Effects of cryopreservation on human sperm deoxyribonucleic acid integrity

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Objective: To evaluate the effect of cryopreservation on sperm motility and viability and to assess sperm DNA fragmentation and oxidation in men undergoing infertility investigation before and after cryopreservation in liquid nitrogen.

Design: Analysis of cryopreservation effects on sperm DNA integrity.

Setting: Laboratory of Histology–Embryology of medicine faculty, Sfax, Tunisia.

Patient(s): Fifteen semen samples from men undergoing infertility investigation.

Intervention(s): Neat semen samples were cryopreserved in liquid nitrogen using a commercial freezing medium (SpermFreeze, Fertipro, Belgium) according to the manufacturer’s instructions. Samples were thawed at room temperature.

Main Outcome Measure(s): Sperm DNA fragmentation was assessed using terminal deoxynucleotidyl transferase (Tdt) mediated dUTP nick end labeling and sperm DNA oxidation was determined using a fluorescent assay (OxyDNA test) for the detection of 8-oxoguanine. Evaluation of DNA fragmentation and oxidation rates was carried out before and after cryopreservation using flow cytometry.

Result(s): A significant decrease in sperm motility and viability was observed after cryostorage. In addition, sperm DNA fragmentation and DNA oxidative damage increased significantly after cryopreservation/thaw.

Conclusion(s): Cryopreservation has deleterious effects on sperm DNA by inducing DNA fragmentation and oxidation but the mechanisms underlying such damages need to be elucidated by further investigations. (Fertil Steril® 2010;93:159–66. ©2010 by American Society for Reproductive Medicine.)

Key Words: Cryopreservation, human sperm, DNA integrity, 8-oxoguanine, TUNEL assay, oxidative stress

Progress in assisted reproductive techniques (ART) has enhanced the use of frozen-thawed semen. In fact, radiotherapy, chemotherapy, some malignant diseases, or even invasive surgery can induce testicular failure or ejaculatory dysfunction (1, 2). Semen cryostorage is the only proven method that may offer couples a chance to have children in the future after such conditions. Sperm cryostorage is also indicated in ART programs in case of donor insemination procedure or for men with severe oligozoospermia likely to deteriorate.

Nevertheless, cryopreservation is known also as a cell damaging procedure and may lead, due to temperature variations, to deleterious changes in sperm structure (e.g., membrane, mitochondria, DNA) (2–5). Therefore, sperm can lose its function and some of its fertility potential. It was largely reported that freezing-thawing results in decrease in sperm viability and motility (6–8) and, the impact of cryostorage on sperm DNA was also investigated in many studies, but results remain controversial. In fact, in some studies, sperm DNA integrity was altered after cryopreservation/thaw (8–10), whereas it was not affected in other studies (4, 7).

Evaluating of the impact on sperm chromatin after cryopreservation is of extreme importance as DNA integrity is essential in achieving fertilization and embryo development; it is crucial to guarantee the success of ART (11, 12).

Oxidative stress was studied as a possible mechanism for the origin of sperm DNA damage. It was widely reported that reactive oxygen species (ROS) are associated with poor semen quality and defective functional competence of human spermatozoa (13, 14). Reactive oxygen species are also known to play an important role in the pathophysiology of damage to human spermatozoa (15, 16). Therefore, it is thought that sperm DNA damages including DNA fragmentation, base modifications, chromatin cross-linking, and other alterations, are linked to oxidative stress (15–17). Among all of these alterations, DNA fragmentation was the most studied and associated with male infertility and sperm parameters defects (11, 13, 15, 18), but its origin is still unclear. It could be attributed to oxidative stress but also to apoptosis and defective chromatin packaging (13, 15, 19). Terminal deoxynucleotidyl transferase (Tdt) mediated dUTP nick end labeling is one of the most used tests to detect DNA fragmentation (11, 18–21) either with fluorescence microscopy or by flow cytometry (21).
The oxidative DNA biomarker 8-oxoguanine commonly used to evaluate oxidative DNA alterations due to its high specificity and sensitivity, relative abundance in DNA, and potent mutagenicity (22). This oxidative DNA damage is a result of interaction of DNA with ROS, in particular, the hydroxyl radical. In the presence of oxygen guanosine is hydroxylated and transformed to 8-oxoguanine (23). Detection of this oxidized DNA base remains the best direct assessment of sperm DNA oxidative damage (24) and it can be analyzed by high-performance liquid chromatography (HPLC) or by an OxyDNA assay (12, 22).

