

Key Factors to Avoid Cancellation of Trophectoderm Biopsy for Preimplantation Genetic Screening

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Abstract

This study analyzed 1103 cycles of assisted reproductive technology underdoing either intracytoplasmic sperm injection (ICSI) only or ICSI with preimplantation genetic screening (ICSI+PGS). The primary objective was to find the key factors resulting in cancellation of ICSI+PGS cycles. The embryos were cultured in one step media to the blastocyst stage. The trophectoderm biopsies were performed on day-5 or day-6 and the embryos were vitrified. Upon receiving PGS results and availability of at least one euploid embryo, patients were prepared for frozen embryo transfer. The patients were grouped into ≤ 35 , 36-39, 40-42, 43-46 and 47-48 yrs. Out of 1103 cycles, 826 cycles were in ICSI and 129 in ICSI+PGS group, from which 79 cases were cancelled. The cancellation rate increased with increase in the age category. The key factors for ICSI+PGS cycle cancellation were advanced female age and availability of <5 mature oocytes. Other reasons included poor oocyte quality, increased abnormal fertilization and availability of no motile sperm. The study proposes to optimize ovarian stimulation to retrieve about 15 oocytes per retrieval for a success ICSI+PGS cycle if trophectoderm biopsy is to be performed. In advanced age or poor responders, multiple retrievals may be needed to obtain enough blastocyst for PGS. The study highlights that counseling of patients undergoing ICSI+PGS should include not only success rates with PGS, but also information on the factors that may result in cycle cancellation. Therefore, prior to initiation of treatment it is essential to ensure that the patient has clearly understood the advantages and disadvantages of PGS and is prepared for all possible cycle outcomes.

Keywords: Trophectoderm biopsy, aneuploidy, preimplantation, PGS, PGD, PGT, ICSI

INTRODUCTION

Preimplantation Genetic Screening (PGS) refers to detection of chromosomal aneuploidy in the embryo whereas Preimplantation Genetic Diagnosis (PGD) refers to diagnosis of a specific genetic disease in the embryo that may transmit from parents to the offspring [1, 2]. Collectively, they can be referred as Preimplantation Genetic Testing (PGT). The aim of PGS is to improve pregnancy outcome and delivery. Due to the prevalence of aneuploidy in first trimester losses and the increased prevalence of aneuploidy in the repeated pregnancy losses, PGS has been proposed

as a method for reducing miscarriage by selecting euploid embryos for transfer [3]. The IVF-PGS is also cost effective, compared to routine IVF without PGS and subsequent frozen embryo transfer (FET) in <42 year old age group [4].

With technically appropriate blastocyst culture, trophectoderm biopsy, vitrification, cryostorage and later transfer of biopsied blastocysts is a practical and preferable way of PGS compared with cleavage-stage embryo biopsy, being accompanied by a high implantation rate and low twinning and miscarriage rates [5, 6, 7]. The human blastocysts subjected to

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trophectoderm biopsy and vitrification exhibit very high survival, pregnancy and implantation rates [8, 9]. The vitrification, also allows sufficient interval for comprehensive chromosome assessment of the biopsies.

Embryo morphology is a poor predictor of aneuploidy. A systematic review [10] revealed that PGS using comprehensive chromosome screening after blastocyst biopsy compared with embryo selection based on morphology criteria alone, not only significantly improved implantation rate in good prognosis patients but also reduced the multiple pregnancy rate when one single euploid blastocyst was transferred compared with transfer of two untested blastocysts. Improved clinical outcome was achieved with PGS in women between 38 and 41 years of age, with higher implantation and lower miscarriage, resulting in a significant increase in ongoing pregnancy rates per cycle [11]. In <35 yr. patients, the percent of euploid embryos in grade "AA" blastocysts was significantly higher as compared to that in grade "BB" blastocysts. Similarly, the percent of euploid embryos in grade "BB" blastocysts was significantly higher than that in grade "CC" blastocysts. A blastocyst with grade "A" ICM or grade "A" trophectoderm had a significantly higher number of euploid blastocysts as compared to a blastocyst with grade "B" or "C" ICM or trophectoderm. Although, in this study blastocyst morphology was significantly correlated with ploidy status, however, there was 18.2% chance that an aneuploid "AA" embryo could be transferred. For some patients <35 yr, this risk is very high, and they might opt ICSI+PGS. Also, patients with large cohorts of "BB" and "CC" blastocysts, ICSI+PGS is even more important since a higher percentage of aneuploidy embryos is expected. Thus, a patient may reach pregnancy sooner and have greater confidence to transfer a single, chromosomally normal embryo after utilization of PGS technology.

