7</td>
<td>Stenotrophomonas maltophilia, Comamonas testosteroni</td>
<td>Stenotrophomonas maltophilia, Stenotrophomonas maltophilia, Staphylococcus sciuri</td>
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<tr>
<td>8</td>
<td>Bacillus pumilus, Eikenella corrodens</td>
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<td><strong>Commercial tanks</strong></td>
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<td>9</td>
<td>Nd</td>
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<tr>
<td>10</td>
<td>Aspergillus spp.</td>
<td>Aspergillus spp.</td>
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<td>11</td>
<td>Corynebacterium xerosis, Bacillus sphaericus</td>
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<td>34 962</td>
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<td>12</td>
<td>Nd</td>
<td>Stenotrophomonas maltophilia</td>
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<td>14</td>
<td>Aspergillus spp.</td>
<td>Photobacterium damsela</td>
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<td>15</td>
<td>Nd</td>
<td>Bacillus sphericus, Corynebacterium spp., Staphylococcus sciuri</td>
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<tr>
<td>16</td>
<td>Stenotrophomonas maltophilia</td>
<td>Ralstonia pickettii</td>
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Nd, not detected; —, not available for testing.
a ‘clean patient’ with that from potentially infected patients in the same LN2 tank should not be considered. In general, based on guidelines from professional organizations (e.g. ESHRE), each ART unit should develop good laboratory practices. These include policies for the safety of personnel and for the prevention of the transmission of pathogenic agents. In addition, Magli et al. (2008) suggest that processing of gametes from infected patients should be performed in designated facilities, or if such are not available, it should be carried out during allocated times followed by rigorous cleaning and disinfection of a laboratory. In the later scenario, if handling embryos and/or semen from the uninfected and infected patients is unavoidable on a given day, the ‘clean’ patients should be processed before those who are infected.

**Semen factors**

Like embryos, cryopreserved human and animal semen may be contaminated with viral agents [e.g. HIV-1, hepatitis C virus (HCV), Epstein–Barr virus, foot-and-mouth virus (FMDV), BTV, BVDV, BHV-1] and potentially pathogenic bacteria such as *P. aeruginosa*, *Staphylococcus* spp., *Streptococcus* spp., *Corynebactrium* spp., *N. gonorrhoeae*, *E. coli*, Candida species and *Ureaplasma urealyticum*, despite the presence of antibiotics (Garcia et al., 1981; Hare, 1985; Leiva et al., 1985; Wierzbowksi, 1985; Glander et al., 1986; Mazzilli et al., 2006). Interference of glycerol with the antimicrobial properties of antibiotics has been suggested, with the recommendation of adding them directly to the raw ejaculates rather than to the extender with the CP (Bielanski, 2007). The reduced efficacy of antibiotics during cryopreservation may be due to resistance of the bacteria at lower temperatures and by a protective colloid of the egg yolk around the bacteria (Glander et al., 1983; Leiva et al., 1985). It should be noted that despite its cryoprotective properties, egg yolk can be a source of potential bacterial contaminants (*Salmonella pullorum*, *E. coli* and mycoplasmas), thus, due to the lack of standardized quality control, should not be used for patients.

Although the transmission of infectious agents to embryos by contaminated semen has not been proven experimentally or reported as yet, such potential should be not overlooked when cryopreserved semen is used for the generation of embryos using superovulation and AI or IVF (Wrathall et al., 2006). At present, to reduce such a risk, it is commonly the practice to use a discontinuous gradient centrifugation and/or swim-up procedures to remove or reduce the load of various viral and bacterial agents from frozen–thawed semen prior to fertilization. It has been shown that this procedure diminished the risk of disease transmission to recipients and contamination of IVF systems, e.g. for HIV, HCV, porcine reproductive and respiratory syndrome virus (Maertens et al., 2004; Morfeld et al., 2005). It appears that these procedures may be less effective in removing pathogens from animal semen than from human semen (Bielanski, 2007).

**Containers for cryostorage**

The safety of different types of containers and straws used for cryopreservation of semen and embryos was recently discussed by Rall (2003) and Mortimer (2004). Glass ampoules previously used for these purposes have become obsolete on safety grounds due to the explosion hazard and the possibility of personal injury.

