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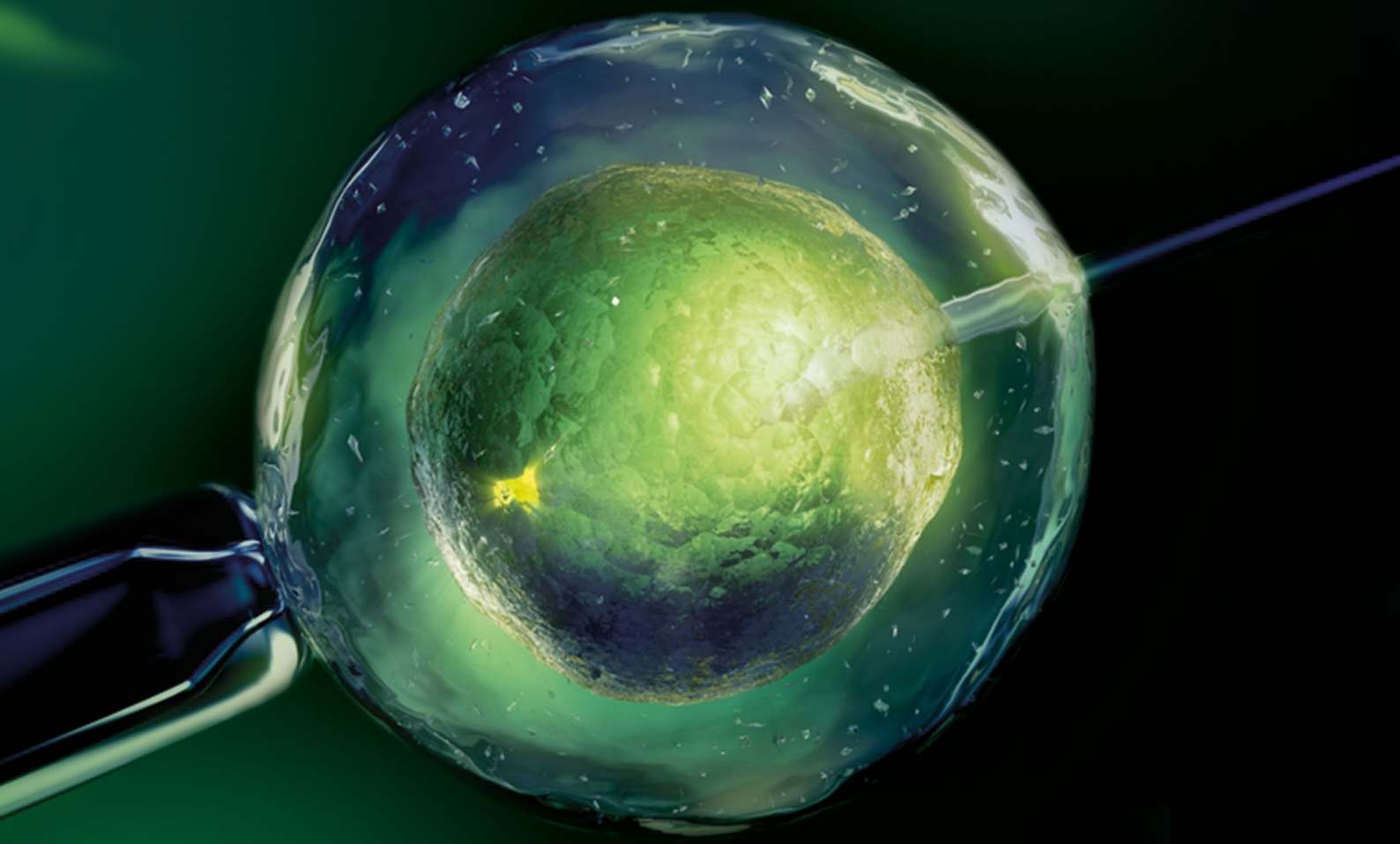

The impact of next generation sequencing (NGS) preimplantation genetic diagnostics (PGD) on pregnancy rate of frozen blastocyst embryo transfer.

