Rapidly cooled human sperm: no evidence of intracellular ice formation

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BACKGROUND: The cellular damage that human spermatozoa encounter at rapid rates of cooling has often been attributed to the formation of intracellular ice. However, no direct evidence of intracellular ice has been presented. Alternatively, the cell damage may be the result of an osmotic imbalance encountered during thawing. This article examines whether intracellular ice forms during rapid cooling or if an alternative mechanism is present. METHODS: In this study, human spermatozoa were cooled at a range of cooling rates from 0.3 to 3000°C/min. The ultrastructure of the samples was examined by cryo scanning electron microscopy and freeze substitution to determine whether intracellular ice formed during rapid cooling and to examine alternative mechanisms of cell injury during rapid cooling. RESULTS: No intracellular ice formation was detected at any cooling rate. Freeze substitution of cells that had been cooled at 3000°C/min and then slowly warmed showed that the cells had become plasmolysed and had evidence of membrane damage. CONCLUSIONS: Cell damage to human spermatozoa, at cooling rates of up to 3000°C/min, is not caused by intracellular ice formation. Spermatozoa that have been cooled at high rates are subjected to an osmotic shock when they are thawed.

Key words: freezing injury/intracellular ice/spermatozoa

Introduction

The physical stresses important in determining the cellular response during conventional cryopreservation are well documented. Following ice formation in the extracellular solution, all solutes and suspended materials, including cells, become localized into freeze-concentrated compartments (Mazur, 2004). During the subsequent reduction in temperature, cells are exposed to increasingly concentrated solutions. This process continues until the freeze-concentrated solution crystallizes as a eutectic or becomes a glass. The hypertonic conditions that cells encounter lead to an osmotic loss of water, the extent of which is dependent on the rate of cooling.

With many cell types, including mammalian oocytes and embryos, the osmotic behaviour of the cell during freezing may be predicted from numerical models. In particular, the probability of intracellular ice formation for different linear rates of cooling may be estimated. The models associate with light cryomicroscopy studies, which allow the visualization of intracellular ice in oocytes and embryos at different rates of cooling (Mazur, 2004). However, experimental observations of mammalian spermatozoa cryopreserved in glycerol have not been in agreement with the results predicted from modelling (Noiles et al., 1993; Curry et al., 1995; Devireddy et al., 2000; Gilmore et al., 2000; Mazur and Koshimoto, 2002). The models assume that cellular damage at rapid rates of cooling is due to the formation of intracellular ice. However, no direct evidence of intracellular ice formation in human spermatozoa has been presented. Morris et al. (2006) proposed an alternative mechanism in which cell damage occurring after rapid cooling rates was caused by an osmotic imbalance encountered during thawing, not the formation of intracellular ice.

Several reports have recently claimed that the vitrification of human spermatozoa occurs after rapid cooling of uncryoprotected cells (Nawroth et al., 2002; Isachenko et al., 2004) and cells suspended in glycerol (Grischenko et al., 2003; Schuster et al., 2003). In these studies, high recovery rates were reported upon warming, and it was argued that damaging intracellular ice formation was avoided by the ultrarapid cooling rates used. No direct evidence of either intracellular ice formation or cellular vitrification was presented.

In this investigation, human spermatozoa suspended in either a glycerol solution or a simple isotonic salt solution were cooled at different rates and examined by cryo scanning electron microscopy (CryoSEM) and freeze substitution. The examination of the ultrastructure was carried out to determine whether intracellular ice forms during rapid cooling and to examine alternative mechanisms of injury, which could apply at rapid rates of cooling.
Materials and methods

Semen samples
Semen was obtained from three patients undergoing fertility assessments, one ejaculate per patient. Patients consented to the use of their spermatozoa in research.

Semen analysis and sperm function testing
Sperm concentration and motility were assessed according to the methods described by the World Health Organization (1992).

Cryoprotectant
Cryoprotectant medium was prepared according to Richardson (1976). A primary buffer was prepared by adding 3 volumes of 0.1 M sodium citrate to 1 volume of 0.33 M fructose and 1 volume of 0.33 M glucose. Four millilitres of fresh egg yolk was added to 3 ml of glycerol and 13 ml of primary buffer. The solution was heat-inactivated at 56°C for 30 min and allowed to cool. A total of 200 mg of glycine was added and the pH adjusted between 7.2 and 7.3.

