INTRODUCTION

The last decade has seen a focused and persistent effort toward developing means and technologies to identify embryos that are most likely to implant and grow till term resulting in the birth of a healthy live baby. At present, morphology evaluation is the mainstay of embryo selection since it is noninvasive and easy to perform. However, it has not proved to be a very efficient method for selecting embryos since implantation rates and clinical pregnancy rates (PR) per transferred embryo continue to be very low. Among many factors, one major reason for poor reproductive potential of embryos generated in vitro is the prevalence of aneuploidies in such embryos.\textsuperscript{1,2}\ The rate

ABSTRACT

\textbf{CONTEXT:} A majority of human embryos produced \textit{in vitro} are aneuploid, especially in couples undergoing \textit{in vitro} fertilization (IVF) with poor prognosis. Preimplantation genetic screening (PGS) for all 24 chromosomes has the potential to select the most euploid embryos for transfer in such cases. \textbf{AIM:} To study the efficacy of PGS for all 24 chromosomes by microarray comparative genomic hybridization (array CGH) in Indian couples undergoing IVF cycles with poor prognosis. \textbf{SETTINGS AND DESIGN:} A retrospective, case–control study was undertaken in an institution-based tertiary care IVF center to compare the clinical outcomes of twenty patients, who underwent 21 PGS cycles with poor prognosis, with 128 non-PGS patients in the control group, with the same inclusion criterion as for the PGS group. \textbf{MATERIALS AND METHODS:} Single cells were obtained by laser-assisted embryo biopsy from day 3 embryos and subsequently analyzed by array CGH for all 24 chromosomes. Once the array CGH results were available on the morning of day 5, only chromosomally normal embryos that had progressed to blastocyst stage were transferred. \textbf{RESULTS:} The implantation rate and clinical pregnancy rate (PR) per transfer were found to be significantly higher in the PGS group than in the control group (63.2\% vs. 26.2\%, \(P = 0.001\) and 73.3\% vs. 36.7\%, \(P = 0.006\), respectively), while the multiple PRs sharply declined from 31.9\% to 9.1\% in the PGS group. \textbf{CONCLUSIONS:} In this pilot study, we have shown that PGS by array CGH can improve the clinical outcome in patients undergoing IVF with poor prognosis.

\textbf{KEY WORDS:} Array comparative genomic hybridization, clinical outcomes, embryo selection, \textit{in vitro} fertilization, preimplantation genetic screening

Preimplantation genetic screening for all 24 chromosomes by microarray comparative genomic hybridization significantly increases implantation rates and clinical pregnancy rates in patients undergoing \textit{in vitro} fertilization with poor prognosis

Gaurav Majumdar, Abha Majumdar, Meena La\textsuperscript{1}, Ishwar C, Verma\textsuperscript{2}, Kallash C, Upadhyyaya\textsuperscript{3}
Center of IVF and Human Reproduction, Institute of Obstetrics and Gynaecology, Sir Ganga Ram Hospital, \textsuperscript{1}Cyto genetics Laboratory, Center of Medical Genetics, Sir Ganga Ram Hospital, \textsuperscript{2}Center of Medical Genetics, Sir Ganga Ram Hospital, New Delhi, \textsuperscript{3}Amity Institute of Molecular Biology and Genomics, Amity University, Noida, Uttar Pradesh, India

Address for correspondence:
Gaurav Majumdar,
Center of IVF and Human Reproduction, Institute of Obstetrics and Gynaecology, Sir Ganga Ram Hospital, New Delhi - 110 060, India.
E-mail: gaurav1979@hotmail.com

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of aneuploidy rises with increasing maternal age, but it is only moderately associated with morphology. Therefore, a significant percentage of even the “most ideal” embryos selected for transfer will be aneuploid, resulting in poor reproductive outcome. Moreover, transferring aneuploid embryos can be potentially dangerous since aneuploidy is the most common cause of miscarriage and the most common genetic abnormality in embryos.