Patient age is a significant factor affecting embryo aneuploidy. An analysis of >6000 embryos on day-3 showed that maternal age, morphology and development parameters had a very large impact on the proportion of normal embryos. The age effect on aneuploidy was highly significant ($P < 0.001$). Embryos with the best morphology and development were 44% euploid in patients <35 yr. but decreased to 21% in patients > 41 yr. The worst morphology group had only 30% normal embryos from patients <35 yr, and 12% from patients >41 yr. [12].

A few other factors that significantly affected the number of genetically normal embryos are the number of biopsied embryos and basal FSH. The authors suggested that there should be at least four day-3 biopsied embryos to obtain at least one normal embryo in a PGD cycle for patients with single gene disorder and under the condition of basal FSH level < 8.0 mmol/L [13].

Majority of the studies on PGS report clinical outcomes only of patients who reach PGS biopsy and/or embryo transfer (ET) and all possibilities of cycle cancellation are not usually captured which may include poor ovarian response to stimulation, recovery of low number of mature oocytes/high number of immature oocytes, poor fertilization/cleavage/blastocyst development and unsuitability of blastocysts for biopsy or cryopreservation. For adequate patient counseling, information on all possibilities that may result in cycle cancellation is important to avoid any unexpected surprises. The purpose of this study was to identify key factors that result in PGS cycle cancellation in a program performing exclusively trophectoderm biopsies.

MATERIALS AND METHODS

The study period was from January to May, 2018. The data from 1103 patients undergoing either intracytoplasmic sperm injection with preimplantation genetic screening (ICSI+PGS) or only ICSI were analyzed. The patients were grouped into ≤35 yrs., 36-39, 40-42, 43-46 and 47-48 yrs. All embryo biopsies for ICSI+PGS group were carried out either on day-5 or day-6.

Ovarian Stimulation

All patients underwent gonadotropin releasing hormone (GnRH) antagonist (Cetrotide, subcutaneous injections; EMD Serono) flexible multi-dose protocol. The gonadotropin subcutaneous injections (Gonal-F, EMD Serono; Merional, IBSA Pharmaceutical or Merional, Ferring Pharmaceutical) were administered in variable doses, depending on patient age and/or ovarian responsiveness in previous cycles, and further adjusted according to vaginal ultrasound measurement of follicular development, every two or three days. HCG (Ovitrelle, Merk Serono or Choriomon, IBSA pharmaceutical) was administered for final maturation of oocytes when the leading follicle reached a diameter >17 mm. Oocytes were aspirated by the transvaginal ultrasonographic route approximately 36 hours after HCG injection.

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ICSI, Embryo Culture and Trophectoderm Biopsy, Embryo Transfer

Oocyte collection, denudation and ICSI were performed per standard protocol. After ICSI, oocytes were cultured in Global Total Medium (Life Global, USA) either in Embryoscope (Vitrolife) or G-210 (K-System) incubators at 37 °C in an environment of 6% CO₂ and 5 % Oxygen. On day-3 assisted hatching was performed using the Zilos Laser (Hamilton Thorne). The embryos were then cultured in a new dish prepared with Global Total (Life Global, USA).