Freezing
The initial concentrations of the semen samples from the three patients were 44 × 10^6, 58 × 10^6 and 108 × 10^6 spermatozoa per millilitre. The samples undergoing testing suspended in cryoprotectant were diluted with equal volumes of cryoprotectant, reducing their concentration by 50%. The osmolality of the diluted semen was measured by freezing point osmometry to be 1430 mOsm/kg water (n = 5). Samples were frozen in 0.25 ml straws (IMV, L’Aigle, France) sealed with polyvinylalcohol powder. All the samples underwent the same freezing process until nucleation: straws were cooled at 20 to −7°C at a rate of 2°C/min in an Asymptote SF100 freezer (Asymptote, Cambridge, UK) and then maintained at −7°C for 10 min, during which time they were nucleated manually by contact against the wall of each straw, with the forceps previously cooled in liquid nitrogen. After ice nucleation, the straws were maintained at −7°C for 5 min to allow the equilibrium amount of ice to form at that temperature. Samples were then cooled at various rates. Samples cooled at rates of up to 10°C/min were cooled on the sample plate of the SF100 freezer until they reached a temperature of −100°C, at which point they were transferred to liquid nitrogen for storage. Samples were cooled at rates of 175, 270, 300, 530 and 1000°C/min using methods described by Morris and Richens (2004), in which straws were placed in direct contact with granules pre-cooled by liquid nitrogen. Samples were also cooled at 3000°C/min by direct immersion in liquid nitrogen. After cooling to below −100°C, the samples were transferred to liquid nitrogen. Some samples that had been cooled at 3000 to −100°C/min were warmed at 1 to −40°C/min before being returned to liquid nitrogen.

Thawing and post-thaw semen assessment
Straws were thawed at room temperature after 72 h storage in liquid nitrogen. Sperm concentration and motility were assessed for each straw according to the methods described (World Health Organization, 1992). All observations were ‘blinded’ with six replicates per sample.

Freeze-fracture electron microscopy
Straws were frozen as above, freeze-fractured and loaded onto a CryoSEM stage (Oxford Instruments XL30-FEG, Oxford, UK) in accordance with methods described by Morris et al. (1999). The stage was warmed from −145 to −90°C for 6 min and the sample etched at −90°C for 6 min before cooling to −145°C. The sample was then transferred to a preparation stage, coated with 10–15 nm gold and loaded back onto the CryoSEM stage for image recording. Estimates of the sample’s ice fraction (external to the freeze-concentrated material) and the glycerol concentration in the freeze-concentrated material were calculated from the micrographs as detailed in Morris et al. (2006).

Freeze substitution
Straws were freeze-substituted in a Reichert automated freeze-substitution device in accordance with methods described by Morris et al. (1999). Samples were maintained at −90°C for 24 h, warmed to −70°C at 3°C/h and then maintained at −70°C for 24 h. Samples were then warmed to room temperature at 3°C/h, rinsed in methyl alcohol and embedded in Spurr’s epoxy resin. Sections 0.5 μm thick were prepared with a Reichert Ultracut S microtome and stained with Methylene Blue.

Results

Viability of sperm
The recovery rate (percentage motile) of human spermatozoa in cryoprotectant decreased as the rate of cooling increased above 100°C/min (Figure 1). At a cooling rate of 3000°C/min, the recovery rate was less than 1%. Some variation in the recovery rate between the three individual samples was observed (Figure 1). Figure 1 also includes data obtained by Grischenko et al. (2003) on the recovery rate of human spermatozoa rapidly cooled on wire loops. Grischenko’s study used several glycerol concentrations, all of which were reported to give the same pattern of recovery: cooling at 3000°C/min gave the optimum recovery rate, whilst cooling at both a faster and slower rate resulted in a decrease in the recovery rate. The recovery rate of human spermatozoa that were not suspended in cryoprotectant was less than 1% at all rates of cooling examined.

Structure of the frozen sample at different cooling rates
Cross fracture of straws frozen in glycerol cryoprotectant, followed by deep etching to remove crystalline ice, revealed the structure of the freeze-concentrated cryoprotectant. After ice nucleation at −7°C and a subsequent cooling rate of 10°C/min, a cross-section of a straw revealed extensive ice formation (Figure 2a). The domains of freeze-concentrated material were homogeneous in structure (Figure 2b). Generally, few
spermatozoa were apparent, although some were associated with the interface between the freeze-concentrated material and ice crystals. At least 100 spermatozoa were observed in each cross-section. Some spermatozoa tails were observed to extend away from the freeze-concentrated material, suggesting that these structures were associated with or entrapped in ice (Figure 2b). Samples which were nucleated at −7°C and then cooled at a rate of 3000°C/min had a freeze-concentrated material which occupied a much larger cross-sectional area (Figure 2c) and contained numerous ice crystals, evident as etched pits and dendrites (Figure 2d). In these samples, spermatozoa cells were not evident. Cross fracture of samples frozen with no additive revealed a greater proportion of ice and a lower proportion of freeze-concentrated material (Figure 2e and f).