Preimplantation genetic screening (PGS), even though highly invasive in nature, started out as a very promising concept allowing embryos to be screened for aneuploidies before being selected for transfer. However, this older version of PGS failed to live up to expectations, as numerous authors failed to show improvement in in vitro fertilization (IVF) outcomes with PGS using fluorescence in situ hybridization (FISH), in which chromosomal analysis of not all but only a few chromosomes was performed.

With the advent of new validated platforms for comprehensive chromosomal screening (CCS) such as single nucleotide polymorphism array, microarray comparative genomic hybridization (array CGH), and quantitative polymerase chain reaction (PCR), capable of analyzing all 24 chromosomes, now an improved version of PGS involving 24-chromosome copy number analysis is being expected to be a likely remedy for the earlier shortcomings.

This retrospective case–control study seeks to examine the efficacy of PGS applied to poor prognosis patients, given the relative paucity of information concerning the use of modern 24-chromosome copy number analysis for this patient group. The objectives of this study were to establish the incidence of aneuploidy in such patients undergoing IVF with poor prognosis and to undertake a retrospective comparative analysis of the clinical outcomes of these patients undergoing IVF-PGS cycles with non-PGS, IVF controls.

MATERIALS AND METHODS

A retrospective case–control study was performed which was approved by the Institutional Ethics Committee. All patients gave their written informed consent before undergoing the IVF-PGS cycles. However, patients were deemed eligible to undergo PGS only if a minimum of four good quality embryos were available for biopsy on day 3. A total of twenty patients underwent 21 cycles of IVF-PGS from July 2014 to March 2015 with the following three indications: Advanced maternal age (AMA) (>38 years), recurrent implantation failure (RIF) (≥2 IVF failures), and repeated miscarriages (RM) (≥2 pregnancy losses). During the same time period, 128 fresh, nondonor IVF cycles were included in the control group that met the same criterion as that of the PGS group, i.e., a minimum of four good quality day 3 embryos, which were all cultured till day 5 without any intervention. This ensured that the patients who were included in the control group were similar to the patients who were offered PGS in terms of number and quality of embryos. Outcome measures studied were blastocyst formation rates postbiopsy, clinical PR per embryo transfer (ET) and per biopsy, implantation rate, ongoing PR, and multiple PR. Only fresh ET cycles were included in the analysis.

All patients underwent controlled ovarian stimulation, using either the standard long protocol or the flexible antagonist protocol. In the long protocol, after confirming pituitary down regulation on day 2 or 3 of the cycle, 150–225 IU of recombinant follicle-stimulating hormone (Gonal F; Merc Serono, Geneva, Switzerland) was administered daily depending on the patients anticipated response, with or without addition of 75 IU recombinant luteinizing hormone (Luveris; Merc Serono, Geneva, Switzerland) in the late follicular phase based on the patients response to stimulation. When at least two follicles reached 18 mm in diameter, recombinant human chorionic gonadotropin (hCG) (250 μg, Ovitrelle, Merck Serono, Geneva, Switzerland) was used to trigger ovulation. Trans-vaginal 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, fertilization media (Vitrolife, Sweden) for culture at 37°C and 6% CO₂ for 3–4 h. In the meanwhile, the semen sample obtained from the husband was subjected to 40:80 double density gradient centrifugation (Puresperm, Nidacon, Sweden) at 300 g for 10 min. The resulting pellet was washed twice (Sperm Rinse, Vitrolife, Sweden) and a subsequent swim-up was performed 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-10X, Vitrolife, Sweden) for 30 s.

