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# DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification

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**BACKGROUND: In contrast to the technique of conventional freezing, the vitrification of spermatozoa requires high cooling rates (720 000°K/min), which could be damaging for spermatozoa. The aim of our study was to compare slowly frozen and vitrified spermatozoa in terms of their post-thaw DNA integrity and motility. METHODS: Semen samples were prepared according to the routine swim-up technique and divided into aliquots for comparison of fresh, conventionally frozen and vitrified spermatozoa from the same ejaculate in the presence or absence of cryoprotectants. Spermatozoa motility and DNA integrity were determined. RESULTS: The motility of spermatozoa conventionally (slowly) frozen with a cryoprotectant was similar to that recorded for spermatozoa vitrified in the absence of cryoprotectant (47 versus 52%). The DNA integrity was unaffected by the cryopreservation method or presence of cryoprotectants. CONCLUSION: The vitrification of human spermatozoa in the absence of conventional cryoprotectants is indeed feasible. The DNA integrity of vitrified sperm is comparable with that shown by standard slow-frozen/thawed spermatozoa, yet the method is quick and simple and does not require special cryobiological equipment.**

*Key words*: comet assay/cryopreservation/human/sperm DNA/vitrification

## Introduction

Cryopreservation is widely used presently as a method of storing different cell types and tissues including male and female gametes and embryos. Since the late 1930s–1940s (Bernschtein and Petropavlovski, 1937; Polge *et al*., 1949; Smirnov, 1949), it has been possible to cryopreserve the spermatozoa of several mammalian species effectively, particularly bovine and human sperm. This type of technique has important applications including the preservation of male fertility before radiotherapy and/or chemotherapy (Sanger *et al*., 1992), which may lead to testicular failure or ejaculatory dysfunction. However, due to the damage induced by freezing, the motility of cryopreserved spermatozoa after thawing is statistically reduced and shows wide interindividual variability (Critser *et al*., 1988; Yoshida *et al*., 1990). To date, the problems of cryoprotectant toxicity due to osmotic stress during the addition and removal of cryoprotectants and possible negative effects on the sperm's genetic apparatus are unresolved (Critser *et al*., 1988; Perez-Sanchez *et al*., 1994; Gilmore *et al*., 1997). Further cryo-damage may also be attributed to the slow thawing process (Mazur *et al*., 1981).

Compared with the conventional 'slow' freezing method, the newly developed techniques of vitrification and ultrarapid freezing, in which cryopreservation is achieved by directly plunging spermatozoa into liquid nitrogen [vitrification (cooling rate ~720 000°K/min) Nawroth *et al*., 2002; Isachenko *et al*., 2003; ultrarapid freezing (cooling rate ~300–600°C/min) Schuster *et al*., 2003)], seem to have certain benefits. This method of cryopreservation does not require the use of classic permeable cryoprotectants, and thus avoids the lethal effects of osmotic shock on the spermatozoa. Moreover, the entire freezing or thawing process only takes a few seconds. Before freezing, the simple 'swim-up' or density gradient centrifugation procedure allows the selection of spermatozoa with progressive motility, normal morphology or even those with non-damaged DNA. This pre-selection has been shown to improve sperm quality after thawing in terms of all the classic markers of quality including DNA integrity (Perez-Sanchez *et al*., 1994; Esteves *et al*., 2000; Sakkas *et al*., 2000; Donnelly *et al*., 2001b; Tomlinson *et al*., 2001; O'Connell *et al*., 2003). Indeed, we were able to report a significant improvement (11.6%; $P < 0.05$) in post-thaw sperm motility when vitrifying swim-up-prepared spermatozoa with no cryoprotectant

(Nawroth *et al*., 2002; Isachenko *et al*., 2003) over best post-thaw results achieved after conventional freezing in the obligatory presence of a permeable cryoprotectant (glycerol). However, the factors 'morphology', 'motile sperm recovery', 'viability after freezing' and 'acrosome-reacted cells' were not statistically different for the two cryopreservation methods ($P > 0.05$). According to these data, the swim-up method of preparing the sperm resulted in a significant improvement in the quality of spermatozoa which was sufficient to match the final results obtained using the conventional freezing procedure.

