A strategy for rapid cooling of mouse embryos within a double straw to eliminate the risk of contamination during storage in liquid nitrogen

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Double packaging, in which an inner straw containing the specimen is inserted into an outer, larger straw (here termed ‘straw-in-straw’) to prevent the inner straw from coming into direct contact with liquid nitrogen provides a simple strategy for reducing or eliminating the potential contamination risk associated with storage in liquid nitrogen. This approach has in the past been used in conjunction with cryopreservation by slow cooling, but has not previously been tested for use throughout an entire rapid cooling and warming procedure. This study determined whether keeping the straw containing the embryos inside a second protecting container throughout the cryopreservation and storage protocol would compromise embryo viability. We established that a cryoprotectant containing a high polymer concentration (35% dextran or Ficoll) together with 25% ethylene glycol (as the penetrating cryoprotectant) was highly effective for day 2 and day 3 mouse embryos in both single and double straws. The survival and development of all cryopreserved embryos, as assessed both in vitro and in vivo, was not statistically different to their untreated controls. This established that a protein/serum-free cryoprotectant solution supplemented with polymers could provide complete protection of mouse embryos. It also shows, for the first time, that embryos can be cooled by direct immersion in liquid nitrogen and warmed by direct immersion into a waterbath within a double straw arrangement to reduce the likelihood of contamination.

Key words: contamination risk/cryobiology/embryo/polymer/vitrification

Introduction

Reducing the incidence of contamination is of high priority for groups using assisted reproduction (Bielskanski, 1997; Bielskanski et al., 1997, 2000; Vanlangendonckt et al., 1997; Sutmoller and Wrathall, 1997a,b; Gardner, 1998; Gook and Edgar, 1999). To protect the animal industry the International Embryo Transfer Society has developed policies and guidelines [IETS web site (http://www.iets.org) and the IETS manual (Stringfellow and Seidel, 1998)] to be applied to reduce the likelihood of disease transmission. Although attempts have been made to ensure that sera are free of infectious agents, one of 37 samples in one study still contained bovine viral diarrhoea (BVD) virus after heat inactivation and gamma radiation (Rossi et al., 1980). Comparable policies have also been developed in some countries for the preparation and use of human semen (British Andrology Society, 1999). In addition to the contaminants that can enter the system through infected donors (Riddell et al., 1993; Sutmoller and Wrathall, 1997a,b; Witz et al., 1999), serum (Rossi et al., 1980, Alberda, 1988), culture medium (Bielskanski and Dubuc, 1993), co-culture cells (Booth et al., 1995), cryopreserved zonae (Bielskanski et al., 1999), and incubators, there is now also significant evidence that the liquid nitrogen, used for frozen storage, can be a source of contamination (Tedder et al., 1995; Bielskanski et al., 2000). Although there are no reported cases of contamination occurring via liquid nitrogen in either human or animal assisted reproduction, there is now documented proof of this occurring with other applications. A variety of viable microbes, including fungi (Aspergillus), virus (hepatitis) and bacteria, can be recovered and cultured from liquid nitrogen. One study (Fountain et al., 1997) showed heavy contamination with Aspergillus sp. in four out of five storage tanks, and low levels of microbial contaminants in four of these five tanks. These authors also found a variety of environmental or waterborne organisms contaminating the outer surfaces of 1.2% (n = 583) previously sterile components. In 1995, it was shown (Tedder et al., 1995) that one liquid nitrogen tank, used to store blood products, could be unequivocally linked to the transmission of hepatitis to patients. The hepatitis virus that was isolated from the infected patients matched that which was isolated from the liquid nitrogen in the tank (Hawkins et al., 1996). Many viruses, including papova virus (Charles and Sire, 1971), vesicular stomatitis virus (Schaffer et al., 1976), herpes simplex virus, adenovirus (Jones and Darville, 1989), may survive direct exposure to liquid nitrogen and therefore have the potential to cause cross-contamination.

