A comparison of DNA damage in testicular and proximal epididymal spermatozoa in obstructive azoospermia

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Testicular and epididymal spermatozoa are used routinely for intracytoplasmic sperm injection (ICSI) to treat men with obstructive azoospermia. Little is known of the effects of obstruction and stasis on the DNA of these spermatozoa, particularly in the epididymis where spermatozoa have been retained for long periods. Surgical epididymal aspiration for ICSI could provide spermatozoa that are senescent or dying. Using the Comet assay, the percentage of undamaged DNA of testicular spermatozoa from 20 men with obstructive azoospermia was significantly better (83.0 ± 1.2%) than from proximal epididymal spermatozoa (75.4 ± 2.3%; P < 0.05). There was no difference between the percentage of undamaged DNA of testicular spermatozoa from 39 men with obstructive azoospermia (84.0 ± 0.9) or from 10 fertile men at vasectomy (86.8 ± 1.8) or from ejaculated spermatozoa from five of the controls (78.9 ± 3.9; P > 0.05). In nine subjects, a second biopsy was carried out 6 months later. There was no significant difference in undamaged DNA on these two occasions (83.5 ± 5.6 and 84.1 ± 4.2; P > 0.05). This confirms the reproducibility of the Comet assay for non-ejaculated spermatozoa. Our data suggest that testicular sperm DNA appears to be significantly less damaged than epididymal sperm DNA, and so testicular spermatozoa should be used in preference for ICSI to treat men with obstructive azoospermia.

Key words: DNA/epididymal spermatozoa/obstructive azoospermia/testicular spermatozoa

Introduction

The advent of intracytoplasmic sperm injection (ICSI) has permitted most types of male infertility to be treated. Since its success does not depend on sperm concentration, motility or morphology (Nagy et al., 1995), the technique has been extended to include injection of epididymal and testicular spermatozoa. Fertilization rates of 47% have been reported (Silber et al., 1995a) for epididymal spermatozoa with ICSI compared with 7% for conventional in-vitro fertilization (IVF). Testicular spermatozoa can only be used for ICSI because of their low numbers and minimal progressive motility. However, they have yielded fertilization, cleavage and ongoing pregnancy rates similar to epididymal spermatozoa (Silber et al., 1995b).

Concerns about the safety of ICSI remain due to its short clinical history and because it was introduced without thorough testing on animal models (te Velde et al., 1998). As ICSI circumvents all the natural barriers to fertilization (adequate motility, morphology and concentration) which select against structurally-defective spermatozoa, there is a theoretical risk of an increase in congenital anomalies by the random injection of spermatozoa with abnormal DNA. In fact, infants conceived of spermatozoa, as well as the total damage in an ejaculate, can be determined. Some degree of baseline damage exists in all samples and we did not examine any sperm sample in which the DNA was 100% undamaged.

Epididymal sperm aspiration is often carried out in preference to testicular sperm aspiration, because it is simpler and
Figure 1. Schematic diagram of a typical comet. The comet head is made up of undamaged DNA, while the comet tail is composed of damaged DNA. The longer the tail, the more damaged is the sperm DNA. Comets with no tails (i.e. no damage at all), have not been seen.

less invasive, but the spermatozoa in the obstructed epididymis are senescent and, therefore, possibly carry compromised genetic material. In this study, we assessed the DNA of testicular and proximal epididymal spermatozoa from men with obstructive azoospermia and compared these with testicular and ejaculated spermatozoa from fertile controls.

Materials and methods
A total of 39 subjects with azoospermia, confirmed on two separate analyses, were recruited. All had blood taken for karyotype, hormone profile and cystic fibrosis (CF) gene status. Written consent for testicular biopsy and epididymal aspiration was obtained from all participants. Ethical approval was obtained from the Queen’s University of Belfast Research and Ethics Committee.

