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First successful pregnancy in Switzerland after prospective sex determination of the embryo through the separation of X-chromosome bearing spermatozoa

Christian De Geyter[a], Oliver Sterthaus[a], Peter Miny[b], Friedel Wenzel[b], Olav Lapaire[c], Maria De Geyter[a], Gideon Sartorius[a]

[a]Clinic of Gynaecological Endocrinology and Reproductive Medicine, University Hospital, University of Basel, Switzerland

[b]Division of Human Genetics, Children's Hospital, University of Basel, Switzerland

[c]Clinic of Fetomaternal Medicine and Obstetrics, University Hospital, University of Basel, Switzerland

Summary

QUESTION UNDER STUDY: The feasibility and the potential advantages of separating X-chromosome bearing spermatozoa for the prevention of a severe X-chromosome linked disorder with the use of intracytoplasmic sperm injection are presented.
METHOD: A carrier of muscular dystrophy type Becker was treated with intracytoplasmic sperm injection, using spermatozoa previously stained with the Hoechst dye 33342 and sorted with flow cytometry.
RESULTS: After transfer of one single blastocyst, an intrauterine pregnancy arose. In the ninth week of gestation, the female sex of the embryo was confirmed with proof of absence of the SRY gene of the Y-chromosome. After normal pregnancy, the patient delivered a healthy daughter.
CONCLUSIONS: The staining of spermatozoa with specific markers and sorting with flow cytometry provides a means of preventing significant disease in the offspring and may help in reducing the number of surplus embryos needed for preimplantation genetic diagnosis.

Key words: *preimplantation genetic diagnosis; assisted reproduction; flow cytometry; semen preparation; X-linked disorders*

Introduction

Assisted reproduction technology (ART) in Switzerland is regulated through a restrictive legislative framework, limiting the number of embryos to be developed to three in one single treatment cycle. This limitation together with a ban on all cryopreservation of embryos has prevented the introduction of preimplantation genetic diagnostics (PGD). Although the Swiss Parliament voted in 2005 in favour of PGD, the adoption of this technique is being considered impractical by virtually all expert institutions and organisations in Switzerland, as long as the number of embryos to be tested is to remain limited [1]. Currently, the debate surrounding the legal framework of both ART and PGD is heavily focused on the ethical and practical implications of surplus embryos. Therefore, the emergence of new technologies reducing the risk of surplus embryos is of interest.
Due to a 2.8% difference in total DNA content the population of either X- or Y-chromosome bearing spermatozoa can be enriched using flow cytometry and sorting (FACS). In flow cytometry single spermatozoa are guided into a small microfluidic channel and illuminated with a laser beam. For this purpose the DNA of the spermatozoa is made visible with the fluorescent signal emitted by the Hoechst 33342 dye, which is able to penetrate into the spermatozoa's nuclei and to bind the DNA in a non-intercalating and reversible fashion. The emitted fluorescent light is detected and quantified. Based upon this quantified signal the single cells can then be gated and collected as enriched populations of either X- or Y-chromosome bearing spermatozoa.
We here present the first successful pregnancy and delivery after the sorting of X- and Y-chromosome bearing spermatozoa in Switzerland.

Material and methods

The female partner is a known carrier of Becker's muscular dystrophy, which is an X-linked recessive disorder characterised by progressive muscular weakness of the lower limbs and the pelvic muscles. One of her two affected brothers had died as a consequence of the disease. From the onset, the couple rejected the option of prenatal diagnostics and legal abortion. For financial reasons PGD in a treatment centre outside Switzerland was considered impossible. Polar body biopsy was considered technically feas-

ible [2], but after long discussions, it was decided to reduce the risk of an affected child through the sorting of X-chromosome bearing spermatozoa for intracytoplasmic sperm injection (ICSI).

In preparation for ICSI the female partner went through a regular fertility checkup, including hormonal analysis of the early follicular phase, ultrasound assessment of the antral follicular count and of the preovulatory endometrial lining. The husband underwent evaluation of his fertility status, including conventional semen analysis. In both partners hepatitis B, hepatitis C, HIV and syphilis were excluded. In addition, the couple was counselled by a medical geneticist on the residual risk of transmission of the genetic condition and about the necessity to confirm the sex of the foetus using prenatal diagnostics such as chorionic villi sampling or amniocentesis.

The case and the treatment plan were presented to the ethical board of the University Hospital of Basel and a formal approval was obtained. Then, we contacted MicroSort, a subdivision of the Genetics and IVF Institute located in Fairfax, Virginia, U.S.A., holding the technology to separate X-bearing from the Y-bearing spermatozoa. After having obtained their written approval, two semen samples of the husband were obtained and cryopreserved. These samples were then sent to the MicroSort facilities, thawed, sorted and cryopreserved again.

In compliance with the Swiss law on ART, the couple signed an agreement to undergo a treatment with ICSI. Ovarian hyperstimulation was carried out with recombinant follicle-stimulating hormone (Puregon, MSD, Luzern, Switzerland) in a long GnRH-agonistic protocol (Decapeptyl Depot, Ferring, Wallisellen, Switzerland) and ovulation was induced with recombinant human chorionic gonadotropin (Ovitrelle, Merck-Serono, Zug, Switzerland). The luteal phase was supported with vaginally applied micronised progesterone (Utrogestan, Vifor, Villars-sur-Glâne, Switzerland).

