Flow cytometric ploidy analysis of testicular biopsies from sperm-negative wet preparations

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BACKGROUND: The use of testicular sperm in assisted reproduction depends on the availability of sperm in wet preparations. It is not always possible to recover sperm from the testis, even with previous sperm-positive histopathological findings. The purpose of this study was to evaluate the sperm-negative wet preparation search results with flow cytometric ploidy analysis and histopathological examination. METHODS: Two pieces of testicular tissue were obtained from azoospermic patients to investigate the spermatogenic status of the testis, and to determine the presence of sperm through a wet preparation. The testicular tissue was shredded and then vortexed; the cellular suspension was then processed for a wet preparation sperm search, while the residual tissue was exposed to enzymatic digestion for flow cytometric ploidy analysis. RESULTS: A total of 38 patients had sperm-negative wet preparation results. Of those, six (16%) were shown to have haploid cells after flow cytometric analysis. Histopathological examination showed three samples with maturation arrest at the spermatid stage, and the other three at the spermatocyte stage. CONCLUSIONS: Flow cytometric ploidy analysis can be used to verify the results of a wet preparation sperm search when no sperm were detected. Flow cytometric ploidy analysis can also reveal the presence of spermatids when no sperm are available.

Key words: flow cytometry/histopathology/spermatozoa/testicular biopsy/wet preparation

Introduction

Azoospermia is detected in ~10–20% of infertile men, the cause being routinely analysed by histopathological examination of testicular biopsies (Hellstorm et al., 1990; Jequier, 1997). The introduction of ICSI for the treatment of male factor infertility (Palermo et al., 1992) has made it possible to use testicular sperm to achieve successful fertilization and pregnancies (Craft et al., 1993; Schoysman et al., 1993; Devroey et al., 1994). The use of testicular sperm for ICSI mainly depends on the availability of sperm in wet preparations. It is not always possible to recover sperm from the testis, even with previous sperm-positive histopathological findings. Furthermore, sperm can be found in almost half of the patients with a negative histopathological report (Devroey et al., 1995; Silber et al., 1995, 1996).

A diagnostic wet preparation of testicular biopsies for the presence or absence of sperm is a part of the patient work-up if testicular failure is suspected in our clinic. Searching for sperm in the wet preparation is a labour-intensive and lengthy procedure, especially if the patient has severe hypospermato genesis, maturation arrest or Sertoli cell-only syndrome. Patients with maturation arrest or Sertoli cell-only syndrome could have focal spermatogenesis (Silber et al., 1995) that yields very few sperm (if any) in a wet preparation and requires hours of searching to find enough sperm to inject.

Flow cytometric ploidy analysis is a powerful technique to detect the presence of haploid cells from testicular biopsies, and has been used to assess testicular function in several studies (Pfitzer et al., 1982; Chan et al., 1984; Kaufman and Nagler, 1987). The quantitative evaluation of spermatogenesis through the percentage of haploid cells has been well correlated with histopathological findings (Hellstorm et al., 1990; Lee and Choo, 1991). Flow cytometry coupled to cell sorting was even utilized to isolate round spermatids from mouse and human testicular tissue (Aslam et al., 1998; Lassalle et al., 1999; Ziyyat et al., 1999). To our knowledge, there is no published report which correlates wet preparation with flow cytometric ploidy analysis. The purpose of this study was...
to evaluate and compare the results of sperm-negative wet preparation with flow cytometric ploidy analysis and histopathological findings.

Materials and methods
The routine procedure in our unit is to refer all non-obstructive azoospermic patients (determined by at least two semen analyses) for testicular fine needle aspiration (FNA) to determine their spermatogenetic status. Patients with sperm-positive FNA results are accepted into the ICSI programme directly. Patients with negative or inconclusive FNA results are referred for diagnostic testicular histopathological examination and wet preparation sperm search. Overall management of non-obstructive azoospermia is shown in Figure 1 for the study. Patients signed an informed consent form for a testicular biopsy. Sperm obtained during diagnostic testicular biopsies were not frozen as sperm freezing is prohibited by local regulations.