The present study was designed to compare the effects of slow freezing and cryoprotectant-free vitrification on the motility and DNA integrity of spermatozoa from fertile men. The effects of cryoprotectants on fresh sperm and when used during the slow freezing and vitrification process were also evaluated.

## Materials and methods

### *Samples*

Ejaculates containing at least $20 \times 10^6$ spermatozoa/ml and showing at least 50% progressive sperm motility were obtained from 18 healthy men by masturbation, after a minimum of 48 h of sexual abstinence. Informed consent was obtained from each donor. Semen analysis was performed according to the guidelines published by the World Health Organization (1999). Each ejaculate was swim-up prepared (SUP) and divided into four aliquots for: conventional slow freezing with (CSF+) or without (CSF–) standard cryoprotectants, and vitrification with (V+) or without (V–) standard cryoprotectants. The cryoprotectants used were glycerol/egg yolk. Swim-up was performed using a standard medium containing 10 mg/ml of human serum albumin (SPM; Scandinavian IVF Science, Gothenburg, Sweden) according to the instructions published by the World Health Organization (1999). In short, an ejaculate was washed twice by centrifugation at 380 *g* for 10 min in a double volume of SPM. After the second washing, 0.8 ml of SPM were pipetted over the pellet. The samples were then incubated for 30 min for swim-up.

Fresh SUP spermatozoa (no cryoprotectant) served as controls for all the experimental groups.

### *Conventional (automated) freezing*

For the conventional, programmable slow freezing method, the cryoprotectant used was test–egg yolk–glycerol (TEYG) freezing medium (Scandinavian IVF Science, Gothenburg, Sweden). After 1:1 dilution in TEYG (final glycerol concentration 6%), 0.25 ml of the spermatozoa suspension was pipetted into standard 0.25 ml insemination straws (MTG, Altdorf, Germany) and kept at room temperature for 10 min. The straws were then placed in a programmable freezer.

Semen samples in both groups (CSF+ and CSF–) were frozen according to Giraud *et al*. (2000). The protocol for conventional freezing was the following: cooling from 22 to 4°C at a rate of 5°C/min; from 4 to –30°C at a rate of 10°C/min; and from –30 to –140°C at a rate of 20°C/min, followed by plunging into liquid nitrogen. After storage of the spermatozoa in liquid nitrogen for a minimum of 24 h, the samples were thawed by plunging the straws into a water bath at 37°C for 50 s. Next, 5 ml of SPM were added to the thawed samples and the sperm suspension was centrifuged at 380 *g* for 5 min. The supernatant was removed and the pellet was resuspended in 100 µl of SPM.

### *Cryopreservation by direct plunging into liquid nitrogen (vitrification)*

The method of vitrification used was described in detail by Nawroth *et al*. (2002). Briefly, the same concentration of TEYG as for slow freezing was used for vitrification in the presence of a cryoprotectant. Drops ($20 \pm 2$ µl) of sperm samples in both groups (V+ and V–) were placed on copper loops of 5 mm diameter. These cryoloops were then plunged into liquid nitrogen and stored for at least 24 h. After the storage period, the samples were warmed by plunging the copper loops into a 15 ml tube containing 10 ml of SPM at 37°C and mixing thoroughly. After warming five loops per tube, the tubes were placed in a $CO_2$ incubator for 5–10 min. The spermatozoa were then concentrated by centrifugation at 380 *g* for 10 min. The pellet was resuspended in 100 µl of SPM.

### *Evaluation of sperm motility and viability*

Sperm motility was assessed immediately after liquefaction (conventional freezing) or sample concentration by centrifugation (vitrification). The Makler chamber was used for motility scoring. Motility was estimated under the light microscope using the ×400 magnification. Only spermatozoa with progressive motility (WHO categories 'a' and 'b') were assessed. Motility was evaluated immediately after thawing. Recovery of motile spermatozoa was defined as the percentage of post-thaw motility × 100% divided by the percentage of pre-freezing motility. To test the effect of the cryoprotectant on sperm motility and DNA integrity before cryopreservation, spermatozoa suspensions were equilibrated in TEYG for 10 min and then washed with SPM for swim-up preparation.

