

Selecting Euploid Embryos for Transfer by Preimplantation Genetic Testing with the Help of Next-Generation Sequencing in Poor Prognosis Patients: A Retrospective Cohort Analysis

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ABSTRACT

Background: The current embryo selection methods rely on subjective grading of embryo morphology or a real-time monitoring of the embryonic development and assessment of multiple quantitative endpoints. Even up to 40% of morphologically normal embryos harbour aneuploidies. Preimplantation genetic testing (PGT) is a technology, which gives opportunity to identify euploid embryos before implantation. **Aims:** This study seeks to determine the role of PGT in poor prognosis patients, i.e., patients with advanced maternal age (AMA) (maternal age ≥ 35 years), recurrent pregnancy loss (RPL) (miscarriages ≥ 2) and recurrent implantation failures (RIFs) (*in vitro* fertilisation failures ≥ 3). **Study Setting and Design:** A retrospective case-control study was done on a group of patients who underwent intracytoplasmic sperm injection for the indications of AMA, RPL and RIF. **Materials and Methods:** In 33 cases who opted for PGT, day 5 blastocysts were subjected to trophoctoderm biopsy with the help of next-generation sequencing. Euploid blastocyst was transferred in hormone replacement cycle at a later date. In 154 controls, blastocyst transfer was done based on morphological grading. Pregnancy outcomes are compared in terms of implantation rate, pregnancy rate, miscarriage rate and multiple pregnancy rate. **Statistical Analysis:** Chi-square test was used for comparisons between the study groups with respect to percentage. $P < 0.05$ was considered statistically significant. **Results:** The highest aneuploidy rate was found in embryos with AMA. Implantation rate was found to be statistically significantly higher in the PGT group as compared to the non-PGT group. However, take-home baby rates were not improved by PGT. There were less number of mean embryos transferred in the PGT group and lower multiple pregnancy rate. **Conclusions:** With the application of PGT, embryo selection rates and implantation rates improved in poor prognosis patients.

KEYWORDS: Advanced maternal age, next-generation sequencing, preimplantation genetic testing, recurrent implantation failure, recurrent pregnancy loss

INTRODUCTION

In vitro fertilisation (IVF) is effective in overcoming various barriers for fertility. One in 2 embryos produced with help of IVF are abnormal.^[1] The current embryo selection methods rely on subjective grading of embryo morphology or a real-time monitoring

of the embryonic development and assessment of multiple quantitative endpoints. However, none of these have helped to improve pregnancy rate.^[2,3] Up

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to 40% of morphologically normal embryos harbour aneuploidies.^[4]

Despite the fact that there are multiple variables involved in the processes surrounding implantation and early foetal development, ploidy status remains an important and requisite component of a successful pregnancy. Preimplantation genetic testing (PGT) is a technology which gives an opportunity to identify euploid embryos before implantation. PGT application has become possible through the development of artificial reproductive technology and sensitive molecular methods allowing genetic analysis at the single-cell level.

The last decade has seen dramatic improvements in the application of PGT and the ability to test blastocysts. Following the discouraging results associated with fluorescence in situ hybridisation (FISH), using 23 chromosome pair evaluation with trophectoderm biopsy has reduced misdiagnosis rates. In FISH evaluations, only a discrete number of chromosomes could be evaluated, typically 9–12 chromosomes at a time.^[5] With the advent of new validated platforms for comprehensive chromosomal screening such as single-nucleotide polymorphism array, microarray comparative genomic hybridisation (array CGH), quantitative polymerase chain reaction (PCR) and Next generation sequencing (NGS) capable of analysing all 24 chromosomes, now, an improved version of PGT involving 24-chromosome copy number analysis has overcome the earlier shortcomings.

The current study seeks to determine the role of PGT in poor prognosis patients, i.e., patients with advanced maternal age (AMA) (maternal age ≥ 35 years), recurrent pregnancy loss (RPL) (miscarriages ≥ 2) and recurrent implantation failures (RIFs) (IVF failures ≥ 3). There is a paucity of data determining the outcome of modern 24 chromosome analysis in this patient group. The objective of study was to undertake retrospective analysis of clinical outcomes of patients undergoing PGT and to compare it with similar group of patients who could not opt for PGT.

