Preimplantation diagnosis for X and Y normality in embryos from three Klinefelter patients*

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In some 47,XXY Klinefelter patients without evidence of mosaicism, testicular spermatozoa can be successfully recovered and used for intracytoplasmic sperm injection (ICSI). To ensure the replacement of embryos with a normal X and Y chromosome pattern, preimplantation diagnosis can be performed. This paper reports on three 47,XXY Klinefelter patients from whom it was possible to retrieve testicular spermatozoa in order to perform ICSI. From their healthy wives a total of 27 oocyte-cumulus complexes were retrieved from which 22 metaphase-II oocytes were obtained and injected; 19 of these were intact after injection. Two distinct pronuclei were observed in eight oocytes (42.1%) 18 h after injection. On day 3 of development, five embryos (62.5%) had reached at least the 6-cell stage and were of sufficient quality to undergo biopsy and subsequent preimplantation diagnosis for sex chromosome analysis by fluorescent in-situ hybridization. The four embryos diagnosed as normal were transferred to three respective patients, resulting in one biochemical pregnancy. The remaining cells of the fifth embryo were analysed afterwards, revealing a normal X and Y chromosome constitution. So far, in the five embryos diagnosed, a normal sex chromosome pattern has been observed.

Key words: FISH/ICSI/ Klinefelter's syndrome/preimplantation diagnosis/testicular spermatozoa

Introduction
In some individuals, with an apparently homogeneous 47,XXY Klinefelter karyotype, focal spermatogenesis and severe oligozoospermia have been observed (Paulsen et al., 1968; Luciani et al., 1970; Tournaye et al., 1996). The introduction of testicular sperm recovery for patients with abnormal tubular function (Devroey et al., 1995a,b; Tournaye et al., 1995) combined with successful intracytoplasmic sperm injection (ICSI) using the testicular spermatozoa opens new perspectives of fatherhood for these Klinefelter patients. Normal fertilization and embryo development in patients with mosaic Klinefelter's syndrome has recently been reported (Bourne et al., 1995; Harari et al., 1995).

Analysis of spermatozoa from mosaic 46,XY/47,XXY Klinefelter patients revealed a significant increase in 24,XY-bearing sperm cells (Cozzi et al., 1994; Chevret et al., 1995). This indicates that 47,XXY cells are able to go through meiosis and to produce hyperploid spermatozoa.

Fluorescent in-situ hybridization (FISH) with chromosomespecific DNA probes is a reliable technique for the detection of numerical aberrations (Jones et al., 1987; West et al., 1988). The FISH method with directly labelled DNA probes specific for the X and Y chromosomes offers an efficient and fast procedure to determine the exact number of sex chromosomes present in one or two blastomeres of a preimplantation embryo. Preimplantation diagnosis, after infertility treatment with ICSI using testicular spermatozoa, in order to ensure the replacement of embryos with normal XX or XY chromosome pattern, is therefore a justified procedure in the procreation of healthy children at this experimental stage.

The present paper reports on our experience of three Klinefelter patients from whom sperm recovery with a view to ICSI was successful.

Materials and methods.

Patient description and testicular biopsy
Three Klinefelter patients with a 47,XXY karyotype without evidence of mosaicism and their healthy wives with a normal fertility checkup form the basis of this report. The testicular-biopsy sperm extraction procedure and patient-related characteristics are described in detail by Tournaye et al. (1995; patient nos. 1, 2 and 9).

Oocyte retrieval and embryo culture
Ovarian stimulation was performed by a desensitizing protocol using the gonadotrophin-releasing hormone agonist buserelin acetate (Suprefact; Hoechst, Brussels, Belgium) in association with human menopausal gonadotrophin (Humegon from Organon, Oss, The Netherlands; or Pergonal from Serono, Brussels, Belgium) and human chorionic gonadotrophin (HCG: Pregnyl from Organon or Profasi from Serono)(Smitz et al., 1988) Luteal phase supplementation was performed by daily intravaginal administration of 3X200 mg micronized progesterone (Utrogestan; Piete, Brussels, Belgium) as described previously (Smitz et al., 1992). Oocyte retrieval was carried

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blastomeres were checked for the presence of nuclei. Either one or two cell stage on day 3 of development were biopsied. Before biopsy, the embryos were assessed at least the 6-<sup>th</sup> cell stage.