Epididymal sperm aspiration
Epididymal and testicular biopsies were performed as previously reported (Craft et al., 1995; Steele et al., 1998). Briefly, the spermatic cord on the left side was injected with 10 ml of 0.5% bupivacaine (without adrenaline). After 10 min, a 23 gauge butterfly needle was introduced into the proximal epididymis (usually on the upper pole of the testis) and gentle suction applied with a 2 ml syringe. The aspirate was then flushed into an Eppendorf tube with Biggers–Whitten–Whittingham medium (BWW; Biggers et al., 1971).

Epididymal sperm preparation
It was intended to prepare epididymal spermatozoa by density centrifugation in order to ensure that assessment was of motile, viable spermatozoa. In practice, however, this was not possible as from an initial pilot group of six patients, large numbers of spermatozoa were obtained from only one subject (900 000 spermatozoa). When an epididymal sample was obtained where at least 50 spermatozoa could be counted in several high power fields from a 5 µl portion of the sample, these samples were concentrated by centrifugation at 1000 rpm for 10 min.

Testicular sperm extraction
For testicular biopsy, the skin and tunica albuginea were first punctured with a scalpel and then a 14 gauge Trucut needle (Baxter Healthcare Ltd., Thetford, Norfolk, UK) was inserted through the puncture site into the testicle and two biopsies taken. Subjects maintained pressure over the skin puncture site for 30 min, while recumbent, before being discharged from the clinic. Diclofenac sodium (100 mg) was given rectally for analgesia and one dose of ciprofloxacin 250 mg was administered orally at the time of the procedure and 12 h later, as antibiotic prophylaxis.

Testicular sperm preparation
A portion of the first testicular biopsy sample from each patient was sent for histological analysis. The seminiferous tubules from the remainder of this biopsy were teased out in BWW and then milked of their contents. When all tubules had been milked, the surrounding culture medium was aspirated and concentrated by centrifugation at 1000 rpm for 10 min. The pellet was resuspended in 100 µl BWW. After performing the first 20 diagnostic biopsies, the numbers of spermatozoa obtained were so large that from then on the spermatozoa were milked out of the second biopsy under sterile conditions and the resulting sperm suspension stored in liquid nitrogen for future treatment cycles to avoid the patient requiring a second biopsy.

Controls
Fertile men having vasectomy under general anaesthetic in the Day Procedure Unit, the Royal Victoria Hospital, Belfast were recruited as controls (n = 10). They were asked to provide an ejaculated semen sample on the day of their operation. Ejaculated samples from fertile controls were prepared by density centrifugation on a gradient of 50 and 100% Percoll at 2000 revolutions per minute (rpm) for 12 min and then after resuspension of the pellet in 300 µl BWW, centrifugation at 1000 rpm for 6 min. The concentration of the final pellet was adjusted, by dilution in BWW, to 10 × 10⁶ spermatozoa/ml because with a sample more concentrated than this the comet tails of each spermatozoon overlap in the assay and analysis is impossible.

Reproducibility
As some of the first patients in the study did not have spermatozoa in storage they underwent subsequent biopsies. Nine subjects who had had diagnostic left-sided Trucut biopsy underwent repeat biopsy from the right testicle 4–6 months later to obtain spermatozoa for an ICSI treatment cycle. On the second occasion, excess spermatozoa were also prepared for and analysed using the Comet assay to assess the reproducibility of the preparation and analysis techniques.

Comet assay
The single cell gel electrophoresis (Comet) assay was then applied to the testicular, epididymal and ejaculated samples as previously reported (Hughes et al., 1997). In summary, normal melting point agarose gel was pipetted onto fully frosted slides and allowed to solidify at 4°C. A top layer was then made by mixing low melting point agarose with 10 µl sperm suspension. This was pipetted onto the first layer and allowed to solidify. The slides were placed in lysis solution for 1 h (22.5 ml 2.5 mol/l NaCl, 100 mmol/l Na₂EDTA, 10 mmol/l Tris at pH 10 with 250 µl of 1% Triton X-100 added just before use). To decondense the DNA, 2.5 ml dithiothreitol (DTT) was then added, to a final concentration of 10 mmol/l, for 30 min at 4°C, followed by 2.5 ml lithium diiodosalicylate (LIS) to a final concentration of 4 mmol/l, for 90 min at room temperature. The slides were removed from the lysis solution and placed in a horizontal electrophoresis tank, filled with freshly made electrophoresis solution, for 20 min to allow the DNA to unwind. Electrophoresis was then conducted for 10 min at 25 V. Subsequently, the slides were drained and flooded with neutralizing solution to remove any alkali and detergents before being stained with ethidium bromide.

