Original Article

Effect of Single Embryo Blastomere Biopsy from Human Frozen Embryos on Assisted Reproductive Outcomes

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Abstract

Objective: Preimplantation genetic testing for aneuploidies (PGT-A) is used to determine chromosomal normality and achieve a successful live birth in infertile couples. There is a possible correlation between chromosomal aneuploidy, embryo development and pregnancy rate. This study evaluated the influence of single blastomere biopsy (SBB) on embryo development and pregnancy rates during frozen embryo transfer (FET) and fresh cycles.

Materials and Methods: This quasi-experimental study evaluated 115 intracytoplasmic sperm injection (ICSI) cycles, including 443 embryos (6-8 cells) with a grade A on day three, following PGT-A in the fresh or FET cycles from February 2018 to June 2020. In addition, the fresh cycles without PGT were included as a control group (n=166 embryos). SBB was done on day three and was grouped as FET-PGT (n=149) and the fresh-PGT (n=128).

Results: There is a more aneuploidy rate in the FET-PGT group compared to the fresh-PGT cycle (36.60% vs. 20.38%, P<0.001). There is a rate of higher development and blastocyst in the control group. While the embryos of PGT groups showed higher degrees of expansion (expansion 5) on day five. 8.6, 8.59, and 9.37% of expansion 3, 4, and 5 in the fresh-PGT embryos, 12.58, 2.78, and 14.84% of expansion 3, 4, and 5 in the FET-PGD embryos compared to 10.84and 33.73% of expansion 3 and 4 in the control group (without expansion 5; P<0.001). There was no significant relationship between 13, 18, and 21 chromosome aneuploidies with blastocyst development competence among the groups (P<0.1). Following embryo transfer (n=97), the spontaneous abortion rate was higher in the FET-PGT cycles compared to the fresh-PGT and control groups (50 vs. 22 and 11%, respectively; P<0.04).

Conclusion: The process of SBB following vitrification significantly decreased embryo development and pregnancy outcomes. Therefore, a morphological analysis could not be reliable in selecting chromosomally normal embryos.

Keywords: Aneuploidy, Biopsy, Blastocyst, Genetic Testing, Pregnancy Outcome

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Introduction

Selecting viable embryos for implantation to achieve successful live birth is a significant concern during intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF) cycles. At present, morphological evaluation is the basic embryo selection approach. Preimplantation genetic testing for aneuploidies (PGT-A) determines embryos' chromosomal normality. Simultaneously, there is no permanent relationship between embryo morphology and chromosomal status. However, a considerable rate of human blastocysts with excellent morphological grades may have an abnormal chromosomal status (1).

Morphological characteristics include evaluating blastomere number, shape, size, and fragmentation rate, multinucleation of blastomeres, perivitelline space, and zona pellucida evaluation, a common approach for embryo quality assessment (2). However, the predictability of embryo morphology for implantation is controversial (3), while studies reported this association to be fragile (4).

Progression in cryopreservation permits almost 100% recovery of warmed embryos with minimum damage and chemical pregnancy rates similar to (or even higher) than fresh embryo transfer (5). In addition, a recent multicenter cohort study demonstrated that freeze-only transfer cycles had better implantation and pregnancy rates than fresh transfer cycles (6), stimulating a dispute on freeze-only IVF competence (7).

The rate of embryo development to the blastocyst stage has been studied in many studies. Many parameters related to semen quality, maternal age, and embryo gender on aneuploidy, as well as the developmental potential of biopsied and non-biopsied embryos in freeze and fresh embryo transfer cycles, were evaluated. The embryo's quality, determined by the morphological parameters, was not associated with the embryo's genetic status (8, 9).

In contrast, most studies evaluated blastocyst embryos with trophectoderm (TE) biopsy on day five. Therefore, physicians and patients can be informed about the

effect of single blastomere biopsy (SBB) on the embryo development potential to choose an alternative embryo transfer approach and diagnostic tests or to use non-invasive chromosome screening (NICS) and less-invasive chromosome screening (LICS) methods (10, 11).

This study evaluated the role of SBB in the fresh and frozen-warmed embryos (day three) on assisted reproductive outcomes. So, the correlation between embryo gender and aneuploidy (13, 18, 21, X and Y chromosomes) with embryo development potential and embryo resistance following the vitrification-warming procedure was evaluated. In addition, the pregnancy and the live birth rate were studied in the FET-PGT, fresh-PGT and fresh (without PGT) cycles.