This hazard, however, to a lower extent may also be applied to various plastic cryovials (Cryo Tube, Nunc A/S, Roskilde, Denmark; Nalgene Nunc International, Naperville, IL, USA) which, despite packages marked ‘for vapour storage only’, are often stored in LN2. The most common cause of contamination is the faulty seal, leak or breakage of these containers in LN2. Clarke (1999) reported that 45% of cryovials without O-ring (Nunc) and 58% of cryovials with an O-ring (Iwaki, Japan) absorbed LN2 during a 3 h immersion in LN2. Although manufacturers strongly recommend the use of a second skin (Cryoflex tubing of Nunc; or Nescofilm, Merk Ltd, Dorset, UK), these measures are rarely applied in everyday practice.

Regarding plastic straws, three main types were available on the market: those made from polyvinylchloride (PVC), polyethylene terephthalate glycol (PETG; IMV, L’Aigle, France) and ionomeric resin (CBS High Security Straws; CryoBioSystem, Paris, France). Recently, PVC straws have been removed from the market due to sterilization problems. It appears that CBS straws are the most suitable for cryopreservation and storage of germplasm. To prevent contamination, these straws are loaded into a special heat sealer (SYMS SEALER, CryoBioSystem, Paris, France) and sealed thermally on both ends. According to the manufacturers claim, the straws are impermeable to pathogenic agents and have recently been validated for sanitary properties by experimental contamination with HIV-1 and HCV (Benifla et al., 2000; Letur-Könirsch et al., 2003; Loskutoff et al., 2005). CBS straws conform to ISO 9002 standards and have been cleared by the US Food and Drug Administration (FDA) for human applications in ART.

The filling and sealing methods [e.g. polyvinyl alcohol (PVA) powder and plastic spheres] may have even more influence on biosafety than the material of the straw. The potential for contamination of samples by straws containing suspensions of *E. coli* and Newcastle disease virus or ethylene blue and sealed with different methods was investigated by Russell et al. (1997). Samples sealed by a traditional ‘dip and wipe’ method of immersing a tip of the PVC straw in the solution with the PVA powder (in a multi-use container) demonstrated a significantly higher degree of contamination when compared with straws filled using an aseptic method such as a syringe. This was probably a consequence of retained residue solution on the inside of the straw tip which subsequently contaminated the sealing plug. It was concluded that in a real scenario, PVA could accumulate microbes from a number of individuals which can then be introduced to the inside of the straws of another donor. Therefore, it is important to aliquot PVA into tubes which can be used for one donor only and leave an air-gap of at least 1 cm to allow for expansion during freezing. According to a publication of Letur-Könirsch et al. (2003), CBS straws prevented safely HIV-1 contamination, whereas some of the samples cryopreserved in PVC and PETG straws became infected. The above authors, however, suggested that the main factor was the different sealing method (thermal versus ultrasonic sealing for CBS versus PVC and PETG straws, respectively).

Thermal sealing of straws by use of a specially designed device would be most recommended from a sanitary point of view. In addition, to reduce the risk of translocation of contaminants, the sample container not only should be closed hermetically, but its outside surfaces disinfected before freezing and after thawing (e.g. 3% hypochlorite and 70% ethanol). When programmed rate alcohol freezers are used, this risk is automatically eliminated. It should also
be mentioned that alcohol (methanol) does not leak into the thermally heat-sealed straws and has been used to freeze mice, ovine and equine embryos (Rall et al., 1984; Czlonkowska et al., 1991; Bass et al., 2004).

**Cooling methods and sterility of liquid nitrogen**

Cryopreservation by slow cooling using static vapour freezers offers relatively less risk of contamination of the samples during cooling than rapid cooling by open vitrification systems, because the latter approach includes direct contact with potentially infected LN₂. The so-called ‘Minimum Drop Size’ technique was originally described by Arav (1992) and later modified by others who used different cryocarrier systems such as electron microscopy grids, open-pulled straws (OPS; Minitüb, Tiefenbach, Germany), cryoloops (Hampton Research, Laguna Niguel, CA, USA), hemi-straws (IMV) or cryotops (Kitazato, Fujinomiya, Japan) for vitrification of oocytes and embryos (for references, see Vajta and Nagy, 2006).

