First Romanian live birth after Preimplantation Genetic Testing in a couple with Severe Oligospermia determined by Y Chromosome Microdeletions

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Abstract

Y chromosome microdeletions (YCM) represent a genetic disorder, frequently responsible for the disruption of the spermatogenesis process. Its prevalence is about 20% among patients diagnosed with oligozoospermia. Here we report the successful live birth of a female baby after morphologically selected sperm injection (IMSI) and PGD for a couple with severe oligospermia related to Y chromosome microdeletions. Blood samples from the couple were obtained and the cytogenetic analysis was carried out on in vitro cultured peripheral lymphocytes. DNA extraction and molecular analysis performed on blood samples from the male patient using multiplex-PCR method enabled the identification of 24 microdeletions in AZFa, AZFb or AZFc loci, the absence of a series of markers being observed. The couple underwent an IVF cycle associated with PGD. The resulting embryos were cultured until day 3 and subsequently, the blastomere biopsy was carried out on 7 of the viable embryos. The entire genome was scanned using the Microarray-CGH technique with the Agilent GenetiSure Pre-Screen test.

The analysis of the 7 embryos obtained through IMSI revealed an affected genotype in four of biopsies and a non-affected genotype in three biopsies. Two of the unaffected embryos were female and one was male. Accordingly, it was decided to carry out the transfer of the most suitable female embryo, which resulted in the birth of a healthy baby.

The results confirm the importance of performing these type of genetic investigations in patients with a history of male infertility with genetic component.

Keywords: Severe Oligospermia, Y Chromosome Microdeletions, Intracytoplasmic Morphologically Selected Sperm Injection, Preimplantation Genetic Diagnosis, In Vitro Fertilization

Introduction

Preimplantation genetic diagnosis (PGD) is a technique performed in conjunction with in vitro fertilisation (IVF), dedicated at identifying possible genetic defects in embryos obtained through human assisted reproductive technology (ART) [1]. Usually, PGD is performed for couples in which one or both partners suffer from a genetic disorder. Therefore, embryos may be tested to reveal whether they are affected by the same abnormalities. Over 350 different medical conditions may be diagnosed, with an accuracy of about 99.5% [2].
Unlike other well-known methods of prenatal diagnosis (e.g. amniocentesis), which is only available when the pregnancy is established, PGD is performed before implantation. It can aid both medical team and the patients in choosing the most genetically suitable embryo(s). In contrast to amniocentesis, where the parents may risk being faced with the dramatic decision to terminate an ongoing pregnancy, PGD may improve the likelihood of obtaining a normal pregnancy [3].

The successful implementation of PGD technique in an IVF clinic involves collaboration between several areas such as IVF, genetics and single cell diagnosis laboratories.

Notwithstanding, the ability of the PGD test to enable parents affected by a genetic disease to have a healthy child, there are some legal, ethical and medical implications in Romania which were highlighted before [4], but unfortunately still remain unanswered.

**Material and Methods**

A 28 years old female patient and her 30 years old male partner presented for fertility advice after unsuccessfully trying to conceive for almost 2 years. The initial consultation aimed at assessing the medical history of the patients, the eventual risk factors, as well as the results of previous investigations. Both patients were submitted to a viral screening for hepatitis and HIV (Ag HBs, Ac HBC, Ac HCV, Anti HIV1+ 2, VDRL), both patients being tested negative.

For the female patient, a series of clinical and paraclinical investigations were carried out. The physical examination revealed pain in the right flank. Consecutive the evaluation of the genital tract through ultrasound with 3D/4D reconstruction, the presumptive diagnosis of bilateral hydrosalpinx was established. In order to confirm the diagnosis, patients undergone a laparoscopy.

Further on, the parasidical investigations involved the determination of endocrine markers (TSH, progesterone, estradiol, FSH, LH, prolactin), the values ranging between the normal values. The ovarian reserve was assessed by determining the anti-Müllerian hormone (AMH), the result being 9.09 ng/ml.

The TORCH screening (Rubella IgG, Varicella IgG, Toxoplasma IgM and IgG, Citomegalovirus IgG, Herpes IgG) revealed that the patient was tested positive for Citomegalovirus (IgG 245.1 UA/ml), Rubella (IgG anti Rubella 162 UI/ml), Herpes (IgG Herpes I+II 5.46 UI/ml).