### *Comet assay*

The comet assay was performed using the CometAssay™ Reagent Kit for Single Cell Gel Electrophoresis Assay (Trevigen, Inc., Gaithersburg, MD) according to the manufacturer's instructions with slight modification by Donnelly *et al*. (2001b). Briefly, the spermatozoa samples were washed twice with SPM and the sediment was resuspended in Dulbecco's phosphate-buffered saline ($Ca^{2+}$- and $Mg^{2+}$-free PBS; Bio-Wittaker, Verviers, Belgium). The samples were then placed on ice to inhibit endogenous damage occurring during sample preparation. During preparation, the cells were handled under yellow light to prevent DNA damage by UV light. Some cells were treated with 25 µmol/l $KMnO_4$ for 20 min at 4°C, as controls for the comet assay (sperm cells with a comet tail have disrupted DNA). Subsequent treatment of DNA-damaged and undamaged cells was performed as follows. Freshly prepared lysis solution supplemented with 1% dimethylsulphoxide (DMSO) was chilled at 4°C for at least 20 min before use. The lysis solution contained 2.5 mol/l sodium chloride, 100 mmol/l EDTA pH 10, 10 mmol/l Tris base, 1% sodium lauryl sarcosinate and 1% Triton X-100. After mixing the spermatozoa suspension (at ~$1 \times 10^5$ cells/ml) with 1% molten low-melting point agarose at 40°C at a ratio of 1:10 (v/v), 75 µl of suspension was immediately pipetted onto the Trevigen CometSlide™, gently spread over the slide area and placed flat in the dark at 4°C for 10 min. The slides were then immersed in the pre-chilled lysis solution for 60 min for dissolution of the cell membranes. To achieve DNA decondensation after cell lysis, the slides were incubated with 10 mmol/l dithiothreitol (DTT; Sigma-Aldrich, Steinheim, Germany) for 30 min at 4°C and then with 4 mmol/l 3.5-diodosalicylic acid lithium salt (LIS, Sigma-Aldrich) for 90 min at 20°C. After tapping the slides to remove excess solution, they were immersed in freshly prepared alkaline solution (300 mmol/l NaOH, 1 mmol/l EDTA, pH >13) in the dark for 20 min at room temperature. A horizontal gel electrophoresis apparatus was filled with the same alkaline solution at 4°C. The slides were placed flat onto a gel tray and aligned equidistant from the

**Table I.** Characteristics of fresh ejaculated and swim-up-prepared spermatozoa before cryopreservation

| Concentration after ejaculation ($\times 10^6$l/ml) | Morphology after ejaculation/liquefying (%) | Motility after ejaculation/liquefying (%) | Concentration after swim-up ($\times 10^6$/ml) | Motility after swim-up without cryoprotectants (%) | Motility after swim-up with cryoprotectants (%) |
|---|---|---|---|---|---|
| 107.7 ± 10.9 | 10.7 ± 1.6 | 51.7 ± 6.1 | 44.8 ± 7.8 | 89.5 ± 7.1[a] | 77.5 ± 8.9[b] |

[a,b]The differences between the parameters (motility after swim-up without cryoprotectants) and (motility after swim up with cryoprotectants) was significant ($P < 0.05$).

electrodes. Electrophoresis was performed at 1 V/cm adjusted to 300 mA by either raising or lowering the buffer level in the apparatus for 10 min. After electrophoresis, the excess solution was gently tapped from the slides, which were then dipped in 70% ethanol for 5 min with subsequent air-drying at room temperature before being stored in an airtight desiccator. The slides were viewed using a Zeiss IM epifluorescence microscope equipped with an excitation/emission filter of 485 nm/520 nm under ×400 magnification. Fluorescent staining was performed using SYBR green stain (working concentration 1:200). In healthy cells, the fluorescence was confined to the nucleoid: undamaged DNA is supercoiled and does not migrate very far from the nucleoid (Figure 1). In cells that have incurred damage to the DNA, the alkali treatment unwinds the DNA, releasing fragments that migrate from the nucleoid (Figure 2). A total of 200 cells were analysed per slide.

