Analysis of Sex Chromosomes Disorders in Preimplantation Genetic Diagnosis

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بررسی ناهنجاری گروه‌های جنسی در تشخیص زننگی قبل از لانه گزینی

مقدمه: تشخیص قبل از لانه گزینی یک روش تشخیص پیش‌ترین ایست که با کمک IBF از طریق توارث بیماری‌های زننگی با حذف یا تغییرات جنین‌های معمول و انتقال جنین‌های سالم به داخل رحم مادران انجام می‌گردد.

روش کار: این مطالعه تجاری در سال 1383 میلادی در مرکز ناباروری تهران انجام شده است. آزمایش تشخیص قبل از لانه گزینی برای 10 زوج که بیماری زننگی وابسته به گروه‌های X و Y داشتند، انجام گردید. مراحل هورمون درمانی، تزریق داخل سیتوپلاسمی اسپرم، شوپه برداری از بلاستوم و آزمایش هیپریپاسیون فلورست گروه‌هایی (فیش) برای نمایش صورت گرفت.

نتایج: از مادران 80 نمونه به دست آمده که از 56 عدد آنها با موقعیت بلاستوم استخراج شد. در آزمایش کل شناسانه درای 29 عدد X و Y که 18 عدد YY و 1 عدد XY و 1 عدد XO شناسانه داده شد. انتقال جنین‌ها به داخل رحم در این 10 مورد باعث ایجاد 2 حاملگی و یک جنین سالم متولد گردید.

بحث: میزان جایگزینی جنین معادل 20% و میزان به سالم به منزل 10% بعد از 10 آزمایش بود. ناهنجاری‌های کروموزومی مشاهده شده در جنین‌های دیسمورفیک و خصوصاً در جنین‌های که از مادران با سن بالاتر متولد شده بودند، بیشتر مشاهده گردید.

کلمات کلیدی: تشخیص زننگی قبل از لانه گزینی، هورمون‌های جنسی، مهار آزمایشگاهی، نمونه برداری جنین
**Introduction**

The advances in genomic information in the last decade has allowed the genetic diagnosis of embryo to be performed even before pregnancy, using preimplantation genetics diagnosis (PGD) (1-4). PGD saving the intended parents the stress of performing chorionic villi sampling (CVS) or amniocentesis and deciding to keep or abort the fetus when fetus is twelve to sixteen weeks old. Genetic testing of embryo is usually carried out using FISH or PCR techniques. PCR is commonly used for genetic diseases diagnosis due to a single gene defect and FISH is a reliable technique for analysis of the most common human chromosome abnormalities. These chromosome abnormalities include aneuploidy screening when mother is over age 35, or if sex linked genetic disease such as haemophilia, fragile X syndrome, most of the neuromuscular dystrophies, Y chromosome microdeletions are present, also when woman previously had a pregnancy with a chromosomal abnormality and couples diagnosed with chromosomal abnormalities (5-14).

At present, PGD is available in approximately 20 developed countries worldwide and has been used for a variety of cases. However, PGD technique needs trained expertise, costly probes, florescence microscope facilities and has been recently started to be used for diagnosis of chromosome numerical abnormalities throughout IVF centers in developing countries as well as Iran. In this study FISH with two fluorescence DNA probes has been used for analysis of two sex chromosomes in single human blastomer in sex-linked recessive disorders. Ten gender determinations of embryo were performed for couples, carrier of sex chromosome disorders. In the case of mother being carrier of an X linked disease girl embryo were chosen to transfer and in the case of father being affected with X linked disease they preferred to have a boy to inhibit transmission of mutation to their children (15-17).

**Material and Methods**

**PGD candidate**

This experimental study was done in the year 2004 in Navid Institute of infertility, Tehran, Iran. Before requesting PGD, candidates were introduced for genetic consultation to evaluate the risk of transferring their genetic abnormality to offspring. The consent form was obtained from the couples. To stop passing X linked mutation from mother carrier of X linked disorder XX embryos were selected and for couples with affected father XY embryos were transferred to uterus. The appropriate couples were introduced for IVF where they prescribed for hormonetherapy and ovarian stimulation in order to produce several good-quality eggs. Multiple oocytes were released with pretreatment cycle of FSH (Follicle stimulating hormone) and LH (Luteinising hormone) for ovarian stimulation. During the 7 to 14 days stimulation period, ultrasound examinations and laboratory tests were performed to monitor the development of the follicles. When follicles were ready for egg retrieval, they were punctured, their contents aspirated under the laparoscopic guidance, and the eggs identified and harvested. The male partner provided a semen sample for injection into mature oocytes. Oocytes were fertilized by intracytoplasmic injection (ICSI) and were transferred into a 30 µl droplet of B2 medium under mineral oil (Sigma) and incubated at 37ºC in an atmosphere of 5% CO₂. The following morning, the eggs are observed for signs of fertilization which usually shows the formation of two pronuclei (PN). These nuclei have the male and female origin of the embryo. On day 3, when the embryo was normally at the 8-cell stage, the embryos were prepared for biopsy. Before biopsy embryo was incubated in Ca²⁺ and Mg²⁺ free medium for disassociation of the blastomeres.

