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Effects of embryo characteristics in frozen-thawed single euploid blastocyst transfers on pregnancy outcomes: a retrospective cohort study

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ABSTRACT

Objectives: Our study examined the effects of the trophectoderm biopsy (TB) day and the presence of necrotic foci (NF) or separate blastomeres (SB) within euploid embryos on in vitro fertilization (IVF) pregnancy outcomes.

Methods: This retrospective cohort study was conducted from January 2017 to September 2021 at Memorial Sisli Hospital, Istanbul, Turkey. The study comprised a total of 2758 frozen-thawed euploid embryo transfer cycles. After thawing, blastocysts were graded using Gardner's classification Top-Quality (TQ), Good-Quality (GQ), Moderate-Quality (MQ), Poor-Quality (PQ) and further divided into groups according to the presence of NF and/or SB and evaluated for pregnancy outcomes.

Results: There were significant correlations between pregnancy outcomes and the degree of blastocoele expansion, as well as the presence of NF or SB in the euploid embryo. Ongoing pregnancy rates were lower in the group with NF in the inner cell mass (ICM) or trophectoderm (TE) than in the group without NF. The presence of SB decreased the rates of ongoing pregnancy and increased the rates of miscarriage. Embryos with expansion grades ≤ 3 had lower rates of ongoing pregnancy and higher rates of miscarriage compared to embryos with expansion grades > 3. TQ and GQ embryos had a higher rate of ongoing pregnancy and a lower rate of miscarriage than MQ and PQ embryos.

Conclusions: When selecting the embryo to be transferred to a patient, careful consideration should be given to the morphological grade of the embryo as well as whether or not it contains NF and SB.

Keywords: Necrotic foci, separate blastomeres, blastocyst morphology, pregnancy, miscarriage

Embryo aneuploidy is one of the most important reasons, causing in vitro fertilisation (IVF) failure [1]. Selecting chromosomally healthy embryos using preimplantation genetic testing for aneuploidies (PGT-A) programmes is very important for a healthy pregnancy [2-4]. However, choosing the best single euploid embryo to transfer is still challenging if the patient has more than one. On the other hand, selecting an embryo depending on ploidy alone does not guarantee a live birth. Therefore, embryo implantation fail-

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ure and pregnancy losses may still occur, although a euploid embryo was selected for transfer and other risk factors were eliminated [5-7].

The effect of blastocyst morphology on pregnancy outcomes is recognized, yet additional research is required to explore the influence of necrotic foci (NF) and/or separate blastomeres (SB) within the embryo on these outcomes. During embryo development, some of the blastomeres are observed to be excluded from the embryo. These separate blastomeres cannot integrate into either the trophectoderm (TE) or inner cell mass (ICM) as the embryo reaches the blastocyst stage, and they remain within the perivitelline space or blastocoel cavity. These blastomeres that are not included display disorganized or reduced expression of gap junction protein. It has been suggested that embryos employ apoptotic pathways to autonomously eliminate abnormal cells or fragments autonomously, thereby initiating a self-correction mechanism [8]. Lagalla *et al*. [9] in 2020, based on the results of chromosome analysis from biopsied embryos in the trophectoderm and separate blastomeres of the same embryo, suggested that separate blastomeres are a possible self-correction mechanism aimed at removing aneuploid cells from mosaic embryos and thus reducing or eliminating aneuploid load [9]. There have been limited studies investigating the effect of separate blastomere (SB) existence in blastocysts on IVF outcomes in the literature [9-11]. Blastocoele expansion degree has been studied in previous studies and was found to be related to higher implantation, ongoing pregnancy and live birth rates in both fresh and frozen embryo transfer cycles [12-14].

This study aimed to investigate whether the presence of necrotic foci (NF) or the SB, the blastocoele expansion degree, and the day of trophectoderm biopsy (TB) affect the pregnancy outcomes of euploid, frozen-thawed embryo transfer (FET) cycles.

METHODS

Patient Selection

A total of 2758 euploid FET cycles were included into this single-centered, retrospective cohort study that was conducted at Memorial Şişli Hospital, Istanbul, Turkey, between January 2017 and September 2021. Patients with ovarian, endometrial, or uterine abnormalities (Müllerian anomalies, severe endometriosis/adenomyosis, Asherman's syndrome, thin endometrium (< 7 mm) were excluded from the study.

Controlled Ovarian Stimulation (COS) Protocol

Gonadotropin-releasing hormone (GnRH) antagonist protocols were used in most of the patients (n = 2642, 95.8%). Others were administered long-stop protocol (GnRH agonist + antagonist) (n = 59), letrozole (n = 50), clomiphene citrate (n = 4), and in a few of the patients (n = 3) natural or semi-natural protocols. To stimulate the ovaries, recombinant follicle-stimulating hormone (rFSH) (Gonalf, Merck, Switzerland) or a combination of rFSH and recombinant luteinizing hormone (Pergoveris, Merck, Switzerland) or human menopausal gonadotropin (Menogon, Ferring, Germany) were used. The starting dosages were determined depending on the individual features of each patient. The oocyte pickup (OPU) was performed 36 hours following the administration of 250 mcg of recombinant human chorionic gonadotropin (rhCG) (Ovitrelle; Merck, Switzerland) or a GnRH analog (Lucrin; Abbott Laboratories, USA), by transvaginal ultrasound guidance.