Additionally, immunological investigations for infectious diseases (Chlamydia IgG + IgA in blood), microbiological investigations (Ureaplasma and Mycoplasma in col) and hematological investigations (hematocritogram, APTT, fibrinogenemia, 25-OH-vitamin D, coagulogram composed of INR, Quick time) were performed and the results were in normal limits based on sex and age of the patient.

Semen quality was assessed by performing a spermogram and semen culture. The patient was diagnosed with severe oligospermia (0.2x 106/ml) and asthenozoospermia. Additionally, the sperm fragmentation index was determined through sperm chromatin structure assay (SCSA), the integrity of the sperm DNA being considered moderate according to the result (22.3%).

In light of the information obtained from the anamnesis and the clinical and paraclinical investigations, the medical team decided to recommend a genetic screening for both patients. It consisted in determining the karyotype for the female patient and detecting the microdeletions in the Y chromosome structure for the male patient.

The study was conducted by an interdisciplinary team between 2015 - 2017. The infertility consult and the medical investigations, as well as the IVF procedure, embryo culture and subsequently embryo biopsy were carried out at Orügn Fertility Center, Iasi, Romania. The genetic investigations and PGD procedure were performed at the Regional Oncology Institute, Department of Molecular Biology, Iasi, Romania.

The antenatal routine monitoring of the patient was performed at the Orügn Fertility Center, while the neonatal evaluation was carried out at Clinical Hospital of Obstetrics and Gynecology “Cuza Voda” Iasi, Romania, where the birth took place.

Blood samples from the couple were obtained and the cytogenetic analysis was carried out on in vitro cultured peripheral lymphocytes. In the case of the female patient, a total number of 33 metaphases were examined and 4 of them were karyotyped. No chromosomal numerical or structural abnormalities were detected.

DNA extraction and molecular analysis was performed on blood samples from the male patient. Multiplex-PCR method was used with the purpose of identifying the presence of 24 microdeletions in AZFa, AZFb or AZFc loci. The analysis revealed the absence of the following markers: SY153, SY155, SY254, SY255, SPGY, SY277, SY269.

The couple signed a consent form according to the internal ethical protocol in our fertility center regarding the procedures of IMSI and PGD after being previously counseled by the medical team with respect to the IVF procedure, the risks associated with the ovarian hyperstimulation stimulation syndrome (OHSS), the probability of obtaining pregnancy, the risks of complications during pregnancy, the necessity of performing the prenatal screening and possible risks associated with the embryo biopsies, the measures to be taken in case of obtaining embryos with genetic disorders, as well as the possibility of cryopreserving the supernumerary embryos obtained following IVF treatment.

The treatment was carried out with gonadotropin-releasing hormone (GnRH) antagonist down-regulated protocol using ganirelix (Orgalutran; Organon, Oss). Ovarian stimulation was
started on the second day of menstruation with a daily dose of 125 IU of recombinant follicle stimulating hormone (rFSH) (Puregon; MSD, Oss) until the 8th day of stimulation. Afterwards, the stimulation was continued with 100IU of rFSH (Puregon; MSD, Oss). Final oocyte maturation was triggered by administration of 0.2mg triptorelin (Diphereline; Ipsen).

The oocyte retrieval was carried out 36 hours afterwards. Subsequently, the oocytes were inseminated using IMSI and the resulting embryos were cultured and monitored until day 3 using a time-lapse system (EmbryoScope, Vitrolife, Denmark). On day 3, the blastomere biopsy was carried out by an embryologist and 7 embryos were biopsied.

Briefly, the embryo biopsy was performed in drops of 20 ml of flushing media containing HEPES-buffered solution (FertiPro, Belgium) under a layer of Mineral Oil (Irvine Scientific). A laser beam (company, details) was used to puncture the zona pellucida, and 1-2 cells were obtained using embryo biopsy micropipettes. Immediately after the biopsy was carried out, the cells were washed carefully using the same flushing media, placed in Eppendorf PCR microtubes in drops containing 3ml of flushing media. The embryos were subsequently cryopreserved according to published vitrification protocols (Kitazato, Japan) and stored in liquid nitrogen.

The entire genome of the embryos was scanned using the Microarray-CGH technique using the Agilent GenetiSure Pre-Screen test (Agilent Genomics, USA). We have used the Agilent Single Cell Recommended Analysis Method. The embryos were compared with both male and female controls and reports considered only the abnormalities identified in comparison with both controls (larger than 5 Mbases). Smaller variants were considered technical artifacts.

