Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa

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BACKGROUND: Sperm DNA damage (fragmentation) is a recently discovered cause of male infertility for which no efficient treatment has yet been found. Previous findings have suggested that clinically relevant sperm DNA damage may occur at the post-testicular level. This study was undertaken to assess the clinical usefulness of ICSI with testicular spermatozoa in this indication. METHODS: The percentage of spermatozoa with fragmented DNA, assessed by terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling assay, and ICSI outcomes were compared in two sequential attempts performed, respectively, with ejaculated and testicular spermatozoa in 18 men with increased sperm DNA fragmentation. RESULTS: The incidence of DNA fragmentation was markedly lower in testicular spermatozoa as compared with ejaculated spermatozoa. No differences in fertilization and cleavage rates and in embryo morphological grade were found between the ICSI attempts performed with ejaculated and with testicular spermatozoa. However, eight ongoing clinical pregnancies (four singleton and four twin) were achieved by ICSI with testicular spermatozoa (44.4% pregnancy rate; 20.7% implantation rate), whereas ICSI with ejaculated spermatozoa led to only one pregnancy which was spontaneously aborted. CONCLUSIONS: These data show that ICSI with testicular spermatozoa provides the first efficient assisted reproduction treatment option for men with high levels of sperm DNA damage.

Key words: DNA fragmentation/ejaculated spermatozoa/ICSI/sperm fertilizing ability/testicular spermatozoa

Introduction

Several studies have shown that male infertility can be caused by sperm DNA damage (Aitken, 1999; Host et al., 2000; Larson et al., 2000; Morris et al., 2002; Tomsu et al., 2002; Benchab et al., 2003; Carrel et al., 2003; Henkel et al., 2004; Tesarik et al., 2004a). However, the pathophysiological mechanism leading to sperm DNA damage is understood only incompletely, and no specific treatment for infertility caused by this condition has yet been proposed.

Even though the pattern of sperm DNA damage (fragmentation) closely resembles that resulting from programmed cell death (also called apoptosis) in somatic cells, several studies have questioned the causal relationship between the activation of the classical apoptotic pathway and DNA fragmentation of mature human spermatozoa (Sakkas et al., 2002; Henkel et al., 2004; Moustafa et al., 2004; Lachaud et al., 2004). We have shown recently that the classical cell death signalling pathway, in which caspase activation is followed by phosphatidylserine externalization marking the cell as target for phagocytosis, is active while germ cells remain in a tight association with Sertoli cells, but most Sertoli cell-free germ cells in the diseased human testis undergo DNA fragmentation without caspase activation and phosphatidylserine externalization (Tesarik et al., 2004b). After release from Sertoli cells, spermatids and spermatozoa thus appear to suffer DNA damage independently of the usual cell death signalling pathways. Previous studies have suggested that oxidative stress can be responsible for sperm DNA damage (Fraga et al., 1996; Barroso et al., 2000; Aitken and Krausz, 2001; Agarwal et al., 2003; Moustafa et al., 2004). The loss of nutritional support by Sertoli cells may aggravate the impact of oxidative stress on sperm cell components (Tesarik et al., 2004b).

If DNA damage detected in ejaculated spermatozoa essentially begins after sperm release from Sertoli cells, it can be hypothesized that the degree of damage increases with time after Sertoli cell release. If this is true, sperm populations recovered directly from the testis could be expected to be less affected by this pathological process as compared with ejaculated sperm populations. To test this hypothesis, this
study compares the incidence of DNA damage in spermatozoa recovered from the ejaculate and from the testis in two sequential assisted reproduction attempts performed in a group of patients characterized by abnormally high levels of ejaculated sperm DNA damage. Moreover, developmental competence of spermatozoa obtained from these two sources was determined by evaluating fertilization rate, the percentage of good morphology embryos, pregnancy rate and implantation rate.

Materials and methods

Study design and participants

This study involved 18 couples who had undergone at least two unsuccessful ICSI attempts with ejaculated spermatozoa and whose male partner had ≥15% of ejaculated spermatozoa with damaged DNA. All of the male partners were non-smokers without any apparent cause of infertility in their medical history. Their age ranged between 28 and 55 years. The female age ranged between 24 and 35 years. None of the patients enrolled in this study received pre-ICSI therapy aimed at improving sperm quality. All of these couples first underwent another ICSI attempt with ejaculated spermatozoa. If no ongoing pregnancy resulted, a subsequent attempt was performed with testicular spermatozoa. The interval between the two sequential attempts did not exceed 4 months.