Testicular biopsy and wet preparation
In total, 38 consecutive patients with sperm-negative wet preparation search were included in the study between May 1998 and March 1999. Biopsies were obtained under local anaesthesia and placed into flushing medium (MediCult, Copenhagen, Denmark) for wet preparation. A second biopsy was taken from the same incision site and placed into Bouin’s fixative for histopathological examinations. All efforts were made to extract a maximum number of sperm for wet preparation before enzymatic digestion for flow cytometric ploidy analysis. Processing of testicular tissues for wet preparation was as described previously (Jaroudi et al., 1999). Briefly, each piece of the testicular tissue was shredded by stretching the tissue with the edges of two glass slides in a 60 mm culture dish. The tissue was collected into 5 ml medium in a 15 ml conical centrifuge tube and vortexed for 5 min. The supernatant was removed and the pellet resuspended in 2 ml of erythrocyte lysing buffer as described previously (Nagy et al., 1997). The reaction was stopped by adding 8 ml of flushing medium after 5 min of incubation. The pellet was resuspended in fresh medium after centrifuging. The presence of sperm was checked under a light microscope by using a Makler sperm counting chamber. If no sperm were identified in the Makler chamber, an extensive search for sperm was performed on thin-layered 5 µl oil-covered drops under an inverted microscope. The sperm search was considered positive if any cells with a tail were identified.

Enzymatic digestion
The residual tissue pieces left from the wet preparation sperm-negative samples were used for enzymatic digestion and flow cytometric ploidy analysis. The method of enzymatic digestion used to obtain a single-cell suspension was described previously (Crabbe et al., 1997), with slight modifications. Briefly, the fresh testicular biopsies were minced using a 25-gauge needle attached to a syringe. Tissue was then enzymatically digested using flushing medium containing 1 mg/ml collagenase A (Boehringer Mannheim, Mannheim, Germany), 0.05 mg/ml DNase-I (Boehringer Mannheim) and 1% bovine serum albumin (low endotoxin; Irvine Scientific, Irvine, CA, USA) and incubated at 37°C for 1 h. The mixture was centrifuged (400 g for 5 min) and the supernatant was removed. The pellet was resuspended in 10 ml MediCult flushing medium using the same centrifugation conditions as above. The supernatant containing the isolated cell suspension was removed and the cell yield and viability were determined on a small aliquot of cells, using a haemocytometer and the Trypan Blue exclusion method. Approximately one-quarter of the cell suspension was kept for a second
analysis under inverted microscopy in case of discrepancy between the first wet preparation and flow cytometric ploidy analysis results. The remaining supernatant was used for flow cytometric analysis.

**Flow cytometric analysis**

The samples were centrifuged after enzymatic digestion, and red blood cells were lysed as described above. An aliquot (200 µl) of DNA-Prep LPR and 1 ml of DNA-Prep stain reagents (Coulter Corporation, Hialeah, FL, USA) were added, and the mixture was incubated at 4°C in the dark for 30 min. All the stained samples were analysed using a FACScan flow cytometer (Becton Dickinson, Immunocytometry System, San Jose, CA, USA) equipped with an argon ion laser emission at 488 nm and using Cell Quest software. A total of 50 000–100 000 total events were acquired, and two parameters were recorded: fluorescence light 2 (FL2)-width and FL2-area by using a 575 ± 10 nm filter for DNA measurement. Histograms of number of cells per fluorescence channel containing 1000 channels were gated on FL2 width/FL2 area dual parameter dot plot by defining the population of interest. Data were expressed as percentages of gated haploid, diploid and tetraploid populations (Figure 2). A total of 15 specimens with sperm-positive wet preparation search were used as positive controls, and all of them showed haploid cell peak by flow cytometric ploidy analysis. Freshly isolated human endometrial cells were analysed by flow cytometry, and no haploid cell peak was detected as expected. Flow cytometric ploidy analysis takes ~1 h to perform. The direct cost of flow cytometry analysis is relatively low if an on-site flow cytometer is available.

