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Comparison between computerized slow-stage and static liquid nitrogen vapour freezing methods with respect to the deleterious effect on chromatin and morphology of spermatozoa from fertile and subfertile men

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Summary

The purpose of this study was to determine the negative effects (cryodamage) on human spermatozoa after freeze-thawing and to determine whether freeze-thawing of spermatozoa with a programmed slow freezer is better than freezing with liquid nitrogen vapour (rapid freezing) with regard to alterations in sperm chromatin and morphology in semen from fertile (donor) and subfertile, IVF/ICSI, patients. Ninety-five semen samples were obtained either from patients attending our IVF unit for treatment ($n = 34$) or from donors ($n = 25$) with proven fertility and normal sperm quality according to WHO guidelines. Each semen sample was divided into two parts after liquefaction and addition of the cryoprotectant. The first part was frozen using a programmed biological freezer and the second part was frozen by means of liquid nitrogen vapour. Smears were made before the freezing and after the thawing procedure to assess morphology (strict criteria) and chromatin condensation (Acridine Orange test). The mean percentage of chromatin condensed spermatozoa in the samples from donors (control group) was 92.4 ± 8.4% before freezing and this decreased significantly ($p < 0.0001$) to 88.7 ± 11.2% after freeze-thawing with the computerized slow-stage freezer and to 87.2 ± 12.3% after using static liquid nitrogen vapour ($p < 0.001$). The corresponding values for semen obtained from patients was 78.9 ± 10.3% before freezing which decreased to 70.7 ± 10.8 and 68.5 ± 14.8%, respectively ($p < 0.001$). On the other hand, the mean percentage of normal sperm morphology in the control group decreased from 26.3 ± 7.5% before freezing to 22.1 ± 6.4% ($p < 0.0001$) after thawing with the computerized slow-stage freezer and to 22.2 ± 6.6% ($p < 0.0001$) after the use of static liquid nitrogen vapour. In the patient group, the mean percentage of normal morphology decreased from 11.7 ± 6.1% after freezing with the biological freezer to 9.3 ± 5.6% and to 8.0 ± 4.9% after freezing with static liquid nitrogen vapour.

This study demonstrates that chromatin packaging and morphology of human spermatozoa decrease significantly after the freeze-thawing procedure, not only after the use of static liquid nitrogen vapour but also after the use of a computerized slow-stage

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freezer. However, the chromatin of semen samples with normal semen parameters (donor sperm) withstand the freeze-thaw injury better than those with low quality semen samples. Therefore, the computerized slow stage freezer could be recommended for freezing of human spermatozoa, especially for subnormal semen samples, for example, ICSI and ICSI/TESE candidates and from patients with testicular tumours or Hodgkin's disease, in order to avoid further damage to the sperm chromatin structure.

Keywords: chromatin integrity, liquid nitrogen vapour, semen cryopreservation computerized slow-stage freezer, sperm morphological alteration

Introduction

Cryodamage to a variety of cell organelles is regarded as being the result of the stresses of cryopreservation, i.e. the cellular dehydration, the formation and dissolution of ice crystals, the effect of elevated solute within the cell or changes in membrane permeability (Gilmore *et al.*, 2000; Mazur *et al.*, 2000). Even the stages of cooling below the freezing point of the medium can impose a stress to the cells (reviewed by Watson, 1995). Therefore, the success of cryopreservation of cells is affected by the rate of freezing as it is by the composition of the solution in which the cells are frozen (Farrant, 1980). Most procedures utilize an initial slow cooling rate between the temperature at which the sample is collected and –5 °C (Foote, 1975; Graham, 1978; Watson, 1979). When the initial cooling rate is sufficiently slow, water is lost from the cells and the formation of ice crystals, which occurs between –5 and –10 °C, is impeded (Mazur, 1963; Farrant, 1980; Fujikawa, 1980; Gao *et al.*, 1994; Muldrew & McGann, 1994). Otherwise, when cells are cooled rapidly, water is not lost fast enough to maintain equilibrium, the cells become increasingly supercooled and freeze intracellulary (Mazur, 1990), a process that might affect the integrity of chromatin and the morphology of spermatozoa.