Sterility of factory-derived LN₂ often is always of concern, although the risk of contamination by human pathogens seems to be rather low. Components of the standard LN₂ production system comprise a compressor, a cryogenerator and containers. From a practical point of view, the complete sterilization and maintenance of sterility in such a robust system might be a very demanding task, if possible at all. Accordingly, some ubiquitous bacterial agents can be expected in any commercially produced LN₂. Nevertheless, it is an ‘in and out’ system and only air-borne contaminants are supposed to enter it (LN₂ compressor) via air used for LN₂ production.

As they are not air-borne, it is unlikely that viral agents of human concern such as HIV, hepatitis and herpes viruses would enter the LN₂ production system. No analysis of factory-derived LN₂ samples has been published yet to confirm this assumption.

For some bacterial micro-organisms and fungal spores, application of proper filters at the outlet of the LN₂ apparatus or filtration of factory-derived LN₂ should ensure the required protection. Filtration of LN₂ and its sterility validation was described by McBurnie and Bardo (2002) using 0.2 μm PTFE filters and Brevundimonas diminuta as a contaminant (ATCC 19146).

It should also be noted that methods exist to produce LN₂ in a completely sterile way (http://www.freepatentonline.com/5737926.html) and there are commercial companies that offer devices for such production, but only for pharmaceutical purposes (Veriseg SLG, Linde Gas Division, Pullach, Germany; Messer Austria GmbH, Gumpoldskirchen, Austria). To the authors’ knowledge, at the present time, there is no commercial supplier of sterile LN₂ or of a portable device producing LN₂ suitable for ART.

To avoid sterilization of large volumes, embryos at first can be cooled in small volume of decontaminated LN₂, followed by placing the original sample into a secondary pre-cooled sealed container before plunging into LN₂ for storage (Vajta et al., 1998). To test the value of this approach, the risk of contaminating embryos vitrified in OPS using the commercial kit Vit-Set (Minitüb, Tiefenbach, Germany) was investigated under experimental conditions (Bielanski and Hanniman, 2007). The Vit-Set consists of three stainless steel chambers for cooling the 0.5 ml protective straws, vitrification of OPS straws and for loading OPS into the protective straws, respectively. The protective straw is pre-cooled by submerging its sealed end in LN₂. The sealing of the other end of protective straws, after the insertion of OPS straws, does not allow LN₂ in, thus preventing the explosion hazard after its removal from cryostorage.

In the experiment, bovine embryos were vitrified and protected against contamination as described originally by Vajta et al. (1997, 1998). Briefly, when embryos contaminated with cultures of E. coli, P. aeruginosa or non-cytopathic NewYork (NY) strain of BVDV, were vitrified in OPS straws and then protected by 0.5 ml CBS straws, no cross-contamination to clean embryos or LN₂ was detected. It was concluded that the potential for cross-contamination of samples by application of the Vit-Set for vitrification of embryos using OPS (and other high-rate cooling techniques requiring direct contact with LN₂ during cooling) is negligible if: (i) LN₂ in the chambers is frequently replaced and the chambers are disinfected between embryo donors, and (ii) protective straws are applied over OPS (or analogue tools) and are hermetically sealed.

Many researchers recognizing the strict sanitary requirements for the generation of cryopreserved oocytes/embryos free of infectious agents developed ‘closed systems’ of vitrification without the necessity of exposing embryos or semen directly to LN₂. Using such a system, mouse pronuclear oocytes (Kuleshova and Shaw, 2000; Isachenko et al., 2005a; Kuwayama et al., 2005), porcine embryos (Hirayama, 2007) and human embryos (Isachenko et al., 2005b, 2007; Stachek et al., 2008) were vitrified. These authors placed embryos in OPS straws or other carriers and then either sealed the carrier or inserted it into large sterile plastic-sealed straws before immersion in LN₂.

Another approach, which is to cryopreserve sperm, embryos or oocytes on pre-cooled metal surfaces (solid surface vitrification or SSV technique), has been reported earlier for the freezing of sperm and embryos in small drops on supercooled aluminium foil or steel blocks before placement into a protective container and immersion in LN₂ (Dinnyes et al., 2000; Isachenko et al., 2005c).