Mature oocytes were injected with sperm according to the centers established intracytoplasmic sperm injection (ICSI) protocol. During ICSI, an elongated 10 μl poly vinyl pyrrolidone (PVP, Medicult, Denmark) drop under oil was used to select spermatozoa with normal morphology for subsequent injection. Fertilization was checked the next morning at 16–18 h postinsemination. 2PN embryos were then cultured sequentially in G1P (Vitrolife, Sweden) from day 1 to 3 and G2P (Vitrolife, Sweden) from day 3 to 5. On the morning of day 3, embryos were graded based on their morphology and cleavage rates, and decision to perform embryo biopsy was taken if at least four reasonably good quality embryos with 7–12 cells and 0–15% fragmentation...
were available. Prior to the biopsy, embryos were placed in a calcium–magnesium-free buffer (PGD Biopsy Media, Vitrolife, Sweden). Biopsy was performed using laser (Hamilton Thorne, UK) to make a hole in the zona pellucida through which a single blastomere with a visible nucleus was gently sucked out, using a biopsy micropipette (TPC, Australia). The individual biopsied embryos were then immediately washed and placed back in culture, and the single blastomere was washed and transferred to a PCR tube containing 2 μl phosphate buffer saline under strictly sterile and DNA-free conditions to avoid any contamination. The PCR tubes containing the single cells were subsequently sent to the genetics laboratory for further genetic analysis.

Once at the genetics laboratory, the single-cell genomic DNA was subjected to whole genome amplification using SurePlex™ kit (Bluegnome, Cambridge, UK) in accordance with the manufacturers’ guidelines to obtain sufficient quantity of sample DNA for array hybridization. 24sure array kit (Bluegnome, Cambridge, UK) was used to perform array CGH. Normal controls were provided with the 24sure kit. The sample and control DNAs were labeled with Cy3 and Cy5 fluorophores using random primers. Labeling mixes were combined and co-precipitated with COT human DNA. The labeled DNA was hybridized under cover slips to V3 slides (3–20 h). The slides were then washed to remove unbound labeled DNA and scanned using a laser scanner. The data were extracted and analyzed using BlueFuse Multi software (Bluegnome, Cambridge, UK) for detection of gains and losses across all 24 chromosomes using detection criteria defined by the 24sure platform.

Meanwhile, the biopsied embryos were cultured till day 5 for fresh transfer. Once the array CGH results were available on the morning of day 5, only those euploid embryos that had progressed to blastocyst stage were chosen for transfer. A maximum of two euploid embryos were transferred after evaluation of the embryo quality and discussion with the patient. In case, all the embryos were found to be aneuploid, the ET was cancelled. Post-ET, any supernumerary “normal” blastocysts were cryopreserved.

All patients received progesterone support daily, from the day of OCR in the form of vaginal tablets (400 mg BD) alternating with injectable natural micronized progesterone (100 g), until day 11 after ET when they were tested for βhCG. A clinical pregnancy was defined as presence of one or more gestational sacs on ultrasound 2–3 weeks after positive βhCG. Ectopic pregnancies were not counted as clinical pregnancies. Pregnancy loss rate was defined as the number of clinical pregnancies lost before 20 weeks of gestation divided by the total number of clinical pregnancies.

Statistical analysis was performed by the SPSS program for Windows, version 17.0 (SPSS Inc., Chicago, Illinois, USA). Continuous variables are presented as mean ± standard error of mean. Data were checked for normality before statistical analysis. Non-normally distributed continuous variables were compared by Mann–Whitney U-test except age. For all statistical tests, a P < 0.05 was considered statistically significant.

RESULTS

Twenty patients who enrolled for 21 IVF-PGS cycles using array CGH were classified into the following primary indications: (1) AMA (n = 1), (2) RIF (n = 11), and (3) RM (n = 9). Some patients had more than one indication. The array CGH results and blastulation rates postbiopsy for patients undergoing PGS for different indications have been summarized in Table 1. There was no significant difference found in the overall blastocyst formation rate for the euploid and aneuploid embryos postcleavage stage biopsy (82.1% vs. 70.2%, respectively, P = 0.273). A higher blastocyst formation rate postbiopsy was found in the RM group as compared to the RIF group (91.9% vs. 61.4%, respectively), but the aneuploidy rate was similar in both the groups.

Eighty-seven embryos were analyzed by array CGH in 21 PGS cycles in the study group, out of which 47 embryos were found to be aneuploid (54%), 39 were reported as euploid (44.8%), and 1 with no DNA amplification. Among the 47 aneuploid embryos [Table 2], majority of copy number abnormalities involved monosomies (42.5%), which were almost 3 times the number of trisomies (14.9%). The remaining aneuploidies involved two or more chromosomes. An embryo with five or more affected chromosomes was labeled as complex aneuploid. Among the reported aneuploidies, chromosome 22 and 18 were found to be most frequently affected.