Materials and Methods

Patients and study design

This quasi-experimental study was conducted at the Mehr infertility and research center, Rasht, Iran, from February 2018 to June 2020. The study included infertile patients undergoing ICSI cycles with/without PGT-A; subsequently, one to three euploid embryos were transferred to the patient. The age range of men and women was 24 to 59 and 21 to 49-year-old, respectively.

Four hundred fourty three embryos with grade A and 6-8 cells from ICSI cycles with or without SBB were included in this study. First, these embryos were freezes on day 3. Then, a comprehensive chromosomal screening was performed using fluorescence in situ hybridization (FISH) for 13, 18, 21, X, and Y chromosomes. Therefore, the study included three groups as follows: group I) embryos from fresh-PGT cycles (n=128), group II) embryos from FET-PGT cycles (n=149), and group III) embryos without biopsy and vitrification procedures (n=166). Each group followed the results, and the embryos' developmental potential was evaluated using the Gardner method.

Ovarian stimulation

Pituitary suppression was performed using a long-acting gonadotropin-releasing hormone (GnRH) agonist protocol based on the ovarian reserve and anti-Mullerian hormone levels. In addition, ovarian stimulation with recombinant follicle-stimulating hormone (FSH, Gonal-F, Serono, Germany) was initiated. The daily dose of either human menopausal gonadotropin (Menopur, Ferring, Germany) or FSH was set due to the ovarian response. Monitoring of follicle development was done with estradiol measurement and transvaginal ultrasonography. The oocytes were retrieved 36 to 39 hours after human chorionic gonadotropin (hCG) injection and final oocyte maturation. Then oocytes by ICSI procedure were fertilized.

Embryo culture and evaluation

The embryos were cultured and evaluated till day three after ICSI. First, the embryo classification was done based on Gardner's method into three groups; A (6 to 8 blastomeres with the exact size and ≤ 10% fragmentation rate), B (6-8 blastomeres with even or uneven size, and 10% to 20% fragmentation rate), and C (low number and uneven blastomeres with more than 20% fragmentation). Then, the frozen-thawed and/or fresh embryos were cultured until day five (blastocyst stage) and scored based on the Gardner method, which explains the extent of blastocoel expansion, establishment of TE, and inner cell mass (ICM) grade of development.

The rate of expansion was determined as follows: 1=early blastocyst; cavity starting to the formation, 2=early blastocyst; cavity less than 50% of the total embryo, 3=full blastocyst; cavity thoroughly loaded with the embryo, 4=expanded blastocyst; cavity bigger than complete blastocyst, zona slimming, 5=hatching blastocyst; TE is splitting via the zona, 6=hatched blastocyst; blastocyst fully exited of the zona. ICM was graded as follows: A=many cells; firmly compacted, B=some cells; firmly compacted, C=some cells; disordered, D=few cells; disordered. The TE grading was defined as follows: A=forming a coherent epithelium in many cells, B=moderate cells making a weak epithelium, C=some cells making a weak epithelium, and D=very few cells.

Vitrification-warming technique

The embryo transfers of patients with homogeneous hyperechogenic endometrium and serum progesterone levels greater than 2 ng/ml on the day of hCG administration were canceled, and embryos with grade A were vitrified on day three to use at subsequent cycles as a FET-PGT cycle. The vitrification and warming procedures were conducted with the suggested protocol in the Vitrification Kit from the Kitazato company. In brief, the embryos were placed in vitrification solutions for 15 minutes and 40-50 seconds in two steps. Then, embryos were transferred with minimal vitrification solution (0.1 µl or less) and lay a Cryotop. Next, the embryos were put into a nitrogen tank. For warming, the frozen embryos were submerged in a warming solution at 37°C for 1 minute. Then they were rehydrated with a 3-step dilution protocol to recover at room temperature. Finally, embryos were transferred to culture media (12).

Evaluation of embryos the following warming

After embryo warming, the quality of embryos was morphologically defined by quantifying the embryo's intact cells. In addition, intact embryos without signs of degeneration and damaged cytoplasm and/or membrane were considered. In this study, embryos were incubated to evaluate blastulation potential for another two days. The embryos were transferred in an embryo culture medium covered with oil (Global total, COOPER) in a 37°C incubator (SANYO, MCO-5AC) with 5% O_2, 6% CO_2, and 89% N_2 atmosphere to day five. Blastocysts scored with a numeric scoring system from 1-6 due to the

expansion grade and hatching situation (12).

Embryo biopsy

The SBB on day three was accomplished based on morphological evaluation. At first, the fresh and warmed embryos, at the cleavage stage of day 3, were transferred into a cation (Na^+ and Mg^{++}) free medium. Then, zona pellucida was hatched locally. So that, just a single blastomere was removed.