In this study, which aimed to investigate the effect of cryopreservation of human semen on sperm motility, viability, and DNA integrity, we used the TUNEL assay and the OxyDNA assay to assess DNA fragmentation and DNA oxidation before and after cryostorage in liquid nitrogen.

MATERIALS AND METHODS

Patients

This study was approved by the Institutional Review Board (IRB) of Medicine Faculty of Sfax, Tunisia.

Semen specimens were obtained from 15 men attending the Laboratory of Histology–Embryology of Medicine Faculty of Sfax, Tunisia, for infertility investigations. The men were aged 29–47 years. Men with azoospermia or severe oligoospermia were excluded from the study. According to the World Health Organization (WHO) criteria (25), semen samples with a sperm count of 20 × 10^6/mL and motility of ≥50% were considered normal.

Semen Analysis

Semen samples were obtained by masturbation into sterile containers after 3–5 days of sexual abstinence and left to liquefy at 37°C. Basic semen analysis were performed within 1 hour of collection and consisted of the measurement of semen volume and sperm concentration, motility, viability, and morphology. All of the parameters were carried out according to the WHO guidelines.

Semen Cryopreservation

Liquefied semen samples were cryopreserved by a standard protocol using SpermFreeze, a commercial cryoprotectant consisting of 15% glycerol in HEPES buffer (Fertipro, Belgium). Two aliquots (200 μL) of each sample were diluted (1:0.7) with freezing medium in a dropwise manner. After 10 minutes of equilibration at room temperature, the mixture was frozen in liquid nitrogen vapor for 15 minutes and plunged into liquid nitrogen (-196°C) in cryovials (Nunc International, Roskilde, Denmark) for storage. Samples were cryopreserved in liquid nitrogen for a minimum period of 7 days.

After cryostorage, the specimens were thawed at room temperature for 15 minutes, then they were immediately analyzed for motility, viability, and DNA fragmentation and oxidation assessment.

TUNEL Assay

A commercial kit (In situ Cell Death Detection Kit, Fluorescein, Roche, Germany), based on an enzymatic reaction of labeling free 3'-OH termini, was used for the evaluation of DNA fragmentation. We followed the manufacturer’s instructions with little modifications: briefly, 3 × 10^6 cells were washed with phosphate-buffered saline (PBS; pH 7.4), then fixed with 200 μL of 4% paraformaldehyde for 1 hour at room temperature. After that, sperm cells were washed with 1 × PBS and permeabilized using 0.1% Triton X-100 in 0.1% sodium citrate for 15 minutes on ice. After washing with PBS, sperm DNA was labeled by incubating spermatozoa with 50 μL of the TUNEL reaction mixture (Tdt enzyme and fluorescein isothiocyanate conjugate [FITC]-labeled nucleotides) in a humidified atmosphere for 60 minutes at 37°C in the dark, with mixing each 15 minutes. Washed and labeled sperm cells were then resuspended in 1 × PBS for flow cytometry analysis. The EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA) was used with an argon ion laser at 488 nm. Analysis of the scatter properties (forward-angle light scatter vs. side-angle light scatter) was performed to gate spermatozoa and exclude debris and other cell types. For each sample, 10,000 events were examined and the percentage of TUNEL-positive cells were estimated from the histograms using the System II software (Coulter). A negative control (sample without the addition of the Tdt enzyme) and a positive control (sample treated with DNase I, 3 U/mL, Invitrogen (Invitrogen, Carlsbad, CA), for 10 minutes at room temperature to generate DNA strand breaks) were also assessed by TUNEL assay. For each semen sample, sperm DNA fragmentation was evaluated before and after cryostorage.

Measurement of 8-Oxoguanine Levels by Flow Cytometry

We used the OxyDNA kit (Biotrin International, Dublin, Ireland), which is specific for detection of 8-oxoguanine as one of the major studied oxidized nucleotides. The test is based on the direct binding of a fluorescent probe (FITC conjugate) to DNA adduct 8-oxoguanine. We used the method described previously by Meseguer et al. (12), with little modifications. One aliquot of each semen sample containing 3 × 10^6 spermatozoa was washed with 1 × PBS, fixed, permeabilized with ice-cold 70% ethanol, and kept for 1 hour at -20°C. Fixed cells were centrifuged at 1,600 rpm for 5 minutes, washed with PBS, resuspended in 1 mL of wash solution (Tris-buffered saline/Tween 20 containing thimerosal), and pelleted at 1,600 rpm for 5 minutes. Twenty-five microliters of diluted (1:9) blocking solution (included in the kit) were added to sperm cells for 1 hour at 37°C to block nonspecific binding sites. After washing with 1 mL of wash solution, 50 μL of FITC conjugate were incubated for 1 hour with pelleted sperm cells in the dark at room temperature, with mixing each 15 minutes. Finally, washed cells were resuspended in 500 μL of 1 × PBS and analyzed in a flow cytometer equipped
with FITC filters (EPICS XL flow cytometer; Beckman Coulter). Debris was excluded by the analysis of the scatter assay properties. At least 10,000 events per sample were analyzed. Data were expressed as percentage of stained cells from histograms using System II software.