Unfortunately, the open system and direct contact might be indispensable to achieve the required cooling and warming rates for very sensitive samples: so far, commercially available closed analogues (Cryotip; Irvine Scientific, Santa Ana, CA, USA), the latest version of the hemistraw system (CBS High Security Vitrification Kit; IMV) and the SSV analogue, Cryohook (Cryologic, Melbourne, Australia) have failed to demonstrate the same efficiency for human oocytes when compared with their counterparts using the direct contact approach (Kuwayama, personal communication; Cobo, personal communication).

**Storage and banking of germplasm**

The risk associated with cryopreservation including serious incidents involving loss or damage of cryobanked germplasm in IVF units was reported recently by Tomlinson and Morroll (2008). To minimize these risks, laboratory staff should be trained and observe safety rules for handling LN₂ according to the appropriate Material Safety Data Sheets (MSDS) and the laboratory Standard Operation Procedure (SOP). SOP should include rules and procedures for safe transportation and refilling Dewar containers with LN₂ within buildings and...
laboratories. Following these rules (e.g. wearing a protective face shield) should prevent the risk of personal injury and contamination through the potential of exploded frozen-thawed specimens. It should also eliminate the hazard of suffocation when handling open Dewars in poorly ventilated areas and freezing injuries through direct body contact with LN (Tomlinson, 2008).

Considering that methods and equipment currently used worldwide are inappropriate to maintain sterility of large amounts of LN2 in cryotanks used for the storage of biological samples, all these tanks should be considered potentially contaminated with micro-organisms. Most germplasm samples are stored in large capacity cryotanks, some of which could accommodate hundreds of thousands of straws or other sample containers. This arrangement may create a possibility for cross-contamination in case of accidental breaking or leaking of containers with infected samples. Also, over the storage time, due to the exposure of cryotanks to the laboratory environment during refilling and handling of specimens, ice crystals are formed on the walls of the vessels. Aggregated ice and sediment may entrap viruses, bacteria, fungal spores and debris pose a risk of microbial transmission to stored samples. Nevertheless, a recent study indicates that long-term banking of germplasm in the LN2 phase, even over 35 years, might be a safe technique for the preservation of genetic materials with a rather low potential risk of cross-contamination when the specimens are properly sealed (Bielanski et al., 2003). No viral agents, but a number of ubiquitous bacterial micro-organisms, were isolated from liquid nitrogen and ice sediments in Dewar containers. In another study, mostly environmental bacteria and fungi were identified in almost all samples of LN2 sediment and ice collected from three Dewars used for the storage of human embryos in sealed glass vials. However, it was pointed out that some of these agents such as Acinetobacter baumannii and Chryseomonas luteola are capable of causing nosocomial infections in humans (Morris, 2005). The microbial load did not correlate with the period for which the Dewar had been used (7–15 years).

**Vapour phase of LN2**

As an alternative to the LN2 phase, the vapour phase of liquid nitrogen (VPLN2) has been proposed as a safer method for the storage of germplasm. Semen and embryos have been stored in VPLN2 providing viability of germplasm after thawing comparable with results obtained from LN2 storage (Clarke, 1999; Tomlinson and Sakkas, 2000). However, these kinds of storage methods are prone to various difficulties during prolonged storage and handling of specimens in attempts to maintain steady temperatures between −150 and −190°C. Frequent opening and high air humidity may also cause an ice formation and frosting on lids and walls and thus attract the environmental micro-organisms. Nevertheless, successful storage of human semen and embryos in the vapour phase has been practiced in some clinics (Clarke, 1999).

The risk of contamination of germplasm in VPLN2 with the use of dry shipper-Dewars during short storage was investigated recently (Bielanski, 2005b). In general, Dewars containing LN2 with biological specimens when moved are considered as hazardous material and their transportation possibilities are restricted accordingly. On the other hand, the so-called ‘dry shippers’ that do not contain free LN2 are classified as non-hazardous throughout the world and can be used for various means of transportation including air. The dry shippers are capable of maintaining nitrogen vapours at approximately −150°C for a few days without the risk of spilling LN2 or explosion during transportation. In our experiments, none of the embryos or semen samples exposed to LN2 vapours in previously contaminated dry shippers tested positive for the presence of selected bovine bacterial and viral agents (BVDV, BHV-1, P. aeruginosa, E. coli and S. aureus). Moreover, there was no transmission of these agents between contaminated and non-contaminated germplasm stored in proximity in open containers in the VPLN2. This finding may suggest that in contrast to the LN2 phase, the VPLN2 can be used as a safer means of short-term storage and transportation of germplasm even in the proximity of pathogenic agents (Bielanski et al., 2005a).