MATERIALS AND METHODS

Inclusion criteria

Couples undergoing ICSI for the following indications: AMA, RIF and RPL from January 2020 to March 2021 were included in study. PGT was offered to all the couples. Thirty-three couples who opted for PGT-A were classified as cases. One hundred and fifty-four couples who underwent 158 non-PGT ICSI cycles were classified as controls.

Exclusion criteria

1. All donor and surrogacy cycles were excluded
2. We excluded patients with established predisposing factors for RPL (antiphospholipid syndrome, hereditary thrombophilia, parental chromosomal abnormalities, uterine structural anomalies, hypothyroidism and polycystic ovary syndrome)
3. Infertile couples with male factor and secondary infertility were excluded.

Thirty-three couples gave their written informed consent 11 for undergoing PGT-A after ICSI. Additional consent was taken from all patients to use their anonymous data for research and educational purpose. Patients were deemed eligible to undergo PGT only if a minimum of four good-quality embryos (3AA) were available for biopsy on day 5. The study protocol adhered to the ethical guidelines of the 1975 Declaration of Helsinki and its later modifications. The ethical permission for the research was obtained from the Ethics Committee, Unique Hospital, Surat, Gujarat, India (Reg No.-ECR/595/Inst/GJ/2014/RR-20) retrospectively on 26/3/22.

All patients underwent controlled ovarian stimulation, using either the standard microdose long protocol or the flexible antagonist protocol. In the long protocol, after confirming pituitary downregulation on day 2 of the cycle, 150–225 IU of recombinant follicle-stimulating hormone (Gonal F; Merck Serono, Geneva, Switzerland) was administered daily depending on the patient's anticipated response, with or without addition of HMG (IVF-M; LG-Chem, Korea) in the late follicular phase based on the patient's response to stimulation. When at least two follicles reached 18 mm in diameter, recombinant human chorionic gonadotropin (hCG) (250 μ g, Ovitrelle, Merck Serono, Geneva, Switzerland) was used to trigger ovulation. Transvaginal ultrasound-guided oocyte retrieval (OCR) was performed 35–36 h later. Following aspiration, the follicular fluid was examined and oocyte cumulus complexes (OCCs) were retrieved and transferred to GIVF, fertilisation media (Vitrolife, Germany) for culture at 37°C and 6% CO₂ for 3–4 h. Meanwhile, the semen sample obtained from the husband was mixed with sperm wash media (Vitrolife, Germany) was subjected to centrifugation for 10 min at 6000 rpm. The resulting pellet was subjected to swim up to obtain motile sperm for injection. OCCs were denuded mechanically using a 150 μ Flexipet (Stripper Tips, ORIGIO, USA) after brief exposure to 80 IU hyaluronidase (Hyase- $\times 10$, Vitrolife, Sweden) for 30 s. Mature oocytes were injected with sperm according to the established ICSI protocol. During ICSI, an elongated

Table 1: The baseline details of intracytoplasmic sperm injections cycles in the study cohort

Indication	Number of cases	Number of cycles	Aneuploidy rate, n (%)	Number of controls	Number of cycles
AMA	8	8	26/40 (65)	18	22*
RIF	10	10	62/114 (54.3)	54	54
RPL	15	15	23/44 (52.28)	82	82
Total	33	33	111/198 (56.06)	154	158

*Embryo pooling was done in 4 patients. AMA=Advanced maternal age, RIF=Recurrent implantation failures, RPL=Recurrent pregnancy loss

Table 2: Abnormalities detected in biopsied embryos

Genetic disorder	n (%)
Gene mutation	44 (40)
Single gene mutation	22
Polygenic mutation	2
Chromosomal abnormalities	60 (54.5)
Aneuploidy	24
Monosomy	6
Trisomy	18
Structural chromosomal abnormalities	36 (32.72)
Mosaic*	6 (5.45)
Total	110 (100)

*Percentage of aneuploid DNA-load in a biopsy specimen is 20%-80%, an embryo is considered mosaic