### *Evaluation of sperm morphology*

Sperm morphology was assessed using strict criteria (Menkveld *et al*., 1991). The sperm were stained using Testsimplets (Roche Diagnostics LTD, Germany). After pre-staining slides with methylene blue and cresyl violet acetate, 5 µl of sperm were dropped onto the centre of a pre-stained slide and covered with a coverglass. Morphological assessment was performed using an oil immersion microscope at ×1000 magnification after 30 min of staining. Results were recorded as the number of normal spermatozoa out of 100 counted on each slide.

### *Statistical analysis*

Treatment effects on sperm variables were assessed by ANOVA. Data are expressed as means ± SD. The level of statistical significance was set at $P \leqslant 0.05$.

## Results

### *Effect of the cryopreservation method on sperm motility*

Table I shows sperm quality parameters determined in fresh ejaculates and SUP sperm in the absence and presence of cryoprotectant before cryopreservation. These data show a slight (12%) reduction in the motility of SUP spermatozoa after 10 min of incubation with TEYG ($P > 0.05$), indicating a detrimental effect of the cryoprotectant. However, this effect could not be correlated with a change in DNA integrity (Figure 2, $P > 0.05$).

Figure 1 indicates that slow freezing with no cryoprotectant gives rise to a 29.1-fold decrease in sperm motility compared with slow freezing with a cryoprotectant ($P < 0.001$). In contrast, sperm motility was 2.87 times lower when vitrification was conducted in the presence of cryoprotectant compared with vitrification with no cryoprotectant (Figure 1, $P < 0.05$). The highest motility rates after vitrification were achieved using SUP spermatozoa on copper loops with no cryoprotectant (51.5 ± 4.5%), though these rates were similar to those shown by sperm slowly frozen using the cryoprotectant (46.7 ± 4.1%; $P > 0.5$).

### *Effect of cryopreservation method on sperm DNA integrity*

We observed no significant differences in the DNA integrity of prepared spermatozoa related to the freezing method or presence of a cryoprotectant (Figure 2; $P > 0.5$). The proportions of sperm showing undamaged DNA were 85.09 and 89.51%, respectively, for fresh sperm treated or not treated with the cryoprotectant, 84.62 and 83.53%, respectively, for the slowly frozen sperm with or without cryoprotectant, and 87.24 and 84.66%, respectively, for the vitrified sperm with or without cryoprotectant.

## Discussion

Sperm cryopreservation is routinely used presently in the management of human male infertility (Holt, 1997; Donnelly *et al*., 2001b). Despite this, the current cryo-techniques used for human spermatozoa are still imperfect. To date, nearly all cryobiological investigations on spermatozoa or routine freezing involve the use of conventional (programmable or standard vapour) freezing. The effectiveness of the cryo-technique is associated with permeable and non-permeable cryoprotectants. These are used to prevent the formation of ice crystals during freezing and, thus, avoid structural damage and motility loss after cryopreservation.

The decline in spermatozoa motility after cryopreservation is a topic of current research since it is one of the factors that are first affected (Critser *et al*., 1987b; Watson, 1995). However, the mechanism through which motility is decreased is still unclear. This mechanism may be mechanical or of a physical–chemical aetiology. Permeable cryoprotectants play a leading role, while the non-permeable protective agents play a supporting role and, in most cases, cannot protect the cells in the absence of a permeable cryoprotectant. The properties of permeable cryoprotectants are directly related to osmotic and toxic damage with concurrent cell saturation before cooling (Sherman, 1973; Watson, 1979; Gao *et al*., 1997) and/or removal after thawing (Watson, 1995; Gao *et al*., 1995, 1997). In conventional freezing, mechanical cell injury could occur by rapid freezing leading to intracellular or extracellular ice crystal formation and signs of osmotic damage (Watson, 1995; Gao *et al*., 1995, 1997). Conventional freezing causes extensive chemical—physical damage to the extracellular and intracellular membranes of the sperm that are attributable to