As a positive control, we used an infertile man’s semen sample that was washed in 1× PBS then incubated in H₂O₂ (4 M) solution for 1 hour at 37°C. The negative control consisted of selected spermatozoa (3 × 10⁶) from healthy men. For each semen sample, oxidative DNA damage was measured before cryostorage and after thawing.

### Statistical Analysis

The SPSS (SPSS Inc., Chicago, IL) software (version 13) was used for statistical analysis. A nonparametric test (Wilcoxon test) from SPSS software (version 13) was used to compare sperm parameters and flow cytometry data before and after cryopreservation. The test was considered statistically significant when \( P < .05 \). The relationship between DNA oxidation and DNA fragmentation was analyzed using linear regression.

### RESULTS

#### Semen Analysis

Within the studied group, 5 patients were considered subjects with normal semen and 10 patients were considered subjects with abnormal semen.

The mean values (±SD) for semen parameters were as follows: volume 3.5 ± 1.2 mL (range 3.0–6.0 mL), sperm concentration 74.7 ± 42.4 × 10⁶/mL (range 11.7–142 × 10⁶/mL), motility 45% ± 7% (range 25%–55%), viability...
77.2% ± 8.9% (range 60%–90%), and normal morphology 10% ± 5.5% (range 2%–22%). Sperm motility and viability decreased significantly after cryopreservation (30.6% ± 12.2% vs. 45% ± 7%; \( P = .001 \) and 45.6% ± 8.9% vs. 77.2% ± 8.9%; \( P = .001 \), respectively).

DNA Integrity
There was no relationship between DNA oxidation and DNA fragmentation in the studied group (\( r = 0.19; P = .26 \)) after adjustment of sperm motility and count.

**DISCUSSION**
Cryopreservation of human sperm is a useful tool in ART, although it has detrimental effects on sperm competence. Decrease in sperm motility is the most reported effect and our study supports this finding (26, 27). The mechanism behind this damage is still unclear but may have a physical–chemical etiology. It may be related to osmotic stress and...
intracellular ice crystals formation during cryopreservation (28, 29), but also to osmotic damage due to extensive cell shrinkage (7). It was also hypothesized that cryopreservation of sperm induces significant damage to sperm chromatin, morphology, and membrane integrity in both fertile and infertile men (30).

Because cryopreservation may have deleterious effects on sperm chromatin, evaluation of the DNA integrity of frozen/thawed spermatozoa is of great interest. Many techniques evaluating DNA integrity have been driven with the growing use of ART and the awareness that sperm DNA integrity has a key role in IVF (31). In this work, we used two techniques to evaluate the influence of cryopreservation on sperm DNA integrity (TUNEL and OxyDNA assays). We found that cryostorage in liquid nitrogen of neat semen from infertile men induced a significant increase both in sperm DNA fragmentation and oxidation.

Before cryopreservation, abnormal semen exhibited higher levels of DNA fragmentation than normal semen, which is in agreement with the studies of Lopes et al. (11) and Muratori et al. (20) showing that spermatozoa from patients with abnormal semen motility and morphology had increased levels of DNA damage as assessed by TUNEL assay after swim-up selection. These findings could be explained by the presence of a high percentage of immature spermatozoa with several abnormalities, including DNA defects, in men with abnormal semen parameters (20). It was also reported that infertile men have a greater percentage of spermatozoa with fragmented DNA than semen from fertile men (4, 13, 18). Concerning oxidative DNA damage, our results did not show any significant difference between normal and abnormal semen before cryopreservation. Other studies reported that levels of 8-hydroxy-2'-deoxyguanosine (using HPLC) in ejaculated sperm DNA were significantly higher in infertile men than in control patients (17, 22).

After cryopreservation, both DNA fragmentation and oxidation increased significantly in the entire studied group, but only the increase in DNA fragmentation remained significant in the normal and abnormal semen groups.