In the case of freezing semen, the cryodamage of spermatozoa can be reduced dramatically by cooling semen slowly (≤10 °C/min) and the use of optimum cooling rates is a critical factor for cryopreservation as it influences the extent and rate of dehydration. Nevertheless, the liquid nitrogen vapour technique (which is considered rapid cooling) for the freezing of human semen with subsequent storage at –196 °C is in widespread use today (Sherman, 1986). Morphological and ultrastructural evaluation of spermatozoa following rapid cooling reveal extensive damage to the plasma membrane, leading to disruption of the acrosome (Hammerstedt *et al.*, 1990; Alvarez & Storey, 1992). Moreover, cooling of spermatozoa may result in latent injury not detected by conventional laboratory assays. While the proper condensation of chromatin is related to fertility (Hammadeh *et al.*, 1999) and there is some evidence that cytoskeletal elements of spermatozoa may be sensitive to cooling (Holt & North, 1991), the consequences of cooling technique on chromatin integrity and morphology of spermatozoa are not clear. Many reports have described the association between disturbances in sperm chromatin condensation, morphology and male infertility (Bach *et al.*, 1990; Foresta *et al.*, 1992).

Recently, a slow, computer-controlled method with a step-wise gradual temperature decrease was used, before the sample was plunged into liquid nitrogen. This method has been reported to limit cryodamage, especially for subnormal semen samples (Ragni *et al.*, 1990). Therefore, this study was undertaken to determine and compare the effect of the freeze-thawing procedure with the use of either static liquid nitrogen vapour or computerized slow-stage freezer on chromatin and morphology integrity of human spermatozoa from normal (donor) and subnormal semen samples.

Materials and methods

Human ejaculated spermatozoa were obtained by masturbation from patients attending our Andrology and IVF-Laboratories for semen analysis (Group 1, $n = 34$) or from donor semen with proven fertility (Group 2, $n = 25$). The semen samples were liquefied at room temperature. The semen analysis was performed within 20–30 min of delivery to the laboratory according to WHO guidelines (WHO, 1999), except for morphology which was evaluated according to strict criteria (Krüger *et al.*, 1988).

After the examination of the semen, each sample was mixed with the cryoprotectant (TEST-yolk cryoprotectant, Irvine Scientific, Santa Ana, CA, USA, cat. no. 9972). The semen buffer mixture was placed into 0.25 mL straws and divided into two parts. The first part was frozen with liquid nitrogen vapour as follows: first, the straws were placed in a horizontal position, parallel to the surface of the liquid nitrogen at 25 cm above the surface, for 15 min and then plunged into liquid nitrogen for storage, whereas the other straws were placed into the chamber of a computerized slow stage freezer (Planer, serie 10, Sunbury, Middlesex, UK) and were cooled according to the following program: (i) cooling

rate of −1 °C/min from +22 to +5 °C; (ii) 10 °C/min from +5 to −80 °C; (iii) 25 °C/min from −80 to −130 °C and (iv) plunged into liquid nitrogen. The samples were thawed by removing them from liquid nitrogen and putting them into a warm chamber (37 °C) for 5 min. Eight smears were made of each sample before freezing and 16 smears after thawing (eight after freeze-thawing with liquid nitrogen vapour and eight after freeze-thawing with computerized slow-stage freezer). The slides were air-dried and fixed in ethanol before being stained. The Papanicolaou staining method was applied according to the standard procedure as described in the WHO manual (1999).

Assessment of sperm morphology

Sperm morphology was evaluated by the same observer, according to strict criteria (Krüger *et al.*, 1988) and taking into consideration the specific effects, for example, size alteration of a specific staining method (Menkveld *et al.*, 1990). Bright field illumination and a magnification of 100× under an oil immersion objective lens was used for evaluation. For each semen sample 100–200 spermatozoa were evaluated.

