

A novel approach to sperm cryopreservation

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Human spermatozoa have unusual cryobiological behaviour and improvements in their survival have not been achieved by the standard approaches of cryobiology. Conventional approaches to cryopreservation impose a linear change of temperature with time; however, the stresses that cells encounter during cryopreservation are all non-linear with time. In this paper it is shown that improved methods of cryopreservation may be developed by specifically manipulating the manner in which cells experience physical changes instead of imposing a linear temperature reduction. Several treatments were compared: control of solidification to achieve constant ice formation with time was more damaging than the standard linear reduction in temperature. However, treatments which followed a chosen non-linear concentration profile, referred to as 'controlled concentration' allowed recovery of almost all the cells which were motile before freezing. The biophysical basis of these different responses was examined using the cryostage of a scanning electron microscope and freeze substitution and it was found that, surprisingly, all samples of spermatozoa in the frozen state were neither osmotically dehydrated nor had any visible intracellular ice. Viability on thawing did not appear to correlate with conventional theories of cellular freezing injury, which suggests that for human spermatozoa other factors determine viability following freezing and thawing.

Key words: cryopreservation/electron microscopy/freeze fracture/freeze substitution/spermatozoa

Introduction

The methods currently employed in freezing human spermatozoa are crude compared with those used for human embryos (Royere *et al.*, 1996). In particular, control of the cooling rate is often primitive: samples are commonly suspended in the vapour above liquid nitrogen, resulting in significant differences in cooling rate between different samples. The resulting straw-to-straw variation and loss of viability may not be important where sperm counts are normal, but in the case of oligozoospermic or asthenozoospermic samples these losses may be highly significant. With the development of intracytoplasmic sperm injection and the availability of techniques for

surgical sperm retrieval, there is an increased need to store low numbers of sperm and therefore to improve freezing techniques in order to maximize survival (Cohen *et al.*, 1997).

Improvements in cryopreservation of human spermatozoa have been attempted in the past by the use of different cryoprotectants and extenders, and in particular, by altering the cooling rate, usually a linear reduction in temperature with time (Serafini and Marrs, 1986; Ragni *et al.*, 1990; Henry *et al.*, 1993; Gilmore *et al.*, 1997). With many cell types, and mammalian embryos provide a well documented example, a well defined 'optimum' rate of cooling exists, with survival decreasing at both faster and slower rates. Intriguingly, similar studies demonstrate that spermatozoa are relatively insensitive to the magnitude of the linear rate of cooling during freezing. With human spermatozoa, a very broad response curve exists with little difference in survival observed following cooling at 1°C/min up to 100°C/min (Henry *et al.*, 1993). This response is unusual for mammalian cell types, but has received surprisingly little comment. Furthermore, the recovery of viability is comparatively low, with typically less than 60% of cells retaining motility on thawing, which given that this is achieved with such a wide range of linear cooling rates would suggest that the linearity of temperature reduction may not be appropriate.

The cooling rate dependency of cell recovery of many cell types may be predicted from computer models of their osmotic behaviour during freezing. However the predicted results with spermatozoa have not been in agreement with experimental observations (Noiles *et al.*, 1993; Curry *et al.*, 1995). For example, although conventional models have suggested that human sperm cells should survive cooling rates up to 10 000°C/min (Noiles *et al.*, 1993), experimentally the survival rate begins to decline beyond 100°C/min (Henry *et al.*, 1993). It is clear that human spermatozoa have unusual cryobiological behaviour and improvements in their survival have not been amenable to conventional approaches of cryobiology.

Many of the changes in physical properties which occur in an aqueous cryoprotectant following ice nucleation are not linear with temperature. Parameters such as the ice fraction, concentration of ionic species, osmolality, pH, viscosity and gas solubility, all vary in a non-linear manner with temperature (e.g. Franks, 1985). In addition, the biophysical characteristics of cells which determine the response to freezing, for example the cellular permeability to water, also change in a non-linear manner with temperature. Conventional approaches to cryopreservation thus impose a linear change of temperature with time whilst the stresses that cells are encountering are all non-linear with time. It is therefore appropriate to examine whether improved methods of cryopreservation may be

developed by specifically manipulating the manner in which cells experience physical changes rather than imposing a linear temperature reduction. In order to implement the required control of external conditions a new cell freezer has been specifically developed to achieve the desired protocols.

In this investigation, human spermatozoa suspended in a standard cryoprotectant were frozen using various protocols that manipulated different aspects of the physical conditions and the effects on post-thaw survival and function were assessed in comparison with conventional techniques. In order to increase our understanding of the physical behaviour of both the spermatozoa and the cryoprotectant during freezing, fractured straws were examined using the cryostage of a scanning electron microscope and freeze substitution. In addition, some simple computational modelling of the osmotic behaviour of the spermatozoa during freezing was carried out.