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Routine use of next generation sequencing (NGS) for preimplantation genetic diagnostics (PGD) of blastomeres obtained from embryos on day 3 in fresh IVF cycles – a prospective case control study.

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The impact of next generation sequencing (NGS) preimplantation genetic diagnostics (PGD) on pregnancy rate of frozen blastocyst embryo transfer.

Abstract

Objectives:
To determine pregnancy rate after comprehensive chromosome screening (CCS) using PGD NGS in frozen embryo transfer cycles after blastocyst's trophoectoderm biopsy.

Material and methods:
40 patients who decided to undergo PGD on 24 chromosomes on blastocystes with vitrification and frozen embryo transfer were included in the study as a investigated group. As a control group we adjusted 74 cases which were comparable in age, anty-mullerian hormone level (AMH), antral follicle count (AFC) and causes of infertility. First IVF cycles were taken under consideration.

Results:
On 40 cycles we received on average 6.7 blastocystes per patient. We received 30.4% normal results, which allowed us to transfer 1.4 ± 0.6 vitrified blatocystes in frozen embryo transfer. The pregnancy rate was more than 30% higher when compared to control group with fresh blastocystes without PGD transfers (50.0% vs. 18.9%, p<0.006 respectively). The difference in implantation rate was more than 20% (32.4% vs. 11.5%, p<0.05). The difference in pregnancy loss was more than 8% (11.1% vs. 3%, p<0.52).

Conclusion:
NGS PGD technology is very useful and should replace aCGH technology soon. It allows to personalize the method and could be in parallel used for all PGD indications. The performing time is the biggest limitation of NGS.

Key words:
/ next-generation sequencing
/ preimplantation genetic diagnosis
/ genotyping, aneuploidy screening
/ semiconductor-based sequencer
/ frozen embryotransfer

Introduction

Aneuploidy is the leading cause of implantation failure and early spontaneous abortion in humans [1],[2]. Testing embryos prior to transfer could increase the pregnancy rate, decrease miscarriages and prevent preforming multiple IVF cycles in a patient with a very high incidence of aneuploidy and consequently prevent a low or zero chance of achieving pregnancy with her own oocytes [3].
Most of the selection methods used before were based on the detailed morphology of embryos. First of the technologies, still used nowadays, is microscopic morphology analysis. It was shown that the optimal morphologic characteristics correlate with higher implantation rates, but morphology is a weak predictor of implantation rate and ploidy [4],[5]. The introduction of the time lapse system for continuous morphokinetics analysis increased the implantation rates.
Nowadays methods base on aneuploidy screening rather than just embryo morphology evaluation. Most of the current preimplantation genetic screening tests for aneuploidies are based on low quality and density of comparative genomic hybridization arrays. Their results are based on less than 2700 probes. It means, that it is rather a multiple FISH method than a real array one. New technical possibilities, such as next generation sequencing methods, allow to improve genetic

diagnostics [6],[7].

Aim

The study explored the usefulness of comprehensive chromosomal screening using next generation sequencing (NGS) on biopsed, vitrified/thawn blastocystes in improving the pregnancy rate.

Materials and Methods

Study design

A prospective study was performed at a private fertility clinic. The study population consisted of 621 patients treated by ICSI in our IVF Center between 08.2013 and 01.2014. 40 patients who decided to undergo PGD on 24 chromosomes on blastocystes with vitrification and frozen embryotransfer were included in the study. First IVF cycles were taken into consideration. Patients were provided with counseling regarding the study, and signed written consents were obtained. The average maternal age of patients was 38. The patients who were asked to participate in the comprehensive chromosome screening were at an advanced reproductive age (>35 y).