Then, the blastomere was gently aspirated using an appropriate biopsy microneedle. Next, the blastomeres were studied by the FISH assay. Biopsy samples were maintained in the hypotonic buffer and deferred for analysis. Following blastomere biopsy sampling, embryos were re-cultured. PGT results were described as aneuploid or euploid due to FISH outcomes. After detecting the desired signals in the PGT and control groups, a maximum of three embryos was transferred on day five.

Embryo selection for transfer

Euploid blastocyst embryos with the expansion grades 2, 3, 4, and 5 were selected for transfer. In addition, the embryo grade on day five was considered for embryo selection among the biopsied embryos by evaluating expansion, TE, and ICM morphology (13). In the control group, 1-3 embryos with the best quality were transferred. Embryos were transferred with a Cook catheter under abdominal ultrasound conduction on day five.

Embryo transfer outcomes

The primary outcomes were chemical, clinical, ongoing pregnancy, and lived birth rates. The chemical pregnancy is an early pregnancy that occurs shortly after implantation and was defined as a positive test of serum βhCG level 14 days after embryo transfer. In addition, the sonographic evidence of fetal heartbeat confirmed the clinical pregnancy. Ongoing pregnancy was determined as a viable intrauterine pregnancy observed after eight weeks of pregnancy. Live birth was defined as a live birth after 28 weeks of pregnancy. Secondary outcomes were spontaneous abortion or early pregnancy loss (EPL) and clinical pregnancy loss (CPL) rates. EPL was determined as a pregnancy loss occurring before an intrauterine gestational sac was discovered using ultrasound. Following the fetal heartbeat detection using ultrasound, a lost pregnancy was described in CPL (13).

Statistical analysis

For the pregnancy outcomes, the mixed logistic regression model was used for parameters of TE grade, ICM grade, and embryo expansion as final prophesiers to assess the effect of grades on embryo development and pregnancy outcomes. All data were adjusted for covariates, containing necessary information of semen and oocyte and the day of embryo biopsy during FET or fresh ICSI (with or without PGT-cycles) cycles. An OR table was designed to evaluate the composite development grade to define each pregnancy outcome's likelihood. Statistical analysis was done using a statistical package of the social sciences version 26 (SPSS Inc. Chicago, IL, USA). The Chi-square test for classified variables was used. A statistically significant P value was considered less than 0.05.

Ethics consideration

The Ethics Committee approved this original research at the Islamic Azad University, Rasht Branch (IR.IAU. RASHT.REC.1398.053). In addition, all participants were informed about this study.

Results

Study population and cycle characteristics

Totally 443 embryos with grade A were obtained from 115 patients (1265 mature oocytes) who underwent ICSI with or without PGT cycles. Then, 277 embryos were evaluated following blastomere biopsy in FET and fresh transfer cycles (Fig.1). The cycle characteristics and demography of patients are presented in Table 1. Less than 0.05 was considered statistically significant.

No significant difference was seen in cycle characteristics, including retrieved or fertilized oocytes, good-quality embryos, and euploid blastocysts. In addition, the blastocyst rate is not influenced by parental age, sperm parameters, and oocyte number. However, there is a significant difference between pregnancy outcomes, including chemical, clinical, and ongoing pregnancy, and live birth rate among the three groups ($P<0.001$). Also, there was a significant difference in the blastocyst rate in the three groups ($P<0.002$, Table 2).

Embryo morphology and ICSI outcomes

In the present study, 42.1% of embryos screened with X, Y, 13, 18, and 21 probes were aneuploidy, but 57.9% were euploid. However, there was no relation between 13, 18, and 21 chromosomes aneuploidy and development rate to the blastocyst stage ($P<0.12$).

Among the embryos evaluated for aneuploidy, normal chromosomes were detected in 65.40% of embryos in the FET cycle and 79.62% in the fresh process. This difference can confirm the possible adverse effect of vitrification and warming processes in the FET cycle on the embryo's 13, 18, 21, X, and Y chromosomes (Fig.2). On day five of the embryo development, the blastocyst expansion rate was observed at 26.56% of fresh PGT embryos (8.6% blastocyst expansion 3, 8.59% blastocyst expansion 4, and 9.37% blastocyst expansion 5), 30.20% of FET-PGD embryos (12.58% blastocyst expansion 3, 2.78% blastocyst expansion 4 and 14.84% blastocyst expansion 5) compared with 44.57% in the control group (10.84% blastocyst expansion 3 and 33.73% blastocyst expansion 4, $P<0.001$). Although the expansion rate increased in the control group (OR: 75% vs. 0%), the highest expansion

quality (expansion 5) was observed in the PGT groups (fresh- and FET-PGT).