Oocytes with 2PN were assessed on day 2 and day 3 after injection for embryonic development, and the embryos reaching at least the 6-cell stage on day 3 of development were biopsied. Before biopsy, the blastomeres were checked for the presence of nuclei. Either one or two blastomeres with a distinct nucleus were removed, depending on the stage of the embryo.

The biopsy procedure started with zona drilling. For this, the embryo was maintained in a stationary position by gentle suction through the holding pipette (outer diameter of 60–80 μm and inner diameter of ±15 μm) so that the fragments were at the 3 o'clock position. If no fragments were present, the blastomeres were kept as far as possible from the drilling site. Zona drilling was performed by gently blowing acid (pH 2) Tyrode's solution from a small pipette with a maximum outer diameter of 10 μm. The pipette was moved from the 2 to the 4 o'clock position to thin the zona over a given area. This movement decreased as the zona became thinner. Towards the end, drilling was done very carefully. At the moment when the zona was perforated, blowing was stopped immediately to prevent the acid Tyrode's solution from entering the embryo. The dish was removed from the inverted microscope. The embryo was then aspirated into a hand-pulled pipette, rinsed in a fresh droplet and transferred to a new dish with droplets of HEPES-buffered Earle's medium. Thereafter, the acid Tyrode's pipette was replaced by a biopsy pipette (inner diameter 30–35 μm) on the micromanipulator. Subsequently, each embryo was rotated by means of the holding pipette to bring the previously made opening to the 6 or 12 o'clock position and in such a way that one blastomere containing a nucleus was close to the opening. Using the biopsy pipette, one blastomere was pushed through the opening and out of the zona. After the retraction of one blastomere, the pressure inside the zona pellicula was restored by gently retracting the biopsy pipette while preventing the blastomere from re-entering through the opening. If a second blastomere had to be removed, the procedure was repeated. The biopsied blastomeres were removed from the embryo using a hand-pulled pipette and transferred to a dish with culture-medium drops under oil. After biopsy, the embryos were returned immediately to normal culture conditions.

**Spreading of the interphase nuclei**

Using a mouth-aspirated pipette, the individual blastomeres were first rinsed in medium and thereafter transferred to a 1–2 μl droplet of 0.01 N HC1/0.1 % Tween 20 solution (Coonen et al., 1994) on a slide. A small circle was etched on the back of the slide to facilitate localization of the nuclei after fixation and hybridization. During spreading, the blastomere was constantly observed by means of an inverted phase-contrast microscope (Olympus CK2, with ×5, ×10, ×40 objectives). After spreading, the slides were left to dry for ~20 min, washed in phosphate-buffered saline (PBS) for 5 min and dehydrated through an ethanol series.

**FISH procedure**

The FISH procedure as described by Coonen et al. (1994) was used. Briefly, the nuclei were digested with pepsin (from porcine stomach.

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### Table 1. Details of numbers of oocytes retrieved, fertilization and embryo development for each patient

<table>
<thead>
<tr>
<th>Patient no</th>
<th>No of cumulus–oocyte complexes</th>
<th>No of MII oocytes injected/ intact after ICSI</th>
<th>No of oocytes with 2PN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1)</td>
<td>6</td>
<td>5/4</td>
<td>2</td>
</tr>
<tr>
<td>2 (2)</td>
<td>11</td>
<td>10/9</td>
<td>4</td>
</tr>
<tr>
<td>3 (9)</td>
<td>10</td>
<td>7/6</td>
<td>2</td>
</tr>
</tbody>
</table>

*The numbers in parentheses are the corresponding identification numbers for these patients in the study reported by Tournaye et al. (1996)

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### Table 2. Details of biopsy and results of fluorescent in-situ hybridization (FISH) technique on biopsied cells

