Comparison of the effects of two methods of cryopreservation on testicular sperm DNA

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Objective: To assess the effects of two methods of freezing on testicular sperm DNA from subjects with obstructive azoospermia and to compare these with samples of fresh and freeze-thawed testicular sperm from fertile men.

Design: The Comet assay was used to determine the percentage of undamaged DNA in fresh testicular sperm, testicular sperm freeze-thawed in suspension and in a biopsy sample (men with obstructive azoospermia), and in fresh and freeze-thawed testicular sperm (fertile men).

Setting: The Regional Fertility Center, Royal Maternity Hospital, Belfast, Northern Ireland, United Kingdom.

Patient(s): Twelve males with obstructive azoospermia (normal testicular volume and hormone profiles) and nine fertile control subjects.

Intervention(s): Trucut needle testicular biopsy under local anesthetic.

Main Outcome Measure(s): Measurement of the percentage of undamaged DNA in fresh and freeze-thawed testicular sperm using the Comet assay.

Result(s): No significant difference was found between the percentage of undamaged DNA of fresh testicular sperm and of both types of freeze-thawed testicular sperm. There was also no significant difference between the percentage of undamaged DNA in fresh or freeze-thawed testicular sperm from controls. Control ejaculated sperm DNA was significantly more damaged than testicular sperm DNA from control men.

Conclusion(s): Cryopreservation of testicular sperm does not increase baseline levels of DNA damage.

Since 1993, obstructive azoospermia has been successfully treated using sperm extracted from the testis or epididymis for intracytoplasmic sperm injection (ICSI) (1). However, concerns have been raised about the risk of testicular damage following any form of testicular biopsy, especially open (2). Schlegel and Su (2) reported that because of the circumferential nature of the sub tunical endarteries, it is not possible to incise the tunica albuginea at any point without risking vascular trauma and devascularization of the testis. Ron-El et al. (3) have also suggested that blind, fine-needle biopsies have the same potential for vascular damage as open biopsies.

When open biopsies are performed, pieces of tissue are removed following extrusion through a tunical incision and the yield of sperm has been shown to be significantly greater than from fine-needle biopsy, permitting excess sperm to be stored for future use (4). Fine-needle biopsy is a simple, local anesthetic procedure that is used to obtain a few sperm for fresh ICSI treatment cycles. There are usually not enough sperm retrieved from this type of sampling to permit cryopreservation. Therefore, these blind procedures must be repeated for each treatment cycle.

We previously reported that the use of the Trucut needle that allows large numbers of sperm to be retrieved under local anesthetic from men with obstructive azoospermia (5). Therefore, sperm can be frozen for subsequent treatment cycles. However, there is no consen-
sus as to whether it is better to freeze sperm in suspension or within a sample of testicular tissue. Aslam et al. (6) proposed that during the cryopreservation of testicular sperm suspensions, secreted toxins from cell lysis could compromise the viability of the spermatids. Allan and Cotman (7) suggested that seminiferous tubules afforded some protection to sperm during cryopreservation because the post-thaw survival rates of sperm stored in this way were better than for those stored in suspension.

If freezing damages sperm DNA it is possible that it will cause DNA fragmentation. The single cell gel electrophoresis assay, as described by Ostling and Johanson (8), permits assessment of fragmentary DNA damage in individual cells. The method is based on the electrophoresis of cells embedded and lysed in agarose on a microscope slide. Following lysis, the cell is stripped of all but its nuclear DNA (9). When placed in alkali, the DNA begins to unwind from sites of strand breakage.

The assay is more commonly known as the “Comet assay” because of the appearance of cells with DNA damage. Although undamaged DNA appears as a spherical mass occupying the cavity formed by the lysed cell, damaged DNA can be seen streaming as a comet tail in the direction of the anode (10) (Figure 1).

The Comet assay has been modified, by this laboratory, to enable assessment of sperm DNA with high reproducibility (11–13). During preparation for the Comet assay, sperm membranes are lysed, disulfide bonds are broken, and the DNA is unwound at sites of DNA strand breaks. During electrophoresis, broken strands of DNA are drawn out of the sperm nucleus, with smaller fragments usually traveling further than the larger fragments. The DNA is stained with ethidium bromide to facilitate analysis by fluorescence microscopy.

Sperm comets are viewed with a fluorescence microscope; fifty images are captured onto a television monitor and then analyzed, using an image analysis package, to calculate the length and fluorescent intensity of the tail. This is proportional to the amount of damaged DNA (the comet tail) that the sperm contains and is expressed as a percentage of the total DNA in that sperm by the computer software. Thus, the percentages of damaged and undamaged DNA of individual sperm as well the total damage in an ejaculate can be determined. Some degree of baseline damage exists in all samples, and we have not analyzed any sperm sample in which the DNA is completely undamaged.