Stimulation protocol

All women were treated on long agonist protocol starting from oral contraceptive pills (OC) (Ovulastan, Adamed, Czosnow, Poland) from 2nd-5th day of the cycle. Triptorelin acetate 0.1 mg (Gonapeptyl, Ferring, Saint-Prex, Switzerland) was administered 14 days after the beginning of OC.

Fourteen days later (7 days after the end of OC administration) the administration of urinary gonadotropins (Menopur, Ferring, Saint-Prex, Switzerland) for ovarian stimulation commenced. The dosage, was dependant on AMH level (from 150 to 300 IU daily) [8].

Monitoring of follicular growth was carried out on day 8 by ultrasonographic scan and assays of serum estradiol (E2), progesterone (P) and luteinizing hormone (LH). Oocyte pick-up was performed 36 hours after the administration of 5,000 IU of hCG (Choragon, Ferring, Saint-Prex, Switzerland). Luteal phase was supplemented by transvaginal progesterone (P) (3x100 mg Lutinus, Ferring) and transvaginal estradiol (E2) (3x2 mg, Estrofem, Bagsvaerd, NovoNordisk) administration. The hormones (E2, P and HCG) levels were verified every 3-4 days.

Laboratory procedure

Blastomeres biopsy (from 3 to 8 blastomeres from trophoectoderm collected) was performed on day 5 of culture. Embryos underwent biopsy after assisted hatching. The biopsy was performed by 1488 nm 300 mW Anritsu laser for doped fiber amplifier implemented into Saturn 3 system (Research Instruments, Falmouth, UK). After biopsy the embryos were washed and transferred to blastocyst growing medium (Vitrolife, Vastra Frolunda, Sweden). Then vitrified with Cryotop carrier as described by Kuwayama [9]. The collected blastomeres were washed in phosphate-buffered saline (PBS)/polyvinyl-pyrolidone (PVP).

Next Generation Sequencing

Aspirated blastomers were transferred into thin walled 0.2mL PCR tubes in total volume of 5 µl. Blastomeres were subjected to lysis and whole genome amplified prior to construction of library. WGA DNA concentration was quantified with Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay. Ion Xpress Plus gDNA Fragment Library Kit (Life Technologies, California, USA) was used for library preparation according to manufacturer's protocol. Libraries were clonally amplified with The Ion PGM™ Template OT2 200 Kit (Life technologies, California, USA) using Ion One Touch 2 System. After chip loading the sequencing was performed using Ion PGM™ Sequencing 200 Kit v2 (Life technologies, California, USA) on Ion 316 chip. Preliminary analysis e.g. basecalling and reads mapping against human genome reference sequence (Hg19) was performed with Ion Torrent Suite Software. The Invicta Bioinformatics Team Script was used for further computational calculations. Read coverage for each chromosome was corrected for GC-bias and aneuploidy detection was performed using sample results in comparison to baseline values obtained from 85 male and 83 female samples processed beforehand with established protocol described above. The Invicta algorithm was introduced to eliminate the influence of sample-to-sample reads coverage variance on false-positive calls. Male control sample was processed together with blastomers and underwent the same computational analysis to exclude any performance malfunctions. Additionally, negative control sample was processed to exclude any contamination. Next generation sequencing method was designed in Invicta Fertility Clinic Molecular Laboratory Department.

Institutional Review Board

We obtained a written consent from each couple to perform ICSI on at least some of the retrieved oocytes. All data were de-identified and analyzed anonymously. Moreover, we obtained informed consent to present our data in any publication as long as confidentiality was maintained. These conditions were met and study was approved by the University of Varmia and Masuria, Olsztyn, Poland Institutional Review Board.

Statistical analysis

The statistical package StatSoft, Inc. (2011) STATISTICA (data analysis software system), version 10 (www.statsoft.com) was used for data analysis. The clinical characteristics in investigated group were compared using Mann Whitney U test. The Pearson Chi-square and Fisher test was used to assess differences among groups with regard to different rates of development. A value of $P<.05$ was considered statistically significant.