In the food industry, experiments have shown that bacteria could be transmitted between carcasses which were rapidly chilled by immersion in liquid nitrogen (Berry et al., 1998). Although there are studies which have analysed the liquid nitrogen from sperm storage tanks (Piascek-Serafin, 1972), and others which have calculated the risk of transmission of diseases in association with international movement of livestock embryos (Sutmoller and Wrathall, 1997a,b), it is...
Rapid cooling in double-straws to protect embryos

Unclear how likely disease transmission by liquid nitrogen is in IVF.

As some, but not all, contaminating pathogens can be removed from embryos by rigorous washing steps (Bielanski and Jordan, 1996; Bielanski et al., 1997; Stringfellow et al., 1997; Kim et al., 1998; Trachte et al., 1998), it is best to adopt strategies which minimize the likelihood of either the straws or their contents from becoming contaminated. Several strategies aimed at minimizing the likelihood of disease transmission during storage have been suggested. Contamination could be reduced by storing spermatozoa, eggs and embryos in nitrogen vapour, but the risk of accidental thawing or the effects of greater fluctuations in storage temperature may counter the benefits of using this storage procedure (Wood, 1999). Russell et al. (1997) and Wood (1999) showed that properly sealed insemination straws do not leak dye, *Escherichia coli* or Newcastle Disease virus, and should therefore provide good protection. However, some sealing strategies were better than others (Russell et al., 1997). Therefore extra protection, e.g. wrapping the straws in a plastic film, may be needed to further reduce the risk of leakage/contamination (Wood, 1999).

Developing protocols to prevent contamination of very rapidly cooled or vitrified materials may prove more difficult. One strategy for achieving very rapid cooling rates has been to minimize the size of the sample, e.g. by placing the specimen on electron microscope grids (50 000–180 000 °C/min, Martino et al., 1996; Park et al., 1999; Hong et al., 1999), loops (Lane et al., 1999a,b) or aluminium foil (Choo et al., 1999; Dinnyes et al., 2000), or inside heat-softened and pulled straws (–18 000 °C/min, Vajta et al., 1998). The disadvantage of each of these approaches is that the vitrification solution comes in direct contact with liquid nitrogen during cooling or storage. Storage of such rapidly cooled specimens in nitrogen vapour would reduce the likelihood of cross-contamination, but the storage temperature is higher and more variable than in liquid nitrogen. Small specimens warm significantly faster than large specimens, with the consequence that current practices, such as pulling canes partially out of a tank to check the information on a specimen before removing it, could inadvertently thaw the specimens. Alternative strategies for reducing contamination should therefore be sought. The risk of contamination by larger pathogens could be reduced by filtering the liquid nitrogen through a 0.2 μm filter (Vajta et al., 1998), UV irradiation of the liquid nitrogen, or by inserting the cryopreserved specimen into an additional outer protective container before they are moved to a storage tank (Vajta et al., 1998). The latter approach has disadvantages, as it is difficult to manipulate small specimens and to seal containers at low temperatures.

This study was therefore aimed at developing a simpler protocol, which would allow rapid cooling of embryos with glass-like solidification (vitrification), while simultaneously reducing the risk of contamination from the liquid nitrogen. Although blastocyst transfer has become more popular in human in-vitro fertilization, we deemed day 2 and day 3 mouse embryos to be an appropriate animal model. To minimize the likelihood of contamination, we aimed to develop protein/serum-free cryoprotectants, which would allow embryos to be immersed directly into liquid nitrogen while packaged inside a double straw.