In parallel to the treatment with ICSI, fluorescence in-situ hybridisation (FISH) was performed on surplus spermatozoa not used in ICSI to verify the relative numbers of X- and Y-chromosome bearing spermatozoa. For that purpose spermatozoa were fixed with 3:1 methanol/acetic acid on superfrost slides. For FISH the slide was then incubated in 1 M NaOH at room temperature for 2 minutes in order to render the chromatin of the spermatozoa accessible to the DNA probes: cepX: DXZ1, cepY: DYZ3, and cep6: D6Z1 for control purposes (ABBOTT/Vysis). After co-denaturation at 80 °C for 10 minutes the samples were hybridised for 16 hours at 37 °C. Thereafter, the signals were analysed using a ZEISS Axioscope microscope.

Results

None of the various infertility investigations showed any abnormality in either partner. The husband was found to be normogonadotropic normozoospermic. After ovarian hyperstimulation 14 oocytes in the metaphase II stage were harvested for ICSI. After thawing, none of the spermatozoa were found to be motile. All 14 mature oocytes were then micro-injected with immotile spermatozoa. The next morning 3 oocytes presented two pronuclei and were left in culture. One embryo developed to the blastocyst stage and was then transferred into the uterine cavity under ultrasound guidance. Fourteen days after oocyte collection an intrauterine singleton pregnancy was diagnosed.

The sex-chromosome status of the heads of 203 surplus spermatozoa was evaluated with FISH, and showed that 190 (93.6%) were carrier of the X-chromosome, whereas 13 (6.4%) carried the Y-chromosome.

As the sorting procedure cannot assure full certainty with respect to the sex of the offspring, prenatal diagnostics were considered essential for confirmation. However, in order to avoid the associated risks of an invasive prenatal procedure, full blood containing cell-free fetal DNA was taken from the pregnant patient in the 9th week of gestation to confirm the sex of the developing embryo. Using polymerase chain reaction (PCR) the maternal blood was typed for the absence of SRY and DYS14. In the presence of a negative signalling for both genes, the blood of both parents was typed again for a set of bi-allelic genes to identify informative paternal marker systems. Then, DNA was again isolated from the plasma and tested for the presence of a paternal marker system. After confirming the absence of SRY, a female embryo was diagnosed within two weeks after the initial blood sampling.

Further pregnancy evolved well and the patient delivered a healthy girl at term.

Discussion

Although the Swiss law on ART specifically allows gamete selection in determining the sex of the offspring for the prevention of severe and incurable disease (Fortpflanzungsmedizingesetz, FMedG, Art. 5, subheading 2), the technology of separating X- and Y-chromosome bearing spermatozoa has not yet been reported for this purpose in Switzerland so far. In the light of the current debate surrounding the introduction of PGD into clinical medicine, the first successful case of sperm sorting for the prevention of a severe X-linked disease in Switzerland helps to illustrate new strategies how to reduce the number of surplus embryos deriving from assisted reproduction. Furthermore, as compared to PGD this method is non-invasive and therefore less risk of exerting damage to the embryo, which has become more evident after the freezing and thawing of previously biopsied embryos [3, 4].

The technology for the separation of X- and Y-chromosome bearing spermatozoa was developed in veterinary medicine for breeding purposes and in humans for either family balancing or for the prevention of X-linked diseases. The use of flow cytometry for the separation of spermatozoa according to their sex chromosome content was first described by Pinkel and collaborators in 1982 [5]. They used spermatozoa of the creeping vole, which are characterised by a 9% difference in DNA content between Y-chromosome bearing spermatozoa and the “female” spermatozoa lacking any X-chromosomal material. Early trials were hampered by the discoid form of the spermatozoa, necessitating two simultaneous measurements at two angles around the flow-channel and subsequent correction of the detected signals. Another problem was posed by the staining of the DNA of the spermatozoa, which was at that time

inevitably toxic to the cells. The first problem is now overcome by the microfluidic orientation of the spermatozoa in the microchannel flow with the help of a nozzle, which due to the specific form of the spermatozoa needs to be adapted to each species in particular. The nozzle developed for the sorting of human spermatozoa has been filed as a patent and MicroSort is the owner of it.