In the embryos with detected signals in the FET-PGT cycle, 39.39% and 60.61% of blastocysts were aneuploid and euploid, respectively. Also, in the fresh PGT cycle, 3.57% of blastocysts were aneuploid, and 96.43% were euploid ($P<0.001$). The control group had no blastocyst with expansion 5 on day five. On the other hand, 14.76% and 9.37% of embryos had blastocysts with expansion 5 in FET-PGT and fresh PGT groups, respectively ($P<0.01$).

The blastocyst expansion grade was a better prognosticator of pregnancy outcomes than the TE and ICM grades separately (Table 3). Embryos with an expansion of grade 4 compared with grade 5 did not show significantly different successful pregnancy rates ($P<0.05$). But there were significant differences in the gender ratio, morphology, and development rates in the PGT groups (fresh- and FET-PGT groups). A total of 55.65% (48.15% in FET-PGT and 63.16% in fresh-PGT) of euploid embryos with expansion 4 and 5 on day five were female, and 44.35% (51.85% in FET-PGT and 37.84% in fresh-PGT) were male embryos. Also, 90.48 (92.86% in FET-PGT and 85.71% in fresh-PGT) of male blastocyst embryos developed to blastocyst expansion 5 compared with 36% (38.46% in FET-PGT and 33.33% in fresh-PGT) of female embryos ($P<0.04$). Thus, it reveals the higher development rate of female embryos and the faster development rate in male embryos.