The slides were viewed using a Nikon Eclipse E600 epifluorescence microscope which was fitted with an excitation filter of 515–560 nm from a 100 W mercury lamp and a barrier filter of 590 nm. The slides were analysed from front to back and from left to right to prevent re-analysis of any of the spermatozoa. Images of the first 50
spermatozoa to be visualized on each slide were captured and analysed by an image analysis system using Hewlett Packard Super VGA and Komet software (version 3.1, Kinetic Imaging, Liverpool, UK). This image analysis calculates the length and fluorescent intensity of the comet tail. This represents the percentage of damaged DNA in that particular spermatozoon 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 analysed was carried out using the Komet software to provide the mean percentage undamaged DNA in the sample.

**Results**

The mean age of azoospermic subjects in this study was 37 years (range 25–55 years) and of controls was 36 years (range 28–42 years). There were only four azoospermic subjects who had abnormal hormone profiles. One who had had a vasectomy had a slightly reduced testosterone level of 9.9 nmol/l (normal range 12–30 nmol/l) and three had elevated concentrations of follicle stimulating hormone (FSH) (15.9, 15.0 and 11.2 IU/l; normal range 1.5–8.0 IU/l) but normal values of luteinizing hormone (LH) and testosterone. However, in all the azoospermic and control samples the histological sample was graded as A1 and there was no active inflammation or evidence of malignancy (Dajani, 1998).

The cause of the azoospermia was unknown in 22 subjects. Of the rest, nine were as the result of a vasectomy and eight were due to congenital absence of the vas deferens (CBAVD). One of these subjects had cystic fibrosis (CF), five were CF gene carriers and two were negative for any of the common CF genes.

Paired samples of epididymal and testicular spermatozoa were obtained from 20 subjects; testicular samples only were obtained from the other 19 subjects. In all 39 cases of epididymal aspiration in this study, fluid was aspirated into the butterfly tubing but spermatozoa were found in <50% of the samples. The concentrations of epididymal spermatozoa were not calculated because the volume of epididymal fluid was so tiny that when a 5 µl portion was viewed to confirm the presence of spermatozoa a quick count was carried out to ensure that >50 spermatozoa could be seen in several high power fields and the remainder of the sample was all used to prepare slides for the Comet assay. The numbers of epididymal spermatozoa were too low to permit preparation by density centrifugation. In one case, <20 spermatozoa were obtained whilst there were 54,000 spermatozoa in the testicular biopsy. One of the largest epididymal counts was 900,000 spermatozoa; this was still not sufficient to prepare by density centrifugation for analysis by the Comet assay. Testicular samples were obtained from all 10 controls and only five of them produced ejaculated samples on the day of surgery.

Results are given in terms of the percentage of undamaged DNA (head DNA) that was present in the head of the comet. The overall mean percentage head DNA (±SEM) for testicular spermatozoa from all biopsied subjects (n = 39) with obstructive azoospermia was 84.0 ± 0.9. In contrast, for the 20 subjects where both epididymal and testicular spermatozoa were available the mean percentage head DNA (±SEM) in epididymal spermatozoa was 75.4 ± 2.3, and in testicular spermatozoa 83.0 ± 1.2 (P < 0.05; Figure 2). Photographs of testicular (Figure 3) and epididymal spermatozoa (Figure 4) comets are shown. The length and fluorescent intensity of the tail of the epididymal comet is clearly much greater than either of the very small tails of the testicular sperm comets, thus showing that there is more DNA damage in epididymal spermatozoa than in the testicular spermatozoa.

