Effect of cryopreservation on sperm DNA integrity in patients with teratospermia

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Objective: To test whether sperm with abnormal head morphology are more likely to undergo DNA damage and/or chromatin modification during the process of freeze-thawing.
Design: In this prospective study, the semen samples from forty-four men attending the infertility clinic were included. Samples were divided into aliquots to allow direct comparison of fresh and frozen spermatozoa from the same ejaculate. The sperm morphology and the sperm DNA damage were evaluated before and after cryopreservation. The relationship between sperm head abnormalities and freeze-thaw–induced DNA modification was assessed.
Setting(s): University hospital fertility center.
Patient(s): Men attending infertility clinic for semen analysis.
Intervention(s): The normospermic and teratospermic semen samples were evaluated for DNA damage before and after cryopreservation by comet assay and acridine orange bindability test.
Main Outcome Measure(s): Elucidation of association between sperm morphologic defect and cryodamage.
Result(s): A threefold increase in the amount of DNA damage was observed in teratospermic samples compared with their normospermic counterparts, indicating a higher susceptibility of morphologically abnormal sperm to cryodamage.
Conclusion(s): The susceptibility of morphologically abnormal sperm to DNA damage/chromatin modification during the freeze-thaw process is significantly higher than that of sperm with normal morphology. (Fertil Steril® 2008;89:1723–7. ©2008 by American Society for Reproductive Medicine.)

Key Words: Cryopreservation, teratozoospermia, DNA damage, chromatin denaturation

During cryopreservation, sperm undergo dramatic changes in their intracellular and extracellular environment owing to exposure to cryoprotectants, cooling, freezing, and thawing. The chemical and physical effects of these reagents/processes are known to have a detrimental effect on sperm structure and functional capabilities. The most predominant effects of cryopreservation on sperm are loss of viability, reduction in motility, morphologic changes, and alteration in chromatin structure (1–4).

An intact chromatin structure is extremely important for the fertilizing ability of the sperm, and it has been shown that defective spermiogenesis is associated with abnormal remodeling of sperm chromatin and membrane components, which in turn results in morphologically abnormal spermatozoa (5). Sperm chromatin structure, once believed to be stable during the cryopreservation process (6), is now found to be altered during freezing-thawing of spermatozoa (7, 8). In addition, cryopreservation appears to reduce the ability of sperm chromatin to decondense during fertilization (9), suggesting the detrimental effect of freeze-thawing on sperm chromatin.

Several factors, such as type of cryoprotectant used, freezing/thawing rate, seminal antioxidant level, and physical properties of the sperm membrane can influence sperm cryosurvival. Lipid composition has been associated with tolerance of the sperm to cryopreservation (10). Greater resistance of mammalian spermatozoa to cold shock has been noted for species in which the cholesterol-to-phospholipid molar ratio and the degree of saturated fatty acids in the phospholipid fraction were high (10, 11). Therefore, we hypothesize that sperm with head abnormalities may have an altered membrane physical property and thereby have an altered tolerance to cold stress. Increased susceptibility of DNA to denaturation corresponds to the heterogeneity of chromatin structure, which in turn has been associated with disturbances in spermiogenesis (12, 13). Although earlier studies have demonstrated the cryopreservation-induced alteration on various sperm functional capabilities, there are no studies conducted to know whether or not a morphologically abnormal sperm can retain its chromatin integrity during cryopreservation. Therefore, the present investigation was undertaken to elucidate the association between sperm morphology and freeze-store-thaw–induced DNA damage during semen cryopreservation.

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MATERIALS AND METHODS

Subject Selection

This study included 44 men undergoing semen analysis at the university infertility clinic between November 2004 and May 2007. Those samples with a minimum sperm concentration of 20×10^6/mL and motility of 50% were included for the study.

Semen Analysis

Semen specimens were collected by masturbation after 72 hours of ejaculatory abstinence. The sperm count, motility, and morphology were assessed in liquefied semen according to World Health Organization criteria (14). Normal values for semen variables were sperm density $>20 \times 10^6$/mL, sperm motility >50% (a + b), and normal morphology >30%. Semen samples containing $>1 \times 10^6$ WBC/mL were excluded to avoid reactive oxygen species (ROS) generation and its possible influence on sperm chromatin. Samples were divided into aliquots to allow direct comparison of fresh and frozen spermatozoa from the same ejaculate.