Materials and methods

**Animals**

Female C57BL×CBA F1 hybrid mice were superovulated with an intraperitoneal (i.p.) injection of 10 IU pregnant mare’s serum gonadotrophin (Folligon®; Intervet, NSW, Australia), followed 48 h later by 10 IU human chorionic gonadotrophin (Chorulon®; Intervet). Females were mated with C57BL×CBA F1 hybrid males. Embryos were collected from the oviducts 42–44 and ~66 h later (day 2, 2-cell and day 3, 8-cell embryos respectively) in Dulbecco’s phosphate-buffered saline (PBS, GibcoBRL Life Technologies, Melbourne, Australia) supplemented with 4 mg/ml bovine serum albumin (BSA, AlbuMAX I®, GibcoBRL) (handling medium), washed in fresh handling medium and then placed in 30 μl drops of M16 or MTF (mouse tubal fluid) medium (Gardner and Lane, 2000) overlaid with mineral oil which had been pre-equilibrated at 37°C in an atmosphere of 5% CO2 in air overnight.

**Embryo transfer procedures**

Mature C57BL×CBA F1 females were placed with vasectomized C57BL×CBA F1 males to induce pseudopregnancy. Females with a mating plug were anaesthetized with 0.4 mg Rompun (Troy Laboratories, NSW, Australia) and 2 mg ketamine (Parnell Laboratories, Alexandria, NSW, Australia) per mouse and 5–6 embryos transferred to each oviduct. Females were killed on day 15 of gestation and the number of fetuses and implantation sites assessed.

**Cryopreservation procedures**

**Preparation of solutions**

The solutions for pre-equilibration and cryopreservation were prepared as follows. Pre-equilibration solution: 2.5 ml ethylene glycol 62.07 mol. wt (EG, Sigma-Aldrich, NSW, Australia), and 7.5 ml handling medium. Two different final cryopreservation solutions were made: (i) 2.5 ml EG, 3.5 g Ficoll 70 000 mol. wt (Sigma), 4 ml protein free PBS. This solution is referred to as EG25:F35; (ii) 2.5 ml EG, 3.5 g dextran 60 000–90 000 mol. wt (clinical grade, Sigma), 4 ml protein-free PBS. This solution is referred to as EG25:D35.

**Cryopreservation protocol**

Two-cell mouse embryos were pre-equilibrated in 25% volume ethylene glycol in PBS + 4 mg/ml BSA in 35 mm tissue culture dishes (Falcon; Becton Dickinson, Melbourne, Australia) at room temperature for 2–3 min and then inserted in plastic 0.25 ml straws containing 30 μl of a final solution (EG25:F35 or EG25:D35) at the same temperature. After a 30 s equilibration period with the final solution, the straws were either sealed and immersed directly into liquid nitrogen (experiment 1), or sealed and placed inside a 0.5 ml straw which in turn was sealed (‘straw-in-straw’) before being immersed into liquid nitrogen (experiments 2 and 3). In experiment 1, the straws were warmed by holding them in air for 5 s before immersing them into a 31°C waterbath. In experiment 2, the larger (outer) straw was removed before the thawing step. In experiment 3, the outer straw was kept in place until after they were warmed by immersion in a waterbath at 38°C. The larger (outer) straw was removed before the dilution procedure. A two-step dilution protocol was used for all experiments, followed by in-vitro culture in M16 or MTF. All treatments were carried out with 3–10 replicates in each experimental group (>28 embryos/group). Control embryos were placed in culture without treatment.
Protocol for sealing the straws
The 0.25 ml straws for the embryos were shortened before the start of the experiment so that they could fit inside the outer straw. In order to save time in the straw-in-straw protocol, one end of the outer 500 µl straw was sealed before the start of the experiment. Two sealing strategies were examined, heat sealing and sealing with polyvinylalcohol (PVA) sealing powder. The former, which flattened and widened the straw, was very effective for the outer straw, but was less suitable for the inner straw as it made it difficult to insert into the outer straw. It also caused stretching of the outer straw, which may compromise its strength and integrity. With practice, effective seals could be created with either method.