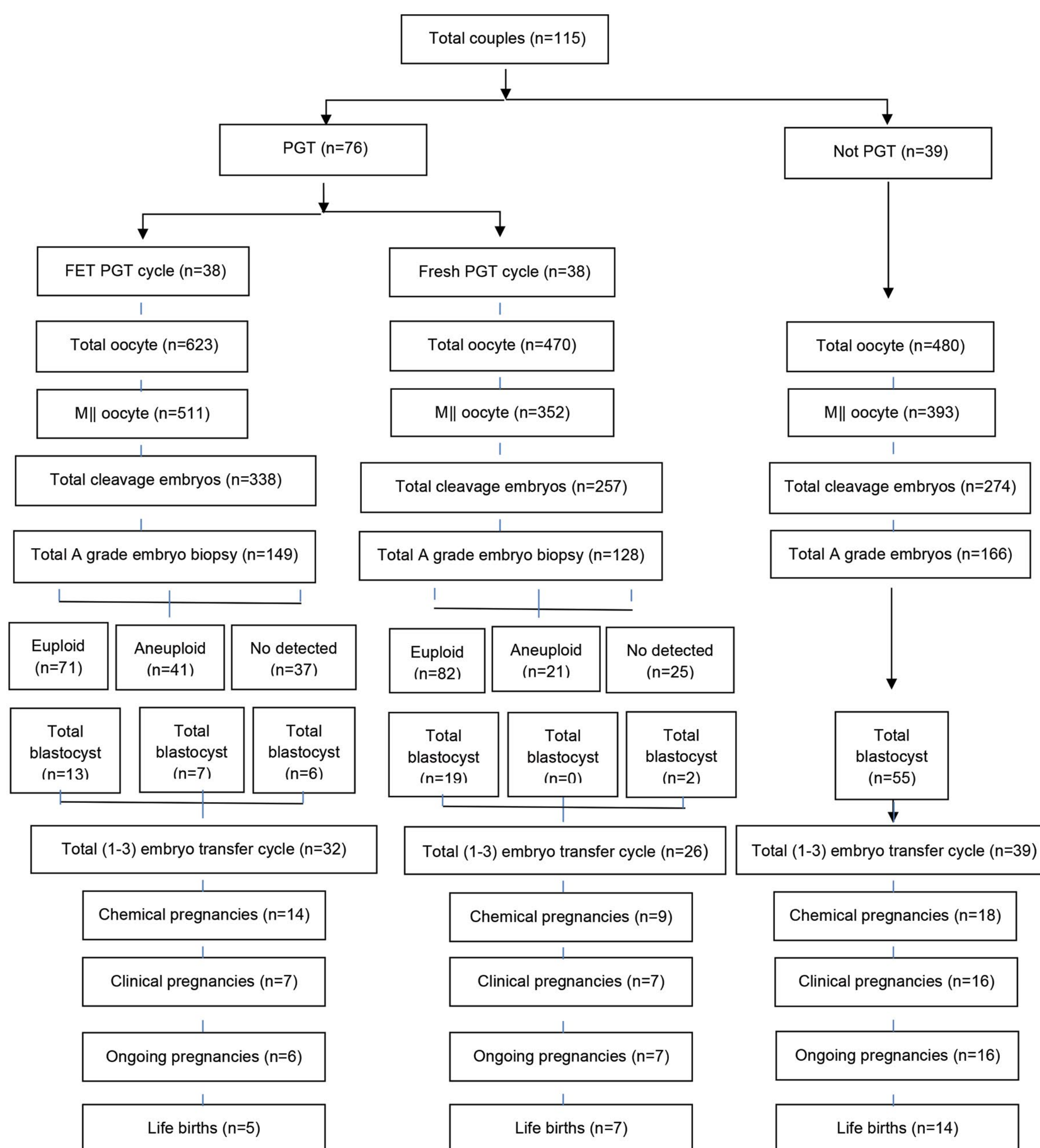

Fig.1: Schematic representation of study design and workflow (total blastocyst; blastocyst expansions 4 and 5). PGT; Preimplantation genetic testing and FET; Frozen embryo transfer.

Table 1: Demography and cycle characteristics of 115 patients

Cycle characteristics	Fresh PGT (n=38)	Freeze PGT (n=38)	Fresh without PGT (n=40)	P value
Maternal age (Y)	35.88 ± 5.18	35.66 ± 6.50	35.26 ± 7.51	0.174
Paternal age (Y)	39.85 ± 5.98	42.06 ± 6.44	38.72 ± 7.62	0.324
No. of oocytes retrieved	10.58 ± 4.06	15.41 ± 8.16	12.31 ± 9.37	0.427
No matured oocytes	9.46 ± 4.03	12.87 ± 6.75	10.8± 7.00	0.184
No cleavage embryos	7.15 ± 2.69	8.88 ± 4.77	7.03 ± 4.74	0.058
Count of sperm (million/ml)	34.71 ± 22.85	27.81 ± 16.93	30.81 ± 24.12	0.772
Motility of sperm (%)	55.69± 20.19	51.25 ± 24.16	48.44 ± 26.94	0.790
Morphology of sperm (%)	11.46 ± 5.37	10.31 ± 5.30	9.58 ± 5.70	0.027
No. of biopsy embryos	5.38 ± 1.92	5.56± 2.17	-	0.107
No. of the transferred embryo(s)	1.62 ± 0.70	2.69 ± 0.69	2.56 ± .75	0.107

Data are presented as mean ± SD. There is no significant difference among groups. N.S.; Not statistically significant and PGT; Preimplantation genetic testing.

Table 2: Embryo development and assisted reproductive outcomes following PGT and/or vitrification-warming compared with the control group

Variables	FET PGT (n=149)	Fresh PGT (n=128)	Fresh without PGT (n=166)	P value
Aneuploidy rate (%)	41/112 (36.60)*	21/103 (20.38)	-	0.001
Blastocyst rate (%)	45/149 (30.20)	34/128 (26.56)	74/166 (44.57)*	0.002
Chemical pregnancy (%)	14/32 (43.87)	9/28 (36.61)*	18/39 (46.15)	0.001
Clinical pregnancy (%)	7/3221.87))	7/28 (26.9)	16/39 (41.02)*	0.001
Ongoing pregnancy (%)	6/32 (18.75)	7/28 (26.9)	16/39 (41.02)*	0.001
Live birth rate (%)	5/32 (15.6)*	7/28 (26.9)	14/39 (35.89)*	0.001
EPL	7/14 (50)*	2/9 (22.22)	2/18 (11.11)	0.04
CPL	2/7 (28.57)*	0/7 (0)*	2/16 (14.28)	0.04

Data are presented as n (%). PGT; Preimplantation genetic testing, EPL; Early pregnancy loose, CPL; Clinical pregnancy loose, FET; Frozen embryo transfer, and *; Is meaning differences (P<0.05).

Fig.2: Specific probes of X, Y, 13, 18, and 21 chromosomes in the In-situ hybridization technique. Chromosome X is blue, chromosome Y is green, chromosome 13 is orange, chromosome 18 is purple, and chromosome 21 is red. **A.** Blastomere of normal male (X, Y, 13, 13, 18, 18, 21, and 21), **B.** Blastomere of trisomy 13 female (X, Y, 13, 13, 18, 18, 21, and 21), and **C.** Blastomere of normal female (X, X, 13, 13, 18, 18, 21, 21) (magnification: 100x).