Sperm samples

Ejaculated spermatozoa were obtained by masturbation after 3–5 days of sexual abstinence. After liquefaction, semen samples were divided into two aliquots to be used for the evaluation of DNA fragmentation and ICSI, respectively. For ICSI, sperm samples were diluted 1:10 with Gamete 20 medium (Scandinavian IVF Science, Gothenborg, Sweden), centrifuged (200 g; 10 min), and spermatozoa were allowed to swim up from the resulting pellet to overlayed Gamete 20 medium. This procedure previously was shown to result in a better selection of DNA-intact spermatozoa than a discontinuous density gradient technique (Lachaud et al., 2004). Basic sperm parameters (concentration, motility and morphology) were evaluated in all samples according to World Health Organization criteria (World Health Organization, 1992).

Testicular spermatozoa were obtained either by open testicular biopsy or by fine needle aspiration as described (Ubaldi et al., 2004). After disintegration with sterile microscope slides, the presence of spermatozoa in the wet preparations was assessed under the inverted microscope at ×200 or ×400 magnification. Spermatozoa to be used for the ICSI attempt were selected with the use of an assisted hatching micropipette (Humagen, Charlottesville, VA). The rest of the sample was divided into two aliquots, one of which was used for the evaluation of sperm DNA fragmentation. The other aliquot was cryopreserved for eventual future use.

Evaluation of sperm DNA fragmentation

Liquefied semen samples were centrifuged at 200 g for 10 min. After supernatant removal, samples of the remaining sperm pellet were smeared on microscope slides, air-dried, fixed with 4% paraformaldehyde in phosphate-buffered saline at 4°C for 25 min and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The smears were then processed for the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay which was performed using a Cell Death Detection Kit with tetramethylrhodamine-labelled dUTP (Roche, Monza, Italy) and according to the manufacturer’s instructions. The percentage of spermatozoa with fragmented DNA was determined in a fluorescence microscope as described (Tesarik et al., 2004a).

For evaluation of DNA fragmentation in testicular spermatozoa, disintegrated testicular tissue was smeared on slides and processed further as for ejaculated sperm smears. Two hundred spermatozoa per sample were analysed for both the ejaculated and the testicular sperm specimens.

Assisted reproduction techniques

Controlled ovarian stimulation, oocyte recovery and ICSI were performed as described (Tesarik et al., 2001). Briefly, patients were treated with recombinant FSH (Puregon; Organon, Oss, The Netherlands) after pituitary desensitization with triptorelin (Decapeptyl; Ipsen, Slough, UK) started in the mid-luteal phase. The overall dose of FSH per stimulated cycle varied between 1800 and 3500 IU depending on the individual response. As soon as at least three follicles of >18 mm were detected, ovulation was induced with 10 000 IU of HCG (Profasi; Serono, Geneva, Switzerland). Oocytes were recovered by transvaginal ultrasound-guided follicle aspiration 36 h later.

Zygotes and embryos were evaluated with the use of previously described scoring systems (Tesarik and Greco, 1999; Tesarik et al., 2000; Mendoza et al., 2002) on days 1, 2 and 3 after ICSI. Two to four best scoring embryos were transferred to the patient’s uterus on day 3 after ICSI.

Statistics

Differences between groups were assessed by two-tailed $\chi^2$-test with Yates’ correction and Fisher’s exact test All analyses were performed using the Statistica 5.0 package (Statsoft Version 5.1, Hamburg, Germany).

Results

Incidence of DNA fragmentation in ejaculated and testicular spermatozoa

Basic sperm parameters (concentration, motility and morphology) showed a high variability among individual patients, ranging between normal values and severe oligoasthenoteratozoospermia (Table I). All 18 men involved in this study showed >15% spermatozoa with fragmented DNA in ejaculated sperm samples used for the first ICSI attempt (Table I). In all of these men, a second ICSI attempt was performed with testicular spermatozoa. The percentage of spermatozoa with fragmented DNA in testicular sperm samples obtained on this occasion was <6% in all but one of these men (Table I).

The overall incidence of DNA fragmentation in the testicular sperm samples was 4.8 ± 3.6% (mean ± SD), which was significantly lower ($P < 0.001$) compared with the ejaculated sperm samples from the same individuals (23.6 ± 5.1%).

Developmental competence of ejaculated and testicular spermatozoa

Comparison of fertilization outcomes of the two sequential ICSI attempts, the first of which was performed with ejaculated spermatozoa and the second with testicular spermatozoa, showed no significant difference either in fertilization and cleavage rates or in the proportion of embryos with good
The morphological appearance (Table II). The ovarian stimulation protocol was the same in these two attempts, and no apparent differences in oocyte quality and quantity were noted between them. However, only one pregnancy (patient 6 in Table I), which was later spontaneously aborted, was established by using ejaculated spermatozoa (Table III). This contrasted with outcomes of the attempts using testicular spermatozoa which resulted in eight clinical pregnancies (patients 1, 3, 5, 8, 10, 13, 15 and 16 in Table I), four single- and four twin, all of which are ongoing. The number of gestational sacs with cardiac activity was thus 12, leading to an implantation rate of 20.7%, which was significantly higher than for ICSI attempts with ejaculated spermatozoa (Table III).