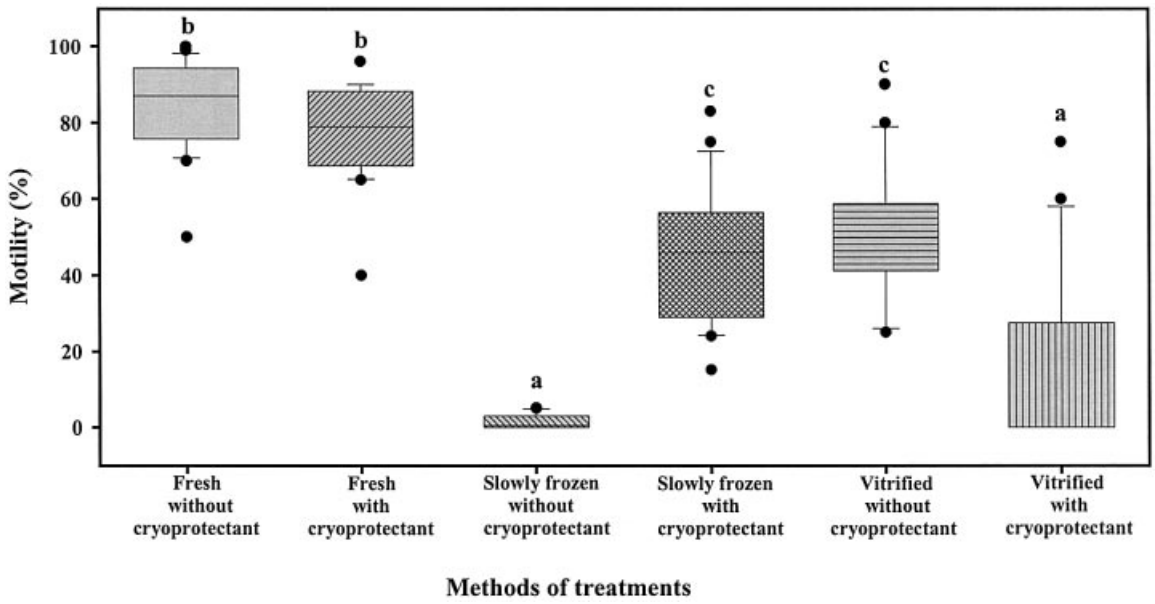


**Figure 1.** Motility of spermatozoa according to treatment and cryopreservation method. Results are given as values after thawing, compared to pre freezing values. Each bar represents the median, 25th and 75th percentile, minimum and maximum values. Bars with different letters within each treatment group indicate a significant difference ($P < 0.001$).