Materials and methods

Semen samples

Semen was obtained from patients undergoing fertility assessments who had consented to the use of their spermatozoa in research. In experimental series #1, #2 and #3 semen from several patients was pooled in order to provide sufficient material for the range of experimental treatments. In experimental series #4, semen from individual patients was examined under a limited range of freezing conditions.

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 0.33 M fructose and 1 volume of 0.33 M glucose. Four millilitres of fresh egg yolk was added to 3 ml glycerol, and 13 ml of primary buffer. The solution was heat inactivated at 56°C for 30 min and allowed to cool. Finally, 200 mg glycine was added, and the pH adjusted to between 7.2 and 7.3.

Freezing

Semen samples were diluted with equal volumes of cryoprotectant. The osmolality of the diluted semen was measured by freezing point osmometry to be 1430 mOsm/kg water. Samples were frozen in 0.25 ml straws, sealed with polyvinylalcohol powder. Ten straws were frozen using each experimental protocol. Freezing was carried out using a variety of methods:

Method 1. Suspension in nitrogen vapour, 20–25 cm above the surface of liquid nitrogen in an open Dewar, no manual nucleation of ice.

Method 2. Within a vapour phase controlled rate freezer (Planer cryo10/16; Planer Products, Sunbury-on-Thames, UK). Straws were held vertically and cooled from 20°C to –5°C at a rate of 2°C/min. Straws were maintained at –5°C for 10 min, during which time they were nucleated manually by touching the wall of each straw with forceps previously cooled in liquid nitrogen. Straws were then cooled at a programmed linear rate of cooling of 10°C/min to –100°C and then transferred to liquid nitrogen for storage.

Method 3. Using the Planer controlled rate freezer programmed as in Method 2, but with no manual nucleation of ice.

Method 4. In the Asymptote SF100 freezer (Cook IVF, Letchworth, Herts, UK), in which straws are held horizontally, programmed as in Method 2, with manual nucleation.

Method 5. In the Asymptote SF100 freezer as Method 4, but without manual ice nucleation.

Method 6. In the Asymptote SF100 freezer to control various extracellular physical conditions following nucleation of ice at –5°C.

To simplify these preliminary studies it was assumed that the extracellular solution was entirely glycerol, and from the measured osmolality the concentration was equivalent to a 13.5% solution. From the phase diagram of glycerol (Lane, 1925) it was then possible to calculate the required temperature change in the sample during cooling in order that the cells were exposed to defined external physical changes. The temperature profiles were programmed into an Asymptote SF100 freezer to control the temperature of the aluminium sample plate during cooling. The close contact of the straws with the sample ensured good heat transfer, and the temperature within samples closely followed the programmed temperature. The following three protocols were compared. (i) Controlled rate of change in extracellular concentration—referred to in the text as ‘controlled concentration’. This was chosen such that the rate of change of solute concentration in the liquid phase decreases for more than 90% of the time taken to lower the temperature from –5°C to –45°C. (ii) Linear change in ice fraction with time. (iii) Constant heat extraction with time.

The required temperature changes to achieve these extracellular conditions were controlled in these experiments from the nucleation temperature (–5°C) to –45°C, (close to the eutectic temperature for an aqueous solution of glycerol). In all tests the elapsed time taken to cool from –5°C to –45°C was 4 min (i.e. an average cooling rate of 10°C/min, consistent with the linear cooling rate, Method 2 above). All samples were then cooled from –45°C to –100°C at a linear rate of cooling of 10°C/min and then transferred to liquid nitrogen for storage.

Thawing and post-thaw semen assessment

Straws were thawed at room temperature after 72 h storage in liquid nitrogen. Straws from each experimental treatment were pooled for estimation of sperm concentration, motility, viability staining, hypo-osmotic swelling (HOS) testing, and the acrosome reaction ionophore challenge (ARIC) test.

Semen analysis and sperm function testing

Sperm concentration and motility were assessed according to the methods described by the WHO, (1992). Viability was assessed by staining with 5% eosin (WHO, 1992).

Membrane function was assessed using the HOS test (Jeyendran *et al.*, 1984). Briefly, an aliquot of spermatozoa was diluted 10:1 with hypo-osmotic medium (7.35g sodium citrate and 13.51g fructose in 1000 ml, 150 mOsm/kg), and incubated at 37°C for 30 min. Spermatozoa were scored for the presence or absence of swelling in the tail region.

Acrosome function was assessed using a modified ARIC test (Cummins *et al.*, 1991). Briefly, washed sperm were incubated in 10 µM calcium ionophore, A23187. The presence or absence of the acrosome was identified by staining with FITC linked to *Pisum sativum* lectin, and viable spermatozoa identified using bis-benzimide (Hoechst H33258). A score was derived by subtracting the number of viable, acrosome-reacted spermatozoa in the control sample from the percentage of viable, reacted spermatozoa in the ionophore-treated sample.