**Histopathological examination**

Testicular tissue for histological examination was immediately fixed in Bouin’s solution, embedded in paraffin and stained with haematoxylin and eosin and trichrome stains. One pathologist (A.T.) who was blinded to the flow cytometry results reviewed all specimens. During the histological review, the following were evaluated: (i) total number of tubules in the section; (ii) relative percentages of germ cell-containing tubules; and (iii) the relative proportion of atrophic tubules. The state of spermatogenesis was assessed by using modified Johnsen score of testicular biopsies (Table I) (Jezek et al., 1998). When the score was variable in the same biopsy, the highest score was assigned to the specimen.

**Results**

Samples from 38 patients showed no sperm in wet preparation analysis. Six of these samples (16%) had a haploid cell peak in flow cytometric analysis. Re-examination of the unstained portion of enzymatically digested testicular cells revealed the presence of sperm in one sample, while the other five samples contained no sperm, even after an extensive search under an inverted microscope.

The distribution of histopathological classification according to the Johnsen score is shown in Table II. Twenty-five samples with a Johnsen score of 1, 2 or 3 had no haploid cell peak in flow cytometric ploidy analysis. There were 13 samples with a Johnsen score of 4, 5 or 6, among which six samples were shown to have a haploid cell peak in flow cytometry. Histopathological examination of these six wet preparation sperm-negative and flow cytometry haploid-positive samples showed that three were in maturation arrest at the spermatid level (Johnsen score 6; Figure 3); one had many spermatocytes and no spermatids (Johnsen score 5), and two were in maturation arrest at the primary spermatocyte stage (Johnsen score 4).

**Discussion**

Testicular sperm can be used successfully to obtain fertilization and pregnancy in azoospermic patients (Craft et al., 1993; Schoysman et al., 1993; Devroey et al., 1994). Finding either sperm or late spermatids is not always easy—or possible—in a wet preparation, and in some specimens hours of searching may be required to find a few sperm to inject. On numerous occasions, no sperm can be recovered after a long search in a wet preparation. In these cases, it might be questioned whether the wet preparation search was performed correctly. Hence, in
Figure 3. A haematoxylin and eosin-stained seminiferous tubule, showing maturational arrest at the spermatid stage. This was classified as Johnsen score 6. Original magnification, ×400.

Table I. Modified Johnsen score for histopathological examination of testicular biopsies

<table>
<thead>
<tr>
<th>Score</th>
<th>Histopathological appearance</th>
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<tbody>
<tr>
<td>1</td>
<td>Tubular sclerosis</td>
</tr>
<tr>
<td>2</td>
<td>Sertoli cell-only</td>
</tr>
<tr>
<td>3</td>
<td>Spermatogonia-only</td>
</tr>
<tr>
<td>4</td>
<td>Arrest at primary spermatocyte</td>
</tr>
<tr>
<td>5</td>
<td>Many spermatocytes, no spermatids</td>
</tr>
<tr>
<td>6</td>
<td>No late spermatids, arrest at spermatid stage</td>
</tr>
<tr>
<td>7</td>
<td>No late spermatids, but many early spermatids</td>
</tr>
<tr>
<td>8</td>
<td>Few late spermatids</td>
</tr>
<tr>
<td>9</td>
<td>Many late spermatids, disorganized tubular epithelium</td>
</tr>
<tr>
<td>10</td>
<td>Full spermatogenesis</td>
</tr>
</tbody>
</table>

Table II. Histopathological examination of testicular biopsies of sperm-negative wet preparation search and results of flow cytometric (FC) ploidy analysis

<table>
<thead>
<tr>
<th>Johnsen score</th>
<th>No. of patients</th>
<th>No. of patients with haploid peak by FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>1</td>
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<tr>
<td>6</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

the present study flow cytometric ploidy analysis was used to compare results with wet-preparation findings. Flow cytometric ploidy analysis has been reported to be an objective method for the investigation of spermatogenesis in infertile men (Kostakopoulos et al., 1997).