Although the detailed mechanism of the microbial movements within dry shippers is unknown, it could be assumed that there is no, or very limited, circulation of frozen particles within the Dewar chamber by the LN2 vapours in the absence of LN2 phase. This contrasts to the environment within the conventional LN2 Dewars where extensive movement of both vapours and boiling LN2 take place particularly during temperature changes and tank refilling. This view could be supported by the report of Fountain et al. (1997) who detected environmental or water-borne bacteria and fungi in the vapour phase above LN2 in the Dewar that was tested. This vapour contamination reflected the findings of bacterial cultures isolated from LN2 and was likely the result of aerosolization by boiling LN2 collecting contaminants over a long period of Dewar service without its periodic decontamination. It remains to be established whether more advanced LN2 vapour freezers and mechanical freezers which can provide air-phase at −140 and −150°C without the need for LN2 can prevent cross-contamination of germplasm over a long period of storage without compromising germplasm post-thaw viability.

**Practical considerations for germplasm storage and transportation**

Although it is cumbersome from a practical standpoint, cryotanks require periodic decontamination using an efficient disinfectant to decrease the risk of cross-contamination. Also, as a rule, it could be recommended that germplasm suspected of carrying infectious micro-organisms should not be stored in a cryotank with other, supposedly not contaminated gametes to avoid cross-contamination in the event of the accidental breakage of a freezing container.

From information obtained from the manufacturers and suppliers, regular cryotanks can be sanitized with any solution that does not react with aluminium or stainless-steel. In most cases, bleach, any household detergent or even a mild soap solution is suitable. Other cleaners and disinfectants that can be safely used include 3–6% hydrogen peroxide and 37% denaturated alcohol. It is important that after exposure to disinfectant (15–30 min), the inner vessel is thoroughly rinsed with sterile water and all cleaner residues have been removed. Spraying the solution into the inner vessel is the preferred method, although agitation of the solution inside the inner vessel will also suffice. Other disinfectants, e.g. Virkon S (sodium chloride/potassium peroxymonosulfate; Antec International, UK), are also approved for use against viruses of highly contagious diseases...
such as FMDV, avian influenza, African swine fever virus, vesicular stomatitis virus, retroviruses, adenoviruses and bacterial and fungal agents (e.g. *S. aureus*, *P. aeruginosa*, *E. coli*, enterobacter spp., *Candida albicans* and *Aspergillus fumigatus*). To the authors’ knowledge, there is no data published on the efficacy of disinfectants or detergents for LN₂ cryo-tanks decontamination. It could only be assumed that the application of some detergents and sanitizers used in food and dairy industry for cleaning of aluminium and steel containers could be used safely for washing LN₂ Dewars. Dewars, which are not in immediate use, should be washed and stored dry with cups tightly sealed to prevent the growth of environmental microbes. However, upon returning Dewars to service from storage additional cleaning would be advisable prior to having them refilled with fresh LN₂.

In contrast, decontamination of dry shippers is more difficult due to their inner structure.

The effect of two different types of an LN₂ absorbent for decontamination of dry shippers was investigated recently (Bielanski, 2005b; Table II). Dry shippers were heavily contaminated with high titres of cultures of *P. aeruginosa*, *E. coli*, *S. aureus*, BVDV and BHV-1 to create a worst-case scenario. The concentrations of bacterial and viral cultures to which the vapour shippers were exposed exceeded potential titres which can be expected to occur during the storage and transportation of germplasm or other contaminated biological material inadvertently harbouring infectious pathogens. It has been concluded that shippers containing a hydrophobic absorbent were suitable for disinfection using liquid biocide solutions (e.g. sodium hypochlorite) by filling the inner vessel to its full capacity with the cleaning mixture and then rinsing and drying thoroughly by inverting them under a laminar flow hood. Shippers with a non-hydrophobic insert could only be disinfected by the application of vapour sterilization, as attempts to use liquid solutions on the latter resulted in permanent damage to the LN₂ absorbent. It should be pointed out, however, that it remains unknown whether the disinfectants selected in these experiments would be effective against the causal agents of transmissible spongiform encephalopathies, bacterial spores or very small viral agents (e.g. porcine parvovirus or FMDV).