Table 3: The evaluation of chemical, clinical, ongoing pregnancy, and live birth following PGT and/or vitrification-warming

Groups			Expansion grade			TE grade			ICM grade		
			3	4	5	A	B	C	A	B	C
FET PGT	Chemical pregnancy	No./Obs.no	9/13 (67.4)	6/9 (61.0)	8/11 (75.0)	7/10 (70.0)	10/15 (66.6)	2/4 (50.0)	7/10 (70.0)	10/15 (66.6)	2/4 (50.0)
		Adjusted OR	0.69 (0.22-.12)	0.52 (0.18-1.50)	REF	REF	1.11 (0.39-3.18)	0.51 (0.07-3.81)	REF	1.11 (0.39-3.18)	0.51 (0.07-3.81)
		P value	NS			NS			NS		
	Clinical pregnancy	No./Obs.no	6/13 (44.2)	5/9 (57.6)	6/11 (55.4)	5/10 (50.0)	8/15 (52.6)	1/4 (25.0)	5/10 (50.0)	8/15 (52.6)	1/4 (25.0)
		Adjusted OR	0.79 (0.29-2.15)	1.36 (0.52-3.52)	REF	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)
		P value	NS			NS			NS		
	Ongoing pregnancy	No./Obs.no	5/13 (41.9)	5/9 (57.6)	4/11 (37.5)	5/10 (50.0)	8/15 (52.6)	¼ (25.0)	5/10 (50.0)	8/15 (52.6)	1/4 (25.0)
		Adjusted OR	1.20 (0.43-3.34)	1.36 (0.52-3.52)	REF	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)
		P value	NS			NS			NS		
	Life birth	No./Obs.no	4/13 (37.2)	5/9 (52.5)	4/11 (37.5)	5/10 (50.0)	8/15 (52.6)	1/4 (25.0)	5/10 (50.0)	8/15 (52.6)	1/4 (25.0)
		Adjusted OR	0.98 (0.35-2.77)	1.84 (0.69-4.87)	REF	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)
		P value	NS			NS			NS		
Fresh PGT	Chemical pregnancy	No./Obs.no	11/15 (67.4)	8/11 (61.0)	8/11 (75.0)	9/12 (70.0)	12/17 (66.6)	3/6 (50.0)	7/10 (70.0)	10/15 (66.6)	2/4 (50.0)
		Adjusted OR	0.69 (0.22-.12)	0.52 (0.18-1.50)	REF	REF	1.11 (0.39-3.18)	0.51 (0.07-3.81)	REF	1.11 (0.39-3.18)	0.51 (0.07-3.81)
		P value	NS			NS			NS		
	Clinical pregnancy	No./Obs.no	8/15 (44.2)	7/11 (57.6)	8/13 (55.4)	7/11 (50.0)	8/15 (52.6)	2/6 (33.3)	7/11 (50.0)	8/15 (52.6)	2/6 (33.3)
		Adjusted OR	0.79(0.29-2.15)	1.36 (0.52-3.52)	REF	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)
		P value	NS			NS			NS		
	Ongoing pregnancy	No./Obs.no	7/15 (41.9)	7/11 (57.6)	6/13 (37.5)	7/11 (50.0)	8/15 (52.6)	2/6 (33.3)	7/11 (50.0)	8/15 (52.6)	2/6 (33.3)
		Adjusted OR	1.20 (0.43-3.34)	1.36 (0.52-3.52)	REF	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)
		P value	NS			NS			NS		
	Life birth	No./Obs.no	7/15 (41.9)	7/11 (57.6)	6/13 (37.5)	7/11 (50.0)	8/15 (52.6)	2/6 (33.3)	7/11 (50.0)	8/15 (52.6)	2/6 (33.3)
		Adjusted OR	0.98 (0.35-2.77)	1.84 (0.69-4.87)	REF	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)	REF	1.00 (0.37-2.68)	0.35 (0.03-3.51)
		P value	NS			NS			NS		
Fresh without PGT	Chemical pregnancy	No./Obs.no	24/36 (66.6)	11/19 (61.0)	No sample	20/31 (64.5)	13/19 (68.5)	2/5 (40.0)	20/3 (64.5)	13/19 (68.5)	2/5 (40.0)
		Adjusted OR	0.69 (0.22-2.12)	REF	No sample	REF	1.11 (0.39-3.18)	0.41 (0.07-3.81)	REF	1.11 (0.39-3.18)	0.41 (0.07-3.81)
		P value	NS			NS			NS		
	Clinical pregnancy	No./Obs.no	16/36 (44.6)	11/19 (57.7)	No sample	16/31 (52.3)	10/19 (52.7)	1/5 (20.0)	16/3 (52.3)	10/19 (52.7)	1/5 (20.0)
		Adjusted OR	0.79 (0.29-2.15)	REF	No sample	REF	1.00 (0.37-2.68)	0.25 (0.03-3.51)	REF	1.00 (0.37-2.68)	0.25 (0.03-3.51)
		P value	NS			NS			NS		
	Ongoing pregnancy	No./Obs.no	15/36 (41.9)	11/19 (57.6)	No sample	16/31 (52.3)	10/19 (52.7)	1/5 (20.0)	16/3 (52.3)	10/19 (52.7)	1/5 (20.0)
		Adjusted OR	1.20 (0.43-3.34)	REF	No sample	REF	1.00 (0.37-2.68)	0.25 (0.03-3.51)	REF	1.00 (0.37-2.68)	0.25 (0.03-3.51)
		P value	NS			NS			NS		
	Life birth	No./Obs.no	14/36 (37.2)	10/19 (52.5)	No sample	16/31 (52.3)	10/19 (52.7)	1/5 (20.0)	16/31 (52.3)	10/19 (52.7)	1/5 (20.0)
		Adjusted OR	0.98 (0.35-2.77)	REF	No sample	REF	1.00 (0.37-2.68)	0.25 (0.03-3.51)	REF	1.00 (0.37-2.68)	0.25 (0.03-3.51)
		P value	NS			NS			NS		

