Evaluation of Cryoinjury of Spermatozoa After Slow (Programmed Biological Freezer) or Rapid (Liquid Nitrogen Vapour) Freeze–Thawing Techniques

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Purpose: *This study was initiated to determine the negative effect (cryodamage) on human spermatozoa after freeze–thawing and to find out whether freezing of spermatozoa with a computerized biological freezer is more advantageous than freezing above static liquid nitrogen vapour with regard to spermatozoa vitality, chromatin normality, morphology, and membrane integrity.*

Methods: *Forty-four semen samples were obtained from patients attending andrology laboratory, and each sample was divided into two aliquots. One aliquot was frozen using static liquid nitrogen vapour (G.II) and the second with a computerized biological freezer (G.III). Acridine orange was used for assessment of chromatin cryoinjury, whereas the morphology was evaluated according to WHO criteria. Hypoosmotic swelling test was used to identify membrane integrity and eosin–nigrosin staining was used to determine the vitality of spermatozoa.*

Results: *The mean percentage of normally condensed chromatin in the native semen sample (G.I) decreased significantly ($p < .001$) after freeze–thawing by using either liquid nitrogen vapour (G.II), or a biological freezer (G.III), which was significantly higher ($p < .001$) after freezing with liquid nitrogen vapour than after freezing with the biological programmed freezer. Morphologically normal spermatozoa decreased significantly ($p < .001$) in both freezing methods in comparison to the native semen samples. In addition, membrane integrity of spermatozoa (HOS-test positive) was significantly lower ($p < .001$) after the freeze–thawing procedure in G.II and G.III compared to G.I. In both these parameters the deterioration was similar among the two freezing procedures. Finally the mean percentage of live spermatozoa decreased significantly ($p < .001$) in both freezing techniques in relation to the mean value in the neat semen samples.*

Conclusions: *Freeze–thawing procedure has a detrimental effect on chromatin, morphology, membrane integrity, and vitality of human spermatozoa not only by freezing above static liquid nitrogen vapour but even by using a computerized biological freezer. However, the chromatin deterioration rates are significantly higher by freezing above static liquid nitrogen vapour in comparison to freezing with a programmed biological freezer. Therefore, we recommend the use of this technique for freezing semen especially when ICSI technique is considered as the main therapeutic procedure.*

KEY WORDS: computerized biological freezer; freeze–thawing procedure; spermatozoa chromatin and morphology; static liquid nitrogen.

INTRODUCTION

Spermatozoa represent the first cell type to be successfully frozen and thawed (1). Nevertheless, the successful cryopreservation of cells is affected by the rate of freezing and the composition of the solution in which the cells are frozen (2,3). Therefore, the use of optimum cooling rates is also critical for cryopreservation of many cell types as it influences the extent and rate of dehydration. The cryodamage of spermatozoa can be reduced dramatically in many species by cooling semen slowly; so most procedures utilize an initial slow cooling rate ($\leq 10^{\circ}$C/h) between the temperature at which the sample is collected and $+5^{\circ}$C (4–6). As cells are cooled to about -5°C, both cells and

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the surrounding medium remain unfrozen and supercooled. Between −5 and −10°C ice forms in the external medium but the cell contents remain unfrozen and supercooled; the supercooled water in the cells has a higher chemical potential than that of water in the partially frozen extracellular solution, and thus water flows out of the cells osmotically and freezes externally.

It becomes obvious that the cooling rate will affect the subsequent physical events within the cells and determine the outcome of the freezing process. If cells are cooled rapidly, water is not lost fast enough to maintain equilibrium; the cells become increasingly supercooled eventually attaining equilibrium by freezing intracellulary (7). In most cases, cells undergoing intracellular ice formation are killed (8–10). If cooling is slow, the cells will lose water rapidly enough to concentrate the intracellular solutes sufficiently to eliminate supercooling. As a result, cells dehydrate and do not freeze intracellulary. If cells are cooled too slowly, they will experience a severe volume shrinkage and long-time exposure to high solute concentrations before eutectic is achieved. Both factors could cause cell injury. As in other cells, plots of the survival of sperm versus the cooling rate during the freezing are in the form of an inverted "U" (11–13).

Theoretically, any relatively slow cooling rates that ensures less than 2°C intracellular supercooling and produce 90% loss of isotonic intracellular water before the cells have cooled to the intracellular ice nucleation temperature should prevent intracellular freezing (7).