Table 3: Comparison of variables between cases and controls

Variables	Mean±SD		P
	PGT group	Non-PGT group	
Age	31.24±4.17	30.62±6.69	0.23
Peak serum estradiol	3061.58±1701.2	2728.73±1961.5	0.524
Oocyte retrieved	21.93±13.3	16.8±9.46	0.297
Oocyte fertilised	15.25±9.3	9.93±6.3	0.312
Endometrial thickness on day of transfer	9.84±1.5	10.16±1.65	0.452
Embryos transferred in FET	1.8±0.5	2.44±0.67	0.001

SD=Standard deviation, PGT=Pre-implantation genetic testing, FET=Frozen embryo transfer

10 µl polyvinylpyrrolidone (Medicult, Denmark) drop under oil was used to select spermatozoa with normal morphology for subsequent injection. Embryos were then cultured till day 3 in one-step media (Vitromed, Germany). On the morning of day 3, embryos were graded based on their morphology and cleavage rates. Day 3 embryos were transferred to fresh culture dish for blastocyst progression. Blastocyst scoring was done based on trophoectoderm and inner cell mass. For patients who opted for PGT, blastocysts (3AA) were chosen for biopsy. Biopsy was performed using laser to make a hole in the zona pellucida through which 8–10 trophoectoderm cells were gently sucked out,

using a sterile biopsy pipette. The individual biopsied embryos were then immediately washed and placed back in culture, and trophoectoderm cells were washed and transferred to a PCR tube containing 2 µl transport media under strict sterile and DNAase-free conditions to avoid any contamination. The PCR tubes containing the cells were subsequently sent to the genetic laboratory for further genetic analysis by new-generation sequencing (NGS) technique. None of embryos failed to be amplified.

At the genetic laboratory, whole-genome amplification and DNA barcoding were performed using Ion ReproSeq PGT kit (Thermo Fisher scientific, MA, USA). Template preparation and chip loading were automated with IonChef™ (Thermo Fisher Scientific). Sequencing steps were subsequently performed in a PGM sequencing machine using 318 chip or in a S5™ XL sequencer (Thermo Fish Scientific) using a 530 chip. Data analysis was performed using version 5.4 of Ion Reporter Software (Thermo Fisher Scientific). Embryos were diagnosed with euploid, aneuploid or chaotic abnormal.

All embryos including biopsied embryos were vitrified as per the standardised vitrification protocol by laboratory. After results of genetic tests were obtained maximum two euploid day 5 frozen thawed blastocysts were transferred after endometrial preparation by HRT. Following embryo transfer, any supernumerary ‘normal’ blastocysts were cryopreserved. For patients who didnt opt for PGT upto maximum 3 day 5 grade 1 frozen thawed embryos were transferred after endometrial preparation by HRT.

All patients received progesterone support daily, from the day of OCR in the form of vaginal tablets (400 mg BD) or injectable natural micronised progesterone (100 g), until day 11 after ET when serum beta-hCG (βhCG) was tested. There were no ectopic pregnancies in both the groups (cases and controls).

A clinical pregnancy was defined as the presence of one or more gestational sacs on ultrasound 2–3 weeks after positive βhCG divided by the total number of patients. Implantation rate was defined as number of gestational sacs observed divided by the total number of embryos transferred, expressed in percentage.^[6] Miscarriage rate was defined as the number of clinical pregnancies lost before 20 weeks of gestation divided by the total number of clinical pregnancies. Live birth rate was defined as the number of deliveries that resulted in a live born neonate divided by the total number of clinical pregnancies in percentage.^[7] Ongoing pregnancy rate is

Table 4: Comparison of clinical pregnancy rates

Indication	Clinical pregnancy rate		P
	PGT (n=33), n (%)	Non-PGT (n=154), n (%)	
AMA	6/8 (75.7)	10/18 (55.5)	0.34
RPL	8/10 (80)	38/54 (26.31)	0.0001
RIF	13/15 (86.6)	56/82 (68.29)	0.14
Total	27/33 (81.8)	104/154 (67.5)	0.1

AMA=Advanced maternal age, RIF=Recurrent implantation failures, RPL=Recurrent pregnancy loss, PGT=Pre-implantation genetic testing