### Conclusion and recommendations

Safe and successful cryopreservation of semen, oocytes and embryos requires (i) screening of patients for infectious diseases, and (ii) application of the appropriate sanitary and cryo-procedures to ensure high post-thaw survival of germplasm and to minimize the risk of disease transmission to recipients. Since the LN₂-mediated transmission of infective agents between samples has been demonstrated under experimental conditions, accordingly the application of hermetically sealed containers or a secondary enclosure (for cryovials and open vitrification systems) is suggested. To ensure rapid cooling in some vitrification techniques requiring direct contact, sterile LN₂ should be used, then samples should be safely sealed into pre-cooled secondary containers. Temporary storage of samples in dry shippers in the VPLN₂ appears to reduce the potential risk of samples cross-contamination; however, the safety of large-scale long-term storage of samples in vapour of LN₂ has to be investigated. Periodic disinfection of cryo-Dewars should be considered. As an additional precaution, storage of germplasm in a quarantine cryotank until the donors have been tested for seroconversion and/or samples for the presence of infectious agents is advisable.

<table>
<thead>
<tr>
<th>Biocide (manufacturer)</th>
<th>Concentration or dilution used for disinfection</th>
<th>Hydrophobic membrane (HM) permeability and silica absorption to disinfectant</th>
<th>No. positive/total microbial tests from dry shipper*</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hypochlorite</td>
<td>30%</td>
<td>—</td>
<td>0/5 0/11</td>
<td>(Household bleach 5.64%)</td>
</tr>
<tr>
<td>Rocol (L &amp; F, Canada)</td>
<td>0.02%</td>
<td>—</td>
<td>0/5 3/3</td>
<td>Foaming</td>
</tr>
<tr>
<td>I-Stoke (A.P.A., USA)</td>
<td>1.250%</td>
<td>—</td>
<td>0/5 3/3</td>
<td>Foaming</td>
</tr>
<tr>
<td>Virkon (Antek Inter, Inc., UK)</td>
<td>1%</td>
<td>—</td>
<td>0/5 3/3</td>
<td>Red stain residue; foaming</td>
</tr>
<tr>
<td>Expel (Indeq, USA)</td>
<td>100%</td>
<td>—</td>
<td>0/5 0/5</td>
<td>Possibly corrosive</td>
</tr>
<tr>
<td>Expel Odor Neutralizer (Indeq, USA)</td>
<td>1:1</td>
<td>—</td>
<td>0/5 3/3</td>
<td>Foaming</td>
</tr>
<tr>
<td>Paracetic acid (FMC Corp., USA)</td>
<td>30%</td>
<td>—</td>
<td>0/5 0/5</td>
<td>Acrid odour</td>
</tr>
<tr>
<td>Ethanol (BDH)</td>
<td>70%</td>
<td>Yes</td>
<td>—</td>
<td>Caused gel, possible damage</td>
</tr>
<tr>
<td>Viralex (Alda Pharmac. Inc., Canada)</td>
<td>100%</td>
<td>Yes</td>
<td>—</td>
<td>Caused gel, possible damage</td>
</tr>
<tr>
<td>Formalin (BDH)</td>
<td>10%</td>
<td>—</td>
<td>0/3* 3/3*</td>
<td>Potential carcinogen</td>
</tr>
<tr>
<td>Ethylene oxide (AmCO)</td>
<td>100%</td>
<td>—</td>
<td>0/6 0/10</td>
<td>Potential carcinogen</td>
</tr>
<tr>
<td>Airchem (Ecolab Inc., USA)</td>
<td>1%</td>
<td>—</td>
<td>3/3</td>
<td>—</td>
</tr>
</tbody>
</table>

*Formalin solution.
*Formalin vapours.
*Each test represents experimental replication with three bacterial and two viral agents.
Acknowledgement

Authors thank to Dr John Yovich for his professional and language corrections and suggestions.

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Submitted on December 16, 2008; resubmitted on March 30, 2009; accepted on April 6, 2009.