However, cooling may result in latent injury not manifested by conventional laboratory assays, which generally are not reliable measures of fertility. While there is some evidence that cytoskeletal element of the sperm may be sensitive to cooling (14), several reports have also shown that the freeze–thawing process reduces the metabolic state of spermatozoa and damages their plasma membrane, resulting in a small number of functional spermatozoa available for assisted reproduction (15,16). Other investigators have placed more emphasis on sperm morphology alteration, coiled tails, damaged membranes, and damaged acrosomes (17–20). More sensitive criteria than the objective assessment of motility, such as energy status, damage to the plasma membrane or to subcellular elements, chromatin stability, and chromosomal damage, have been proposed as complementary ending points to better assessment of sperm cryopreservation (21). However, the consequences of the cooling technique on chromatin integrity and morphology of spermatozoa are not clear. Therefore, this study was undertaken "firstly" to determine the relationship between the cooling rate either with a computerized biological freezer or above static liquid nitrogen vapour and the cryodamage of human spermatozoa of fertile men after the freeze–thawing procedure and "secondly" to find out which cryopreservation technique is superior with regard to preservation of chromatin, morphology, vitality, and membrane integrity of spermatozoa from cryodamage.

MATERIALS AND METHODS

Human ejaculated spermatozoa were obtained by masturbation from patients and donors, attending our assisted reproduction unit and andrology laboratory for semen analysis ($n = 44$). The semen samples were liquefied at room temperature. A basic semen analysis was performed within 20–30 min after delivery according to WHO guideline (22). After the examination of the semen, each sample was divided into two aliquots. The semen sample was mixed with the cryoprotectant containing glycerol, glycin, and glucose (human sperm preservation medium (HSPM, 1 : 1). The semen–cryoprotectant mixture was drawn into 0.25 mL straws. The first aliquots were frozen by holding the straws horizontally 20 cm above the liquid nitrogen liquid (static liquid nitrogen vapour) for 20 min and the second was frozen in a computerized biological freezer (Planer Kryo 10 series III) according to the following cooling rate program:

(i) −1C°/min from +22C° to +5C°
(ii) −10C°/min from +5 to −80C°
(iii) −25C°/min from −80C°to −130C°
(iv) Plunged into liquid nitrogen (LN)

The samples were thawed later by removing them from liquid nitrogen (LN) and putting them into the water bath (37C°) for 2–4 min. Smears were made from each sample before freezing and after thawing without washing, to remove the cryoprotectant for the evaluation of the investigated parameters.

Assessment of Sperm Morphology

A small drop of semen was used to prepare a very thin smear for the assessment of sperm morphology as described (23). The slides were air-dried and fixed in ethanol before being stained. The Papanicolaou staining method was applied according to

standard procedure as described in the WHO manual (22). Sperm morphology was evaluated by one observer, according to WHO criteria (22) and taking into consideration the specific effects, e.g. size alteration, of the specific staining method (22). Bright field illumination and a magnification of 100× under an oil immersion objective was used for the evaluation. For each semen sample 100–200 spermatozoa were evaluated.

Assessment of Chromatin Injury (Acridine-Orange Staining)

Nuclear integrity of the spermatozoa was assessed by acridine-orange fluorescence method (24). Different smears were fixed overnight in freshly prepared Carnoy's solution (3 parts of methanol and 1 part glacial acetic acid) and let to air-dry for a few minutes before being stained with acridine orange (Sigma, Germany). The smears were stained for 5 min, gently rinsed under tap water, and mounted. The slides were evaluated immediately using a fluorescence microscope (Leitz, Oberkochen, Germany) equipped with a 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 were evaluated per smear.)

Hypo-Osmotic Swelling Test (HOS)

The HOS test was performed, before and after the freeze–thawing procedure, according to the method described by Jeyendran *et al.* (25). It was performed by mixing 0.1 mL of sperm suspension with 1 mL hypo-osmotic solution (equal parts of 150 mOsmol fructose and 150 mOsmol sodium citrate solutions) followed by 60-min incubation at 37C°. After incubation, one drop was observed in a light microscope to evaluate the results of the test. A minimum of 200 spermatozoa were examined in each sample.

Eosin–Nigrosin Test (Vitality Assessment)

The eosin–nigrosin test in the present study was used as described by Eliasson and Treich (26). Briefly one drop of semen was mixed on a slide with one drop of the 0.5% aqueous yellowish eosin solution and covered with a cover slip. After 1–2 min, the spermatozoa were stained red (dead spermatozoa), which were distinguished from the unstained spermatozoa (alive). Nigrosin (10% in distilled water) was used as a counterstain to facilitate visualization of the unstained live cells (27).

Statistical Analysis

Because not all variables are symmetrically distributed, nonparametrical analysis was applied for all variables. The variables were presented as mean± standard deviation (SD). Parameter were compared by the Wilcoxon Signed-Ranks Test in paired samples. A p value of $\leq .05$ was considered to be statistically significant.