Data are presented as n (%) and CI. NS; No statistically significant; REF; Reference, CI; Confidence interval, OR; Odds ratio, Obs.no; Observer, PGT; Preimplantation genetic testing, FET; Frozen embryo transfer, TE; Trophectoderm, ICM; Inner cell mass, and *; P<0.05.

Embryo grade and ICSI outcomes

In the present study, about 97 participants reached the embryo transfer step. In pentaplex PGT of embryos, pregnancy-related data could not be cited due to the tiny statistical population (n=38), and accurate statistical analysis could not be obtained from them. Therefore, their data were excluded from the pregnancy outcomes. We analyzed the pregnancy outcome according to the quality of the transferred embryos. The results showed that 34.61, 43.75, and 46.15% of patients from fresh-PGT, FET-PGT, and control groups had a positive chemical pregnancy test. Also, 26.92, 15.62, and 3 8.46% of patients from fresh-PGT, FET-PGT, and control groups had a live birth, respectively. EPL frequency in the fresh-PGT, FET-PGT, and control groups was 22, 50, and 11%, respectively. Therefore, there is a significant increase in the FET-PGT group.

Sixty percent of blastocyst transfer cycles in the control group showed chemically positive pregnancy. However, 94.44% of chemical pregnancies had blastocyst with expansions 3 and 4. In the fresh-PGT group, 57.14% of positive results had blastocyst embryos, and 44% of blastocyst transfer cycles were positive for a chemical pregnancy. In the fresh-PGT group, 75% of positive results had blastocyst embryos, and 42.85% of blastocyst transfer cycles had a clinically positive pregnancy (Fig.3).

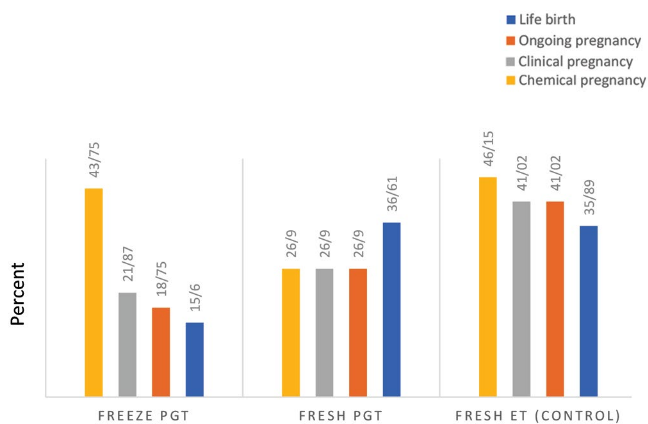

Fig.3: Pregnancy outcome in study groups. A significant difference in pregnancy results in the three groups was seen. Fresh embryo transfer has the highest rate in chemical (P=0.002), clinical (P=0.001), ongoing pregnancy (P=0.001), and live birth rate (P=0.001). PGT; Preimplantation genetic testing and ET; Embryo transfer.