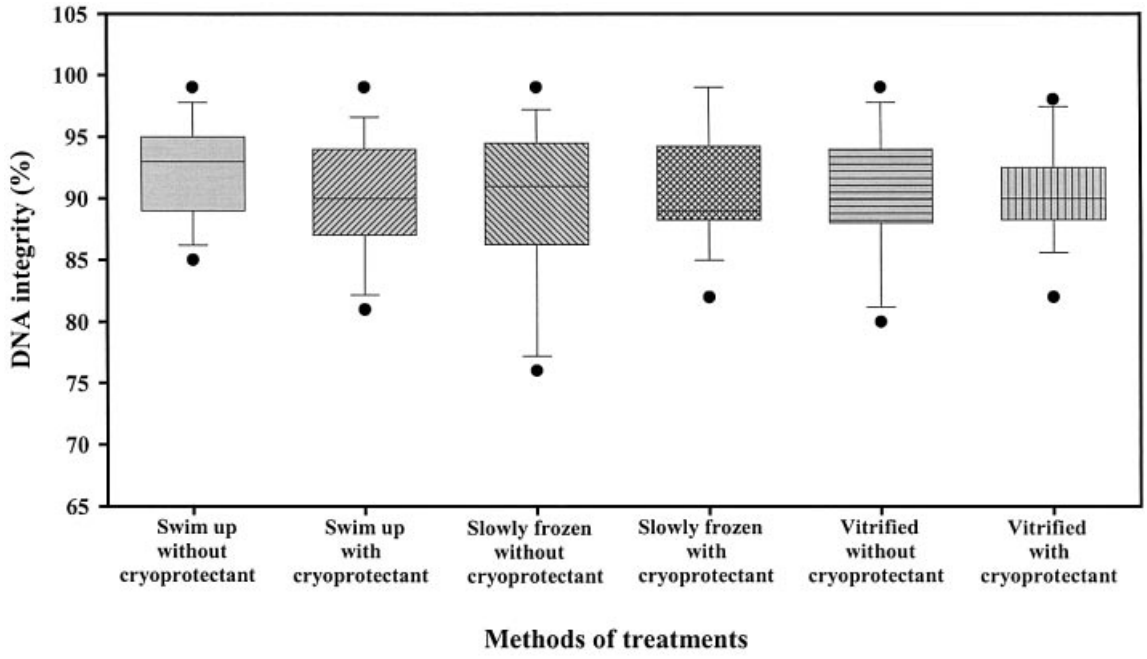


**Figure 2.** DNA integrity of spermatoza according to treatment and cryopreservation method. Each bar represents the median, 25th and 75th percentile, minimum and maximum values. Bars with different letters within each treatment group indicate a significant difference ($P < 0.05$).

changes in the lipid phase transition and/or increased lipid peroxidation (Alvarez and Storey, 1992, 1993; Mossad *et al*., 1994) during cooling or after thawing, with the consequence of a decrease both in sperm velocity and in the percentage of motile spermatozoa (Critser *et al*., 1987a,b; Keel *et al*., 1987; Mossad *et al*., 1994; Watson, 1995; Leffler and Walters, 1996). It has been established that the production of reactive oxygen species leads to increased lipid peroxidation after cryopreservation (Alvarez and Storey, 1992) and is significantly associated with a loss of sperm motility (Aitken *et al*., 1989; O'Connell *et al*., 2002). As previously suggested (Alvarez and Storey, 1992, 1993; Chatterjee and Gagnon, 2001), the injury to human spermatozoa induced by cryopreservation mainly occurs during thawing. This damage could, at least in part, be related to reduced antioxidant defence activity during cooling and/or structural damage to the cytoskeleton and/or antioxidant enzymes during cryopreservation (Alvarez and Storey, 1992, 1993). All these findings suggest that the slow freezing of

sperm, aside from ice crystal formation, is intrinsically deleterious. Egg yolk, a natural complex mixture of cholesterol, phospholipids and antioxidants, has been used in sperm cryopreservation for many years to reduce the negative effects of osmotic shock. How this protective effect is produced is not entirely clear, since egg yolk is such a complex mixture, but it may play a role in reducing the deleterious effects of hyperosmotic salt solutions on membrane structures during rapid cooling (Watson, 1976, 1995; Ostashko, 1978, 1995; Pursel *et al*., 1978; Holt *et al*., 1988, 1992; Katkov *et al*., 1996). However, its most important role could be fortification of the cell membrane by the lipid components of the egg yolk (Ostashko, 1978). Some of these components (low-density lipoprotein fraction, glycolipids, cholesterol) may also become incorporated into the membranes, reducing their tendency to gel during cooling, as described for sperm and erythrocytes (Watson, 1976; Parks and Lynch, 1992; Ostashko 1978). Further, Chatterjee and Gagnon (2001) demonstrated that egg yolk–Tris–glycerol cryoprotectant medium (EYTG) is an efficient scavenger of $NO^-$, $O_2^-$ and $H_2O_2$ radicals. Thus, we may be dealing with a dual action whereby the cell membrane becomes coated and isolated from direct contact with the cryoprotective agents while preserving its fluidity and flexibility at a lower temperature, thereby reducing cytoskeletal damage. In a previous study (Isachenko and Nayudu, 1999), we showed that by combining the two factors, an increased temperature and the inclusion of egg yolk in both the vitrification and dilution media, the survival of mouse germinal vesicle oocytes was significantly improved after warming. The inclusion of egg yolk in the vitrification/dilution medium also improved the maturation rate and the proportion of normal metaphase forms, suggesting a major effect on reducing internal cell damage. However, the effectiveness (avoiding intracellular ice formation) of permeable and non-permeable cryoprotectants during conventional freezing is only revealed when we use a slow cooling rate (Gao *et al*., 1997). All these negative effects of freezing on cells can also lead to chromatin damage. The assessment of sperm nucleus integrity due to such causes is very important, since, as recently described (Spano *et al*., 1999; Sakkas and Tomlinson, 2000), chromatin abnormalities affect sperm quality and male fertility status. Fraga *et al*. (1991) correlated damaged sperm DNA with mutagenic effects. It has also been shown that, besides having significant effects on sperm morphology and membrane integrity, freezing/thawing the sperm of fertile and infertile men also leads to significant chromatin damage (Royere *et al*., 1988, 1991; Hammadeh *et al*., 1999; Donnelly *et al*., 2001a,b). Other studies have demonstrated (Balhorn *et al*., 1988; Manicardi *et al*., 1995) that any defects in sperm chromatin structure in infertile men with increased DNA instability are sensitive to denaturing stress. This denaturing stress may be induced by a treatment such as freezing. Despite all this, the oocyte is able to repair a small amount of sperm DNA damage (>8%; Ahmadi and Ng, 1999), though this repair seems to be insufficient to support subsequent embryo development (Ahmadi and Ng, 1999) and can lead to decreased conception rates or failed conception (Hunter, 1976; Royere *et al*., 1988). Proportions of spermatozoa with fragmented DNA have been negatively correlated with fertilization rates in IVF (Sun *et al*., 1997) and ICSI (Lopes *et al*., 1998).