Flow cytometric ploidy analysis was conducted prospectively with sperm-negative wet preparation samples only. There was a 16% discrepancy between wet preparation and flow cytometric ploidy analysis. Re-examination of samples after enzymatic digestion under an inverted microscope revealed the presence of sperm in only one sample. It has been reported that, in non-obstructive azoospermia, enzymatic digestion of testicular tissue might be more successful in finding sperm compared with mincing of the tissue (Crabbe et al., 1998).

Histopathological examination revealed that no sperm were present in all biopsies taken from the same area as the tissues of the wet preparation. This gives a 97.4% agreement with the wet preparation, as only in one sample were sperm found after enzymatic digestion (this patient was classified as Johnsen score 5; many spermatocytes, no spermatids). The same specimen also had a haploid cell population as shown by flow cytometry. There were five other samples in which no sperm were detected after either mechanical or enzymatic digestion of testicular tissue, while haploid cells were detected by flow cytometric ploidy analysis. Histopathological examination of these samples showed three of them to be Johnsen score 6 (no late spermatids, arrest at the spermatid stage), and this was in agreement with data obtained from the flow cytometric ploidy analysis. However, the other two samples were classified as Johnsen score 4 (arrest at primary spermatocytes, no spermatids). The discrepancies of histopathology with flow cytometric ploidy analysis might be due to different locations of the biopsies. Although the tissues were taken from the same incision site, they may have contained different tubules, with different spermatogenic activity.

None of samples classified as Sertoli cell-only in histopathological examination had a haploid peak in the flow cytometric ploidy analysis (100% agreement). It has been reported that focal spermatogenesis might be present in the testes, even if the diagnosis is Sertoli cell-only or maturation arrest (Devroey et al., 1995; Silber et al., 1995, 1996). Moreover, microdissection of testicular tubules has been reported to improve sperm retrieval in patients who failed to yield sperm with standard testicular biopsy (Schlegel, 1999). The absolute correlation in the present study between histopathology and wet preparation can be explained by the experimental design, as all patients
with positive sperm search in wet preparation were excluded, and only sperm-negative patients were included. Some of the excluded patients with positive sperm from a wet preparation might have been diagnosed histopathologically as Sertoli cell-only.

The presence of a haploid peak in flow cytometric ploidy analysis, while no sperm were found in a wet preparation, leads to two possibilities concerning these specimens: first, that there were very few sperm present to detect; or second, that round spermatids were present. The first possibility is unlikely, as an intensive search using inverted microscopy was conducted before flow cytometric analysis and again after enzymatic digestion, and the results of the flow cytometric analysis were known. Another search of the haploid cell population after cell sorting of the haploid peak might offer an alternative means of avoiding an extensive search, though this requires additional equipment and expertise. The second possibility appears more likely, that there were round spermatids present which could not be differentiated from other round cells in the present settings. It has been reported that the identification of a round spermatid requires special microscopy other than that used routinely for ICSI (Verheyen et al., 1998). The presence of only round spermatids without having sperm in the testicular biopsies remains a subject of controversy. It has been stated (Silber and Johnson, 1998) that sperm (or at least mature spermatids) could be recovered if round spermatids are found; in other words, there would be no maturation arrest at the spermatid level. A recent report (Verheyen et al., 1998) showed that no round spermatids could be found in several biopsies from patients with nonobstructive azoospermia when no sperm were retrieved after a long search. These authors also discussed the possibility of a rare form of maturation arrest at round spermatid level (two or three among 800 cases). Findings in the present study suggest that round spermatids might exist in the absence of sperm or elongated spermatids in the wet preparation, and this was confirmed by both flow cytometric ploidy analysis and histopathological examination in at least three patients. Molecular studies in mice and humans have also shown that the cAMP responsive element modulator (CREM) plays a role in spermogenic arrest, and CREM mutant mice show round spermatid arrest (Peri and Serio, 2000; Fimia et al., 2001). CREM expression was reduced or undetectable among patients with predominant round spermatid arrest, and CREM-negative spermatids did not progress beyond stage III of spermatogenesis, meaning that no elongation had occurred (Weinbauer et al., 1998).