Assessment of chromatin condensation (acridine orange staining)

Acridine orange was used to distinguish between spermatozoa with native DNA (green fluorescence) and single-stranded DNA (orange-red fluorescence) as a marker for abnormal chromatin (strand breaks) (Tejada *et al.*, 1984). Many smears were fixed overnight in freshly prepared Carnoy's solution (1:3; glacial acetic acid : absolute methanol). Slides were then stained in acridine orange at pH 2.5 for 5 min at ambient temperature in the dark. The staining solution comprised 10.0 mL 0.1% (w/v) acridine orange (CI 46005) in reagent water, 40 mL 0.1 M citric acid and 2.5 mL 0.3 M disodium orthophosphate. The slides were kept in the dark and evaluated on the same day using a fluorescence microscope (Leitz, Oberkochen, Germany) equipped with 490 nm excitation filter and 530 nm barrier. A normal DNA content exhibited green fluorescence over the head region while abnormalities of DNA content were indicated by a spectrum of fluorescence varying from yellow to red (a total of 200 spermatozoa was evaluated per smear).

Statistical analysis

The variables were presented as mean ± standard deviation (SD). Parameters were compared by the Wilcoxon signed-ranks test in paired samples and by Mann–Whitney *U*-test in independent samples. A p-value of ≤ 0.05 was considered to be statistically significant.

Results

Table 1 shows the mean ± SD of the classical semen parameters for the subfertile (Group 1) and fertile (Group 2) men who participated in this study, before (native) and after application of the freeze-thawing procedure using either liquid nitrogen vapour or computerized slow-stage freezing.

The mean percentage of spermatozoa with condensed chromatin in the group of patients with subnormal semen parameters (Group 1) decreased significantly ($p = 0.001$) after the freeze-thawing procedure from 78.9 ± 10.3 to 68.5 ± 14.8% using static (uncirculated) liquid nitrogen vapour 78.9 ± 10.3 to 68.5 ± 14.8% and to 70.7 ± 10.8%

Table 1. Semen parameters of subfertile and fertile men before and after the freeze thawing process

Parameters	Native semen sample	After freeze-thawing with	
		Static liquid nitrogen vapour	Computerized slow-stage freezer
Subfertile group (n = 34)			
Volume (mL)	3.7 ± 1.8		
Concentration of spermatozoa (millions/mL)	41.1 ± 37.1	22.5 ± 17.2	25.0 ± 18.7
Eosin-test (vitality) (%)	56.0 ± 23.0	27.9 ± 9.5	37.8 ± 21.2
Motility (%)	28.3 ± 22.5	13.3 ± 9.9	18.2 ± 8.1
HOS-test (%)	60.5 ± 17.2	46.5 ± 15.0	50.2 ± 15.5
Fertile group (n = 25)			
Volume (mL)	3.0 ± 1.3		
Concentration of spermatozoa (millions/mL)	53.0 ± 14.1	28.3 ± 16.1	30.0 ± 15.5
Eosin-test (vitality) (%)	72.6 ± 18.3	28.0 ± 17.3	41.8 ± 14.1
Motility (%)	52.8 ± 27.3	32.7 ± 19.1	38.5 ± 12.0
HOS-test (%)	66.7 ± 2.9	45.7 ± 9.2	52.3 ± 2.7

Table 2. Relationship of chromatin structure and morphology in spermatozoa from fertile and subfertile men to subsequent chromatin damage after freeze-thawing with static liquid nitrogen vapour or a computerized slow-stage freezer