Results

A prospective study was performed at a private fertility clinic. Clinical pregnancy rate was our primary outcome. Secondary outcomes were the implantation, and miscarriage rates. Embryos of 40 patients underwent biopsy on day 5 with PGD and blastocystes freezing. Embryos were transferred in the consecutive cycles. We selected 114 patients who underwent ICSI cycles simultaneously. They were adjusted according to age, AFC, AMH and hormones levels (Table 1).

Finally, we performed 40 cycles in the investigated group. The average number of retrieved oocytes was not high - 6.7 oocytes per patient, this was not sufficient to find normal embryos in all of the 40 cases. However, the pregnancy rate per embryo transfer was more than 30% higher compared to control group. The increase in implantation rate was over the level of 20% (Table 2).

Discussion

It was shown that 32% to even 85% of embryos at blastocyst stage are chromosomally abnormal [10]. Since the vast majority of aneuploidies are lethal, it has been argued that IVF outcomes could be improved if embryos are tested for aneuploidy and those chromosomally normal given priority for transfer [11]. This concept is referred to as preimplantation genetic screening (PGS).

Preimplantation genetic screening at blastocyst stage has become a trend in recent years. It is mostly caused by lower mosaicism incidences at this stage than in cleavage stage and the largest increase in implantation and live birth rates reported after blastocyst embryo transfer. Analysis of trophoectoderm biopsy material using 24-chromosome screening platforms requires the freezing of embroys and using different cycle preparation for frozen embryo transfer. It complicates the procedure and causes logistic and psychological problems for couples. For that reason we introduced both possibilities for our patients. They decide which material they want to analyze and whether they prefer fresh or frozen embryotransfer. Obviously, it is very difficult and not always possible to perform blastocyst PGD in fresh cycle. That is why numerous couples decide to perform PGD at blastocyst stage with vitrification. There is a major debate in literature concerning consequences of embryo freezing. Chamayou et al. studied consequences of freezing/ thawing process on mRNA in oocytes indicating overall decrease of mRNA extracted from cryopreserved oocytes compared to fresh ones. However, it was shown that vitrification/warming protocol, as the one used in our study, is more conservative than slow-freezing/rapid thawing resulting in a level of mRNA sufficient to maintain biologic functions in the subsequent fertilized oocyte [12]. Simultaneously, there is no evidence that any children conceived from warmed blastocysts show an increased risk of chromosomal anomalies and chance of becoming pregnant as a result of frozen blastocysts transfer is comparable to the replacement of fresh embryos [13], [14].

The study described in this paper examined the feasibility of utilizing emerging NGS methods for the detection of aneuploidy in single blastomeres biopsied at the blastocyst stage with vitrification. Utilization of NGS has several potential advantages over existing methods, including significantly lower costs than existing commercial aCGH method offered to us by BlueGnome. It could open up the possibility of preimplantation genetic screening for many more patients. For instance, PGS could be proposed to younger women. Typically, preimplantation diagnosis is not recommended in their case. But our previous results show that even in 31 years old and younger women 32% of blastocystes were aneuploid [10]. NGS is a superior technology over aCGH because it relies on actual DNA sequences rather than signal hybridization, resulting in a vast reduction in the false positive and false negative rates. In aCGH, as a consequence of low coverage potential, big chromosome deletions may be undetected, which leads to misinterpretation of the aneuploidy status. NGS provide reliable detection of chromosomes abnormalities under high coverage (over 60 000 reads per sample, which represents 10 times more reads than in aCGH method). Thereby, NGS leads to improved selection of viable embryos and better chance to achieve pregnancy. This study led to the first pregnancies following screening of human embryos using next generation of genome sequencing.

Pregnancy and implantation rates achieved in cycles utilizing NGS exceeded expectation and suggest that this approach is improving identification of viable embryos. However, the relatively small size of the investigated group and lack of randomization are acknowledged weaknesses of this investigation.