Computational modelling

Estimates of the local undercooling were made by simple computation of the mass transport across the cell membrane. The membrane permeability was assumed to be temperature dependent, and the mass transport was assumed to be driven by the difference between

Table I. Recovery of sperm function following freezing and thawing by various experimental treatments, as described in the text

	A Motility (%)	A Motility (normalized)*	B Motility (%)	Hypo-osmotic (%)	Eosin (%)	Acrosome (%)
<i>Experiment #1</i>						
Fresh	25		15	71	83	19
Passive vapour phase cooling (Method 1)	15	60	11	67	73	15
Planer freezing + nucleation (Method 2)	16	64	10	67	74	14
Planer freezing no manual nucleation (Method 3)	10	40	6	36	42	16
SF100 linear freezing + nucleation (Method 4)	18	72	11	69	79	18
SF100 linear freezing no manual nucleation (Method 5)	18	72	9	67	76	15
SF100 linear ice fraction freezing (Method 6.ii)	12	48	6	100*	54	7
SF100 controlled concentration freezing (Method 6.i)	22	88	13	69	81	16
<i>Experiment #2</i>						
Fresh	33		17	66	73	21
Passive vapour phase cooling (Method 1)	12	36	5	51	60	12
SF100 linear heat extraction (Method 6.iii)	4	17	2	32	30	8
SF100 controlled concentration (Method 6.ii)	18.5	56	12	59	64	13
<i>Experiment #3</i>						
Fresh	68		10	64	94	24
Passive vapour phase cooling (Method 1)	54	79	7	60	88	14
SF100 linear freezing -10°C/min (Method 4)	33	49	28	61	82	16
SF100 linear freezing -18°C/min	35	51	27	60	76	15
SF100 linear ice fraction freezing (Method 6.ii)	18	26	18	92	61	11
SF100 controlled concentration freezing (Method 6.ii)	69	100	10	62	92	21
SF100 modified controlled concentration freezing	59	87	9	63	86	19

*All spermatozoa displayed a swelling of the tail region immediately on thawing.

Table II. Recovery of sperm motility following freezing and thawing; spermatozoa from three patients (non-pooled) frozen by two methods as described in the text

Patient no.	A Motility (normalized)	
	'Controlled concentration'	Linear cooling
1	71	35
2	98	62
3	10	7

the intracellular and extracellular concentrations of solute. The intracellular solute concentration changed only as a result of mass transport.

Freeze fracture electron microscopy

Straws were prepared as described above and following the various methods of solidification were cross fractured under liquid nitrogen and then loaded onto the cryostage of a scanning electron microscope (cryoSEM; Oxford Instruments XL30-FEG; Oxford Instruments Ltd, Abingdon, Oxon, UK). The stage was warmed from -145°C to -90°C over 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 and coated with 10-15 nm gold and then loaded back onto the cryoSEM stage for image recording.

Freeze substitution

Straws were refractured into 1 mm thick segments and transferred under liquid nitrogen to substitution chambers in a Reichert automated freeze substitution device. The substitution medium contained 2% osmium tetroxide and 1% uranyl acetate in methyl alcohol. 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 warmed to room temperature at 3°C/h, rinsed in methyl alcohol and embedded in

Spurr's epoxy resin. Sections 0.5 µm in thickness were prepared with a Reichert Ultracut S microtome and stained with methylene blue. Photomicrographs were taken using a Zeiss Axiophot microscope.

Results

Sperm recovery and function testing

Results for the three experimental series with pooled spermatozoa are presented in Table I. These are summarized below in two parts, those from linear cooling rate and passive vapour freezing, and those where the extracellular conditions were specifically controlled.

Linear cooling rates and passive vapour freezing (Methods 1-5)

In experiment #1, samples cooled in the Planer freezer using method (3), at a linear rate without manual ice nucleation showed a significantly lower recovery rate (recovery and viability) than samples cooled linearly with ice nucleation (method 2) and those cooled linearly in the Asymptote SF100 freezer with manual nucleation (method 4) and without manual nucleation (method 5). In experiment #3, where two different rates of linear cooling were used (10°C/min and 18°C/min) using method (2) there was no significant difference between the two rates. Passive vapour phase cooling (method 1) resulted in similar recovery rates to programmed linear cooling (methods 2, 4 and 5).

