Comparison of the aneuploidy frequency in embryos derived from testicular sperm extraction in obstructive and non-obstructive azoospermic men

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BACKGROUND: The use of ICSI has been a major breakthrough in the treatment of male infertility. Even azoospermic patients with focal spermatogenesis in the testis (not sufficient to spill over into the ejaculate) may benefit from the technique. Previous reports suggest a higher pregnancy rate after ICSI treatment in patients with obstructive azoospermia (OA) compared to their non-obstructive azoospermia (NOA) counterparts, which could be due to a higher aneuploidy frequency in the embryos of the latter group. We therefore conducted a prospective cohort study to compare the aneuploidy frequency of the screened embryos between the two groups.

METHODS: From May 2001 until September 2003, we offered couples with an OA or NOA partner ICSI in combination with preimplantation genetic diagnosis for aneuploidy screening. RESULTS: No difference in age (30.6 and 33.5 years) or stimulation parameters was observed between the two groups; 53 and 60% of the embryos were abnormal in the NOA and OA groups respectively ($P$ = not significant). CONCLUSIONS: The aneuploidy frequency in embryos obtained from NOA as well as OA men is similar and very high, despite the young age of their female partners.

Key words: azoospermia/chromosomal aneuploidy/FISH/PGD/TESE

Introduction

ICSI has become a widely used technique for the treatment of male infertility and it has even been successfully applied to treat infertility due to azoospermia by using surgically retrieved sperm. In this regard, injection of testicular sperm has been used to treat male infertility caused by both obstructive azoospermia (OA) (Craft et al., 1993; Schoysman et al., 1993; Devroey et al., 1994) and non-obstructive azoospermia (NOA) (Devroey et al., 1995, 1996; Tournaye et al., 1995). Previous studies show a significantly lower fertilization rate (Tournaye et al., 1996; Palermo et al., 1999; De Croo et al., 2000) and pregnancy rate (Vernaeye et al., 2003) in cases of NOA. This might be due to the fact that testicular sperm obtained from patients with testicular failure score lower with regard to quality (motility and morphology) and quantity, resulting in a lower number of embryos for selection at the day of embryo transfer, than testicular sperm derived from cases with OA (Nagy et al., 1998).

A further explanation of the differences in pregnancy rate could be the higher aneuploidy frequency in embryos where testicular sperm was used from NOA men. Several sperm studies support an association between morphological (In’t Veld et al., 1997; Bernardini et al., 1998; Viville et al., 2000b) or motility aberrations (Vegetti et al., 2000) and sperm chromosome aneuploidy. Moreover, several studies (Bernardini et al., 2000; Martin et al., 2000; Viville et al., 2000a; Levron et al., 2001; Burrello et al., 2002; Mateizel et al., 2002; Palermo et al., 2002; Sukcharoen et al., 2003) report that the aneuploidy frequency of testicular sperm from azoospermic men suffering from severe testicular failure is higher compared to sperm from azospermic patients with a normal spermatogenesis. The same studies also report that the aneuploidy frequency from the latter group is significantly higher than ejaculated sperm from donors.

From May 2001 until September 2003, we therefore conducted a prospective cohort study, offering couples with an OA or NOA partner ICSI in combination with preimplantation genetic diagnosis for aneuploidy screening (PGD-AS). Our aim was to compare the aneuploidy frequency of the screened embryos between the two groups. We also included a group of fertile patients who underwent the same PGD treatment for gender selection because of an X-linked disease as a historical control group.
Materials and methods

Patients

All patients included in the study were shown to be azoospermic on the basis of at least two semen analyses, including a centrifugation step at high speed. They all had a clinical work-up that included a physical examination, hormonal assessment (FSH, LH and testosterone levels) and assessment of biochemical seminal markers. All patients with an abnormal genetic work-up (karyotype analysis and assessment for Yq microdeletions) were excluded from the study, to keep the group uniform and to exclude confounding factors. All patients (with obstructive and NOA) had a histological confirmation of their normal or deficient (maturation arrest, germ-cell aplasia and tubular sclerosis/atrophy) spermatogenesis. Patients with hypospermatogenesis were excluded from the study, as we wanted to compare a well-defined group with testicular failure with patients with a normal spermatogenesis. Another group of patients, for which the male partners had a normal sperm count, who underwent PGD with the same number of FISH probes to determine fetal gender with regard to sex-linked diseases, was selected for comparison.