The embryologists graded the blastocysts on the mornings of day 5 and day 6 based on the degree of expansion and the morphology of inner cell mass and trophectoderm according to Gardner's grading scale [14]. One very experienced embryologist confirmed the scores of all day 5 and day 6 embryos. The degree of expansion included the following six grades: 1: a non-expanded embryo with the blastocele filling <50%; 2: the blastocele fills >50% of the embryo; 3: the blastocele fills the entire blastocyst; 4: an expanded blastocyst with a thin zona pellucida; 5: a hatching blastocyst; and 6: a hatched blastocyst. The ICM was graded as follows: A: tightly packed cells; B: loosely gathered cells; and C: no identifiable cells. The three TE grades were: A: many cells establishing a cohesive epithelial layer; B: few uneven cells creating a loose epithelium; and C: few large cells pushed to the side.

On day 5 or 6, at the blastocyst stage, approximately 4-6 trophectoderm cells protruding out of the breach were biopsied, placed in a tube and sent for PGS analysis. All blastocysts survived the biopsy procedure and were vitrified utilizing Kitazato vitrification

solutions (Kitazato Corporation, Japan) and Cryotop device (Kitazato Corporation, Japan). Biopsies were analyses at on-site genetic analysis laboratory using 24sure PGS Microarray (Illumina).

In ICSI+PGS cases, the patients were prepared for frozen embryo transfer and only euploid embryos after PGS testing were transferred. The pregnancy test was carried in fresh transfers without PGS, 14 days post oocyte retrieval and in ICSI+PGS cases, 9 days after vitrified-warmed blastocyst transfer.

Statistical Analysis

The data were analyzed by Statistical Package for Social Sciences (SPSS), Version 23. Continuous variables were expressed as Mean ± SD and range. The percentages were compared with the chi-square. The t test was used for parametric data. The P< 0.05 was considered to be statistically significant.

RESULTS

During the study period of Jan to May 2018, out of 1103 total patients, 826 patients were treated by ICSI only and 219 patients by ICSI+PGS. The trophectoderm biopsies were performed either on day-5 or day-6 for 140 patients in ICSI+PGS group and 79 patients were cancelled. From the ICSI group, 58 cycles were cancelled due to multiple reasons. The cancellation reasons are shown in **Table 1**. Significantly high number of patients did not have either blastocyst available for trophectoderm biopsy or it was not suitable for vitrification due to extremely poor morphology. In the age group of 47-48 yrs., none of the embryo was biopsied. For majority of the patients ICSI+PGS procedure had to be cancelled when ≤5 mature oocytes were retrieved.

Table 1. Parameters of cancelled ICSI+PGS cycles in different age groups (N = 219).

Age Group (yr.)	≤ 35 (n=86)	36 – 39 (n=75)	40 – 42 (n=31)	43 – 46 (n=22)	47 – 48 (n=5)	Total (n=219)
PGS cancel reasons						
No (0) egg	1	2	0	1	0	4
No (0) M II	0	1	0	1	0	2
One M II	4	0	1	1	2	8
Two M II	5	3	8	4	0	20
Three M II	3	2	1	1	1	8
Four M II	0	3	1	4	0	8
Five MII	1	0	0	0	0	1
Abnormal fertilization	1	1	2	2	0	6

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Non-motile sperm	2	1	0	0	0	3
Poor quality oocytes	4	3	0	0	2	9
Blastocyst unfit for vitrification	2	2	3	0	0	7
No blastocyst when > 5 MII	0	2	0	0	0	2
Cancelled by patient	1	0	0	0	0	1
Total	24	20	16	14	5	79

The percent cancelled ICSI+PGS cases in each age group are shown in **Fig 1**. Significantly high number of cases were cancelled in 40-42, 43-46 and 47-48 yrs. patients as compared to patients in ≤35 and 36-39 yrs.

age categories. None of the patients in 47-48 yrs. age category succeeded to trophectoderm biopsy stage, due to either very poor oocyte quality or <5 mature oocytes retrieved.