Control of extracellular freezing (Methods 6.i, 6.ii, 6.iii)

The highest recovery of grade A motility was seen in all three experiments when cooled using the 'controlled concentration' method described above (method 6.i). A modification of

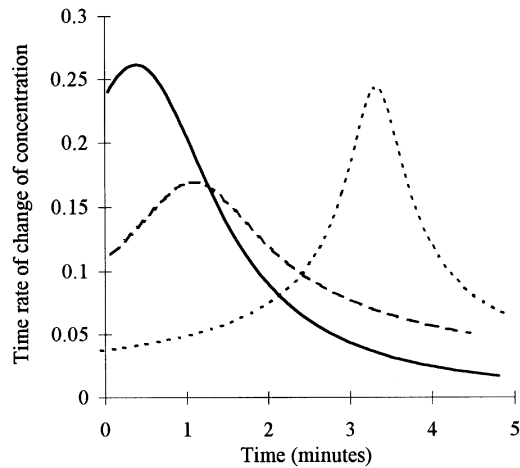


Figure 1. The rate at which the extracellular concentration is changing as a function of time during three treatments. Linear change of temperature with time (---); 'controlled concentration' (—); linear change in ice fraction with time (- - - -).

this protocol, giving an even greater initial rise in solute concentration, was attempted in experiment #3, but gave rise to slightly lower recovery rates.

Those samples cooled such that the ice fraction increased at a linear rate (method 6.ii) had the poorest recovery in terms of motility. In addition, this protocol appeared to have a significant effect on membrane function with hypo-osmotic swelling apparent in all samples immediately on thawing (100% in experiment #1), and a significant decrease in the ARIC score in all three experiments.

Linear heat extraction (method 6.iii), which was carried out only in experiment #2, gave rise to a significantly lower recovery rate in terms of motility, viability and function, compared with passive cooling, and 'controlled concentration'. It should also be noted that the sample used in experiment #2 appeared to be more susceptible to cryodamage than the samples used in experiments #1 and #3.

Experimental series #4—non-pooled sperm

Spermatozoa from three patients were frozen by both the controlled concentration (method 6.1) and conventional linear cooling (method 4) and the results are presented in Table II. These three samples varied in their sensitivity to freezing injury when frozen by conventional linear cooling. When frozen by the controlled concentration method these samples retained their relative ranking, however motility compared with linear cooling was increased by a factor of at least 50%.

Temperature histories within the straws

The concentration–time histories of the samples during freezing by the various methods could be derived from the measured temperature changes. It is of particular importance, and the purpose of the work described here, to control the rate at which the extracellular solute concentration is changing. This rate of change is illustrated in Figure 1, for three of the most distinct cases: 'controlled concentration' (method 6.i), linear ice fraction (method 6.ii) and the standard linear cooling rate (method 4). The proportion of time for which this rate of

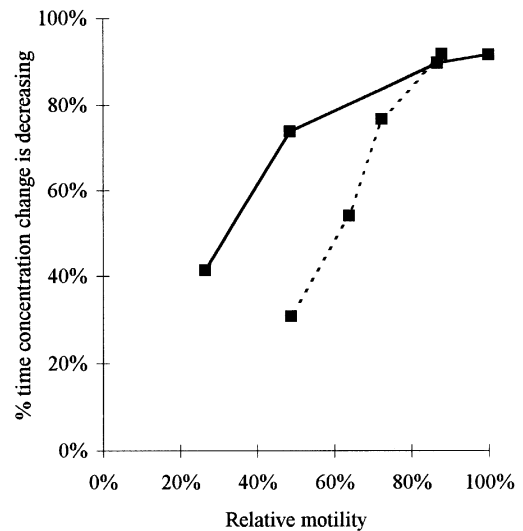


Figure 2. Cell recovery (normalized %) as a function of the proportion of the time that the rate at which the extracellular concentration is changing is decreasing. Experiment #1 (—); experiment #3 (- - - -).

change is decreasing is critical, as shown in Figure 2 where the percentage of time that the rate of change is decreasing was calculated and plotted against post-thaw recovery of motility in spermatozoa for experiments #1 and #3 (experiment #2 was excluded as there were too few data points). There was a clear direct correlation between these parameters. (Data from passive vapour freezing and from treatments where no manual nucleation took place was also excluded because without manual nucleation there would be significant unknown straw-to-straw variation.)

Electron microscopy

Cross-fracture of the straws followed by deep etching to remove ice revealed the structure of the freeze-concentrated glycerol (Figure 3a, b) which had a uniform appearance across the straw. The revealed ice crystal structure showed a similar structure and spacing of ice crystals in all materials frozen by the different methods in which ice was manually nucleated at -5°C .