Another challenge is given by the need to reversibly stain the DNA of the gonosomes of the spermatozoa to be sorted. This was achieved by the use of the Hoechst dye 33342, which is a synthetic fluorescent bisbenzimidazole, which readily permeates the cellular membrane and selectively binds to the AATT-base pairs of the DNA double helix [6] (2001). As the Hoechst dye 33342 is not intercalating, it is considered safe for supravital staining purposes and is not regulated under the European Union directive guidelines. At the concentrations used for the purpose of sorting of human spermatozoa (e.g. 9 μM), no effect of the Hoechst dye 33342 on the motility of the spermatozoa nor in the mutation rate of the β-globin gene, which was chosen to represent a portion of the genome, was observed [7]. Although the dye in the unwashed spermatozoon has been shown to diffuse within the oocyte, to pass from the male pronucleus to the female pronucleus and to remain detectible in the developing embryo up to the 16-cell stage [8], the safety of the dye in combination with FACS has been amply documented, as approximately a million mammalian offspring have been produced following the sorting process using the Hoechst dye [8, 9]. Large trials comparing the gestational outcome and the offspring resulting from sorted spermatozoa and those from control semen demonstrated no changes in abortion rate, duration of gestation, and offspring characteristics of bovine [10] and pig [11]. Experience with more than 5,000 sorting procedures in human ART has also failed to find a difference from the general population with respect to the complication rates during pregnancy and to neonatal abnormalities [12].

Flow cytometry for the selection of X- and Y-chromosome bearing spermatozoa was pioneered in the early nineties [13] and has meanwhile led to the births of 924 babies [12]. The efficiency of purifying X-chromosome bearing spermatozoa is higher than purifying Y-chromosome bearing spermatozoa, 80% to 90% versus 60%to 70% [14], making it the ideal procedure for the prevention of X-chromosome linked diseases. X-linked diseases may be either dominant or recessive (table 1). Females having an X-linked recessive mutation will not present with significant clinical manifestation of the disorder, but 50% of their male offspring will be affected and 50% of the female offspring will be a carrier of the mutation. Except muscular dystrophy type Duchenne or type Becker most of these diseases are very rare or are of lesser medical concern (table 1A).

There are, however, also a number of X-linked dominant disorders (table 1B), in which both sexes are affected, but typically the male more severely. A good example is given by the full mutation of FMR1, which is characterised by the presence of more than 200 repeats of the base pair triplet CCG in the promotor region of the gene and which causes severe disability in the male offspring, known as the fragile X-syndrome. Prolonged expansion of the CGG repeat number of the FMR1-gene are also associated with primary ovarian insufficiency and infertility in women and both the American College of Obstetrics and Gynaecology (ACOG) and, to a lesser extent, the European Society of Human Reproduction and Embryology (ESHRE), have therefore recommended or advocated screening of women with primary ovarian insufficiency [15, 16]. Whereas the incidence of FRM1-premutation (55 to 200 CGG-repeats) and of intermediate repeat length (45 to 54 CGG-repeats) is as high as 5.6%, resp. 7.4% among women with primary ovarian failure[17], an incidence of 0.2%, resp. 1.3% were found among infertile women with normal ovarian reserve [18]. In the light of the current controversy concerning the cost-effectiveness of a widespread screening for the carrier status of FMR1 among infertile women or at the population level [19], the clinical importance of the sorting of X-chromosome bearing spermatozoa should receive more attention.

In achieving a separation rate of up to 90% of X-chromosome bearing spermatozoa [14], this method reached efficacy rates clearly inferior to those reached by PGD: among 61 viable pregnancies for X-linked disease, only 4 were found to be misdiagnosed (6.6%) [20]. However, the implantation rates of embryos after manipulation for PGD are also lower than after regular assisted reproduction (after PGD for X-linked diseases 14.0% [20] versus approximately 20.0% to 25.0% with unselected embryos). In view of the limited number of embryos available for PGD in many couples, the targeted creation of more suitable embryos using sorting of X-chromosome bearing spermatozoa prior to PGD will undoubtedly contribute to increase the life birth rate in PGD cases. In addition, it will help to reduce the number of surplus embryos in PGD.

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Correspondence: *Professor Christian De Geyter, MD, Klinik für Gyn. Endokrinologie und Reproduktionsmedizin, Spitalstrasse 21, CH-4031 Basel, Switzerland,* *cdegeyter[at]uhbs.ch*

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Table 1: The most common X-linked disorders are listed according their prevalence.

Name of the syndrome	Prevalence
Recessive disorders	
Glucose-6-dehydrogenase deficiency	Common
Duchenne muscular dystrophy	1:3,600
X-linked bulbospinal neuropathy, Kennedy's disease	1.3:8,500
Hemophilia A and B	1:5,000
Ichtyosis, X-linked	1:2,000 to 1:6,000
Aarskog-Scott syndrome	1:25,000
Fabry's disease	1:40,000
Wiskott-Aldrich syndrome	1:100,000
Bruton's agammaglobulinaemia	1:100,000
Hunter syndrome	1:130,000
Androgen insensitivity syndrome	1:200,000
Menkes disease, kinky hair syndrome	1:254,000
Lesch-Nyhan syndrome	1:380,000
Severe combined immunodeficiency	Rare
Sideroblastic anaemia	Rare
Leigh syndrome, X-linked	Extremely rare
Dominant disorders	
Charcot-Marie-Tooth disease	1:2,500
Fragile X-syndrome	1:3,600
Alport syndrome	1:5,000
Rett syndrome	1:12,500
Hypophosphataemic rickets	1:20,000
Ornithine transcarbamylase deficiency	1:80,000
Incontinentia pigmenti type 2	Rare
Lujan-Fryns syndrome	Rare
Bazex-Dupré-Christol syndrome	Rare
Aicardi syndrome	Very rare