Statistical analysis
Differences between individual replicates in vitro were determined using 2×2 contingency analysis for proportions. Development rates of non-vitrified and vitrified warmed embryos were compared to the untreated control embryos using simple factor analysis of variance (ANOVA) for the replicates of embryos developing into blastocysts.

In vivo data are expressed as the proportion of live fetuses from the total number of embryos transferred with between 4–10 replicates in each experimental group. Data were analysed by one-tailed Fisher exact test to determine if the number of live fetuses from vitrified embryos was significantly less than the number of fetuses in control experiments. All statistical analyses were computed using Microsoft Excel and P < 0.05 was taken as a significant result in all cases.

Results
The survival rates of day 2 mouse embryos pre-equilibrated in 25% by volume EG at room temperature followed by vitrification in two polymer-based solutions (EG25,D35 and EG25,F35) are summarized in Table I. There was no difference in the proportion of day 2 embryos which developed to the blastocyst stage in vitro following cryopreservation in the single or straw-in-straw configuration when the outer 0.5 ml straw was removed before warming (Table I). Survival, as assessed by the developmental potential in vitro, was not affected by the vitrification solution or the process of immersion into liquid nitrogen. In total 100% of the cryopreserved embryos were recovered with an intact zona pellucida and developed to the expanded blastocyst stage in culture.

The viability of day 2 embryos cooled and warmed within a single straw, or embryos cooled within a double straw but warmed after the removal of the outer protective 0.5 ml straw was verified with additional embryos by performing embryo transfers at the 2-cell stage (Table II).

Fetus formation after cryopreservation by immersion in liquid nitrogen, followed by warming in either a single straw throughout, or a double package on cooling and a single package on warming were not significantly different from each other or from the untreated controls. The results showed that the cryopreservation protocol did not alter the proportion of control and cryopreserved 2-cell embryos that formed blastocysts in vitro, or fetuses in vivo.

The survival rates of day 3 mouse embryos (8-cell stage), pre-equilibrated in 25% by volume EG at room temperature and then vitrified by three different protocols in the above mentioned Ficoll- and dextran-based solutions are shown in Table III. There was no difference in the proportion of day 3 (8-cell) embryos which developed to the expanded blastocyst stage in vitro following cryopreservation in the single or straw-in-straw configuration, irrespective of the warming protocol. The results were not influenced by whether the larger (outer) straw was left in place or removed before thawing (Table III). All day 3 embryos that were vitrified-warmed, using the three different protocols, were recovered with an intact zona pellucida and developed into expanded blastocyst stage in culture.

Discussion
We have developed a rapid cryopreservation procedure which can be used in combination with straw-in-straw packaging without reducing the viability of day 2 or 3 (2- and 8-cell) mouse embryos. We believe that this protocol would, providing that both straws were effectively sealed, be effective in preventing liquid nitrogen contamination, and would as a result help reduce the risk of cross-contamination during cryopreservation.

The cryoprotectant used in this study which contains a high concentration of high molecular weight polymers was developed for mouse embryos. The concentration of penetrating cryoprotectant in this solution (25%) is more than double that used for conventional slow cooling (~11%) but is only half

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**Table I. In-vitro development of cryopreserved day 2 mouse embryos. The embryos were within a standard 0.25 ml straw and plunged either without (single) or inside a sealed outer protective 0.5 ml straw (straw-in-straw)**

<table>
<thead>
<tr>
<th>Freezing procedure</th>
<th>Cryoprotectant composition</th>
<th>Cooled as single straw</th>
<th>Cooled as straw-in-straw</th>
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<tr>
<td></td>
<td></td>
<td>Warmed as single straw</td>
<td>Warmed as straw-in-straw</td>
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<tr>
<td></td>
<td>Concentration</td>
<td>Total no. of day 2</td>
<td>Expanded blastocysts no.</td>
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<tr>
<td></td>
<td>of ethylene glycol (%)</td>
<td>embryos (%)</td>
<td>no. (%)</td>
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<td></td>
<td>and type of polymer (%)</td>
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<tr>
<td>Liquid nitrogen</td>
<td>25</td>
<td>28</td>
<td>28 (100)</td>
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<tr>
<td>Liquid nitrogen</td>
<td>25</td>
<td>31</td>
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<tr>
<td>Liquid nitrogen</td>
<td>25</td>
<td>32</td>
<td>32 (100)</td>
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Fresh control 30/30 = 100%.