At higher magnification the freeze-etched samples revealed that the sperm cells had migrated into the freeze-concentrated material during solidification: spermatozoa were not entrapped within ice crystals. However some sperm tails were observed to extend away from the freeze-concentrated material, suggesting that these structures were associated with or entrapped in ice (Figure 3c). Occasionally spermatozoa were observed with the sperm head entrapped in one portion of freeze-concentrated matrix with the sperm tail tethered in a distinct zone with the intervening tail bridging a void that would have contained an ice crystal (Figure 3d). Some spermatozoa were associated with the interface between the freeze-concentrated material and ice crystals (Figure 3e), but generally few spermatozoa were apparent, but these spermatozoa did not appear to be osmotically shrunken. Occasionally very distorted spermatozoa were observed (Figure 3f); these were apparently entrapped between ice crystals rather than osmotically dehydrated.

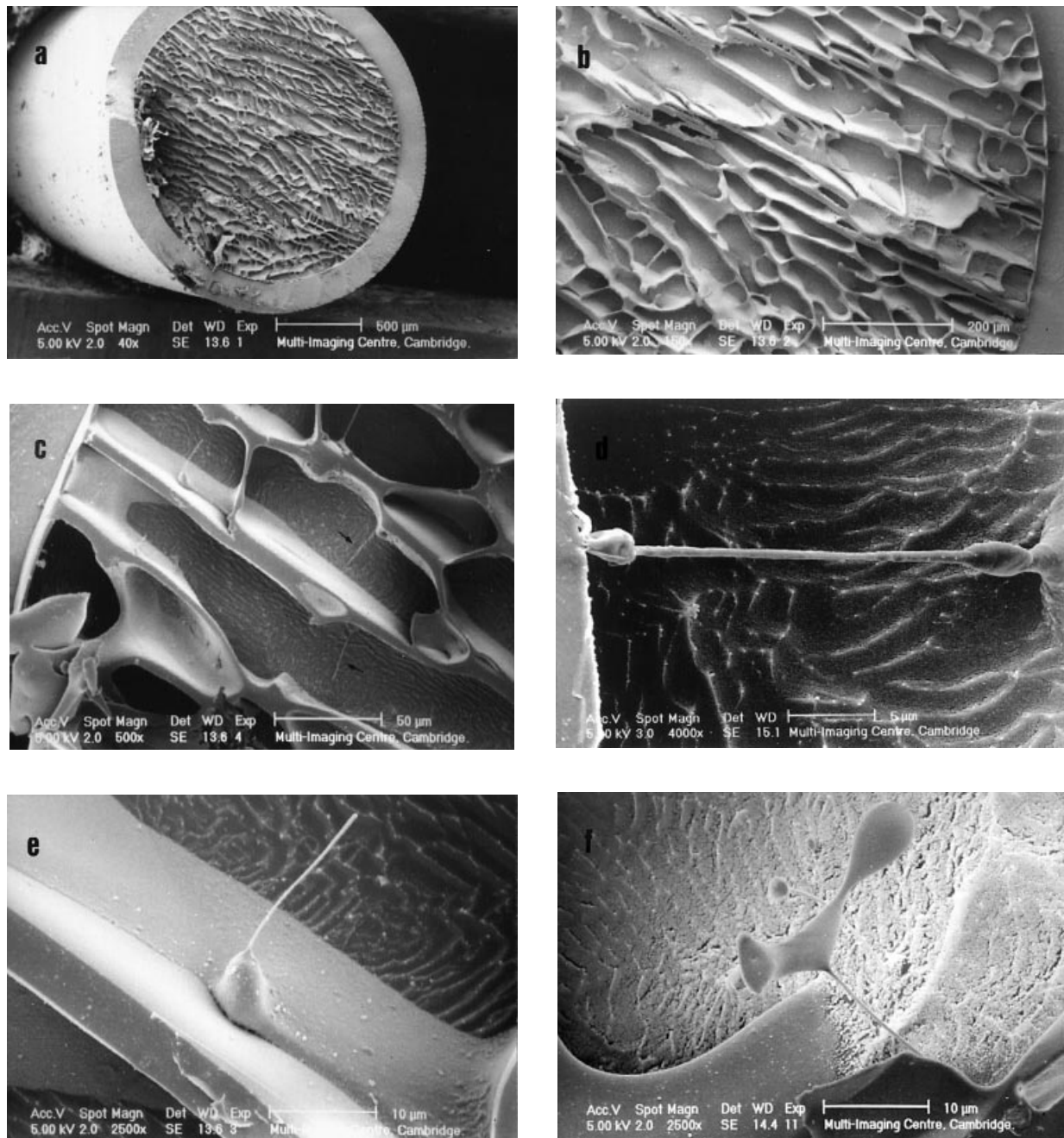


Figure 3. Freeze-fracture electron microscopy of sperm suspensions cryopreserved within conventional straws (0.25 ml capacity). (a) Cross-fracture of whole straw—low magnification. (b) Detail of the etched freeze-concentrated glycerol. (c) Sperm tails (arrows) extending from freeze-concentrated matrix into the space previously occupied by an ice crystal. (d) Detail of a sperm bridging an ice crystal void. (e) Cell at the interface between ice and the freeze-concentrated matrix. (f) Distorted sperm cell.