Testicular sperm recovery

In patients with a clinical diagnosis of NOA, open excisional testicular biopsy samples were obtained under general anaesthesia or occasionally under loco-regional anaesthesia, as described previously (Tournaye et al., 1997). The testicular samples were processed by mechanical shredding (Verheyen et al., 1995); microscopic examination of the wet preparations was carried out at ×400 magnification under an inverted microscope. When no sperm were found after 1 h of initial searching, enzymatic digestion of the testicular tissue with collagenase type IV was performed (Crabbé et al., 1998) in order to digest the tissue and release the few sperm that might be present. During surgery, if no histological diagnosis was still available, a single, randomly taken biopsy of each testis was sent for histological examination. In patients with OA, sperm were obtained by fine needle aspiration (FNA) or (if no histological diagnosis was available) testicular biopsy under local anaesthesia.

Ovarian stimulation and ICSI procedure

All female partners underwent ovulation induction using a GnRH analogue suppression protocol (short or long) or a GnRH antagonist protocol and human menopausal gonadotrophins or recombinant FSH. Oocyte–cumulus complexes (OCC) were recovered 36 h after the administration of 10 000 IU of hCG. The surrounding cumulus and corona cells were then removed and the nuclear maturation of the oocytes was assessed under an inverted microscope. Only metaphase II oocytes were injected with, preferably, morphologically normal motile sperm into the ooplasm. Fresh as well as frozen–thawed testicular sperm were used. These procedures have been described previously (Van Steirteghem et al., 1993; Joris et al., 1998).

Assessment of fertilization, embryo development and biopsy

Further culture of injected oocytes was performed in 25 μl microdrops of culture medium under lightweight paraffin oil. Fertilization was confirmed after 16–18 h by the observation of two distinct pronuclei (2PN). Oocytes with 2PN were assessed for embryonic development on day 2 and day 3 after injection, and the embryos reaching at least the 5-cell stage on day 3 of development were biopsied. The selection criteria for embryo biopsy were similar to those used to decide whether an embryo was transferable on day 3 in the regular ICSI programme without PGD. Before biopsy, the blastomeres were checked for the presence of a nucleus. From the 6-cell stage onward, two blastomeres per embryo were removed (Van De Velde et al., 2000; De Vos et al., 2001).

FISH procedure

The individually biopsied blastomeres were spread onto a Superfrost Plus glass slide (Kindler GmbH, Freiburg, Germany) using 0.01 M HCl/0.1% Tween 20 solution (Coonen et al., 1994; Staessen et al., 1996). Both blastomeres from the same embryo were fixed on the same slide in very close proximity.

A two-round FISH procedure, as described previously (Staessen et al., 2003), allowed us to detect the chromosomes X, Y, 13, 18, 21 (round 1) and 16, 22 (round 2).

In short an aliquot (0.2 μl) of the probe solution (DXZ1, Spectrum Blue; DYZ3, SpectrumGold; LSI13, SpectrumRed; D18Z1, SpectrumAqua; LSI21 SpectrumGreen; Multivision PGT Probe Panel; Vysis, Inc.) was added to the nuclei, covered with a round cover slip (4 mm diameter), denaturated for 5 min at 75°C and left to hybridize for between 4 h and overnight at 37°C in a moist chamber. After washing in 0.4×standard saline citrate solution (SSC)/0.3% Nonidet P40 at 73°C for 5 min and 2×SSC/0.1% Nonidet P40 for 60 s at room temperature. Antifade solution (Vectashield) was added and fluorescence signals were evaluated. The nuclei were then examined using a Zeiss Axiostop fluorescence microscope with the appropriate filter sets. The FISH images were captured with a computerized system.

Following the analysis of the first set of probes, the cover slips were gently removed and the slides rinsed in 1×phosphate-buffered saline at room temperature, denaturated in 0.0625×SSC for 7 min at 57°C, and then dehydrated (70, 90, 100 and 100% ethanol at −18°C, 60 s each). The second hybridization solution was prepared by mixing a probe for chromosome 16 (Satellite II DNA/D16Z3 probe, spectrum Orange; Vysis) and a probe for chromosome 22 (LSI 22, 22q11.2, SpectrumGreen; Vysis). The probes were denaturated separately in a hot water bath at 75°C for 5 min. An aliquot (0.2 μl) of the probe solution was then added to the nuclei, covered with a round coverslip (4 mm diameter), sealed with rubber cement and then hybridized overnight in a water bath at 37°C. Finally, the slides were washed for 2 min in 0.4×SSC solution at 73°C and 2×SSC/0.1% Nonidet P40 for 60 s at room temperature. The washed slides were then mounted with DAPI in antifade solution and analysed, and the results interpreted by two independent observers.