Semen Cryopreservation

Semen samples were carefully mixed with 10% glycerol–based cryoprotective medium (glycerol egg yolk citrate medium) at room temperature. The equilibrated samples were transfered to cryovials (Nunc) and subjected to static cooling at 4°C for 5 minutes and then vapor-phase cooling for 5 minutes before being plunged into liquid nitrogen. After 2 weeks, thawing was accomplished at 37°C followed by the addition of equal volume of EBSS medium. The samples were centrifuged at 1800 rpm for 8 minutes to remove all cryoprotective medium before their use for analysis.

Evaluation of DNA Fragmentation

The DNA fragmentation in spermatozoa was assessed by comet assay as described by Singh et al. (15) with minor modifications. Briefly, the spermatozoa were removed from the seminal plasma and the resulting sperm pellet mixed with sterile phosphate-buffered saline (pH 7.4). The sperm density was kept constant by appropriate dilution to maintain the uniform distribution of the sperm during electrophoresis. The sperm suspension was mixed with equal volume of 0.75% low-melting agarose (Sigma Aldrich, Bangalore, India) and layered on a slide precoated with normal agarose (1%). A third coat of agarose was layered over the second coat, followed by enzymatic lysis at 37°C under alkaline conditions for a minimum of 4 hours and then DNA unwinding in the electrophoresis buffer (pH >13) for 20 minutes. The fragmented strands of DNA were drawn out by electrophoresis for 15 minutes at 30 V. The slides were stained with ethidium bromide and observed under a fluorescent microscope with an image analyzer (Olympus). The damaged sperm attain the shape of a comet with the fragmented DNA in the tail region and intact DNA in the head region. The comet scoring was done in the captured images using Kinetic Imaging 5.5 software. The Olive tail moment (OTM), representing the degree of DNA damage, was calculated in at least 50 comets per slide.

Acridine Orange Staining of Sperm

Smears were prepared from fresh and frozen-thawed spermatozoa on a clean grease-free glass slide and air dried. The slides were fixed in Carnoy's fixative (1:3 glacial acetic acid and absolute methanol) for 2 hours and then stained with 0.1% acridine orange (AO) in citrate buffer (pH 2.5) for 1 minute in the dark as described by Royere et al. (16) with minor modifications (17). Excess stain was removed by repeated washing in citrate buffer and observed under the fluorescent microscope. At least 500 spermatozoa were evaluated from each slide and the percentage of normal and abnormal chromatin condensation calculated. Orange-red fluorescence is an indication of the presence of single-stranded DNA and green fluorescence of sperm with native DNA.

Statistical Analysis

The data represents mean and standard error (mean ± SE) of the values. The statistical significance level was calculated by Student *t* test using Graphpad Instat software (GraphPad Software, Inc, San Diego, CA) and analysis of variance (ANOVA) using SPSS 9.0.0 (SPSS Inc, Chicago, IL). The values were considered to be significant if $P<.05$. Pearson correlation coefficient was used to determine the association between sperm head defect and DNA damage. The graphs were plotted using Origin 6.0 (Microcal Software, Inc, Northampton, MA).

RESULTS

Out of the 44 semen samples analyzed in this study, 20 were normozoospermic and 24 were from teratospermic subjects (<30% morphologically normal sperm). No differences ($P>.05$) in average sperm motility (65.50 ± 2.6 vs. 56.84 ± 2.61), sperm density (49.30 ± 4.28 vs. 39.12 ± 5.43), or percentage viability (63.95 ± 2.42 vs. 55.87 ± 3.86) were observed between normo- and teratospermic samples, respectively. The mean sperm head abnormality among normal samples and teratospermic samples was 64.75 ± 0.85 and 79.63 ± 1.28, respectively, which was a statistically significant difference ($P<.0001$). The post-thaw sperm quality (motility and vitality) decreased significantly irrespective of sperm morphologic abnormalities (data not shown).

DNA damage was observed in fresh ejaculate of both normozoospermic and teratospermic subjects, and there was no significant difference in OTM between the two types of samples. The association between the amount of sperm DNA damage and head defects in normozoospermic and teratospermic samples was analyzed. A strong correlation (R = 0 .77) between sperm head abnormalities and DNA damage was observed for teratospermic samples; for normozoospermic samples, a similar trend was observed with low positive correlation (R = 0.1; Fig. 1). However, when these samples were subjected to cryopreservation, the amount of DNA damage was increased in the post-thaw normospermic samples