Light microscopy of freeze-substituted sections showed that spermatozoa were entrapped within the freeze-concentrated material (Figure 4a, b). Occasional spermatozoa were observed to bridge across two freeze-concentrated zones. At this magnification the freeze-concentrated matrix was observed to be relatively homogeneous in appearance following both linear cooling and linear ice fraction solidification (methods 4 and 6.ii). However following ‘controlled concentration’ (method 6.i) areas of granularity were evident (Figure 4b), which may be interpreted as substituted ice crystals within the freeze-concentrated matrix.

When observed by electron microscopy (Figure 5) the

freeze-concentrated matrix was electron dense, presumably due to the protein components of the egg yolk included in the cryoprotectant. All samples were observed to contain substituted ice crystals in the freeze-concentrated matrix. Frozen spermatozoa in the freeze-concentrated matrix were surprisingly similar to unfrozen controls. There was no evidence of osmotic shrinkage or of the presence of ice voids within the heads of the spermatozoa for any of the freezing methods examined. Occasionally some shrinkage was observed in distal sections. Many sections of sperm head and tails were surrounded by a zone, less than 0.1 μm wide, of low electron density material.



Figure 4. Light microscopy of thin-sectioned freeze-substituted straws. (a) Straw solidified to achieve constant ice fraction with time, a sperm tail bridging two freeze-concentrated zones is apparent (arrow); also, sperm tails extending from the freeze-concentrated matrix (arrowhead). (b) Straw solidified by the 'controlled concentration' method, areas of granularity within the freeze-concentrated matrix are arrowed. Scale bar = 1 µm.



Figure 5. Transmission electron microscopy of a thin-sectioned freeze-substituted straw solidified by the 'controlled-concentration' method; a zone of low electron density around the cell is arrowed. Substituted ice crystals in the freeze-concentrated matrix are also apparent (arrowheads). Scale bar = 1 µm.

Discussion

The results presented here demonstrate that the recovery of cryopreserved human spermatozoa can be significantly improved by controlling the concentration gradients experienced by the cells during freezing. The rate of change in solute concentration is clearly a major factor affecting sperm recovery. In those experiments where the only independent variable was the time rate of change of the solute concentration of the

unfrozen fraction, cell survival was directly and closely correlated with the percentage of time this time rate of change was decreasing, as shown in Figure 2. The 'controlled concentration' method (6.i) where the rate of change of solute concentration was decreasing for about 90% of the time, shows sperm recovery of 88% and 100% in experiments #1 and #3 respectively, where pooled spermatozoa were used. Spermatozoa from individual patients demonstrated similar behaviour.

When linear cooling was applied the recovery was significantly less than that achieved with 'controlled concentration' and was similar to that reported in other studies (Serafini and Marrs 1986; Ragni *et al.*, 1990; Henry *et al.*, 1993). It is significant to note that using the vapour phase cooling apparatus samples which were not manually nucleated had significantly lower motility than nucleated samples, which is consistent with the report by Crister *et al* (1987). It has been suggested that different sub-populations of spermatozoa may differ in their freezing sensitivity (Gao *et al.*, 1993; Curry and Watson, 1994) and it appears that the 'controlled concentration' protocol may provide successful cryopreservation of sensitive sub-populations.

This investigation provides significant insights into the response to freezing and thawing of spermatozoa. Freeze fracture electron microscopy and freeze substitution extends previous observations made by light cryomicroscopy (Korber *et al.*, 1984; Holt *et al.*, 1992) that spermatozoa and solutes migrate either entirely into the freeze-concentrated matrix or are entrapped near to the interface of the ice and the freeze-concentrated material. In some cases sperm tails may be associated with ice crystals whilst the sperm heads are within the freeze-concentrated matrix. In extreme cases the head of the sperm was situated in one region of the freeze-concentrated

matrix with the end of the sperm tail in another zone with the tail bridging through an ice crystal. This may occur because the dimensions of the freeze-concentrated matrix make it difficult to accommodate the sperm head and tail except when lying in the plane of the matrix, or because the tail section of the spermatozoon has surface properties which made it less likely to be excluded from the ice crystal matrix. It is important to note that the relative sperm cell recovery on thawing is not correlated with the structure of the ice crystal network because this structure is essentially fixed by the temperature of ice nucleation in the undercooled straws. All samples nucleated at the same temperature (-5°C) formed a similar initial ice structure upon which all additional ice was subsequently deposited.