<table>
<thead>
<tr>
<th>Patient no</th>
<th>Embryo no</th>
<th>Biopsied blastomeres</th>
<th>FISH result</th>
<th>Embryo transferred?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1)</td>
<td>(1)</td>
<td>2</td>
<td>XX, XX</td>
<td>yes</td>
</tr>
<tr>
<td>2 (2)</td>
<td>(1)</td>
<td>1</td>
<td>XY</td>
<td>yes</td>
</tr>
<tr>
<td>(2)</td>
<td>1</td>
<td>2 nuclei XX, XX</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>(3)</td>
<td>1</td>
<td>XY</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>3 (9)</td>
<td>(1)</td>
<td>1</td>
<td>yes</td>
<td></td>
</tr>
</tbody>
</table>

*The numbers in parentheses are the corresponding identification numbers for these patients in the study reported by Tournaye et al. (1996)

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*Only one blastomere was biopsied and it showed two nuclei, both XX

*No reliable result due to the prometaphase stage of the nucleus

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**Intracytoplasmic sperm injection procedure**

The details of microtool preparation, equipment and technique used for the injection procedure have been described previously (Van Steirteghem et al., 1993, 1995). The ICSI procedure was carried out on the heated stage of an inverted microscope (Diaphot, Nikon Corporation, Tokyo, Japan) at ×400 magnification. The testicular spermatozoa were frequently seen to be embedded in Sertoli cell cytoplasm and needed to be carefully extracted. For the injection, a motile sperm cell, if present, was aspirated into the injection pipette and injected into the metaphase-II oocyte which had extruded the first polar body. Injected oocytes were washed and incubated in 25 μl droplets of B2 medium in a Petri dish at 37°C in an incubator containing 5% CO2, 5% O2 and 90% N2. Fertilization was checked 16–18 h later (Nagy et al., 1994). Normal fertilization was confirmed by the presence of two distinct pronuclei and polar bodies at ×400 magnification using an inverted microscope.

**Embryo development and biopsy**

Oocytes with 2PN were assessed on day 2 and day 3 after injection for embryonic development, and the embryos reaching at least the 6-cell stage on day 3 of development were biopsied. Before biopsy, the blastomeres were checked for the presence of nuclei. Either one or two blastomeres with a distinct nucleus were removed, depending on the stage of the embryo.
mucosa: 100 μg/ml; Sigma) in 0.01 N HCl for 20 min at 37°C. The slides were rinsed in MilliQ water followed by 1× PBS and fixed for 10 min in 1% paraformaldehyde in PBS at 4°C. After fixation, the slides were first rinsed in 1× PBS and then in MilliQ water and dehydrated through an ethanol series.

Double target FISH was performed using directly labelled DNA probes specific for chromosomes X and Y. The X (Vysis, Alpha Satellite DNA probe, spectrum Green) and Y (Vysis, Alpha Satellite DNA probe, spectrum Orange) probes were used for gender determination. The probe mixture was added to the slide under a coverslip and the nuclear and probe DNA were denatured simultaneously for 4 min at 75°C. The slides were then incubated in a moist chamber at 37°C for 30-60 min to allow hybridization of the DNA probes. After hybridization, the slides were washed for 5 min with 60% formamide/2X SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7) and 5 min with 2X SSC at 42°C, followed by additional 5 min wash at room temperature with 4X SSC/0.05% Tween 20 solution. The slides were dehydrated through an ethanol series and mounted in the antifade medium Vectashield (Vector) containing 1.25 ng/ml 4’,6-diamidino-2-phenylindole (DAPI) to counterstain the nuclei. The nuclei were then examined using a Zeiss Axioskop fluorescence microscope with the appropriate filter set [filter 10 for fluorescein isothiocyanate (FITC), filter 02 (DAPI) and Omega filter (FITC/Texas red)]. All nuclei were observed and FISH results interpreted by two independent observers.

Results
A total of 27 oocyte–cumulus complexes were retrieved from the three patients, as shown in Table I. Twenty-two metaphase-II oocytes were injected with testicular spermatozoa and 19 oocytes remained intact after injection. Eight oocytes (42.1%) showed two distinct pronuclei at 18 h after injection. On day 3 of development, five embryos (62.5%) had reached at least the 6-cell stage and were of sufficient quality to undergo biopsy. The nuclei from the retrieved blastomeres were subsequently used for preimplantation diagnosis with the FISH technique using X and Y chromosome-specific probes.