### Table 1: Aneuploidy, blastulation rates, and clinical pregnancies in patients with different indications for preimplantation genetic screening

| Primary indication | Number of cycles | Number of embryos | array CGH results (%) | Blastulation rate (%) | Number of clinical pregnancies/ET |
|--------------------|------------------|-------------------|-----------------------|----------------------|-------------------------------|
|                    |                  |                   | Euploid | Aneuploid | Euploid | Aneuploid | Euploid | Aneuploid | |
| AMA                | 1                | 6                 | 0/6 | 6/6 (100) | - | - | - | - | |
| RIF                | 11               | 44                | 21/44 (47.7) | 22/44 (50) | 14/21 (66.7) | 12/22 (54.5) | 7/9 (77.8) | 7/9 (77.8) | |
| RM                 | 9                | 37                | 18/37 (48.6) | 19/37 (51.4) | 18/18 (100) | 16/19 (84.2) | 4/6 (66.7) | 4/6 (66.7) | |
| Total              | 21               | 87                | 39/87 (44.8) | 47/87 (54) | 32/39 (82.1) | 33/47 (70.2) | 11/15 (73.3) | 11/15 (73.3) | |

ET=Embryo transfer, array CGH=Microarray comparative genomic hybridization, AMA=Advanced maternal age, RIF=Recurrent implantation failure, RM=Repeated miscarriages
There were no significant differences in female age, peak serum estradiol ($E_2$), endometrial thickness on the day of hCG, mean number of oocytes retrieved, and number of fertilized embryos between the PGS and control group [Table 3]. However, mean number of embryos transferred in the PGS group was significantly lower as compared to the control group.

The implantation rate and clinical PR per transfer were found to be significantly higher in the PGS group than in the control group (63.2% vs. 26.2%, respectively, $P = 0.001$ and 73.3% vs. 36.7%, respectively, $P = 0.006$) [Table 4]. When cycles that could not reach the stage of ET (because of absence of a euploid blastocyst) were taken into account ($n = 6$), the clinical PR per embryo biopsy was still higher as compared to the control group (52.4% vs. 36.7%, respectively, $P = 0.172$), but statistical significance was lost. The multiple PRs sharply declined from 31.9% to 9.1% in the PGS group with only one twin pregnancy and no triplets.

Table 2: Number of monosomies, trisomies, and complex aneuploidies among the aneuploid embryos ($n=47$) on microarray comparative genomic hybridization analysis

| Type of aneuploidies          | n (%) |
|------------------------------|-------|
| Monosomy                     | 7 (14.9) |
| Trisomy                      | 20 (42.5) |
| Two abnormal chromosomes     | 8 (17) |
| Three abnormal chromosomes   | 6 (12.8) |
| Complex abnormal embryos     | 6 (12.8) |

Table 3: Comparison of cycle characteristics between the study and control group

|                      | PGS group | Control group | $P$  |
|----------------------|-----------|---------------|------|
| $n$                  | 21        | 128           |      |
| Age (mean±SD)        | 33.17±1.19| 31.86±0.36    | 0.216|
| Peak serum $E_2$     | 1951.06±210.09 | 2064.84±87.09 | 0.891|
| Endometrial thickness on day of hCG (mm) | 7.98±1.24 | 9.38±1.95 | 0.001* |
| Number of eggs retrieved | 15.18±1.34 | 12.66±0.486 | 0.082|
| Number of eggs fertilized | 9.65±0.742 | 7.70±0.327 | 0.018|
| Number of embryos transferred | 1.21±0.114 | 1.81±0.049 | <0.001*|