Major differences in the eutectic structure between different treatments were made apparent by freeze substitution. Following 'controlled concentration' freezing the freeze-concentrated matrix contained large ice crystals, which were absent from the matrix of the other less successful freezing treatments. Further studies are required to determine at what temperature these ice crystals form within the freeze-concentrated matrix, but it is apparent that sperm cells within this matrix are in close association with ice crystals (Figure 4b). It is of interest that gross ice formation within the eutectic is observed in the sample with the highest recovery on thawing. The establishment of spatial gradients within freeze-concentrated materials has been clearly demonstrated by light cryomicroscopy (Korber *et al.*, 1984) and is well documented in metals (e.g. Davies, 1973).

Studies of freeze-substituted lymphocytes (Farrant *et al.*, 1977) have demonstrated that intracellular ice may be visualized and correlates with loss of viability on thawing. Freeze-substitution electron microscopy of frozen sperm has been restricted to an analysis of mouse spermatozoa frozen in the tail of the epididymis (Sherman and Liu, 1982). In this study, intracellular ice crystals were apparent and cellular dehydration was evident, particularly as voids between the acrosome and nuclear membrane and in the midpiece and tail. However as there were no motile cells in any sample following thawing it was not possible to correlate injury with cellular structure. In the current study freezing appeared to have little effect on the cell morphology as revealed by freeze fracture and freeze substitution. No osmotic shrinkage was evident nor was intracellular ice apparent. The possibility exists that cells contain micro-crystalline intracellular ice, beyond the limits of resolution of the ultrastructural techniques employed. Further studies are being undertaken to thermally cycle the samples, which would be expected to increase the dimensions of any intracellular ice present. The only significant structure observed in these studies was the ring of material surrounding sectioned spermatozoa, apparent by electron microscopy. The nature of this material and its formation require further investigation.

It has been demonstrated (Du *et al.*, 1993) that human spermatozoa behave as ideal osmometers, within the range 250 to 1500 mOsm of sodium chloride and that 13% of the isotonic water is osmotically inactive. Using models of the osmotic behaviour of spermatozoa during freezing it has been suggested (Curry *et al.*, 1995) that following a linear cooling

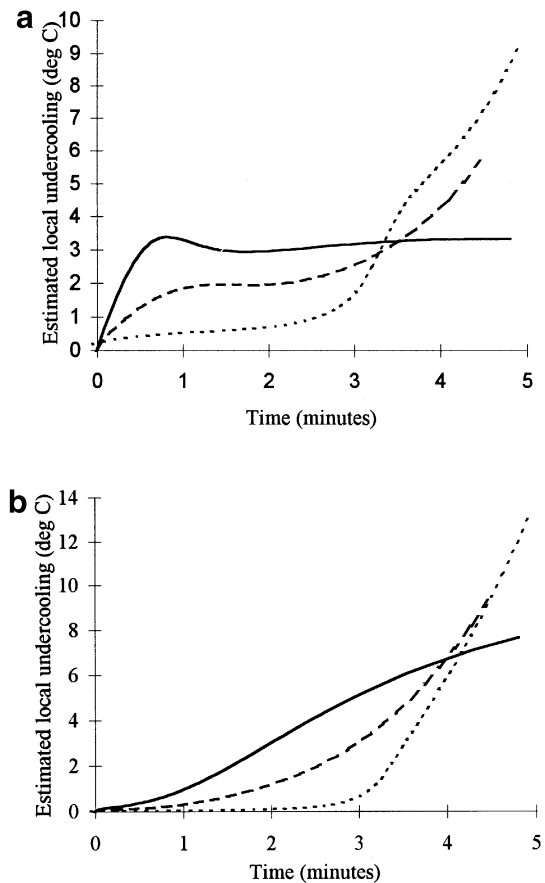


Figure 6. (a) Estimated local undercooling as a function of time—water movement only. (b) Estimated local undercooling as a function of time—dominated by glycerol movement. 'Controlled concentration' (—); linear ice (- - -); linear rate of temperature change (· · ·).

at $10^{\circ}\text{C}/\text{min}$, less than 10% of the cellular water would remain in the cell at -10°C . However, this major loss is not observed here: spermatozoa in Figures 3 and 5 exhibit no osmotic shrinkage. Although the low water content of sperm cells, combined with their flat, non-spherical shape, could allow large changes in cellular water content to cause little modification in the surface area, there is no evidence of any membrane alterations consistent with osmotic dehydration in any of the micrographs examined. However, in this study spermatozoa were frozen in the presence of glycerol, which has a high permeability to human spermatozoa (Gao *et al.*, 1992), and has also been demonstrated to reduce the water permeability of human spermatozoa (Noiles *et al.*, 1992). It is also of potential significance that an aquaporin (AQP7) which mediates water permeability in spermatozoa is also involved in glycerol transport (Ishibashi *et al.*, 1997). The lack of cellular shrinkage would be consistent with the cells effectively being in equilibrium with glycerol at all temperatures during freezing. Similarly the apparent absence of intracellular ice (Figure 5) could also be associated with a high intracellular glycerol concentration.