The mean percentage head DNA (±SEM) for testicular spermatozoa from control subjects of proven fertility (n = 10) was 86.8 ± 1.8 (Figure 5). This was not significantly different from the value for men with obstructive azoospermia (n = 39) (P > 0.05) or from the mean percentage head DNA for ejaculated spermatozoa of 78.9 ± 3.9 (n = 5) (P > 0.05).

There was no difference between the mean percentage head DNA (±SD) for spermatozoa from the left testis (n = 9) 83.5 ± 5.6 and right testis (n = 9) 84.1 ± 4.2 (P > 0.05). As the second biopsy was obtained, prepared and analysed 6 months later this confirms the reproducibility of both the preparation and analysis techniques (Table I).
Figure 4. Photograph of an epididymal sperm comet from a subject with obstructive azoospermia due to congenital bilateral absence of the vas deferens (CBAVD) (CF negative). Original magnification $\times 400$.

Figure 5. Percentage undamaged DNA of testicular spermatozoa from men with obstructive azoospermia and testicular and ejaculated spermatozoa from fertile controls. Values are mean percentage undamaged DNA and SEM. There was no significant difference between the three groups ($P > 0.05$; Mann–Whitney $U$ test).

**Discussion**

This is the first comparative report of DNA damage in testicular and epididymal spermatozoa. It comes against a background of concerns about the use of spermatozoa from these sources in ICSI treatments (te Velde et al., 1998). We have demonstrated that testicular sperm DNA is significantly less damaged than proximal epididymal sperm DNA in men with obstructive azoospermia. Spermatozoa from distal portions of the chronically obstructed epididymis were found to be immotile (Silber et al., 1995a), probably due to senescence, whereas spermatozoa in the proximal epididymis were motile. They also commented that the quality of proximal epididymal and rete testis spermatozoa were similar to ejaculated spermatozoa and superior to spermatozoa from the distal epididymis which was found to contain necrotic and degenerating spermatozoa as well as large numbers of macrophages. In contrast we have shown that despite phenotypic appearances, proximal epididymal spermatozoa are not comparable to testicular spermatozoa in terms of DNA damage. We aspirated spermatozoa from the proximal epididymis for two reasons. Firstly, to mimic clinical practice and secondly to obtain the most recently produced epididymal spermatozoa. However, in the chronically obstructed epididymis even the proximal epididymis will contain substantial proportions of dead and dying spermatozoa. It has been suggested that in the normal epididymis, epididymal fluid prevents enzymes released from the acrosomes of degenerating spermatozoa from acting upon other spermatozoa (Moore, 1996). The numbers of degenerating spermatozoa in the obstructed epididymis must be a great deal larger than in the normal epididymis and it may be that the amount of epididymal fluid produced cannot compensate for the effects of the increased concentration of enzyme. Testicular spermatozoa are, in comparison even with proximal epididymal spermatozoa, both recently produced and have not been exposed to the enzymes released from the dead and dying spermatozoa in the obstructed epididymis. These are factors that may explain the decreased DNA damage seen in testicular spermatozoa in this study.

Our findings may have significant clinical implications. Firstly, percutaneous epididymal spermatozoa aspiration is a popular, simple, procedure performed under local anaesthetic, and large numbers of mature spermatozoa have been obtained using this technique (Craft et al., 1995). However, using the technique of Craft et al., we failed to obtain spermatozoa in nearly 50% of cases and in those epididymi where spermatozoa were found, the numbers were too low to permit preparation for the Comet assay. Testicular spermatozoa extraction has not been as popular as percutaneous epididymal sperm aspiration because of problems with sample processing and poor sperm yields (Silber et al., 1995b). There are also concerns regarding the risk of testicular vascular damage (Schlegel and Su, 1997). We believe that both of these problems can be circumvented by using a Trucut needle (Steele et al., 1998) to perform the biopsy followed by milking of the seminiferous tubules. This gives a yield of $5 \times 10^5$ to $5 \times 10^6$ spermatozoa per 10 mg biopsy in obstructive azoospermia. These data support a change to the use of testicular needle biopsy rather than epididymal aspiration to provide spermatozoa for ICSI.