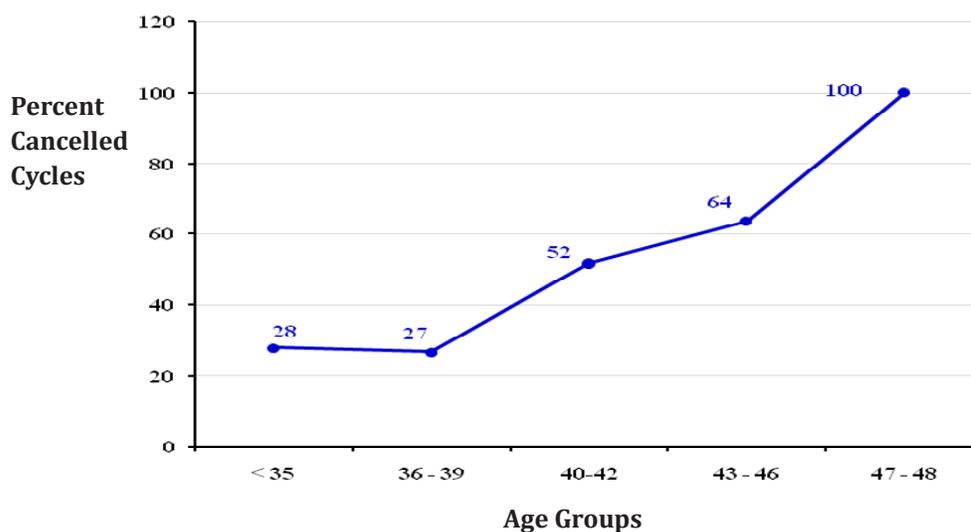


Fig 1. Percent cancellation of ICSI+PGS cases in different age groups (N=140)

The women in our study were significantly older in ICSI+PGS group as compared to the women in ICSI only group (**Fig 2**). For example, in ≤35 yrs. age group 64% women were in ICSI only group

whereas they were 39% in ICSI+PGS group. Also, significantly less women of 36-39 yrs. age were in ICSI only group (17%) as compared to 34% in ICSI+PGS group.

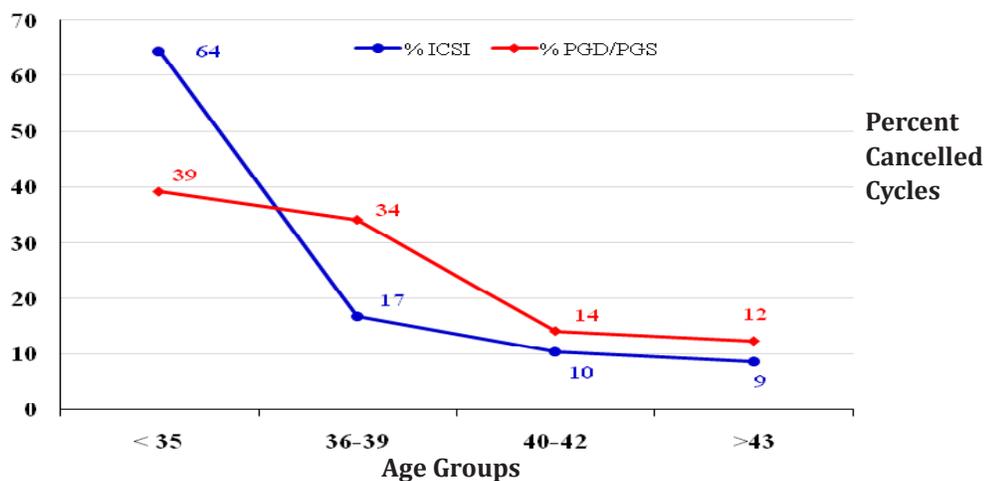


Fig 2. Percent distribution of patients in routine ICSI and ICSI+PGS in each age category (Total ICSI cases = 826 and ICSI+PGS cases =219).

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The parameters of cases that succeeded in trophoctoderm biopsy and PGS are presented in **Table 2**. Most of the cases were from ≤ 35 and 36-

39 yrs. age categories. Out of 219 total ICSI+PGS cases, 79 (36%) did not proceed to trophoctoderm biopsy and PGS.

Table 2. Parameters of successful PGS cycles resulting in blastocyst biopsy in different age groups (N = 140).