Table 4: Comparison of clinical outcomes between preimplantation genetic screening and control groups

|                      | PGS group ($n=21$) (%) | Control group ($n=128$) (%) | $P$  |
|----------------------|------------------------|-----------------------------|------|
| Fertilization rate   | 139/211 (65.9)         | 99/1631 (60.8)              | 0.151|
| Clinical PR/ET       | 11/15 (73.3)           | 47/128 (36.7)               | 0.006*|
| Clinical PR/biopsy   | 11/21 (52.4)           | 47/128 (36.7)               | 0.172|
| Ongoing PR/biopsy    | 9/21 (42.9)            | 41/124 (33.1)               | 0.382|
| Implantation rate    | 12/19 (63.2)           | 59/225 (26.2)               | 0.001*|
| Multiple PR          | 1/11 (9.1)             | 15/47 (31.9)                | 0.127|

DISCUSSION

This retrospective case–control study seeks to examine the efficacy of PGS with CCS using array CGH in patients undergoing IVF with poor prognosis. Our study showed that transfer of embryos reported as euploid by array CGH resulted in a significantly higher implantation rate compared to the non-PGS control group. Traditionally, transfer of fewer morphologically selected embryos has been associated with a reduction in IVF success rates. However, in our study, the transfer of fewer embryos in the PGS group as compared to the control group resulted in a steep rise in implantation rates while concomitantly causing a sharp decline in the multiple PRs, thus proving that genetic screening for all 24 chromosomes resulted in improved embryo selection as compared to traditional morphology-based embryo selection. The clinical PR per transfer was also found to be statistically higher in the PGS group as compared to the control group. However, when all cycles where embryo biopsy was performed were taken into account, including those cycles where ET could not be done as a result of all embryos being aneuploid, the clinical PR per embryo biopsy was higher, but no longer statistically significant between the PGS group and the control group. Nevertheless, our study found an improved outcome after PGS despite including cycles with no euploid embryos to transfer.

In 2012, Yang et al. were perhaps the first group that reported a major improvement in clinical outcome after PGS using CCS in good prognosis patients. The basic objective of their study was to find whether PGS could be used as a tool to augment elective single ET cycles and consequently reduce the risk of multiple gestations. Even though a significant improvement in PRs was reported after PGS, the seemingly beneficial intervention perhaps proved to be effective in only reducing the time to pregnancy by the way of improved embryo selection. Since only good prognosis patients were investigated where already a significant proportion of embryos are expected to be euploid, it was only a matter of time before the “right” embryo was transferred. Since then, there have been a few other randomized controlled trials that have also reported an improved implantation rate in good prognosis patients undergoing IVF with CCS. Forman et al. reported equivalent ongoing PRs between single euploid blastocyst transfer and a two untested blastocysts transfer, but the multiple PR sharply declined from 48% to 0% in the single euploid blastocyst transfer group. Scott et al. also reported significantly higher delivery rates in the CCS group as compared to the control group, when equal number of embryos was transferred in both the groups. However, these results stem from good prognosis patients undergoing IVF with PGS. Our study sought to investigate whether these findings could be extrapolated to poor prognosis patients as well.
The main limitation associated with infertile couples undergoing PGS with poor prognosis is the lack of availability of sufficient number of good quality embryos for biopsy. This is perhaps the biggest drawback associated with PGS where a minimum number of good quality embryos are a prerequisite before any meaningful genetic analysis can be performed. A substantial percentage of poor prognosis patients undergoing IVF tend to have either poor embryo quality or a poor ovarian reserve, which leads to fewer numbers of oocytes retrieved during IVF. As a result, many cycles in this group are unable to fulfill the eligibility criterion for undergoing embryo biopsy, either because of lack of numbers or the minimum required quality. Conversely, patients who do reach the stage of embryo biopsy become favorably selected. Therefore, to remove any such selection bias for patients undergoing PGS, our study included only those patients in the control group who fulfilled the same inclusion criterion as for the PGS group, i.e., a minimum of four good quality day 3 embryos.