**Discussion**

The present data suggest that, in infertile men with a high incidence of DNA damage in ejaculated sperm populations, the percentage of spermatozoa carrying DNA damage is much lower in the testis. This observation confirms the working hypothesis that most of the DNA damage observed in ejaculated spermatozoa results from alterations occurring at the post-testicular level, although it cannot be excluded that spermatozoa may be selectively predisposed for this kind of damage during earlier developmental phases.

More importantly, these data also show that the use of testicular spermatozoa for ICSI can compensate for the reproductive disadvantage associated with the use of ejaculated spermatozoa for ICSI in this category of patients. Interestingly, the improvement of ICSI outcomes with the use of testicular spermatozoa only concerned ongoing clinical pregnancy and implantation rates, whereas fertilization rate and embryo morphology score were similar for the treatment attempts with ejaculated and testicular spermatozoa. This is in agreement with previous studies showing that spermatozoa with DNA damage can still fertilize oocytes and give rise to embryos with good morphological appearance, although these embryos mostly fail to implant or are miscarried shortly after implantation (Twigg et al., 1998; Ahmadi and Ng, 1999; Tomlinson et al., 2001; Morris et al., 2002; Carrell et al., 2003; Henkel et al., 2004; Tesarik et al., 2005).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Basic sperm parameters</th>
<th>% Spermatozoa with fragmented DNA</th>
<th>Concentration (x 10^6/ml)</th>
<th>Motility (%)</th>
<th>Normal forms (%)</th>
<th>Ejaculate</th>
<th>Testis</th>
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**Table II.** Fertilization and embryo development after ICSI with ejaculated and testicular spermatozoa

<table>
<thead>
<tr>
<th>Sperm source</th>
<th>Attempts</th>
<th>Oocytes injected</th>
<th>Normal zygotes</th>
<th>Fertilization rate</th>
<th>Cleaved embryos</th>
<th>Good-morphology embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejaculate</td>
<td>18</td>
<td>185</td>
<td>131</td>
<td>70.8%</td>
<td>124</td>
<td>59 (47.6%)</td>
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<tr>
<td>Testis</td>
<td>18</td>
<td>187</td>
<td>140</td>
<td>74.9%</td>
<td>133</td>
<td>68 (51.1%)</td>
</tr>
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</table>

*aWith two equal-sized pronuclei.
*bPercentage of injected oocytes that developed to normal zygotes.
*cPercentages are calculated from the number of normal zygotes.
*dEmbryos with normal pronuclear morphology on day 1, ≥6 cells on day 3, equal sized blastomeres and <10% of the intrazonal space occupied by fragments. The percentages are calculated from the number of cleaved embryos.
*eThe differences between data for the two sperm sources are not significant (P > 0.05).

**Table III.** Implantation and pregnancy after ICSI with ejaculated and testicular spermatozoa

<table>
<thead>
<tr>
<th>Sperm source</th>
<th>Attempts</th>
<th>Embryos transferred</th>
<th>Clinical pregnancies</th>
<th>Pregnancy rate</th>
<th>Gestational sacs</th>
<th>Implantation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejaculate</td>
<td>18</td>
<td>56</td>
<td>1</td>
<td>5.6%</td>
<td>1</td>
<td>1.8%</td>
</tr>
<tr>
<td>Testis</td>
<td>18</td>
<td>58</td>
<td>8</td>
<td>44.4%</td>
<td>12</td>
<td>20.7%</td>
</tr>
</tbody>
</table>

*aWith at least one gestational sac with cardiac activity.
*bPercentage of attempts resulting in a clinical pregnancy.
*cWith cardiac activity.
*dPercentage of embryos transferred that gave rise to a gestational sac with cardiac activity.
*eP < 0.05.
*fP < 0.01.
pregnancies (four of which were twin) were achieved in a spermatozoa in this group of 18 couples, whereas eight late. We obtained no ongoing pregnancy with ejaculated with at least 15% TUNEL-positive spermatozoa in the ejacu-

Based on the absence of apparent negative consequences for cleaving embryo morphology, the term ‘late paternal effect’ has been suggested recently for the developmental disadvan-
tage conferred to embryos by spermatozoa carrying damaged DNA, as opposed to the ‘early paternal effect’ which is also of sperm origin, is reflected by poor embryo morphology but is not associated with sperm DNA damage (Tesarik et al.,