Table 5: Pregnancy outcomes

Variable	Pregnancy outcomes		P
	PGT (n=33), n (%)	Non-PGT (n=154), n (%)	
Clinical pregnancy rate	27/33 (81.8)	104/154 (67.5)	0.1
Implantation rate	40/60 (66.6)	166/376 (44.1)	0.001
Ongoing pregnancy rate	10/27 (37.03)	22/104 (21.15)	0.11
Multiple pregnancy rate	4/27 (14.8)	36/104 (34.61)	0.04
Miscarriage rate	4/27 (14.8)	20/104 (22.15)	0.5
Live birth rate	13/27 (48.14)	62/104 (59.61)	0.19

LET was cancelled as all embryos were aneuploid. y. ET=Embryo transfer, PGT=Pre-implantation genetic testing

Table 6: Indication-wise comparison of miscarriage rate

Indication	Miscarriage rate		P
	PGT (n=33), n (%)	Non-PGT (n=154), n (%)	
AMA	2/8 (25)	5/10 (50)	0.27
RPL	2/10 (20)	8/38 (21.08)	0.94
RIF	2/15 (13.3)	8/56 (14.28)	0.94
Total	6/27 (20)	21/104 (22.15)	0.81

AMA=Advanced maternal age, RIF=Recurrent implantation failures, RPL=Recurrent pregnancy loss, PGT=Pre-implantation genetic testing

defined as pregnancies which have completed 20 weeks of gestation divided by the total number of clinical pregnancies in percentage. Multiple pregnancy rate is defined as the total number of multiple pregnancies divided by the total number of clinical pregnancies in percentage.

Statistical analysis

Sample size was calculated based on results obtained in earlier publication.^[8] Minimum sample size required for the case group was 17 and for the control group was 68, with a total of 85.

Statistical analysis was performed using the SPSS program for Windows, version 17.0 (SPSS Inc., Chicago, Illinois, USA). Chi-square test was used for comparisons between the study groups with respect to percentage. $P < 0.05$ was considered statistically significant.

RESULTS

Thirty-three case underwent PGT for following indications- (1)AMA ($n = 8$).

(2) RPL ($n = 10$) (3) RIF ($n = 15$).

Controls were classified indicationwise as (1) AMA ($n = 18$), (2) RPL ($n = 54$) and (3) RIF ($n = 82$) [Table 1].

Total 198 embryos were biopsied in 33 PGT cycles. Out of these, 88 (44.4%) were euploid and 110 (55.5%) were aneuploid. Percentage of aneuploid DNA-load in a biopsy specimen is 20%-80%, an embryo is considered mosaic. Below 20%, a biopsy is considered 'normal', while above 80%, it is deemed "aneuploidy".^[9] Aneuploidy rate in AMA was 65%, which was the highest amongst three groups (54.3% in RIF and 51.8% in RPL) [Table 2]. Embryo damage rate in biopsy was 2%.

There was no statistical significance in both the groups in terms of age, oocyte retrieved, oocytes fertilised, peak serum E2, E2 on day of transfer and ET on day of transfer in both the groups. However, embryos transferred in the PGT group were statistically significantly lower than the non-PGT group (1.8 ± 0.5 vs. 2.44 ± 0.67 , $P = 0.001$) [Table 3]. Patient who had previous recurrent pregnancy losses had significantly more clinical pregnancy rate after PGT [Table 4]. To some extent, patients with advanced maternal age and RIF were also benefitted with PGT, but difference was not statistically significant among cases and controls in AMA and RIF categories. Clinical pregnancy rate among PGT cases was 81.8% versus 67.5% in controls. Implantation rate in cases undergoing PGT was higher and multiple pregnancy rate was lower than the controls. This difference was statistically significant. Miscarriage rate in PGT and non PGT groups were similar (20% in PGT versus 22.5% in non PGT) [Table 5]. Miscarriage rate was reduced after transfer of PGT tested euploid embryos, but difference was not found statistically significant [Table 6].

DISCUSSION

This retrospective cohort study sought to determine the efficacy of PGT in poor responder patients undergoing ICSI. There is an increased implantation rate, but a similar live birth rate was observed in the PGT group. Women of AMA and RPL are benefitted to some extent because of PGT. Women with RIF have not shown any clear-cut benefit.