In contrast to slow freezing, vitrification as a rule involves the use of very high concentrations (3.5–8 mol/l) of permeating cryoprotectants and high cooling rates (up to $10^{13}$°K/min). According to the literature, the critical cooling speed for the vitrification of pure water varies dramatically depending on the method used, from $10^7$ to $10^{13}$°K/min (for references see Figure 9 in Karlsson and Cravalho, 1994). Given that high concentrations of cryoprotectants have a marked toxic effect (Fahy, 1986; Pegg and Diaper, 1988; Shaw *et al*., 2000), it is possible to decrease this toxicity using a combination of two cryoprotectants (e.g. ethylene glycol and DMSO), and/or to expose cells to pre-cooled concentrated solutions in a stepwise manner (Fahy *et al*., 1984; Fahy, 1986). Another strategy is to reduce the amount of cryoprotectant and simultaneously increase the cooling and warming rates (Liebermann and Tucker, 2002).

Luyet (1937) first mentioned the possibility of using the vitrification technique (a small specimen cooled very rapidly was vitrified without substantial loss of viability (for references see, for example, Fahy, 1988). The following year, Luyet and Hodapp (1938) reported the survival of frog spermatozoa vitrified in liquid nitrogen, and a few years later, Schaffner (1942) successfully vitrified fowl spermatozoa using a modification of Luyet technique. Nevertheless, all subsequent attempts to vitrify mammalian spermatozoa using this approach resulted in low or null survival (Hoagland and Pincus, 1942; Smith, 1961) mostly because of the critical speed of freezing and warming, which is very high for low concentrations of cyroprotectants. Such high speeds were unattainable by investigators at this time. Unfortunately, the high concentrations of cryoprotectants (30–50% compared with 5–7% for slow freezing) used in classic vitrification cannot be applied to spermatozoa due to their lethal osmotic effects (Holt, 1997; Katkov *et al*., 1998; Mazur *et al*., 2000). However, it has been established as dogma that the vitrification of large cells, tissues and even organs can only be achieved using high concentrations of combinations of permeable and impermeable cryoprotectants (Fahy, 1988). The total concentrations of such substances must be at least 50% (w/w) (if vitrification is conducted at atmospheric pressure) to reach the zone of stable vitrification. Concurrently, the speed of cooling and warming should be relatively low. These conditions can be very damaging for cells and lead to subsequent biochemical alterations and lethal osmotic injury (Fahy, 1984), although some of the deleterious effects of cryoprotectants on mammalian sperm can be avoided by adopting optimal regimes of addition and removal of the cryoprotectant (Sherman, 1973; Watson, 1979; Critser *et al*., 1988; Gao *et al*., 1995; Leffler and Walters, 1996; Katkov *et al*., 1998; Katkov, 2002). These regimes are, however, ineffective for human and animal spermatozoa treated with high concentrations of cryoprotectants. Thus, at present, the only alternative to this is the use of very rapid cooling and warming rates along with a very small specimen size. Such were the conditions used in the present study. The sample size can be minimized using different carrier systems [open-pulled straws (OPS; Vajta *et al*., 1997),

flexipet-denuding pipette (FDP; Oberstein *et al*., 2001; Liebermann *et al*., 2002), micro drops (Papis *et al*., 2001), electron microscopy copper grids (Martino *et al*., 1996; Hong *et al*., 1999), hemi-straw system (Kuwayama and Kato, 2000), small nylon coils (Kurokawa *et al*., 1996), nylon mesh (Matsumoto *et al*., 2001) or cryoloop (Oberstein *et al*., 2001)] such that the duration of solidification of the liquid phase during freezing is reduced.