Of the 87 embryos that were biopsied on day 3, 74.7% embryos progressed to the blastocyst stage and there was no difference in the blastocyst formation rates between euploid and aneuploid embryos (82% vs. 70%, $P = 0.2$). Our data suggest that a good quality embryo selected on the basis of morphology showed an equal propensity to develop to the blastocyst stage irrespective of its chromosomal status. This finding also implied that extended culture to blastocyst stage did not result in any favorable selection of euploid embryos over aneuploid embryos. In addition, some authors have investigated the association between morphology and aneuploidy. They were able to demonstrate only a weak association between blastocyst morphology and aneuploidy status. Consequently, morphology analysis cannot be relied on to ensure transfer of chromosomally normal embryos.

Currently, PGS with CCS is being evaluated to determine which patient group is most likely to benefit from it. Patients who are typically offered PGS fall into the following three categories of indications: AMA, RIF, and recurrent miscarriages. Secondary indications included previous aneuploid conceptions and translocations and most patients can have more than one indication. In our study, very few patients with AMA could be offered PGS since most of them failed to produce enough embryos suitable for embryo biopsy on account of a poor ovarian response. This requirement of a good responder presents a major limitation in the application of PGS for AMA. Now, there is an increasing evidence that indicates that aneuploidy is one of the main causes that leads to poor implantation and PRs in patients with AMA. Furthermore, the high aneuploidy rates in older women are also associated with higher miscarriage rates. Hence, AMA has been an important target patient population for PGS. Some authors have reported an improvement in implantation rates after PGS with CCS in women with AMA whereas others have reported a reduction in miscarriage rate. However, most of these improved outcomes have been reported per ET cycles, without taking in to account those cycles where no euploid embryos were available for transfer. Since women of advancing age have markedly fewer embryos, coupled with a high incidence of aneuploidy, a large percentage of such women fail to reach the stage of ET.

RIF and RM are the two other major indications that constitute a potential target population for PGS. Most available data on the role of PGS as a treatment option for RIF have been obtained by the use of FISH and has been ambiguous and inconclusive. Recently, Greco et al. have shown that PGS by array CGH led to an improvement in clinical outcome for patients with multiple failed IVF cycles. Similarly, a beneficial effect of PGS by array CGH has been confirmed in both idiopathic recurrent miscarriages as well as translocation carries with a history of pregnancy losses resulting in a significant decrease in pregnancy loss rates in both groups. Majority of patients who were offered PGS at our center belonged to the category of RIFs or repeated spontaneous miscarriages. Out of 9 cycles in patients with RM, euploid embryos were available for transfer in only 6, out of which 4 patients conceived. However, two of these conceptions resulted in a miscarriage. This suggests that there will be always a subset of idiopathic recurrent pregnancy losses, which may occur due to factors other than chromosomal abnormalities in embryos, and hence PGS may not be a solution for such cases.

One of the main limitations of the study was the considerably smaller number of patients in the PGS group as compared to the control group. The high cost associated with genetic analysis proved to be a major deterrent for patients to undertake PGS, since opting for PGS resulted in doubling the cost of the IVF cycle. Another reason for the low recruitment was probably the nature of the patient population being investigated, since a high percentage of patients tend to drop out after the first few IVF failures or miscarriages. Moreover, many patients who did give consent to undergo PGS failed to fulfill the minimum criterion required to undergo biopsy. The retrospective design was another limitation of the study.

**CONCLUSIONS**

Our study showed that PGS by array CGH resulted in a marked increase in implantation rates and clinical PRs in patients undergoing IVF with poor prognosis, but further prospective, randomized clinical studies with a larger sample size are required to validate these preliminary findings. In addition, majority of patients who qualified to...
undergo PGS were good responders despite having poor prognosis. Therefore, we realize that the application of PGS remains limited to only a small subset of poor prognosis patients who are good responders or make good quality embryos. In addition, many patients undergoing PGS may end up with all aneuploid embryos and hence may never reach the stage of ET. However, in such cases, PGS may help indirectly in avoiding the transfer of potentially dangerous embryos as well as help in justifying the option of gamete donation for the couple. Despite pregnancy being the ultimate measure of success, achieving it in the shortest possible time with the fewest number of miscarriages is also highly desirable, which may be a point in favor of PGS.

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Conflicts of interest
There are no conflicts of interest.

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