**Table 1.** A comparison of percentage intact head DNA for two testicular sperm samples obtained from each of nine patients 6 months apart.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Undamaged DNA (%) (original result)</th>
<th>Undamaged DNA (%) (reproducibility result)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82.0</td>
<td>83.6</td>
</tr>
<tr>
<td>2</td>
<td>92.7</td>
<td>83.1</td>
</tr>
<tr>
<td>3</td>
<td>87.8</td>
<td>82.6</td>
</tr>
<tr>
<td>4</td>
<td>76.1</td>
<td>77.6</td>
</tr>
<tr>
<td>5</td>
<td>90.3</td>
<td>78.9</td>
</tr>
<tr>
<td>6</td>
<td>80.6</td>
<td>87.3</td>
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<tr>
<td>7</td>
<td>82.8</td>
<td>89.5</td>
</tr>
<tr>
<td>8</td>
<td>78.2</td>
<td>85.3</td>
</tr>
<tr>
<td>9</td>
<td>80.9</td>
<td>89.3</td>
</tr>
<tr>
<td>Mean $\pm$ SD</td>
<td>83.5 $\pm$ 5.6</td>
<td>84.1 $\pm$ 4.2</td>
</tr>
</tbody>
</table>

$P > 0.05$ (Wilcoxon matched pairs test).
The original result was obtained using spermatozoa from the left testis and the second result was using spermatozoa from the right testis.
If the yield of spermatozoa from epididymal aspiration could be increased sufficiently to allow DNA analysis it would be desirable to repeat the Comet assay assessment on prepared epididymal samples to ensure that preparation techniques are in fact removing the damaged spermatozoa to prevent their use for ICSI. However, by using only proximal epididymal spermatozoa we have analysed the best quality spermatozoa available in the epididymis.

The effects of the use of spermatozoa with abnormal DNA can be masked initially in assisted reproduction as DNA damage, sperm nuclear decondensation and pronucleus formation can occur normally (Twigg et al., 1998). This is because events up to the 4-cell stage are controlled by maternally-inherited DNA (Braude et al., 1988). Sperm DNA damage appears only to manifest its effects at the embryo implantation stage (De Croo et al., 1998).

It must be remembered that it is not currently possible to examine the degree of DNA damage of a spermatozoon and then to use it clinically. Therefore, until this is possible and based on the data presented here, we suggest that testicular spermatozoa should be used in preference to epididymal spermatozoa. It is reassuring to find that the degree of testicular sperm DNA damage is the same in men with obstructive azoospermia as in fertile controls. This suggests that testicular sperm DNA is not being altered by the obstructive pathology. Like previous authors (Gottlieb et al., 1991), we found that spermatogenesis is normal in men with obstructive azoospermia. Our study also provides evidence that even in men with obstructive azoospermia spermatogenesis yields spermatozoa with no difference in the levels of DNA fragmentation between the testicles. In addition we have confirmed the reproducibility of the Comet assay for testicular spermatozoa, as was previously demonstrated for ejaculated spermatozoa (Hughes et al., 1997).

Even though the difference in testicular and epididymal sperm DNA damage in this study was small, it was nonetheless significant. However, there may be some variation in the percentage of undamaged DNA in individual testicular sperm samples on different occasions (Table I). However, variations in the percentage of undamaged DNA in epididymal spermatozoa may also occur which could in fact increase the significance of the results.

As ICSI bypasses all of the natural selection processes, which prevent defective spermatozoa from fertilizing an oocyte, we suggest that testicular spermatozoa with less damaged DNA should be used in preference to epididymal spermatozoa to treat men with obstructive azoospermia.

References


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