Whilst experimental treatments give significantly different levels of viability any correlation with cell morphology in the frozen state is lacking, and it is the controlled parameter, namely the rate of change in solute concentration, which is

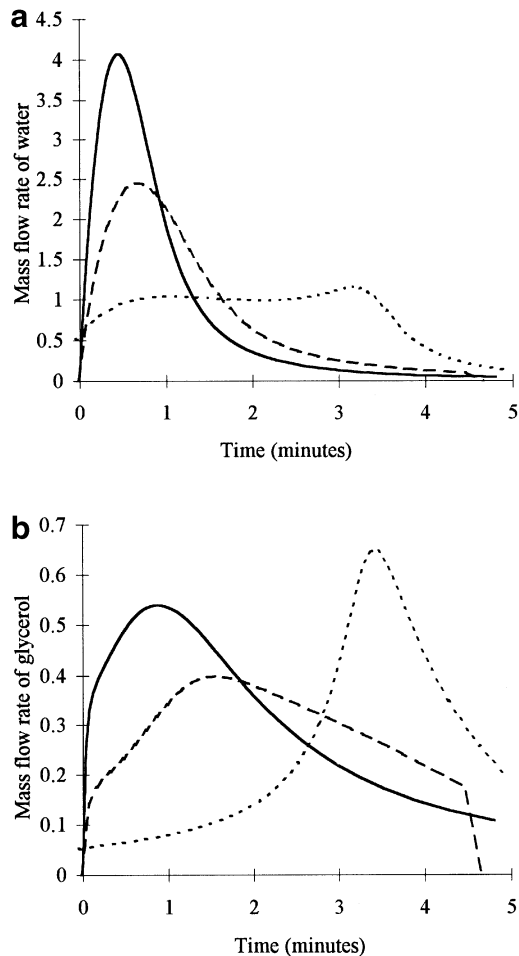


Figure 7. (a) Estimated mass transport as a function of time—water movement only. (b) Estimated mass transport as a function of time—dominated by glycerol movement. Controlled concentration (—); linear ice (---); linear rate of temperature change (· · ·).

the major factor affecting sperm recovery. It is of interest to speculate precisely why this is the case, and Figure 6a and b shows the estimated form of the local undercooling of the cells during the freezing process for the three distinct cases ‘controlled concentration’ (method 6.i), linear ice fraction (method 6.ii) and standard linear cooling (method 4), whose concentration histories are shown in Figure 1. These estimates of local undercooling have been made by a simple computation of the mass transport across the cell membrane. Figure 6a shows the form of the undercooling when the mass transfer is dominated by water transport, and Figure 6b shows the effect when the glycerol transport dominates.

From Figure 6a it can be seen that although in the successful ‘controlled concentration’ the undercooling rises to a higher level initially, it remains approximately constant throughout the rest of the freezing whereas the linear cooling rate and linear ice cases rise significantly towards the end of the freezing. This would be expected to increase the likelihood of intracellular ice in these two cases. If instead the mass transport is dominated by the transport of glycerol then it can be seen from Figure 6b that all cases remain close to equilibrium at the start, but the local undercooling subsequently increases. This increase is almost linear in the ‘controlled concentration’

case but stays low and rises rapidly in the later stages in the other two cases. The values of the local undercooling given in Figure 6a and b are comparable for similar membrane permeabilities in the two cases. The local undercooling would be comparatively higher in the glycerol case if the permeability were less.

However, it must be noted that intracellular ice is not apparent and it is therefore likely that other physical events determine viability during freezing and thawing. The corresponding estimated changes in mass transport are shown in Figure 7a and b for water movement and glycerol movement respectively. These values are again comparable for similar membrane permeabilities, but would scale relatively for different relative permeabilities. These estimates show the relative form of mass transport where the transport is dominated by either the water or glycerol transfer, and it would be expected that the cells may experience a combination of the two effects over the course of the freezing. Cell viability may be determined by a combination of potential cytotoxic events at high sub-zero temperatures together with restrictions on transport at low temperatures due to low permeability, high viscosity etc. The ‘controlled concentration’ treatment would minimize the time of exposure at high sub-zero temperatures and allow extended periods at lower temperatures to compensate for reduction in transport processes.

Finally, in addition to providing a novel insight to understanding the cellular response to freezing and thawing, the data presented here clearly demonstrate that a simple method exists for the cryopreservation of ejaculated human sperm, which gives better results than vapour freezing or by freezing at a linear cooling. The general applicability of this new approach to cryopreservation is being examined for other cell types including human testicular and epididymal spermatozoa, spermatozoa of different species, and also for embryos and oocytes.

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