Age Group (yr.)		≤ 35 (n=62)	36 - 39 (n=55)	40 - 42 (n=15)	43 - 46 (n=8)
Number of oocytes retrieved	Mean \pm SD	15 \pm 11	10 \pm 6	14 \pm 8	10 \pm 5
	Range	1 - 60	2 - 28	3 - 36	2 - 19
Number of M II oocytes	Mean \pm SD	12 \pm 8	8 \pm 6	11 \pm 6	8 \pm 3
	Range	1 - 41	1 - 26	3 - 28	1 - 12
Number of blastocysts	Mean \pm SD	3 \pm 1	3 \pm 2	2 \pm 1	2 \pm 1
	Range	1 - 7	1 - 8	1 - 5	1 - 3

Based on our observations and experience, we suggest that for ideal situations and for success in ICSI+PGS cycles, the optimum number of oocytes retrieved is 15, these are expected to produce 12

metaphase II oocytes, resulting in fertilization of 9 eggs and production of approximately 4 blastocysts, from which 1-2 euploid blastocysts will be available for transfer (**Fig 3**).

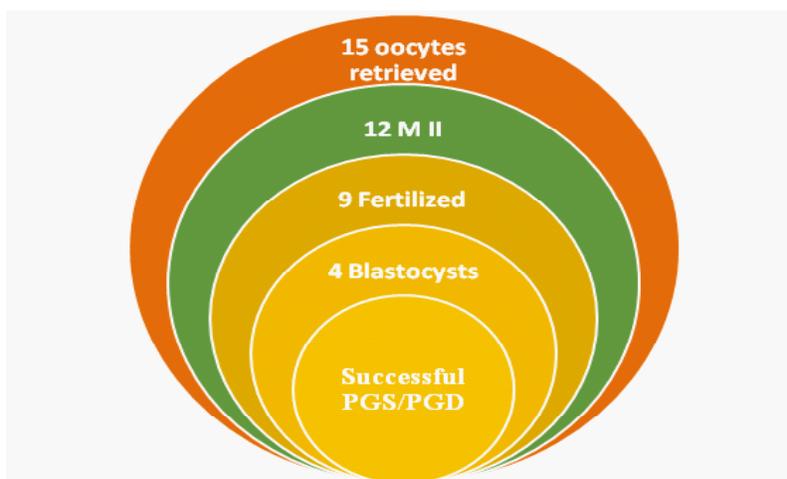


Fig 3. Suggested scheme for success in ICSI+PGS cycles in ideal situations.

Pregnancy rates for ICSI only group are shown in **Table 3**. The highest pregnancy rate is observed in 36-39 yrs. age group. The most likely reason for lower pregnancy rate in ≤ 35 yrs. group is presence of large number of severe male factor infertility cases resulting in poor quality embryos. All these patients utilized their own oocytes and partner's sperm and did not accept donated sperm or oocytes.

Table 3. Pregnancy rates for ICSI only cycles after fresh embryo transfer

Age Group	# Patients	# Embryos/ ET	% Positive/ ET
≤ 35	532	2.03	46
36-39	138	1.7	57
≥ 40 years	156	3	17
All ages	826	-	38

Pregnancy rates for patients undergoing ICSI+PGS are shown in **Table 4**. The biopsies were performed either day-5 or day-6. Higher pregnancy rates were observed when embryo biopsies were performed on day-5. The patient number for day-6 biopsies is low, therefore, caution is needed to draw a solid conclusion.