**Results and Discussion**

The analysis of the 7 embryos revealed an affected genotype in 4 of biopsies and a non-affected genotype in 3 of the biopsies. Two of the unaffected embryos were female and one was male (Figure 1,4,6).

![Figure 1: Male embryo, normal genetic analysis.](image1)

![Figure 2: Male embryo with multiple genetic anomalies: add-chr2 p25.3-p11.2; add-chr2 q11.1-q37.3; del-chr5 p15.33-p13.2; add-chr5 p13.1-p11; add-chr9 p24.3-p13.1; add-chr9 q21.11-q21.13; del-chr9 q21.2-q33.3; add-chr10 q11.21-q26.2; del-chr11 p15.5-p14.3; add-chr11 q12.3-q13.2; add-chr16 p13.3-p11.2; add-chr16 q12.1-q24.3.](image2)
Figure 3: Female embryo with genetic anomalies: add-chr8 q21.2-q22.1 (8.4M).

Figure 4: Female embryo, normal genetic analysis.

Figure 5: Male embryo with genetic anomalies: add-chr19 p13.3-p12 (23M).

Figure 6: Female embryo, normal genetic analysis (with very small regions of additions but only in comparison with one of the controls female or male).

Figure 7: Male embryo with genetic anomalies: del-chr5 q23.1-q23.2 (5.7M), del-chr9 p13.1-p11.2 (8.2M), del-chr14 q31.1-q31.3 (5.4M).
At couples’ request and considering the reproductive impact of a deleted Y chromosome in a male offspring, it was decided to carry out the transfer of the most suitable female embryo.

Embryos W1 and W5 (Figure 1 & 4), respectively were considered as good quality embryos, whereas embryo W7 (Figure 6) was considered as having satisfactory quality due to the presence of very small regions of additions (artefacts). Since from the 3 embryos suitable for transfer, only W5 and W7 were female, we choose number W5 for transfer (Figure 8).

Embryo W3 (Figure 3), which presented a small addition on chromosome 8 was considered as an alternative for transfer in case the transfer of the aforementioned embryos would fail.

The other embryos presented multiple abnormalities such as trisomies of chromosomes 2, 10 and 16, and other four chromosomes with large deletion, and/or additions (chromosomes 3, 5, 9 and 11) (Figure 2, 5 & 7).

A hormone replacement therapy was used for embryo transfer. Endometrial preparation was initiated in the first day of menstruation with oral estradiol valerate (Cyclo-Progynova; Bayer, Germany) 2mg/t.i.d and a first transvaginal ultrasound was performed in day 10 of the treatment when endometrial thickness was 10mm and from day 11 natural micronized progesterone (Utrogestan; Lab. Besins Int., France) was started at a dose of 600mg/day. Embryo transfer was performed in day 5 of progesterone administration.

The chosen embryo was thawed, evaluated and transferred, the biochemical pregnancy being confirmed by beta-hCG determination 15 days after the embryo transfer. A clinical intrauterine singleton pregnancy was confirmed by ultrasound 21 days post-transfer. Following uncomplicated antenatal care the patient delivered through Caesarean section, at 39 weeks, a female baby, with an APGAR score of 10, weighting 3300 grams. The baby was evaluated by the neonatologist and successfully adapted to extrauterine life.

Y chromosome microdeletion (YCM) is a genetic disorder characterised by the absence of certain gene(s) in the Y chromosome. The Y chromosome is composed of a short arm and a long one, which in turn is divided into 3 regions, AZFa, AZFb and AZFc, each of them with a specific role.

It has been shown that Y chromosome related infertility is characterised by azoospermia (absence of spermatozoa) or different forms of oligozoospermia (reduced number of spermatozoa) [5]. According to a study conducted in Romania, the incidence of genetic defects is higher in infertile patients diagnosed with severe oligospermia [6].

Over 60% of microdeletions observed in the structure of the Y chromosome are located in the AZFc region. Since this region is not close to the center of the Y chromosome as the AZFa or AZFb regions, the microdeletions at this level have a less profound impact on sperm production and thereby, some patients diagnosed with AZFc deletions present with a reduced total number of spermatozoa (oligospermia) [7].