Parameters	Percentage of spermatozoa with normal morphology and condensed chromatin before freeze-thawing (native)*	Percentage of spermatozoa with normal morphology and condensed chromatin after freeze-thawing with morphological alteration and chromatin		Significance of difference between the two methods in relation to damage
		Static liquid nitrogen vapour*	Computerized slow-stage freezer*	
Subfertile group (n = 34)				
Morphology (%)	11.7 ± 6.1	8.0 ± 4.9	9.3 ± 5.6	
Decrease in spermatozoa with normal morphology		−3.6 ± 0.4[a]	−2.4 ± 0.2[a]	p = 0.002[a]
Chromatin (%)	78.9 ± 10.3	68.5 ± 14.8	70.7 ± 10.8	
Decrease in spermatozoa with condensed chromatin		−10.8 ± 2.6[b,c]	−7.6 ± 1.6[c,d]	p < 0.06[c]
Fertile group (n = 25)				
Morphology (%)	26.3 ± 7.5	22.1 ± 6.4	22.1 ± 6.4	
Decrease in spermatozoa with normal morphology		−4.2 ± 0.6[e]	−4.1 ± 0.6[e]	p = 0.914[e]
Chromatin (%)	92.4 ± 8.4	87.2 ± 12.3	88.7 ± 11.2	
Decrease in spermatozoa with condensed chromatin		−5.5 ± 1.1[b,f]	−3.7 ± 1.2[d,f]	p = 0.140[f]

* Values are given as mean ± SD.
a = Significance of difference between the two freezing method with regard to morphological alteration of spermatozoa in the subfertile group (p = 0.002).
b = Significance of difference between the mean percentage of chromatin damage of fertile and subfertile group after freeze-thawing with liquid nitrogen vapour (p = 0.04).
c = Significance of difference between the two freezing method with regard to chromatin damage in the subfertile group (p = 0.069).
d = Significance of difference between the mean percentage of chromatin damage of fertile and subfertile group after freeze-thawing with computerized slow stage freezer (p = 0.023).
e = Significance of difference between the two freezing method with regard to morphology in the fertile group (p = 0.914).
f = Significance of difference between the two freezing method with regard to chromatin damage in the fertile group (p = 0.140).

after freeze-thawing using the computerized slow-stage freezer ($p = 0.001$) (Table 2). In Group 2 (semen samples with normal parameters) the percentage of chromatin condensed spermatozoa (92.4 ± 8.4) was also significantly ($p = 0.001$) decreased, both after freezing with static liquid nitrogen vapour (87.2 ± 12.3%) and also after freezing using the computerized slow-stage freezer (88.7 ± 11.2%). Moreover, the mean percentage of spermatozoa from subnormal semen samples (Group 1) with chromatin damage was significantly higher in comparison with those from normal semen samples, not only when freezing with liquid nitrogen vapour ($p = 0.04$) but also when using the computerized slow-stage freezer ($p = 0.023$) (Table 2).

Additionally, the mean percentage of morphologically normal spermatozoa decreased significantly ($p = 0.001$) after the freeze-thawing procedure (in both freeze-thawing methods) when compared with the value observed in the native semen sample or in the subnormal semen sample and in the normal semen samples in both freezing methods.

However, the mean percentage of morphologically normal spermatozoa observed in the subnormal semen sample (11.7 ± 6.1%) decreased significantly ($p = 0.002$) after employing static liquid nitrogen vapour (8.0 ± 4.9%) in comparison to the computerized slow-stage freezer (9.3 ± 5.6%). In contrast, the alteration to morphology of spermatozoa from normal semen samples after rapid freezing (static liquid nitrogen vapour), was comparable to that obtained with the computerized slow-stage freezer (from 26.3 ± 7.5 in the native samples to 22.1 ± 6.4% vs. 22.2 ± 4.6%, respectively, $p = 0.247$) (Tables 1 and 2).

Discussion

The most commonly described adverse effect of the freeze-thawing procedure is severe impairment of sperm motility (Critser *et al.*, 1988; Yoshida *et al.*, 1990). Emphasis has also been placed on altered sperm morphology, coiled tails, structurally damaged membranes and damaged acrosomes (Critser *et al.*, 1987; Check *et al.*, 1991). However,

the consequences of cryopreservation on the integrity of the sperm nucleus, chromatin stability and centrosomes are less clear. Normal condensation and stabilization of sperm chromatin in the nucleus, which allows safe transport of the male genome and decondensation after sperm penetration or injection into the cytoplasm of the oocyte are pre-requisites for fertilization (Flaherty *et al.*, 1995).