In addition to suggesting novel clues for a better under-
standing of male infertility and the processes involved, this is the first study to propose an efficient assisted reproduction treatment option for men whose fertility is compromised by sperm DNA damage. However, the recovery of testicular spermatozoa is an invasive intervention, and its usefulness must be carefully justified in each individual case. It is thus increasingly important to achieve a consensus as to the cri-
tera predicting assisted reproduction failure due to sperm DNA damage. However, different studies have suggested different threshold values for the percentage of damaged spermatozoa in the ejaculate above which sperm development-
mental competence is compromised, apparently because of the use of different techniques for DNA damage visualization. For instance, using acridine orange staining, the percentage of spermatozoa with double-stranded DNA below which no full-term pregnancies were achieved after ICSI was 50% (Hoshi et al., 1996). Two large independent studies using the sperm chromatin structure assay (SCSA), a method assessing the susceptibility of sperm DNA to undergo acid-induced single-strand breakage in situ (Evenson et al., 1980), were undertaken in the USA and Europe, respectively (Evenson et al., 1999; Spanò et al., 2000). They agree in that male fertility is hampered when >30% of sperm have abnormal chromatin. Moreover, no full-term pregnancies (after ICSI, IVF or intra-uterine insemination) were obtained when the DNA fragmentation index assessed by SCSA was >30% (Larson et al., 2000; Larson-Cook et al., 2003; Saleh et al., 2003). However, no differences in SCSA parameter values between patients initiating pregnancies and not doing so in either ICSI or conventional IVF were found in another recent study (Gandini et al., 2004).

Unlike SCSA, which merely reflects the susceptibility of sperm DNA to fragmentation, TUNEL assay is a direct measure for the presence of endogenous nicks in sperm DNA. With the use of this test, Benchab et al., (2003) analysed ejaculated spermatozoa in 54 ICSI attempts and reported the absence of pregnancy when the sperm subpopu-
lation with DNA fragmentation was >20%. Unlike the study by Benchab et al., (2003), the patient group described in the present study was highly selected by including only patients with at least two previous unsuccessful ICSI attempts and with at least 15% TUNEL-positive spermatozoa in the ejacu-
late. We obtained no ongoing pregnancy with ejaculated spermatozoa in this group of 18 couples, whereas eight pregnancies (four of which were twin) were achieved in a subsequent ICSI attempt with testicular spermatozoa. Thus, it appears that, with the use of TUNEL assay applied to whole ejaculated sperm populations, the threshold levels of sperm DNA fragmentation above which ICSI with testicular sper-

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matozoa should be considered will lay between 15 and 20%.

However, another recent study calculated a cut-off value of 24.3% TUNEL-positive spermatozoa in the ejaculate (Henkel et al., 2003). Thus, the question of clinically relevant threshold for sperm DNA fragmentation still remains open.

In addition to providing an immediate treatment option for men suffering from this as yet untreatable reproductive path-
ology, these findings open up a number of stimulating ques-
tions. First of all, with an incidence of DNA fragmentation in ejaculated spermatozoa of 20–40%, there are still more than half of all spermatozoa whose DNA is expected to be intact.

Why does fertilization with these spermatozoa not lead to normal embryonic development? Apparently the advantage of using testicular spermatozoa in this indication is related to some other aspects. It is possible that detectable DNA frag-
mentation only represents the ‘tip of the iceberg’ and that subthreshold DNA damage is present in most of those sper-
matozoa that give a negative TUNEL reaction when the per-
centage of TUNEL-positive spermatozoa in the given sperm population reaches a certain critical value. Alternatively, the developmental impairment observed in these cases may be related to a deficiency of some of the sperm cytoplasmic fac-
tors involved in oocyte activation and early embryo develop-
ment, such as an oocyte-activating substance or centriole. In both of these situations, the developmentally incompetent sperm population might include substantially more sperma-
tozoa than only those actually showing detectable DNA damage.

Further study is also needed to determine whether fertility of men with sperm DNA damage can be restored by less invasive means than ICSI with testicular sperm recovery. Administration of antioxidants to the patients concerned and the development of in vitro selection techniques capable of enriching ejaculated sperm populations in healthy spermato-
zoa are two ways to be explored.

The use of testicular spermatozoa for ICSI may also be con-
considered in men with borderline figures of sperm DNA damage which are suspected to increase the risk of genetic diseases, birth defects and childhood cancer in the offspring (Fraga et al., 1996; Ji et al., 1997). Further study is needed to determine the correlation between the importance of these health risks and the extent of sperm DNA fragmentation to determine cut-off values for adequate patient counselling.

Regardless of these open questions, this study, though pre-
liminary, describes a new, efficient and ready-to-use treatment for an as yet untreatable reproductive pathology. This is particularly important for those infertile couples in whom sperm DNA damage is combined with advanced female age and who, consequently, need a rapid treatment solution.

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