The deletions in the AZFc region of the Y chromosome are the most frequent cause of spermatogenic failure that can be defined molecularly [8]. In a couple of studies, it has been revealed that the AZFc deletions are responsible for almost 20% of cases with non-obstructive azoospermia or severe oligozoospermia [9,10].

Moreover, a recent study revealed that the development of embryos obtained through ICSI (Intracytoplasmic Sperm Injection) from partners diagnosed with AZFc microdeletions and from those without microdeletions was comparable, suggesting that AZFc microdeletions would not affect the outcome of ICSI procedure [11].

Since female offspring inherit only the X chromosome from the father, they are not at risk. It has been suggested that in IVF therapy for couples where the male has a Y deletion, PGD is recommended in order to establish the gender of the embryos and to clarify the presence of the Y chromosome microdeletions [5].

Surprisingly, two recent studies highlighted the probability of obtaining female embryos is higher when using IMSI (Intracytoplasmic Morphologically Selected Sperm Injection) compared to ICSI (Intracytoplasmic Sperm Injection). Therefore IMSI was considered to be the method of choice in the case of this couple [12,13].

Preimplantation screening aCGH technique is a valuable technique. Although, there are certain considerations that must be taken into consideration. Since the biopsy is performed using a single cell (third day biopsy) or from a small group of trophoectoderm cells (fifth day biopsy), test results may offer an altered image of the entire embryo.
However, the identification of major chromosomal abnormalities such as trisomy or monosomes (aneuploidy) in embryos with good or very good morphology and with satisfactory evolution confirms that preimplantation genetic testing has a defining role in the right and informed choice of the appropriate embryo.

We also have to keep in mind that interpreting the results of aCGH tests should not be rigid, small anomalies that cannot be linked in a clinical context may actually be technical artifacts as they work on an artificially amplified DNA by WGA (Whole Genome Amplification) using MDA technique (Multiple Displacement Amplification).

It is worth mentioning that for this couple, starting from the cause of infertility (Y microdeletion), it would have been enough to achieve a simpler technique that would highlight the presence of the Y chromosome (FISH or PCR). The array technique brought very valuable information about of the entire genome which resulted in a pregnancy and the birth of a normal girl and the avoidance of embryonic transplants by attempting.

Conclusion

In the era of human assisted reproduction, the genetic screening has opened new horizons. Thereby the preimplantation testing should be integrated with other IVF techniques as a companion test that may complement the overall picture and help in making the best decisions in the task of obtaining healthy offspring.

The Y chromosomal deletion assays provides a precise diagnostic, thus offering clinicians the chance to establish an appropriate treatment plan and last but not least to better estimate the couple’s chances of conceiving.

In this case, the PGD procedure enabled the identification of 4 embryos with affected genotype and additionally the sex of all the embryos was established. Therefore the medical team decided to transfer a female embryo to the patient since the microdeletions in the AZFc region of the Y chromosome is a genetically transmitted disorder and is one of the most frequent causes of spermatogenic failure.

To our knowledge, this is the first reported case of IMSI combined with PGD performed entirely in Romania. The results confirm the importance of genetic investigations and counseling in patients with a history of male infertility with genetic component. The implementation of preimplantation testing in assisted human reproduction clinics in Romania will open new opportunities for patients, granting them the right to high quality health care services, customized according to their needs.

However, almost 20 years after the creation of the Eshre PGD consortium, preimplantation genetic testing is still inaccessible to many couples because of the extremely high costs that in many countries, including Romania, are not reimbursed through the public health system.

Future challenges are related to the development of new molecular diagnostic methods, but also the necessity of a well-establish strategy that would ensure accessibility to combined IVF and preimplantation genetic testing for patients through national health programs.

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Not applicable.

Conflict of Interest

The authors declare that they have no competing interests.

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Ethical Approval

We obtained ethical approval for this study from the Ethical Committee at the Origyn Fertility Center (observation sheet 564/2014), the agreement being signed by the Medical Director of center as well. The confidentiality was granted through and after the study. Informed and written consent was obtained and signed (2nd July 2015) from all the participants concerning their participation in this study (for IVF, IMSI and ovarian stimulation on 11th September 2015, trigger administration on 7th December 2015, respectively FET on 12th December 2015).

Author Contributions

B-D and R-M (design of the study), L-N and O-D.I (conceptualisation, investigation, analysis, writing). S-M, G-S, I-V, A-A, E-M and E-M (supervision, validation, investigation - review and editing). All authors have read and agreed this version of the manuscript.

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