Embryo scoring and selection for transfer

The subsequent classification of embryos based on the biopsy result of two blastomeres was followed: (i) when both blastomeres had two copies of each chromosome analysed, the embryo was classified as normal; (ii) when both blastomeres had one chromosome with an abnormal number of copies, the embryo was classified as aneuploid; (iii) when both blastomeres had one, three or more copies of each chromosome, the embryo was classified as haploid or polyploid; (iv) when one blastomere was normal and the second blastomere had one chromosome with an abnormal number of copies, the embryo was classified as mosaic; (v) when at least one blastomere had more than one chromosome with an abnormal number of copies, the embryo was classified as complex abnormal. Only chromosomally normal embryos were transferred on day 5.

Definition of endpoint

A rise in serum hCG on two consecutive occasions from 11 days after transfer indicated pregnancy. A clinical pregnancy was defined as at least one fetus with a positive heartbeat revealed by vaginal ultrasound ~5 weeks after embryo transfer. The implantation rate was defined as the number of viable fetuses, as assessed by ultrasound at 7 weeks.
Table I. Age of the female partners, stimulation and clinical outcome

<table>
<thead>
<tr>
<th></th>
<th>NOA (39 cycles)</th>
<th>OA (23 cycles)</th>
<th>Sexing (14 cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>30.6 ± 4.6</td>
<td>33.5 ± 3.9</td>
<td>33.6 ± 5.4</td>
</tr>
<tr>
<td>No. of oocytes retrieved</td>
<td>12.6 ± 5.7</td>
<td>13.6 ± 8.7</td>
<td>11.9 ± 7.1</td>
</tr>
<tr>
<td>No. of metaphase II oocytes</td>
<td>11.1 ± 5.6</td>
<td>11.0 ± 7.4</td>
<td>10.1 ± 4.7</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>59.1</td>
<td>69.8</td>
<td>76.7</td>
</tr>
<tr>
<td>No. of 2-pronuclear oocytes</td>
<td>6.6 ± 3.6</td>
<td>7.6 ± 5.2</td>
<td>7.9 ± 4.4</td>
</tr>
<tr>
<td>No. of embryos biopsied</td>
<td>203</td>
<td>121</td>
<td>85</td>
</tr>
<tr>
<td>No. of embryo transfers</td>
<td>28</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>% embryos transferred</td>
<td>28.2 * (n = 11)</td>
<td>13.0 * (n = 3)</td>
<td>-</td>
</tr>
<tr>
<td>No. of embryos transfer</td>
<td>1.9</td>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>No. of ongoing pregnancies</td>
<td>4</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>(all singletons) (2 sets of twins)</td>
<td>7.5</td>
<td>18.2</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are means ± SD.
NOA = non-obstructive azoospermia; OA = obstructive azoospermia.

Table II. Chromosomal constitution of blastomeres from embryos derived after ICSI with testicular sperm of obstructive (OA) and non-obstructive (NOA) azoospermic men

<table>
<thead>
<tr>
<th></th>
<th>NOA (203 embryos)</th>
<th>OA (121 embryos)</th>
<th>Sexing (85 embryos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% normal</td>
<td>42.1 ± 27.1 (76)</td>
<td>34.8 ± 15.2 (48)</td>
<td>56.4 ± 26.3 (46)</td>
</tr>
<tr>
<td>% abnormal</td>
<td>52.5 ± 27.8 (118)</td>
<td>60.0 ± 18.9 * (69)</td>
<td>40.5 ± 23.9 (36)</td>
</tr>
<tr>
<td>% no diagnosis</td>
<td>5.2 ± 11.7 (9)</td>
<td>2.0 ± 4.0 (4)</td>
<td>2.9 ± 5.4 (3)</td>
</tr>
</tbody>
</table>

Values are means ± SD (no. of embryos).
*P < 0.05 between obstructive azoospermia group and sexing.