RESULTS

The mean percentage of chromatin condensed spermatozoa in the neat semen samples was $(90.1 \pm 6.2)\%$ and decreased significantly ($p < .001$) to $(74.7 \pm 8.6)\%$ by using liquid nitrogen vapour ($p < .001$) and to $(78.3 \pm 8.9)\%$ after freeze–thawing with computerized biological freezer (Table I). There is a statistically significant difference ($p = .001$) in the percentage of condensed chromatin spermatozoa between the native and the freeze–thawed samples. There is also a statistically significant difference ($p = .001$) between the two freezing methods used (Table II). Furthermore, the mean percentage of morphologically normal spermatozoa in the native semen samples, $(47.2 \pm 17.8)\%$, decreased significantly ($p = .001$) after freeze–thawing in both of the methods employed. The percentage became $(30.5 \pm 12.2)\%$ ($p < .001$) after the use of liquid nitrogen vapour

Table I. Alteration of Some Sperm Parameters After Two Freeze–Thawing Techniques ($n = 44$; Means ± SD)

	Group I (Native semen)	Group II (Liquid nitrogen vapour)	Group III (Biological Programmed freezer)
Chromatin (Acridine orange)	90.1 ± 6.2	74.7 ± 8.6	78.3 ± 8.9
Morphology (Papanicolaou)	47.2 ± 17.8	30.5 ± 12.2	29.8 ± 12.1
Hypo-osmotic swelling (Test)	52.4 ± 19.3	36.5 ± 15.1	36.1 ± 16.2
Vitality (Eosin)	50.9 ± 18.2	19.5 ± 10.2	22.2 ± 10.6

Note. All values are given in percentages.

Table II. Significance of Difference Between All Investigated Groups with Regard to Cryodamage of Human Spermatozoa (p-Value)

	Chromatin (Acridine orange)	Morphology (Papanicolaou)	HOS test	Vitality (Eosin)
G.I–G.II	<.001 (h.s.)	<.001 (h.s.)	<.001 (h.s.)	<.001 (h.s.)
G.I–G.III	<.001 (h.s.)	<.001 (h.s.)	<.001 (h.s.)	<.001 (h.s.)
G.II–G.III	<.001 (h.s.)	<.7 (n.s.)	<.47 (n.s.)	<.022 (s.)

Note. G.I = native semen; G.II = after cryo with liquid nitrogen vapour; G.III = after cryo with biological programmed freezer; h.s. = high significance; s. = significance; n.s. = no significance.

and $(29.8 \pm 12.9)\%$ ($p < .001$) after the computerized biological freezer. However the difference between the two freezing methods was not significant ($p = .07$).

The HOS test (membrane integrity) exhibited the same change after the freeze–thawing as the morphology. In other words there was a statistically significant reduction ($p < .001$) in the percentage of spermatozoa with intact membrane, which was not significant between the two methods ($p = .47$) (from $(52.4 \pm 19.9)\%$ in the native semen to $(36.5 \pm 15.1)\%$ after freezing with above liquid nitrogen vapour and to $(36.1 + 16.2)\%$ with the computerized biological freezer). The recovery rate of vital spermatozoa was significantly lower ($p = .001$) after freeze–thawing procedure either above liquid nitrogen vapour, $(19.5 + 10.2)\%$, or computerized biological freezer, $(22.2 + 10.6)\%$, in comparison to the value observed in the native semen samples, $(50.9 + 18.2)\%$, (Table I). However, computerized biological freezer preserve the vitality of spermatozoa significantly better ($p = .022$) than liquid nitrogen vapour method (Table II).

DISCUSSION

Despite advances in cryopreservation techniques, postthaw sperm recovery remains poor (28). The liquid nitrogen vapour technique for the freezing of human semen with subsequent storage at $-196^{\circ}C$ is in widespread use today (29). Although many investigators have shown that the computerized controlled freezing methods preserve sperm quality better than liquid nitrogen vapour freezing (30–32), others have not confirmed it, at least for human spermatozoa (33–35).

In this study the mean percentage of spermatozoa that were alive, with functional membrane, normal chromatin, and morphology in the native semen, decreased significantly ($p < .0001$) after the freeze–thawing procedure (Table I). The mean percentage of vital spermatozoa (eosin–nigrosin test) in the native semen specimens was $(50.9 \pm 18.2)\%$ and decreased to $(19.5 \pm 10.2)\%$ after freeze–thawing in liquid nitrogen vapour and to $(22.2 \pm 10.6)\%$ by employing the computerized freezer. Besides, the mean percentage of spermatozoa with functional membranes (HOS-test positive) in the native semen samples, $(52.4 \pm 19.3)\%$, decreased significantly either with static liquid nitrogen vapour, $(36.5 \pm 15.1)\%$, or computerized controlled freezer, $(36.1 \pm 16.2)\%$, (Table I). Although a significant difference ($p = .022$) was observed for sperm vitality between the freezing methods, this was not the case for membrane integrity ($p = .47$).