The results of the biopsy and of the FISH diagnosis are given in Table II. From three diagnosed embryos, one was diagnosed as clearly female (Figure 1) and two others as male and were transferred. For one embryo (patient no. 2, embryo no. 2), two nuclei were present in the single blastomere biopsied, both showing two signals for the X chromosome in the absence of a Y signal. This embryo was also transferred. In embryo no. 3 from patient no. 2, the nuclear membrane was weak and ruptured as the blastomere spread, scattering prometaphase-like nuclear contents over the slide. After the FISH procedure, one X and one Y signal were visible. Nevertheless, due to an unreliable overview of the scattered nuclear material and the search for X or Y aneuploidy in this particular case, it was decided not to transfer the embryo. The remaining cells of the embryo were further investigated afterwards and revealed a uniform XY pattern.

In two patients, a transfer of a single embryo was performed, resulting in one biochemical pregnancy (HCG 210 IU/l) and one patient received two embryos but without becoming pregnant.

Discussion
Spermatozoa were recovered from the wet preparations of testicular tissue from only four out of nine 47,XXY patients (Tournaye et al., 1996). ICSI was performed in three couples,
and our observations indicated that testicular spermatozoa obtained from Klinefelter patients can induce fertilization (42.1%) followed by embryo development, as already reported by Harari et al. (1995). Despite the fact of severe disturbed spermatogenesis, the fertilization rate and subsequent cleavage rate obtained for this small number of Klinefelter patients were quite similar to the results reported with testicular spermatozoa from patients with non-obstructive azoospermia (Tournaye et al., 1995).

Data concerning genetic analysis performed on sperm cells of non-mosaic Klinefelter individuals are not available in the literature. Establishing the incidence of abnormal sperm cells with respect to X and Y chromosomes would have been of particular importance in these three cases. However, this was not feasible, owing to the absence of supernumerary spermatozoa after injection. In the three cases described, the injected sperm cells were the only ones found after several hours of searching in several shredded testicular biopsies.

Recent publications (Cozzi et al., 1994; Chevret et al., 1995) on analysis of spermatozoa from mosaic 46,XY/47,XXY Klinefelter patients have revealed a significant increase in 24,XY-bearing sperm cells. This indicates, in contradiction to the previous hypothesis (Steinberger et al., 1965; Paulsen et al., 1968; Luciani et al., 1970), that 47,XXY cells are able to go through meiosis and to produce hyperploid spermatozoa. Fertilization of an oocyte with such numerically abnormal spermatozoa from non-mosaic or mosaic Klinefelter individuals would result in 47,XXY progeny.

This paper is the first to report on the genetic analysis of embryos obtained after fertilizing oocytes with sperm cells of 47,XXY Klinefelter patients. None of the investigated embryos showed an abnormality for the X or Y chromosomes. Obviously, more cases are needed to determine the existence and incidence of abnormal sperm cells and embryos in this particular patient population. One embryo was found to be binucleated, both nuclei showing two X-chromosome signals, this can arise from mitotic replication without cytokinesis (Munné and Cohen, 1993). Multinucleation occurs frequently in arrested as well as in normally developing embryos (Munné and Cohen, 1993).

Preimplantation diagnosis by FISH for the chromosomes X and Y is feasible and recommended for mosaic and non-mosaics Klinefelter patients from whom sperm cells can be recovered and embryos obtained after ICSI. This approach can also be considered for other patients with numerical aberrations of the sex chromosomes, such as XXX females or XYY males.

So far, only one biochemical pregnancy has been obtained in our Klinefelter patient population. However, in a patient group undergoing preimplantation diagnosis for sex determination using the same protocol, out of four cycles two pregnancies were obtained. One resulted in a liveborn child confirming the diagnosis and the other is a twin pregnancy of 10 weeks of gestation with the diagnosis not yet confirmed (a prenatal diagnosis is planned in this patient). Due to the novelty of the preimplantation diagnosis technique, in the case of pregnancy a prenatal diagnosis is also recommended.

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Preimplantation diagnosis after ICSI from 47,XXY patients