*In this experiment the 0.5 ml straw was removed before thawing.
that which is currently used in most vitrification solutions (>40%). This EG concentration was chosen in an attempt to minimize the toxicity associated with the penetrating cryoprotectant, while maintaining a high total solute concentration (Shaw et al., 1997; Kuleshova et al., 1999a, b). While we have not ascertained whether other vitrification solutions can be used with the straw-in-straw configuration described here, the high polymer content solutions developed in this study have several advantages. Most vitrification solutions depend on high concentrations of penetrating cryoprotectants in order to vitrify, with the result that embryos must be processed rapidly in order to reduce toxic effects. By contrast 2-cell mouse embryos can be kept in the solutions used in this study for up to 20 min without loss of viability (unpublished data). It is possible that the viscous solutions used in this study slow the movement of water and solutes, thereby reducing both the rate of cryoprotectant penetration and the toxicity of the penetrating cryoprotectants to the embryo. Vitrification solutions which have a high total solute concentration, but substitute a large amount of the potentially toxic penetrating agents for less toxic sugars or polymers, have recently been effectively employed for human oocyte and embryo cryopreservation (Mukaida et al., 1998; Hong et al., 1999; Kuleshova et al., 1999c; Chung et al., 2000).

The solutions used in this study vitrify on cooling when used in either a single 0.25 ml straw or when used in the straw-in-straw configuration; however, they devitrify in both configurations during warming. It is not known whether human material would be damaged by the ice crystals that form as a solution devitrifies during warming. Although it is thought that solutions that remain vitreous throughout the cooling and warming procedure should be better for embryo cryopreservation as they avoid ice formation, previous studies have found no chromosomal or developmental anomalies associated with solutions which devitrify only on warming (Shaw et al., 1991). Solutions which vitrified on cooling but devitrified on warming also were found to be applicable for rapid freezing of bovine oocytes (Martino et al., 1996; Vajta et al., 1998). By contrast, conditions that allow ice crystal formation during cooling have the potential to cause chromosomal damage (Shaw et al., 1991).

The protocol developed here provides a simple strategy for preventing cross-contamination and has the advantage that the specimen can be warmed while still inside the double straw. While we have used it in combination with vitrifying solutions, it can also be used in combination with slow cooling. In both cases further studies are needed, in particular if this approach is to be applied to human cells, to ascertain the optimal temperature for the waterbath, to avoid overheating of cells during warming. Since the solutions used in this study do not contain components of human or animal origin, there is no risk that it will introduce unwanted infectious contaminants, and should therefore be useful for embryos which are to be shipped internationally. The finding that both dextran and Ficoll were suitable was unexpected. Both polymers have similar effects on the physical vitrification properties of ethylene glycol–saline based solutions (Shaw et al., 1997). Although cryoprotectant solutions containing PVP and EG have been used before (Leibo and Oda, 1993; Murakami et al., 1998), we have found that dextran and Ficoll are both significantly less toxic to embryos than either dialysed or non-dialysed PVP (Kuleshova et al., 1999b). It is not clear how these polymers influence the solution, but it appears to make

| Table II. In-vivo development of cryopreserved straw-in-straw day 2 mouse embryos |
|-----------------|-------------------|-----------------|-----------------|-----------------|-----------------|
| Freezing procedure | Cryoprotectant composition | Cooled as single straw | Warmed as single straw | Cooled as straw-in-straw | Warmed as single straw |
| | | Total no. of 2-cell embryos transferred | In-vivo no. (%) | Total no. of 2-cell embryos transferred | In-vivo no. (%) |
| Liquid nitrogen | 25 | 35 Ficoll | 42 | 32 (76.2) | 25 | 19 (76) |