The aim of this study was to assess the impact of two commonly used freezing methods on the DNA of testicular sperm from infertile men and on fresh and freeze-thawed testicular sperm and fresh ejaculated sperm from fertile men using the single cell gel electrophoresis assay.

**MATERIALS AND METHODS**

Twelve subjects with azoospermia, confirmed in two separate semen analyses, were recruited. Blood was taken for karyotyping and for follicle-stimulating hormone (FSH) levels to exclude subjects with nonobstructive azoospermia. Nine fertile subjects undergoing vasectomy in the Day Procedure Unit of the Royal Hospitals’ Trust were recruited as controls, and each was asked to provide an ejaculated sample on the day of surgery after three days of sexual abstinence.

All subjects gave written consent for the testicular biopsy. Ethical approval was obtained from the Queen’s University of Belfast Research and Ethics Committee. Institutional Review Board approval was not sought, as it is not deemed necessary by the university.

**Local Anesthetic**

The procedure was performed as follows. Ten milliliters of 0.5% bupivacaine was injected on either side of the spermatic cord. After 10-15 minutes, satisfactory anesthesia was confirmed by firm palpation. With the testis held firmly, 0.5 ml of 1% lidocaine was injected into the scrotal skin over the lower pole.

**Testicular Biopsy Procedure**

The skin and tunica albuginea were pierced with a disposable scalpel and a 14-gauge Trucut needle (Baxter Healthcare Ltd.; Thetford, Norfolk, U.K.) was inserted through the puncture site into the testicle and advanced 1 cm. The biopsy specimen was obtained by advancing the inner core of the needle and then closing the outer sheath rapidly over it. A core of tissue was trapped in the specimen notch and was transferred into culture medium (Biggers, Whitten, Whittingham; BWW) (14). The biopsy procedure was repeated once.

**Cryopreservation Protocol**

One sample from each of the azoospermic men was immediately placed in a cryovial containing 30 μL BWW to which 21 μL SpermFreeze (FertiPro N.V.; Sint-Martens-...
Latem, Belgium) was added in preparation for freezing. The other biopsy was milked of its sperm, and the resulting sample, divided into two equal aliquots. The first was analyzed by the Comet assay (11–13) while fresh. The second was freeze-thawed before analysis by the Comet assay. The testicular sperm samples from the fertile men were only freeze-thawed as suspensions of sperm.

Ejaculated samples (1 mL) were prepared by density centrifugation on a gradient of 50% and 100% Percoll at 2,000 revolutions per minute for 12 minutes. The pellet was aspirated and resuspended in 300 μL BWW and centrifuged again at 1,000 rpm for 6 minutes. The supernatant was aspirated and discarded, and the concentration of the final pellet was adjusted, by dilution in BWW, to 10 × 10⁶ sperm per mL because, with a sample more concentrated than this, the comet tails of each sperm overlap in the assay and accurate analysis is impossible.

Each sample for cryopreservation was equilibrated at room temperature for 10 minutes and then suspended in liquid nitrogen vapor for 15 minutes before being plunged into liquid nitrogen. Samples were thawed at room temperature twenty-four hours later. Thawed biopsies were washed with 50 μL fresh BWW and then milked of their sperm. The freeze-thawed testicular sperm suspensions were washed with 50 μL BWW and centrifuged at 1,000 rpm for 10 minutes to remove the cryoprotectant.

Slide Preparation for the Comet Assay

Phosphate-buffered saline (25 mL) was added to 0.125 mg of normal melting point agarose in a 50 mL tissue culture flask, that was then melted by heating in a microwave. The sealed flask was then placed in a waterbath at 45°C. The low melting point agarose was prepared in the same way using 25 mL PBS and 0.125 mg low melting point agarose. It was placed in a 37°C waterbath to keep it liquid.

Normal melting point agarose gel (100 μL) was pipetted onto cooled, fully frosted slides. A coverslip was placed on top to spread the gel flat and it was allowed to solidify at 4°C. A top layer was made by mixing 75 μL low melting point agarose with 10 μL sperm suspension. This was pipetted onto the first layer; a coverslip was placed on top and allowed to solidify at 4°C. After a further 15 minutes, the coverslip was gently removed.

Sperm Preparation for the Comet Assay

The slides were placed gently into a coplin jar containing lysing solution for one hour at 4°C (22.5 mLs of 2.5 M NaCl, 100 mM Na₂EDTA, 10mM Tris at pH 10 with 250 μL of 1% Triton X-100 added just before use).

To decondense the DNA, 2.5 mL dithiothreitol were then added, to a final concentration of 10 mM, for 30 minutes at 4°C followed by 2.5 mL of lithium diiodosalicylate to a final concentration of 4 mM, for 90 minutes at room temperature.

The slides were removed from the solution and placed in a horizontal electrophoresis tank, filled with freshly made electrophoresis solution (60 mL of 300 mM NaOH and 10 mL of 1 mM EDTA made up to 2 liters with double distilled water), for 20 minutes to allow the DNA to unwind. Electrophoresis was then conducted for 10 minutes at 25V.