Discussion

This study was designed to determine the correlated blastocysts morphology with chromosomal state and pregnancy outcome. This study is the first to assay the association between gender, SBB, and vitrification with assisted reproductive outcomes. The study shows that the probability of CPL was not associated with morphology and type of cycle. In contrast, the likelihood of EPL was affected by the cycle, especially for the FET-PGT cycle. In a recent study, Forman (14) proposed that all euploid blastocysts do not have equal implantation potential. They reported an implementation rate of 80.9% for excellent quality blastocysts (4AA, 5AA, and 6AA), which was higher than low-quality euploid blastocysts (56.3%), which is in agreement with our study.

In addition, our study evaluated the association of gender with blastocyst grade and resistance against vitrification, which can affect embryo developmental potential. According to this study, a higher percentage of male embryos reached the blastocyst stage, which shows more resistance of male embryos against freeze and warming procedures than female and aneuploid embryos. Also, male embryos reached the blastocyst stage faster than female embryos. On the other hand, Fang et al. found a relation between gender and blastocyst grade, and faster development of female embryos was reported. These differences in development could be due to the time of blastomere biopsy (1). In our study, the embryos were biopsied on day three, whereas in the mentioned study, the blastocysts were biopsied.

To the best of our knowledge, there are a limited number of studies about the association of the ICSI method with PGT outcomes. Our study refers to the embryos' ability at the cleavage stage to reach the blastocyst phase in which they undergo biopsy procedures on day three. In blastocyst development, a similar development rate was observed in the euploid and aneuploid embryos for 13, 18, 21, X, and Y chromosomes. So that, it means that abnormal chromosomal embryos have equal potential for development as normal embryos. Furthermore, using accurate techniques such as Time Laps and array CGH, it has also been reported that various chromosomal aberrations have different effects on the embryo's morpho-kinetics (15).

Our study's strength includes the number of samples in the FET and fresh-PGT cycle and the evaluation of various items and their effect on aneuploidy, blastocyst development, and pregnancy outcomes. In addition, PGT was performed on the frozen embryos and compared with fresh embryos. Therefore, this study may guide the optimal selection of euploid embryos to improve clinical pregnancy after fresh and FET-PGT cycles.

The limiting of this study was the intrinsic nature

of morphologic grading that could not evaluate the epigenetic effect of vitrification-warming and PGD on embryos. Nevertheless, Phan et al. showed the morphological parameters of the embryo, such as the fragmentation and its concentration, following an analysis of the CGH array in every 23 pairs of chromosomes in one blastomere. Although the symmetry of the blastomeres much reflects the condition of the ploidy of the embryo. However, assisted hatching does not accrue for all embryos; the expansion grades for pregnancy outcomes have predictive value (16). Future studies with embryos from more significant sample sizes and under PGT techniques like Next-Generation sequencing can teach morphology and freeze-warm effect in proposing ICSI and pregnancy outcomes.

In addition, morphology and developmental stages are affected by manipulation approaches such as embryo vitrification-warming and blastomere biopsy for PGT. Zona hatching embryos for blastomere biopsy can increase the blastocyst percentage with ICM 3, 4, 5. On the other hand, some euploid embryos have poor morphology, and a significant proportion of aneuploid embryos can get the highest morphologic scores. The implantation rate is far from the gynecologist's final goal despite transferring high-quality and euploid embryos. The impact of expansion grade after the comprehensive chromosomal screening in an optimal embryo transfer is corroborated. However, analysis of embryo valuation and embryo election for the planning of PGT could help increase healthy pregnancy rates with satisfactory outcomes. These findings offer the morphological and genomic criteria to provide supplementary data to increase the fresh-PGT and FET-PGT outcomes.

Conclusion

A significant correlation was found in the present study between SBB and/or vitrification-warming on day three, decreased embryo development potential, and assisted reproductive outcomes. The embryos' pregnancy outcome was also associated with their development stage to assess their development and live birth ability. Our findings may provide pregestational consulting for couples who decided to use PGT and FET procedures in their assisted reproductive technology (ART) process to have healthy children.

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Authors' Contributions

Sh.A., F.Gh., M.M., A.H.; Participated in study design, statistical analysis, data collection and evaluation, and drafting. Sh.A., M.M.; Performed follicle collection and prepared oocytes for ICSI about this study component. Sh.A., A.S., F.Gh., M.M., A.H.; Contributed extensively to interpreting the data and the conclusion. Sh.A., F.Gh., M.M.; Conducted molecular experiments and PCR and FISH analysis. All authors edited and approved this paper's final version for submission, participated in finalizing the manuscript, and approved the final draft.

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