Table 4. Pregnancy rates in ICSI+PGS group after transfer of euploid embryos

Biopsy Day	# Patients	# Embryos/ ET	% Positive/ ET
Day-5	20	1.5	55
Day-6	5	1.6	40
Total	25	-	52

DISCUSSION

This study revealed possibilities of cancellation of ICSI+PGS cycles. We considered an ICSI+PGS cycle successful when at least one embryo reached blastocyst stage, underwent trophoctoderm biopsy, and PGS results were available. We observed that advanced maternal age (>40 yrs.) and retrieval of <5 mature oocytes are key factors resulting in ICSI+PGS cycle cancellation. Other cancellation factors include, availability of non-motile sperm only, poor oocyte quality, poor or no blastocyst for biopsy and increased abnormal fertilization (**Table 1**). In 23 % ICSI+PGS cycles, biopsy and genetic testing were cancelled due to ≤ 5 oocytes retrieved, resulting in availability of ≤ 5 mature oocytes. In >40 yrs. age group, 52% cycles were cancelled (**Fig 1**). The success in assisted reproductive technology procedures is strongly dependent on women's age and number of oocyte retrieved. The percentage of cycles with normal blastocysts increase significantly with the increase in oocyte number [15, 13]. Also, the live birth rate increases with an increase in number of oocytes retrieved up to 15, plateaued between 15 and 20 oocytes, and rapidly decrease beyond 20 oocytes [13].

We suggest that 15 oocytes per retrieval is an optimum number for success in ICSI+PGS cycle (**Fig 3**). This number is expected to result in 12 metaphase II oocytes, 9 zygotes and about 4 blastocysts for biopsy. However, this number can't be anticipated in poor responders or advanced age patients, therefore, in such cases, for adequately stimulated patients, oocyte retrieval may be continued even with low number of oocytes expected [13], to bank about 4 biopsied embryos from multiple stimulated cycles for subsequent PGT. It is imperative that such patients should be counseled either about strategy of multiple cycles or the option of transferring embryos without PGS followed by prenatal diagnosis. The acceptance of latter option is influenced by patient's ethical and religious beliefs about termination of pregnancy if prenatal testing indicates abnormality in conceptus. Nevertheless, counseling in the PGT setting is beneficial as a whole, regardless of previous access to a genetic counselor [16]. In some situations, all tested embryos may indicate chromosomal aneuploidy. In such cases the embryo transfer is cancelled to avoid an unnecessary procedure and to decrease the miscarriage rate by not transferring abnormal embryos [17].

There is scanty information in the literature on ICSI+PGS cycle cancellation before reaching the biopsy stage. Majority of the studies report clinical outcomes only of patients who reach PGS biopsy and/or embryo transfer and cycle cancellations at earlier stages are not usually captured. In the United States of America, the Society for Assisted Reproductive Technology (SART) publishes assisted reproductive technology cycle outcomes. In SART database, the information on initiated but cancelled PGS cycles is not available because cycles inclusion in the SART database for PGT is defined as an embryo biopsied, thus cycles that were initiated with the intent to perform PGT but were converted to standard cycles are not captured. There is need to modify the database so that clinics are able to report ICSI-PGT cycle cancellation rates for accurate patient guidance. Our study addresses this gap in the information.

The SART 2011 report indicated that the rates of PGT cycle cancellation differed between groups undergoing assisted reproductive technology and PGT procedures. The embryo transfer was not performed in 27.3% of cycles for translocation, 17.3% for aneuploidy screening, 16.7% for elective sex selection and 12.9% for genetic diagnosis. Whereas, the cancellation rate for non-PGT cycles was only 6.2%. [18].

The European Society for Human Reproduction and Embryology (ESHRE), includes PGT cycles from the time of oocyte retrieval. In 2006 data, of the 6,561 planned PGT cycles 5,718 underwent oocyte retrieval resulting in 13% cancellation before oocyte retrieval. Embryo transfers were performed in 3,545 cycles, indicating that 46.0% of planned PGT cycles did not undergo ET [18]. In ESHRE data from Jan 2011 to Dec 2012 for PGS cases, 28% cycles did not undergo ET [19].

Our study indicated in > 40 year old patients, 52% cycles were cancelled (**Fig 1**). In a study of 467 PGD cycles in Spain, 50% cycles were cancelled in advanced maternal age due to chromosomally abnormal embryos [20]. In another study from USA, from 128 cycles, eight (6.3%) had no embryos available for biopsy on D5 or D6 of culture. In the remaining 120 cycles, a total of 677 embryos were biopsied, a mean of 5.2 per cycle. However, only 379 embryos (56%) developed to a stage able to be biopsied on Day 5 of culture and only 53 cycles (41%) received a fresh transfer of normal embryos [21], indicating a very high cycle cancellation rate.