The results in this study showed that the morphology of spermatozoa decreased significantly not only by freezing with liquid nitrogen vapour (rapid freezing) but also by using a computerized slow-stage freezer. This decrease in morphologically normal spermatozoa was observed in subnormal semen as well as normal semen samples. Although a significant difference ($p = 0.002$) in sperm morphology was observed between the two freezing methods for subnormal semen specimens, the difference was not significant ($p = 0.914$) for normal semen samples. These results are in agreement with those of Serafini & Mars (1986) who also demonstrated that freezing and thawing of human semen significantly reduced the total number of spermatozoa with normal head ultrastructure, motility and capacity to fertilize. Moreover, Verheyen *et al.* (1993) demonstrated that for normal semen samples, vapour and computer controlled freezing were equally effective in terms of the recovery of morphologically normal spermatozoa.

The freezing protocol can also affect the motility of post-thaw spermatozoa. Hammitt *et al.* (1989) showed that controlled rate freezing and thawing at 40° resulted in significantly greater post-thaw sperm motility and motility index compared with non-controlled freezing and room temperature thawing. Check *et al.* (1996) also demonstrated that the percentage post-thaw motility was significantly higher when a slower cooling rate was used with a semi-programmable freezer (Cellevator, a device used to freeze lymphocytes) than with liquid nitrogen vapour freezing, as was the mean percentage of spermatozoa showing changes in hyperosmotic swelling. However, Morrell *et al.* (1990) demonstrated that donor semen samples, which were divided into two alquots, that were then either frozen in a 1-mL tuberculin syringe by plunging directly into liquid nitrogen or in straws in a controlled-rate freezer, showed no significant difference in post-thaw motility ($p = 0.217$) or survival ($p = 0.217$) after 30 min.

The mean percentage of spermatozoa with condensed chromatin decreased significantly in fertile men with normal semen samples when using either the computerized slow-stage freezer or liquid nitrogen vapour. In the subnormal semen samples, the percentage of condensed chromatin also decreased significantly after freeze-thawing using either static liquid nitrogen or the computerized slow-stage freezer. However, the computerized slow-stage freezing technique preserved the chromatin from cryodamage better than did the static liquid nitrogen technique not only in the group with normal semen quality but also in the group with subnormal semen quality. These results are contrary to those obtained by Huret & Miquereau (1984), who reported that spermatozoa cryopreserved for subsequent artificial insemination, showed a nuclear stability equivalent to that of fresh semen. However, our findings are in agreement with Royere *et al.* (1988) and Hammamah *et al.* (1990), who also noticed a post-freeze/thaw decrease in chromatin stability assessed by acridine orange and Feulgen-DNA. These data also confirm our previous findings in showing that the freeze-thaw procedure significantly affects chromatin structure and sperm morphology (Hammadeh *et al.*, 1999).

A difference between fertile and infertile men with respect to the percentage of unstable sperm nuclei was also reported by Eliasson & Enquist (1981). Hughes *et al.* (1996) have shown that the DNA of spermatozoa produced by infertile men is more susceptible to damage by irradiation than is the DNA of spermatozoa from fertile men. In the present study, the mean percentage of chromatin damage in the semen samples of the subfertile group was significantly higher than that observed in semen samples of the fertile group after freezing with static liquid nitrogen vapour as well as using computerized slow-stage freezing.

In conclusion, the present results demonstrate that the percentage of morphologically normal and chromatin condensed spermatozoa in subnormal and normal semen samples decreased significantly after the freeze-thawing procedure when using liquid nitrogen vapour or a computer controlled slow-stage freezer. Although normal semen samples withstood the freeze-thaw injury significantly better than did subnormal semen samples with respect to sperm chromatin, neither static liquid nitrogen vapour nor the computerized slow-stage freezing technique protected sperm chromatin sufficiently from cryodamage. Therefore, the computerized slow-stage freezer can be recommended for freezing of human spermatozoa, especially those with initially low quality semen specimens in order to avoid further damage of sperm chromatin.

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