Light microscopy of freeze-substituted samples frozen in glycerol cryoprotectant confirmed the morphology observed.
by freeze fracture but allowed the cells to be visualized. At a cooling rate of 10°C/min, spermatozoa cells had migrated into the freeze-concentrated material during solidification; they were not entrapped within ice crystals (Figure 3a). At a cooling rate of 3000°C/min, the freeze-concentrated material occupied a much larger cross-sectional area (Figure 3b), and ice was trapped inside it. Light microscopy of freeze-substituted samples frozen with no additive showed less freeze-concentrated material. At a cooling rate of 10°C/min, the freeze-concentrated material was uniform in structure (Figure 3c). At a cooling rate of 3000°C/min, areas of the freeze-concentrated material were more diffuse (Figure 3d).

After cooling rates of 0.3 and 10°C/min, the ice fraction (external to the freeze-concentrated material) was approximately 85%. From these estimates of ice fraction, the glycerol concentration in the freeze-concentrated material would be approximately 60%. After rapid cooling (3000°C/min), the ice fraction external to the freeze-concentrated material was approximately 50%, and therefore, the glycerol concentration in the freeze-concentrated material would be approximately 20%. Crystalline ice also formed within the freeze-concentrated material, but it was not possible to quantify this.

**Electron microscopy of cells following different cooling rates**

Freeze-fractured cells entrapped in the freeze matrix were occasionally observed. There was no evidence of any intracellular ice in cells cooled at 3000°C/min with glycerol (Figure 4a) and without additive (Figure 4b).

Electron microscopy of freeze-substituted samples frozen in glycerol cryoprotectant at a cooling rate of 10°C/min showed some cell dehydration and distortion, and the cytoplasm and organelles appeared dense (Figure 5a and b). Similar results were obtained after cooling at lower rates. At a cooling rate of 3000°C/min, there was no evidence of ice within any compartment of the cell, within the resolution limits of the techniques (Figure 5c–f). In all cells, small clear spaces <0.1 μm were observed within the sperm heads after cooling at both 10 and 3000°C/min.

Electron microscopy of freeze-substituted samples cooled at a rate of 10°C/min, with no additive, showed cells entrapped in

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**Figure 3.** Light microscopy of freeze-substituted human spermatozoa sample cooled with glycerol cryoprotectant at 10°C/min (a, mag. ×400) and at 3000°C/min (b, mag. ×400), and with no additive at 10°C/min (c, mag. ×400) and at 3000°C/min (d, mag. ×400).
a relatively small freeze-concentrated compartment, with some evidence of cellular dehydration, particularly of the sperm tails (Figure 6a and b). At a cooling rate of 3000°C/min, cell shrinkage was not observed, but there was no indication of ice within the cell (Figure 6c–f).

Cooling samples at a rate of 3000°C/min to −100°C, and then warming at 1°C/min to −40°C, had an effect on the structure of the ice matrix and on the cellular ultrastructure. Freeze-fracture electron microscopy revealed that extensive ice recrystallization had occurred during the warming phase. The ice fraction external to the freeze-concentrated material was approximately 72%, and the freeze-concentrated material had become more homogeneous (Figure 7a). Cells examined by freeze substitution were plasmolysed, and there was evidence of membrane damage (Figure 7b–d). Large amounts of cell debris were present, compared with a frozen sample that had not been rewarmed. These cells contained small inclusions, but there was no evidence of intracellular ice.

**Discussion**

There have been surprisingly few publications on the effect of cooling rate on the recovery rate of human spermatozoa. There are many reports on the response of embryos and oocytes to different cooling rates but only one for human spermatozoa (Henry et al., 1993), which reported that a very broad response curve exists, with little difference in the survival observed following cooling at 1 or 100°C/min. This insensitivity to cooling rate, which is unusual for mammalian cells, has received little comment. Figure 1 shows that the post-thaw motility of human spermatozoa decreases as the rate of cooling increases above 100°C/min.

The results suggest that cell damage to spermatozoa, at least for cooling rates up to 3000°C/min, is not caused by intracellular ice formation. The small clear spaces <0.1 μm observed within some sperm heads are likely to be small inclusions or vacuoles, not intracellular ice because these are also seen after cooling at a slow rate. From the results, it is highly unlikely that intracellular ice would be present in human spermatozoa at any cooling rate. It must be acknowledged that only a limited number of cells were observed by this direct technique; however, intracellular ice was not observed in any cells at the rapid rate of cooling, even though the viability after cooling at 3000°C/min was zero.

There is also no evidence for intracellular ice upon warming and refreezing samples—conditions which would be expected to result in the recrystallization of any ice present within cells. Samples warmed to −40°C and then returned to liquid nitrogen demonstrate a large recrystallization in the ice structure in the sample. However, there is no evidence of intracellular ice in any cells; rather plasmolysis is observed.