In the literature results concerning cell damage induced by cryopreservation are conflicting. Bell et al. (32) found that cryopreservation of sperm from infertile men induced an

![Flow cytometric 8-oxoguanine detection histograms](image-url)
increase in lipid peroxidation, which could be the result of loss of peroxidation protective enzyme activity (33) and excess of ROS production. In contrast, some investigators did not find a significant enhancement in lipid peroxidation of sperm after cryopreservation (3, 34, 35).

Furthermore, sperm DNA damage has been found to be increased after cryopreservation/thaw (8, 10), whereas it was not affected in other studies (4, 7, 27). This controversy can be explained: the procedures of cryopreservation, the eventual preparation of semen before cryostorage and the tests evaluating DNA integrity differ from one work to another. It was reported that sperm DNA fragmentation was not affected by cryopreservation of highly motile spermatozoa selected by a discontinuous Percoll gradient separation (4). In contrast, Donnelly et al. (9) reported a deleterious effect on sperm DNA after cryostorage of neat or prepared semen by swim-up procedure; however, the addition of seminal plasma to prepared sperm improved DNA integrity after cryopreservation.

Sperm DNA damage has been associated with high levels of ROS in fresh and cryopreserved/thawed semen (31, 36, 37). In a recent study by Li et al. (37), supplementation of antioxidants (ascorbate and catalase) reduced ROS levels and sperm nuclear DNA damage (assessed by comet assay), and improves the human sperm quality in the process of freezing/thawing. In addition, Gadea et al. (38) reported that the addition of glutathione (L-gamma-glutamyl-L-cysteinylglycine) to the thawing medium resulted in a reduction in ROS generation and in DNA fragmentation, and improved the fertilizing capacity of frozen bull spermatozoa.

The connection between oxidative stress and DNA damage has been previously suggested (13, 39). It was established that ROS have the ability to disturb nuclear and mitochondrial DNA, including fragmentation, base modifications, deletions, and other alterations (40). Normally, sperm DNA packaging by protamines protect it from ROS assault; however, deficiency in protamination in infertile men makes sperm DNA vulnerable to ROS attack (41).

Nevertheless, oxidative stress is not the only mechanism advanced to explain the origin of DNA damage, two other molecular mechanisms of sperm damage are proposed: defective chromatin packaging and apoptosis (15, 31, 42). All of these mechanisms could be concomitant leading to DNA damage. It was reported that free radicals could induce apoptosis and lead to sperm DNA degradation through caspase activation (13, 36).

In the present study there was no relationship between DNA fragmentation and DNA oxidation, and the pronounced increase in DNA fragmentation induced by sperm cryostorage in liquid nitrogen was associated with a slight increase in percentage of sperm exhibiting DNA oxidation in the entire studied group. Thus, we suggest that cryopreservation/thaw induces sperm DNA fragmentation through other pathways, besides oxidative stress, such as enhancement of defects already present in sperm cells (2), leading to the activation of apoptosis enzymatic pathways, or defects in DNA repairing enzymes, inducing an increase in apoptotic cells.

Although DNA fragmentation is considered a late marker of programmed cell death, it can not distinguish between apoptosis and necrosis (43). Hence, it should be complemented by the detection of other apoptotic markers such as phosphatidyl serine externalization (44), Fas expression (45, 46), but also the presence of caspases that can be activated, particularly after cryopreservation/thaw (47).

In the literature there is no data about the effect of cryopreservation on the structure of DNA bases. It was reported that levels of 8-hydroxy-2′-deoxyguanosine were increased in spermatozoa of infertile men in comparison with control.
patients, correlated to the number of sperm cells with head defects and inversely correlated to sperm density, total sperm number, sperm motility, and the number of sperm cells with normal morphology (17, 22). Furthermore, an increase in sperm oxidative DNA damage was correlated with poor blastocyst formation in vitro (12, 48).

Actually there is clinical evidence to show that sperm DNA damage is detrimental to reproductive outcomes and extensive data exist on the relationship between DNA damage and ART outcomes, particularly on pregnancy rates (PR) (31, 40).

In conclusion, our study showed that cryopreservation has deleterious effects on sperm DNA including DNA fragmentation and oxidation. To our knowledge this is the first study that evaluates sperm DNA oxidation after cryopreservation, as assessed by the detection of 8-oxoguanine.

Although the origin of cryopreservation-induced DNA damages is not well elucidated, we can presume that DNA fragmentation would be attributed to an ROS assault but also to an apoptotic mechanism, whereas DNA oxidation is mainly a consequence of oxidative stress.

Further investigations of the apoptotic process and oxidative stress should be conducted to elucidate the mechanisms by which cryopreservation/thawing affect sperm DNA integrity.

REFERENCES