One of the major drawbacks in assisted reproductive technology is low live birth rates achieved when assisted reproductive technology is performed on women of advanced reproductive age.^[10] Decline in oocyte quality is associated with aneuploidy.^[11] It has been suggested

that routine PGT for the selection of euploid embryos may improve delivery rates in these older women.^[12] Several studies indicate that chromosomal irregularities increase gradually from the age of 31 years to the age of 43 years, with an aneuploidy rate of approximately 85%. The main limitation in infertile women with AMA is lack of availability of sufficient good-quality embryos for PGT. Hence, in the present study, only eight women with advanced maternal age could opt for PGT. Aneuploidy rate in AMA was 65%, which was the highest amongst three groups (54.3% in RIF and 51.8% in RPL). However, pregnancy rates are comparable in the three groups (75%, 80% and 86.6% in AMA, RIF and RPL, respectively), suggesting that women of AMA are benefited with PGT. To avoid any bias, we included women who had blastocyst transfer and not day 3 transfer in the control group. Similar results were obtained by a small cohort study by Schoolcraft *et al* in women with advanced maternal age.^[13]

Two more RCTs investigated the clinical outcomes of using PGT-A on young and good prognosis patients, demonstrating a benefit in terms of implantation and pregnancy rates in PGT group.^[14,15] The present study was intended to see outcomes in poor prognosis groups. It is observed that in spite of less number of embryos transferred in the PGT group, implantation rates are higher. Clinical pregnancy rates are higher only in RPL cases. Mantravadi *et al.*^[16] have observed a marked reduction in miscarriage rate with PGT-A in RPL patients, but take-home baby rates do not seem to improve. Aneuploidy rates in the study groups are comparable in both the studies, but our study group consisted of patients with overlapping indications such as RPL with RIF or AMA. Hence, no significant reduction in miscarriage rate was seen in the present study.

Patients with RIF are not much benefited by PGT. Other factors, such as an altered ratio of mitochondrial copy number to nuclear DNA suggesting embryonic stress, *de novo* clinically significant deletions or duplications, autoimmune factors, endometrial receptivity, endocrinologic abnormalities, anatomic abnormalities or other factors that are currently unknown or undefined, likely play a role in maintaining a lower pregnancy in cases of RIF.^[17]

Conventionally, transfer of fewer morphologically selected embryos has been associated with a reduction in IVF success rates.^[18] However, in our study, the transfer of fewer embryos in the PGT group as compared to the control group (1.8 ± 0.5 vs. 2.44 ± 0.67 , $P = 0.001$) resulted in a significant improvement in implantation rates (62.5 vs. 44.1, $P = 0.01$) and decline in multiple pregnancy rates. Thus, genetic screening for all 24

chromosomes resulted in improved embryo selection as compared to traditional morphology-based embryo selection. In a study conducted by Forman *et al.*^[19] in women <42 years of age, with AMH >1.2 ng/ml, multiple pregnancy rate declined from 48% to 0% when single euploid embryo transfer was done. Such a drastic decline in multiple pregnancy rates is not obtained in the present study as the study group involved poor prognosis patients.

In a study conducted by Munne *et al.* in 2005, it is stated that PGT with FISH analysis in day 3 embryos reduces spontaneous miscarriage rate in RPL cases, especially in women >35 years of age.^[20] Another study conducted by Sato *et al.* found no significant differences in miscarriage rate amongst PGT and non-PGT patients who had RPL and RIF in the past.^[21] Although miscarriages are less in the PGT group in our study, the difference was not statistically significant. It is worth noting that in the AMA group, miscarriage rate without PGT was 50% and clinical pregnancy rate was 55.5% which means nearly half of ICSI cycles failed and within successful cycles, 50% miscarried. However, in the PGT group, 85.7% became pregnant and 66.7% had successful pregnancy outcome. Another important limitation of our study is that it is inconclusive as far as live birth rates are considered as many women in both the case and control groups have ongoing pregnancies.

It can be concluded that application of PGT in poor prognosis patients can lead to better embryo selection. Implantation rates are improved after PGT in the present study.

Data availability statement

Raw data that support the findings of the study are available from corresponding author, upon reasonable request.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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