*Fetus formation in untreated controls = 47/60 (78.3%).

| Table III. In-vitro development of day 3 mouse embryos cryopreserved in 25EG:35F in single or double straws |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cooled as single straw | Warmed as single straw | Cooled as straw-in-straw | Warmed as single straw | Cooled as straw-in-straw | Warmed as straw-in-straw |
| Total no. of day 3 embryos | Expanded blastocysts no. (% ± SD) | Total no. of day 3 embryos | Expanded blastocysts no. (%) | Total no. of day 3 embryos | Expanded blastocysts no. (%) |
| 40 | 39 (97.5 ± 5.0) | 40 | 40 (100) | 40 | 40 (100) |

Control = 40 (100%).

*Outer 0.5 ml straw removed after storage before thawing.
them less likely to fracture (Shaw et al., 1997). The incidence of fracture damage in mouse embryos vitrified in 0.25 ml straws in Kasai’s vitrification solution (Kasai et al., 1990) is lower with moderate cooling (vapour cooling, ~120°C/min) and warming (in air, 300°C/min) rates, than in straws cooled by direct immersion into liquid nitrogen (~2900°C/min) and/or warmed by direct immersion into a waterbath (1900°C/min) (Kasai et al., 1996). These rates are calculated for the temperature range 0 to ~150°C. A 0.25 ml straw within a 0.5 ml straw which is plunged directly into liquid nitrogen cools at around 400°C/min and warms at 120°C/min (in air) or ~650°C/min (in water). This indicates that when a double straw is plunged directly into liquid nitrogen or directly into a waterbath the inner straw will have a cooling and warming rate which is comparable with the ‘moderate’ rates used successfully for mouse (Kasai et al., 1996) and human (Mukaida et al., 1998) embryos. Vapour cooling has also been shown to be effective for human blastocysts (Yokota et al., 2000). While it was not specifically tested in this study, it is likely that some but not all existing vitrification solutions could also be used in combination with this straw-in-straw procedure.

Rapid cryopreservation protocols are starting to be used for human oocytes and embryos at different stages of development including the blastocyst stage (Mukaida et al., 1998; Choi et al., 1999; Hong et al., 1999; Kuleshova et al., 1999c; Lane et al., 1999b; Chung et al., 2000). Recently, it has been suggested that one of the techniques which promotes the viability of cryopreserved mammalian oocytes may also be appropriate for human oocytes. As a result of these efforts, for the first time in June 1999 the birth of a normal infant was reported (Kuleshova et al., 1999c) following a transfer of an embryo derived from a vitrified mature oocyte. The birth of healthy twin babies following the vitrification of day 2 and 3 human embryos at a significantly lower cooling rate (2500°C/min) in 0.25 ml straws has been reported (Mukaida et al., 1998). Using the same approach, a further 10 pregnancies had been achieved by October 1999 (Dr M.Kasai, personal communication). It remains to be ascertained whether the cryoprotectant solutions and the straw-in-straw configuration developed here can be adapted to human oocytes and embryos.

We conclude that embryo cryopreservation in the proposed glass-like vitrification solutions allows effective cooling in protein/serum-free solutions which minimizes the risk of exposure to biological contaminants. This, in combination with the inner straw placed inside an outer sealed protective container to prevent the inner straw from ever coming into contact with liquid nitrogen, provides a simple, rapid and effective strategy for reducing or eliminating the risk of contamination during cryopreservation.

Acknowledgements

This research was carried out while in receipt of financial support from Monash IVF, the Australian Research Council and the Australian National Health and Medical Research Council.

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Received on May 30, 2000; accepted on September 12, 2000

Rapid cooling in double-straws to protect embryos