When the conditions of vitrification are: a very high speed of cooling [up to 720 000°K/min in the initial phase of cooling, as theoretically calculated (Isachenko *et al*., 2003)], a short cooling time (5–8 s) and a small specimen size (20 µl), not many nuclei of crystallization form and these seem to be insufficiently large to damage human spermatozoa. In these conditions, the probability of substantial devitrification (re-crystallization) of the vitrified solution is also low due to the high speed and short time of warming (direct dissolution in a large volume of agitated warm water), and the small size of the specimen (extracellular recrystallization) and cells (intracellular recrystallization). The substantial compartmentalization of intracellular compounds may contribute to the successful survival of spermatozoa.

Moreover, it has been established that the amount of osmotically inactive water is higher in spermatozoa, where it is bound to several macromolecule structures such as DNA, histones, hyaluronidase, etc., than in oocytes or embryos. According to our calculations (see mathematical equation in Isachenko *et al*., 2003), amounts of high molecular weight components can be 6–8 times higher than in embryos and this will invariably affect the viscosity and glass transition temperature of the intracellular cytosol in sperm, but the probability of lethal ice formation during cooling/warming would be higher for embryos. There is indirect evidence to show that the intracellular components of sperm may act as natural cryoprotectants, including the fact that mice spermatozoa, among the most osmotically fragile of all species (Katkov *et al*., 1998), can be frozen successfully in the absence of permeable cryoprotectants, using only protein- and sugar-rich skimmed milk and raffinose as extracellular non-permeable cryoprotectants (Yokoyama *et al*., 1990; Nakagata and Takeshima, 1992; Koshimoto *et al*., 2000). Our estimations for albumin indicate that, in general, during cooling and especially during rewarming/resuscitation, the small amount of specimen and cells, high viscosity of the solution and high speed of cooling and warming (Isachenko *et al*., 2003) used would avoid devitrification (especially intracellular) (Karlsson and Cravalho, 1994; Karlsson, 2001). Seemingly, the presence of relatively high concentrations of albumin, which is highly efficient at inhibiting lipid peroxidation (Karow, 1997), and sugars substantially raised the viscosity of the solution, especially at the lower temperatures. In addition, the small sample size and number of cells made vitrification stable during both cooling and warming, leading to good results after warming. It would be logical to suppose that sperm DNA would be damaged using such an extreme cryo-protocol for sperm preservation. However, Evenson *et al*. (1991) found no difference in their SCSA results for non-cryopreserved or cryopreserved sperm, and slowly or flash-frozen sperm. Duty *et al*. (2002) confirmed these findings and reported that flash freezing in liquid nitrogen with no cryoprotectants most closely reproduced the results of a freshly obtained human ejaculate. These authors assumed that the particular DNA packaging of human sperm protects the DNA from intracellular fluid shifts and ice crystal formation during cryopreservation. All these data were confirmed by our results.

In conclusion, our results show that the vitrification of human spermatozoa in the absence of conventional cryoprotectants is indeed feasible. The DNA integrity of vitrified sperm is comparable with that shown by standard slow-frozen/thawed spermatozoa, yet the method is quick and simple and does not require special cryobiological equipment.

We presently are conducting studies to determine: how the different stages of vitrification compare with those of slow freezing in terms of inducing lipid cell membrane peroxidation; and the roles played by the different cryoprotective (glycerol, ethylene glycol, DMSO) and supporting (egg yolk and human serum albumin) agents in preventing damage to the cytoskeleton and/or antioxidant enzymes in the presence of reactive oxygen species during slow freezing or vitrification.

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