By micromanipulation one or two blastomeres were obtained from each embryo for genetic analysis. The embryo biopsy combination zona drilling and aspiration of the blastomeres were performed under inverted microscope equipped with micromanipulator. An embryo was immobilized by a holding pipette and a hole of 35-40 µm was made in the zona pellucida using acid Tyrode’s solution (Sigma) using a 10 µm diameter micropipette. Once the hole had been made in the zona pellucida, a blastomer was
aspirated through this hole into a biopsy micropipette 35-40 µm in diameter (18). The isolated single blastomere was transferred into a droplet of HEPES-buffered human tubal fluid medium (H-HTF medium; Irvine Scientific, Santa Ana, USA). The presence of a clear single nucleus of the blastomer was carefully tested under an inverted microscope. After biopsy, the embryo was transferred into a 30 µl droplet of B2 medium under mineral oil (Sigma) and incubated at 37°C in an atmosphere of 5% CO₂.

Blastomers were fixed on glass slides using the procedure described by Coon et al (1994) (6). Briefly, for fixation a single blastomer was put in a premarked circle on a glass slide (Surgipath; Richmond, IL, USA). A solution 0.1% Tween 20 in 0.01 N HCl was used to remove the cytoplasm of the blastomere using a mouth controlled glass pipette. The slides were then dehydrated sequentially in 70, 85 and 100% ethanol at room temperature and analyzed using fluorescence in situ hybridization (FISH) technique.

Fluorescent in situ hybridization
Directly labeled double color Vysis alpha-satellite centromeric probes (Vysis) were used in FISH experiment to analyze chromosomes X and Y in the blastomers. A drop of probe and hybridization buffer mixture were put on the slide and covered with an 18X18 coverslip. The coverslip was sealed with rubber cements. Denaturation of DNA was carried out at 78°C for 5 min and then slides were incubated at 37°C for 30 min for hybridization to occur. After hybridization the rubbercement and the coverslip had been removed and slides were washed for removing any extra probes or unspecific hybridization by sequential washes in 60%formamide/2×SSC (Sodium Chloride/sodium Citrate), 2×SSC and 4×SSC/0.05% Tween 20 in a 42°C water bath for 10 min duration in each solution. When the washing procedures were completed, slides were air-dried and 10µl of DAPI (4',6-diamidino-2-phenylindole ) antifade solution (Vysis) was added to the slides and covered with a 18X18 mm coverslip. The location and color of signals were visualized under a fluorescence microscope at ×1000 magnification. Signals of FISH were captured and stored in computer using applied imaging system for FISH. Green signal was representative of X chromosome and red signal for indicative for Y chromosome (Figure 1).

Figure 1: Signals for X (green) and Y (red) chromosomes after FISH analysis of interphase nuclear blastomer with duple color centromeric alpha satellite probes for X and Y chromosomes; a. XY, b. XXY, c. XO.

Results
Ninety six oocytes were retrieved from the patients and inseminated with the husband’s spermatozoa. Of the oocytes, 80 out of 96 were 2 PN, and 71 of 80 fertilized oocytes became 5-8 cell stage embryos with suitable morphological quality. The reminded 9 embryos had the fair morphology quality. Of the 80 blastomeres, 65 had a clearly visible single nucleus. One blastomere was removed from each of the 80 embryos. Of these 56 nucleated blastomeres were fixed, the nuclei of 9 blastomeres did were observed after fixation, because the membrane of the nucleus was damaged during the process of
fixation. The nuclei of 56 blastomeres fixed on glass slides were used in FISH study. In FISH experiments all nuclei represented green signal for X chromosome and red signal for Y chromosome. Normal diploid blastomers had two sex chromosomes and abnormal embryos had unusual number of sex chromosomes. We observed embryos as 29 XX (Female), 18 XY (Male), 4 XXY (Klinefelter's syndrome) and 1 XO (Turner's syndrome). From 10 PGD cycles, two pregnancies have been occurred, one transferred boy embryo has finished the term and has borned healthy and another male pregnancy has been aborted after 1.5 month due to insufficient FSH hormone consumption by patient. The confirmation of PGD diagnosis was performed both by prenatal molecular genetic sexing of embryo and ultrasound.