Studies on freeze substitution and freeze fracture of cryopreserved pig spermatozoa (Courtens and Paquignon, 1985; Rodriguez-Martinez and Ekwall, 1988) and freeze substitution of slowly frozen testicular spermatozoa within the mouse epididymis (Sherman and Liu, 1982) have been carried out. In these reports, although some intracellular ice formation within sperm tails and midpieces was evident, there was no evidence for gross ice formation within the sperm heads. It should be noted that pig and mouse spermatozoa are more sensitive to freezing injury than human spermatozoa (Leibo and Bradley, 1999).

If intracellular ice does not cause cell damage at rapid rates of cooling, other physical factors must be responsible. The most important factor in the reduction of viability is changes in the physical properties of the extracellular environment.

It has been demonstrated that freezing an aqueous solution of glycerol causes the viscosity of the freeze-concentrated material to increase rapidly (Morris et al., 2006). Following ice nucleation, the growth of ice crystals in an aqueous solution occurs by the diffusion of water molecules from the solution adjacent to the ice crystal to the ice crystal lattice. As the viscosity of the solution increases during freezing, this diffusion process may become limited, and the amount of ice formed then becomes dependent on the rate of cooling (Morris et al., 2006). The relationship between the rate of cooling and the amount of ice formed has been quantified for some other cryoprotectants (Boutron and Kaufmann, 1979; Boutron, 1990).

The ultrastructure of cryopreserved sperm differed according to the rate the sample was cooled at after being nucleated. Samples that were cooled at 10°C/min had a freeze-concentrated...
material, with an overall composition of more than 60% glycerol close to the eutectic concentration. However, at faster rates of cooling (3000°C/min), the concentration of glycerol in the freeze-concentrated material was only approximately 20%. At high rates of cooling, the freeze-concentrated material becomes ‘supercooled’. At some subsequent temperature during cooling, ice nucleation occurs within this material, trapping the water as ice within the freeze-concentrated solution.

During warming of rapidly cooled bulk glycerol solutions, containing metastable solid states, a number of recrystallization patterns have been described (Luyet, 1965; Franks, 1982; Ablett et al., 1992). In this study, CryoSEM (Figure 2c and d) demonstrates that following rapid cooling, ice formation occurs within the freeze-concentrated material at some temperature below the glass transition temperature, Tg (–63°C for glycerol in 300 mOsm NaCl), and that during warming of these
samples to −40°C, migratory recrystallization of the ice occurs (Figure 7a).

Figure 8 illustrates the changes in glycerol concentration as a function of cooling rate and temperature. ‘Slow’ Cooling (below 10°C/min): Following ice nucleation at temperatures close to the melting point, the cells are exposed at all temperatures to the concentration of glycerol predicted from the equilibrium phase diagram. At Tg, the glycerol solution will vitrify. On warming, the cell environment is diluted from the maximally concentrated solution to the original concentration. ‘Rapid’ Cooling (3000°C/min): At any temperature during rapid cooling, the cells are exposed to a concentration of glycerol significantly less than the value predicted from the equilibrium phase diagram. At some temperature below Tg, ice nucleates within the freeze-concentrated material, and the concentration of the glycerol then attains a more concentrated value and vitrifies. On warming above Tg, the cells, which have been in equilibrium with a 20% glycerol during cooling, are initially exposed to a

Figure 6. Freeze substitution of cryopreserved human spermatozoa cooled with no additive at 10°C/min (a, b) and 3000°C/min (c–f).
higher concentration of glycerol (up to 60%). Such osmotic shock may be damaging.

The effects of different rates of warming on the recovery of rapidly cooled spermatozoa have been examined (Henry et al., 1993; Mazur and Koshimoto, 2002). The reduction in survival at slow rates of warming has been used as evidence for the formation of intracellular ice during rapid cooling. However, it may simply reflect a long period of exposure to a hypertonic shock during warming, which in itself may be damaging to cells.

A number of recent reports have claimed high recovery levels of human spermatozoa following ‘vitrification’ in the presence of glycerol (Grischenko et al., 2003; Schuster et al., 2003) and with no cryoprotective additive (Nawroth et al., 2002). These studies all assume that after the rapid rates of cooling achieved in straws, intracellular ice formation damages cells and that the beneficial effects of rapid cooling on wire loops are due to the vitrification of the samples. Our study has first demonstrated that, following rapid cooling, intracellular ice formation is not the lethal event for spermatozoa. Secondly, it suggests that the benefits of rapid cooling on loops are likely to be associated with the effect on both the physical state of the medium around the cells and the rapid rates of warming possible with a small sample size, a rate much faster than that obtained in straws.

The results described in this article suggest that it is now appropriate for new models to be developed that exclude the formation of intracellular ice. This more realistic modelling should predict optimum methods of cryopreservation for human spermatozoa and lead to improved practical cryopreservation protocols.

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