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This study showed that ICSI+PGS cycle cancellation rate increased with increasing age. Similar trend has been reported in SART 2011 data [18]. The females especially in advanced age undergoing PGS usually feel an urgency to conceive because time is not on their side and expect conception on each attempt of PGS. However, the reality is that success rate on each cycle of PGS is limited due to the high incidence of cycle cancellation [22, 20] presence of high number of aneuploidy embryos and low cycle outcome [23, 24]. In a multicenter randomized study, 38-41 yr. old patients were divided into two groups; IVF+PGS (Group 1) and only IVF (Group 2). The patients in Group 1 had significantly higher embryo transfer cancellations (32%) as compared with patients in Group 2 (4.5%) [25].

We observed that women in our study were significantly older in ICSI+PGS group than those in only ICSI group (**Fig 2**). The ICSI only group had 64% women ≤ 35 yrs. as compared to only 39% in the ICSI+PGS group. In the 2011 SART report, the mean age of women undergoing PGS was 37 yrs. The women in this group were older than those undergoing other treatments and had a high percentage (17%) of cycles in which embryo biopsy was performed but no ET occurred [18].

In the present study, only good quality and good morphology blastocysts were selected for biopsy and cryopreservation on day 5 and day 6 (**Table 2**). An analysis of 1,396 non-tested blastocysts and morula embryos revealed that the worst quality embryos had the lowest live birth rate [26]. Also, analysis of 477 euploid blastocysts indicated that blastocyst morphologic grading and particularly inner cell mass grade is a useful predictor of pregnancy. The poor quality euploid blastocysts resulted in higher spontaneous abortion rate compared with better-quality counterparts [27]. Some authors suggested that speed of embryo development also influenced the live birth rate as embryos reaching good-quality blastocysts on day 5 yielded significantly higher live birth rate (72.8% vs. 56.5%) compared with those reaching similar quality blastocysts on day 6. Similarly, day 5 average-quality embryos conveyed a significantly higher implantation rate compared with day 6 embryos of the same quality [28]. Our observations support this concept (**Table 4**). However, another study indicated that only blastocyst morphology was predictive of the comprehensive chromosome screening data as

faster or slower growing embryos showed a similar aneuploidy rate and the euploidy rate was 56.4, 39.1, 42.8 and 25.5% in the excellent, good, average and poor blastocyst morphology groups, respectively [29]. We implemented criteria of vitrification of good quality blastocysts and speed of embryo development as no blastocyst was vitrified on day-7 because no pregnancy was achieved after transfer of day 7 vitrified-thawed blastocyst [30]. Thus, the vitrification of good quality blastocysts is expected to decrease the number of cycles required for a live birth.

In this study all oocytes were fertilized using ICSI as fertilization failure after conventional in vitro fertilization (egg insemination) can be a devastating clinical scenario. Rescue ICSI or delayed ICSI after failed fertilization post egg insemination or in cases of an excessive proportion of immature oocytes has been attempted in IVF-PGS cycles, however, the success rate is very low. In a study from 2003 to 2015, a total of 161 patients underwent delayed ICSI. Of these, only 16 patients had trophoctoderm biopsy and PGS. Out of 32 blastocysts biopsied, 23 (71.8 %) were aneuploid and 9 patients did not have embryo transfer performed due to the absence of euploid embryos. From remaining 7 patients, the live births resulted in only 3 patients [31]. This study supports our observation that presence of immature eggs after retrieval results in very poor cycle outcome.

CONCLUSIONS

The data from this study indicates that advanced maternal age (>40 years) and availability of <5 mature eggs are key factors for cancellation of ICSI with PGS cycles. The study emphasizes the need for adequate counseling of patients considering ICSI with PGS. The information provided here will assist reproductive specialists to optimize ovarian stimulation, the genetic counselors to better counsel the couples and the patients to get mentally prepared for all possible outcomes.

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