These findings are in consistence with the results of Check *et al.* (36) who have reported that sperm vitality was decreased from 70.0 to 33.7% postthaw possibly due to membrane damage. Regarding sperm membrane integrity a decrease of 36% (37) and 16% (38) in the population of sperm with swollen tails has been recorded. Esteves *et al.* (39) observed that only 16–20% of sperm were HOS-test positive after thawing of the cryopreserved samples and the other 74–80% was damaged. Furthermore, Check *et al.* (40) demonstrated that the mean percent of sperm showing HOS changes was significantly higher when cryopreserved with a slower cooling rate using semiprogrammable freezer (Cellevator, a device used to freeze lymphocytes) than with liquid nitrogen vapour freezing, as was sperm motility.

The acridine-orange test revealed that the freeze–thawing procedure also causes deterioration of the chromatin structure of spermatozoa. The mean percentage of normal condensed chromatin spermatozoa, $(90.1 \pm 6.2)\%$, decreased to $(78.3 \pm 8.9)\%$ after freezing with a biological freezer, and to $(74.7 \pm 8.6)\%$ after freezing above liquid nitrogen vapour. However, the mean percentage of the cryoinjury was significantly higher ($p = .001$) after freeze–thawing with static liquid nitrogen vapour in comparison to that with computerized controlled freezer (Table II). These results are in accordance with those of Royere *et al.* (41) and Hamamah *et al.* (42) who showed that the normal DNA content decreased and the sperm nuclear surface area was reduced, which might delay

paternal nuclear decondensation during fertilization. They hypothesized a relationship between an "over-condensation" state of sperm chromatin after freeze–thawing and a lower conception rate for human semen after cryostorage.

Furthermore, many reports have described an association between disturbances in morphology and male infertility (43,44). The present study demonstrated a significant ($p = .001$) decrease of the mean percentage of morphologically normal spermatozoa not only by using liquid nitrogen vapour but also by employing the computerized biological freezer (Table I). The mean percentage of morphologically normal spermatozoa decreased from $(47.2 \pm 17.8)\%$ to $(30.5 \pm 12.2)\%$ and $(29.8 \pm 12.1)\%$, respectively. However, the reduction of the mean percentage of sperm morphology was equivalent for both freezing methods employed ($p = .7$) (Table II). This is in good agreement with our previous study (45) as well as results from Verheyen *et al.* (33) who demonstrated that motility and morphology of normal ejaculated semen was reduced by 50% after cryopreservation in comparison to native semen. Nogueira *et al.* (46) investigated the morphological changes caused by freezing and thawing human testicular spermatozoa, using light and electron microscopy. They concluded that cryopreservation of human testicular spermatozoa within its tissue structure causes similar damage to membrane and to the acrosome as seen in ejaculated spermatozoa.

In view of these findings, and those previously reported (47–49), it seems that vitality, chromatin normality, morphology, and membrane integrity of spermatozoa underwent a severe alteration under the freeze–thawing technique, which might influence their capability to fertilize, the oocyte in assisted reproduction technique and this may explain the lower fertilization rate for human semen after cryopreservation in an IVF/ICSI program. Pederson and Lebech (50), as well as Keel *et al.* (51), suggested that the decreased fecundity of cryopreserved sperm is due to structure damage, which resulted in decreased post-thaw survival and motility. Moreover, the present study suggest that vitality and chromatin integrity of spermatozoa were preserved better by using a computerized biological freezer in comparison to liquid nitrogen vapour. This is in agreement with reports from other laboratories (52,53). On the other hand, the cryodamage of sperm morphology and membrane integrity as detected by the HOS test was similar after freezing either with the computerized controlled freezer or static liquid nitrogen vapour, $(29.8 \pm 12.1)\%$ vs. $(30.5 \pm 12.2)\%$ and $(19.5 \pm 10.2)\%$ vs $(22.2 \pm 10.6)\%$ respectively. These results are in accordance with those of Verheyen *et al.* (33) who also found that for semen samples of high quality, liquid nitrogen vapour and computer-controlled freezing were equally effective in terms of recovery of morphologically normal and motile spermatozoa.

CONCLUSION

This study demonstrates that the mean percentage of spermatozoa that are alive with normal morphology, condensed chromatin, and functional membrane decrease significantly after freeze–thawing in comparison to the percentage observed in the native semen samples. However, the mean percentage of cryodamage of normally condensed chromatin and vitality of spermatozoa were significantly higher after freezing with static liquid nitrogen vapour in comparison to that registered by employing computerized controlled biological freezer. Therefore, in assisted reproduction technology, the programmed biological freezer might be recommended for freezing semen samples in order to avoid further reduction of vital spermatozoa and preserve chromatin, especially in patients undergoing ICSI therapy.

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