In general, the use of round spermatids for ICSI is controversial, and the results are not encouraging (Silber and Johnson 1998; Tesarik et al., 1998a; Vanderzwalmen et al., 1998; Silber et al., 2000). Although several pregnancies have been obtained from round or elongating spermatid injection (Fishel et al., 1995; Hannay, 1995; Tesarik et al., 1995, 1996; Mansour et al., 1996; Antinori et al., 1997; Sofikitis et al., 1998a) and ooplasmic injection of secondary spermatocytes (Sofikitis et al., 1998b), the overall success rate for these procedures is disappointingly low. This could be due to several reasons, including germ cell apoptosis, DNA immaturity, genomic imprinting, abnormality of the centrosomes or the presence of a sperm-derived oocyte activating factor, as well as problems related to the correct identification of round spermatids within the heterogeneous population of other round cells in the preparation (Edwards et al., 1994; Fishel et al., 1996; Vanderzwalmen et al., 1997, Tesarik et al., 1998b, 2000). Studies in mice have shown that genomic imprinting is not the problem with spermatid or secondary spermatocytes nuclei injections; rather, the absence of oocyte-activating substance in spermatids was the main obstacle against pronuclei development after injection (Kimura and Yanagimachi, 1995a,b). Rabbit spermatids gain their fertilizing potential at round spermatid stage 3–5, which occurs after coalescence of the proacrosomal granules (Sofikitis et al., 1997). The pathological nature of human specimens might further contribute to low success rates in humans, as the fertilizing capacity of round spermatids was lower in artificially varicoceolized rabbits when compared with sham-operated animals (Sofikitis et al., 1996a). Fertilization and cleavage in humans after the injection of a Sertoli cell nucleus has also been reported (Silber and Johnson, 1998). Our own experience, as well as other recent experimental evidence, does not indicate the use of round spermatids for ICSI at the present time due to the high developmental failure of embryos generated by this procedure (Ghazzawi et al., 1999; Viedan et al., 2001).

It will be interesting to see whether in-vivo or in-vitro manipulations and treatments could improve the fertilizing potential of round spermatids. The testicular level of testosterone is important during spermiogenesis, and testosterone withdrawal suppresses the conversion of round spermatids between stages VII and VIII, and promotes stage-specific detachment of round spermatids from the rat seminiferous epithelium (O’Donnell et al., 1994, 1996). Patients with low testosterone levels and round spermatid arrest could be treated with testosterone. Alternatively, the in-vitro treatment of sperm precursor cells might also help to improve the outcome of spermatid injection. A culture medium supplemented with FSH and testosterone potentiated meiosis and spermiogenesis in vitro, and sperm obtained from such cultures have been used to achieve fertilization and pregnancy in a patient with premeiotic arrest (Tesarik et al., 1998c, 1999). Media for assisted reproduction have been devised to culture sperm rather than spermatids which, in rats, have been shown to have different energy substrate requirements from sperm (Nakamura et al., 1978). These points need to be considered when culturing spermatids in vitro. It may be possible to modify injection techniques to provide better oocyte activation. Indeed, electrical stimulation before round spermatid nuclei injection had beneficial effects on oocyte activation, fertilization and embryo development in rabbits (Sofikitis et al., 1996b).

In conclusion, flow cytometric ploidy analysis can be used to confirm the presence or absence of haploid cells in testicular biopsy specimens of azoospermic men in parallel to wet preparation. Flow cytometric ploidy analysis can also reveal the presence of round spermatids when there are no sperm available. An extensive and lengthy sperm search in a wet preparation for ICSI can be avoided if flow cytometry shows the absence of haploid cells. However, a flow cytometer is expensive to purchase and to operate, and may not be available in smaller centres.
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References


Flow cytometry for testicular sperm search


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