As expected, the number of embryos available for transfer following NGS was much lower in comparison to cycles without PGS. Very high incidence of chromosomal abnormalities (over 60%) in the investigated group allowed us to transfer embryos only in 18 cases out of 40. Although over half of patients didn't even achieve pregnancy we avoided the abnormal embryos implantation and thereby very high risk of unnecessary miscarriages. Only one miscarriage occurred in the group that received screening using NGS, whereas in control group three miscarriages occurred before 12 week of pregnancy. Further investigations including more patients and randomization should be applied to confirm these findings.

Conclusions

This paper confirms that NGS can be applied clinically for the purpose of preimplantation genetic screening of aneuploidies in human embryos. Data from this trial indicated that blastocyst's trophoectoderm biopsy followed by frozen transfer of euploid embryos was associated with much higher implantation and pregnancy rates and a low miscarriage rate. Our findings suggest that NGS may be a useful tool for embryo selection. Our results require more data from different clinics. Confirmation of the results could change the indications for PGD in general population.

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Table 1. Characteristics of the treatment group.

Variable	PGD NGS group	Control group – no PGD	p value
No. of subjects	40	74	
Mean (SD) age	38.0 (3.6) 38.5 (37-40)	37.6 (2.4) 37 (36-39)	0.12
BMI (kg/m2)	23.7 (3.2)	22.2 (2.5)	0.12
AMH (ng/ml) (SD)	2.3 (2.4) 1.4 (0.65-3.1)	2.3 (2.5) 1.6 (0.6-3.0)	0.9
Inhibin B (pg/ml) (SD)	56.9 (38.4)	59.6 (36.3)	0.74
basal FSH (mIU/ml) (SD)	6.6 (2.1)	7.9 (3.4)	0.08
basal LH (mIU) (SD)	6.5 (2.3)	6.1 (3.3)	0.19
basal E2 (pg/ml) (SD)	51.6 (37.4)	52.2 (38.7)	0.74
DHEAS (µg/dl) (SD)	169.3 (62.3)	174.7 (112.1)	0.68
Testosterone (ng/ml) (SD)	1.3 (1.6)	1.5 (1.7)	0.21
SHBG (nmol/l)(SD)	72.3 (33.2)	68.7 (27.4)	0.87
AFC (SD)	13.2 (9.4) 11 (8.0-14.5)	15.8 (9.1) 14.5 (9.0-21.0)	0.11

BMI – body mass index; FSH – follicle-stimulating hormone; DHEAS - dehydroepiandrosteron-sulfate; SHBG - sex hormone-binding globulin; AFC - antral follicle count

Table 2. In vitro fertilization programmes characteristics within the investigated groups

Variable	PGD NGS group	Control group – no PGD	p value
No. of cycles	40	74	
No. of transfers	18		
Duration of stimulation – days (SD)	9.7 (5.1)	7 (3.3)	0.06
hMGdose (IU)	26.4 (7.9)	23.6 (7.9)	0.18
No. oocytes retrieved (SD)	6.7 (4.8)	8.1 (3.8)	0.03
Fertilization rate (%)	79.6 (18.5)	73.7 (23.1)	0.2
Number of embryos biopsied (mean per cycle)	102 (2.55)	-	
Normal	31 (30.4)	-	
Abnormal	63 (61.8)	-	
Non diagnostic	8 (7.8)	-	
No. of embryos transferred (SD)	1.4 (0.6)	1.6 (0.5)	0.3
Pregnancy rate (per ET - %)	9 (50)	14 (18.9)	0.006
Implantation rate (number of intrauterine gestational sacs) (%)	32.4	11.5	0.05
Multiple pregnancy rate of pregnancies (%)	0	7.1 (1)	0.2
Ectopic pregnancy (%)	0	0	
OHSS (%)	0	0	
Spontaneous abortion rate (%)	1 (11.1)	3 (21.4)	0.52

hMG - human menopausal gonadotropin; ET – emryotransfer; OHSS - ovarian hyperstimulation syndrome

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