Subsequently, the slides were drained and flooded with fresh neutralizing solution (0.4 M Tris at pH 7.5) every five minutes for fifteen minutes to remove any remaining alkali and detergents. One hundred microliters of stock ethidium bromide (20 μg/mL) was diluted with 900 μL double distilled water and then 50 μL was pipetted onto each gel and a coverslip placed on top.

Slide Analysis

The slides were viewed using a Nikon Eclipse E600 epifluorescence microscope that was fitted with an excitation filter of 515-560 nm from a 100 W mercury lamp and a barrier filter of 590 nm. Each slide was analyzed from front to back and from left to right to prevent reanalysis of any of the sperm. Images of the first 50 sperm to be visualized on each slide were captured and analyzed by an image analysis system using Hewlett Packard Super VGA and Komet software (version 3.1, Kinetic Imaging, Liverpool, U.K.). This image analysis package calculates the length and fluorescent intensity of the comet tail. This tail represents the percentage of damaged DNA in that particular sperm, and the undamaged DNA left in the comet head is then calculated and expressed as a percentage by the package. Statistical analysis of the 50 sperm analyzed was performed by the Komet software to provide the mean percentage of undamaged DNA in the sample.

RESULTS

All subjects had normal FSH levels and karyotypes. The cause of the obstruction was unknown in five subjects, because of vasectomy in four, and was due to congenital absence of the vas deferens in three (one had cystic fibrosis and the others were cystic fibrosis carriers).

Results are given in terms of the percentage of undamaged DNA in the comet head ± standard error of the mean. There was no significant difference between the mean percentage of undamaged DNA in fresh testicular sperm (81.7 ± 1.2) and in sperm freeze-thawed either in testicular tissue (81.9 ± 2.8) or in suspension (84.4 ± 2.5).

The percentage of undamaged DNA in fresh testicular sperm from control subjects (n=9) was 89.2 ± 1.9 and in freeze-thawed testicular sperm from controls was 87.6 ± 1.3. There was no significant difference between these values (Wilcoxon matched pairs test).

The percentage of undamaged DNA in the ejaculated samples from the controls subjects (n=9) was 80.6 ± 2.9. The value for the percentage of undamaged DNA in ejaculated sperm was significantly lower than the value for un-
damaged DNA in testicular sperm from the controls (Wilcoxon matched pairs test).

**DISCUSSION**

This is the first paper to assess the impact of different methods of cryopreservation on testicular sperm DNA from subjects with obstructive azoospermia. It was not possible to extend this study to include subjects with nonobstructive azoospermia because a minimum of fifty sperm is required per slide to permit analysis using the Comet assay. So far we have not been able to obtain this number of sperm from a subject with nonobstructive azoospermia to examine the DNA using the Comet assay.

The ability of testicular sperm to survive the freeze-thaw process has had significant implications for the treatment of men with obstructive azoospermia. Patients need only a single biopsy and can, therefore, avoid the discomfort and embarrassment of repeated procedures as well as any potential testicular damage from the disruption of the testicular vasculature. The 14-gauge Trucut needle biopsy has been shown to retrieve up to 427,800 sperm per biopsy, from a sample with a maximum weight of 21 mg in subjects with obstructive azoospermia. We have reported that in 41 subjects who underwent Trucut biopsies under local anesthetic, there were no recorded complications (5).

A follow-up study has demonstrated that there are no detectable scars on ultrasound at three months after biopsy and that antisperm antibodies were not formed or hormone profiles altered as a result of the Trucut biopsy. In most cases we found that following Trucut needle biopsy, sufficient sperm are obtained for at least six straws for storage in liquid nitrogen (each for one ICSI treatment cycle), avoiding the need for repeat biopsy.

The effect of cryopreservation on testicular sperm has been reported previously only in terms of fertilization and pregnancy rates (15). However, data provide no information about the effect of cryopreservation on sperm DNA because events up to the four-cell stage are controlled by maternally inherited information alone and, therefore, the effects of DNA damage would not be apparent at this stage (16).

In this study we found that there was no significant difference between the percentage of undamaged DNA of fresh testicular sperm and that of freeze-thawed testicular sperm from azoospermic men. This was also the case with fresh and freeze-thawed testicular sperm from control subjects. In addition, the percentage of undamaged DNA in testicular sperm from fertile men was significantly greater than the percentage of undamaged DNA in ejaculated sperm from the same men. Further, despite fears that cryopreservation of a mixture of spermatogenic cells may be problematic because toxic by-products of cell lysis may damage testicular sperm (6), or the opposing view that seminiferous tubules are necessary to protect sperm during freezing (7), we have not demonstrated that the method of cryopreservation adds to baseline sperm DNA damage in obstructive azoospermia. As there are still fundamental concerns regarding the safety of ICSI, it is reassuring to know that the process of cryopreservation does not cause increased testicular sperm DNA damage.

**References**