Discussion

Fluorescence in situ hybridization (FISH) allows determination of sex for X-linked diseases, chromosomal abnormalities, and aneuploidy screening in preimplantation genetic diagnosis. This is the first application of FISH in PGD that has been performed in Iran. In this way 10 couples have been offered to undergoing PGD for X-linked diseases and for Y chromosome microdeletion. They were offered genetic counseling, IVF, single blastomere biopsy and two color FISH to establish sex chromosomal status in their embryos. From 65 blastomers obtained, 56 had been fixed and after FISH analysis chromosomal status in their embryos observed to be 29 XX (Female), 18 XY (Male), 4 XXY (Klinefelter's syndrome) and 1 XO (Turner's syndrome). In our experiments embryos with fair morphology and poor quality had more chance of having sex chromosome abnormality. The error rate of sex chromosomes aneuploidy was as 9% due to older age of women studied. Two pregnancies of selected sex chromosomes had occurred following PGD, one borne healthy and one aborted after 1.5 month due to discontinuing in FSH consumption. The rate of success for implantation was 20% which is close to the success rate reported previously equal to 15-30% in optimum conditions (19). The error rate for selected sex chromosomes was zero and both children had male gender. The age of mother and health of reproductive system were important in the success rate. The younger mothers had better chance for pregnancy following PGD as this happens in IVF experiment. PGD is costly and time consuming especially when parents had to perform it abroad. However, developing new techniques in our country would allow having healthy child following PGD with less coast and hassle.

There were some limitations for PGD in our clinical application. This technique has been a newly established method in our institute and the couples undergoing PGD were mostly older than 40 years old with an average age of 38 years old. Older women have low numbers of good quality IVF embryos or oocytes and a lower success rate compared to the younger women. A case could be made for transferring more embryos to improve the chance of a live birth following PGD treatment, especially in older women. Furthermore, in the case of older women more genetically unbalanced embryos were observed in our FISH results. Before a PGD treatment cycle is started, the importance of confirmatory testing either by chorion villus sampling (CVS) or by amniocentesis should be stressed to the patients. This is useful not only to confirm the diagnosis but also to counsel the couple (20).

PGD is still a technically challenging, multistep, labour intensive method, which needs the close collaboration of a team of specialists. Other limitations to the wider application of clinical PGD cycles consist of the necessity to involve IVF, even if the couples are not infertile, the relatively low pregnancy and birth rate and the high cost of a complete PGD cycle (21). Approximately half of the PGD cycles carried out to date, have been for age related aneuploidy. The selection of embryos following aneuploidy assessment in women of advanced maternal age or with repeated IVF failure may help to increase reproductive success. For many couples, PGD has already proved to be a valuable and worthwhile procedure (22-28).
Introduction: Preimplantation genetic diagnosis (PGD) is an advanced diagnostic tool in the assisted reproductive technologies (ARTs) to avoid inheritance of genetic disease by transferring unaffected embryos. The presence of numerical sex chromosome abnormalities was studied using fluorescence in situ hybridization (FISH) with two sex chromosome specific probes for X and Y chromosomes.

Material and Methods: In this experimental study, PGD has been performed for 10 couples, who were carriers of inherited X linked disorders. Conventional hormone therapy, intracytoplasmic sperm injection, blastomer biopsy and fluorescent in situ hybridization were carried out for all candidates.

Results: Data was analyzed by descriptive statistics. In FISH analysis 93% of cells provides clear signals for X and Y chromosomes from that 29(XX), 18(XY), 4(XXY), 1(XO) were diagnosed. Transferring of embryos into uterus was performed in 10 cycles resulting into two pregnancies and one born baby. The implantation rate of embryos was 20% and the take home baby rate was 10% following ten PGD experiments.

Conclusion: Chromosomal disorders more frequently in embryos with dismorphology especially in the embryos from older women.

Key words: Preimplantation Genetic Diagnosis, Sex chromosomes, Invitro fertilization, Embryo biopsy, FISH

Abstract

References