Statistical analysis

The mean age of the female partner at the time of oocyte retrieval for each cycle and the mean number of retrieved oocytes, metaphase II oocytes, normally fertilized oocytes and the mean number of abnormal embryos of each cycle was first calculated per couple. In a second step, the mean of all previous parameters of all the couples was calculated. The comparison of the variables was performed by means of two-way analysis of variance, with Bonferroni t-test to perform pairwise comparison of the three groups. P < 0.05 was considered statistically significant.

Results

In all, 62 cycles of TESE–ICSI with embryo biopsy were included in this study: 39 cycles were with testicular sperm from 23 NOA men and 23 from 13 OA men. The historical control group consists of 10 couples who performed 14 cycles. The results are summarized in Tables I and II. The mean (±SD) age of the female partners was similar in the obstructive (33.5 ± 3.9 years), non-obstructive (30.6 ± 4.6 years) and control (33.6 ± 5.4 years) group.

There was no difference in the mean number of retrieved, metaphase II and 2PN oocytes in all groups. The fertilization rate and mean number of embryos available for biopsy (203, 121 and 82 in respectively the NOA, OA and sexing groups) was similar in the three groups. Fifty-three chromosomally normal embryos were transferred in 28 NOA cycles, resulting in four ongoing pregnancies (all singleton) with an implantation rate of 7.5%. In the OA group, 33 chromosomally normal embryos were transferred in 20 cycles, resulting in four ongoing pregnancies (with two sets of twins) with an implantation rate of 18.18%.

In the embryos, no differences in aneuploidy frequency were observed between the TESE groups; there were, however, significantly more abnormal embryos in the OA group compared to the control group. Subanalysing the chromosomal abnormal embryos (Table III) into the different categories (described in Materials and methods) and for each chromosome did not reveal any significant differences between the three groups, probably due to the small numbers.

Discussion

Our results confirm the findings from a previous study (Silber et al., 2003) indicating that embryos derived from TESE in normal ongoing pregnancies (with two sets of twins) with an implantation rate of 7.5%. In the OA group, 33 chromosomally normal embryos were transferred in 20 cycles, resulting in four ongoing pregnancies (with two sets of twins) with an implantation rate of 18.18%.

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NOA men have a high incidence of aneuploidy. The authors found, after analysing 100 embryos derived from testicular sperm (from NOA men), that 78 were abnormal. There was, however, no control group of OA men. The fact that in our study a high percentage of embryos from OA men are also abnormal is unexpected, although this is perhaps not so surprising because, in FISH studies analysing sperm from OA, NOA men and donors (Bernardini et al., 2000; Martin et al., 2000; Viville et al., 2000a; Levron et al., 2001; Burrello et al., 2002; Mateizel et al., 2002; Palermo et al., 2002; Sukcharoen et al., 2003), the aneuploidy frequency of sperm originating from OA men was much higher than from ejaculated sperm.

An explanation for the high aneuploidy frequency in embryos derived from non-obstructive as well as obstructive sperm could therefore be the compromised testicular environment (obstruction, no natural selection in the epididymis, high FSH values, low testosterone values etc.). Some authors have described the degeneration of the testicular tubes and even a decrease in testicular volume after vasectomy (Lohiya et al., 1987). This environment could make these germ cells particularly susceptible to meiotic abnormalities. Earlier data from studies in XXY mice (Mroz et al., 1999) have already suggested this. An alternative explanation could be that abnormal spermatozooids are usually sequestrated along the way between the testis and the tip of the urethra; we would therefore be comparing selected (through the epididymis, prostate etc.) with non-selected sperm.

It is known that aneuploidy appears to increase with maternal age and is related to defects in the oocyte resulting in meiotic disjunction errors. There is no difference between the groups in the embryos categorized as aneuploid, as the age in the OA groups or alternatively to other intrinsic factors in the NOA group, especially as the mean age in the OA group was older (not statistically significant); this could be due to the small groups or alternatively to other intrinsic factors in the NOA group which are not seen after PGD-AS screening.

In conclusion, we think that there is a place for PGD-AS for NOA as well as OA patients in view of the high aneuploidy frequency and the large pool of embryos that can be screened, to allow us to select one or two embryos for transfer. However, the ultimate confirmation that PGD-AS improves the pregnancy rate/live birth rate in this group of patients will only happen after a study that is randomized and probably multicentre (as the number of NOA men with embryos to biopsy is quite small).

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