Embryo Culture and Morphology Assessment

Embryos were cultured in single-step IVF medium (LifeGlobal, Cooper Surgical, Brussels, Belgium) for 5-6 days at 6% CO2, 5% O2, 37 ˚C, with pH 7.26-7.30 until embryo biopsy. On day 3, the culture medium was refreshed. Prior to vitrification and subsequent thawing, blastocysts were evaluated based on Gardner's classification and categorized into distinct groups based on their quality as follows; Top-Quality (TQ): Hatched AA, 6AA, 5AA, 4AA; Good-Quality (GQ): Hatched AB/BA/BB, 5AB/BA/BB, 4AB/BA/BB, 3AA; ModerateQuality (MQ): 3AB/BA, 2AA; and Poor-Quality (PQ): the rest of the embryos.

Embryo Biopsy and Genetic Analysis

A diode laser (RI Saturn 3, England) was utilized on the third day of embryo culture to create an artificial opening in the zona pellucida. This procedure aimed to promote the protrusion of trophectoderm cells following blastulation. A total of five and eight trophectoderm cells were extracted by the flicking technique, involving the use of a pipette with a 30 mm inner diameter (Origio, Denmark), which facilitated a mechanical incision. The ReproSeq kit (Ther-

moFisher, USA) was utilized for Next Generation Sequencing (NGS) in accordance with the manufacturer's guidelines. Initially, the PGM (Ion Personal Genome Machine, ThermoFisher, USA) was employed, followed by the S5 (ThermoFisher, USA) at a later stage. The genetic studies were conducted utilizing the Ion Reporter software package versions 5.2 and 5.6 (ThermoFisher, USA).

Embryo Freezing and Thawing Protocols

After the biopsy, the vitrification cryotops® procedure was performed with the Kitazato vitrification medium. Blastocysts were thawed in accordance with the manufacturer's instructions using Kitazato warming medium. 30 minutes and 2 hours after thawing, embryos were examined for viability. Each embryo was assessed for re-expansion and the presence of necrotic foci and separated blastomeres. Then, blastocysts that are eligible with at least 80% re-expansion and > 90% vitality were transferred.

Endometrial Preparation for Frozen-Thawed Euploid Embryo Transfer Cycles

All patients were evaluated by transvaginal ultrasonography on the second day of the menstrual cycle to check for ovarian, uterine and endometrial abnormalities. And FET cycles were started in cases where no pathology was detected. Patients who started endometrial preparation in mNC-FET cycle were checked 7-9 days after the first examination to determine the dominant follicle that developed spontaneously. When the dominant follicle reached 16-20 mm and LH levels were > 1 5 IU/L, r-hCG (Ovitrelle, Merck-Serono, Switzerland) was given. 2 days after hCG triggering, vaginal progesterone gel 1x1 (Crinone® Merck Serono, Switzerland) or vaginal progesterone tablets 2x1 (Lutinus® Ferring, Germany) were given as luteal phase support. The thawed embryo was transferred 5-6 days after the trigger. In the ERT-FET cycle, a 2 mg Estradiol tablet (Estrofem®, Novo Nordisk, Denmark) or a 3.9 mg Estradiol patch (Climara®, Bayer Turk, Turkey) was used for endometrial preparation. Luteal phase support was started after at least 12 days of Estradiol use and when the endometrium was > 8 mm. For this support, vaginal progesterone gel 2x1 (Crinone® Merck Serono, Switzerland) or vaginal progesterone tablets 2x2 (Lutinus® Ferring, Germany) were given. 9 days after embryo transfer, serum β-hCG was tested. Luteal phase support was given until the 10th week of gestation. At 7 weeks, patients were scheduled for ultrasonography and fetal heart rate was checked.

Ethical Approval

This stady was appoved by Istanbul Memorial Şişli Hospital Ethics Committee (approval number: 003, Date: 03.03.2023).

Statistical Analysis

The demographics and clinical characteristics of patients were evaluated using the t-test. The chi-square test was used for the analysis of frequencies. The logistic regression analysis was performed for the risk assessment by selecting dependent variables such as ongoing pregnancy and total miscarriage.

RESULTS

The demographic and clinical characteristics of patients were presented in Table 1. Semen samples were used for ICSI (93.5%) and most of embryos were biopsied, vitrified, and transferred on Day 5 (92.8%). The morphology of transferred euploid embryos was TQ 59.8%, GQ 33.4%, MQ 4.9%, PQ 1.8%, respectively (Table 1). After thawing, all viable euploid blastocysts were categorized into groups according to the presence of necrotic foci (Fig. 1), the presence of separate blastomeres (Fig. 2), the absence of necrotic foci or separate blastomeres (Fig. 3) and the degree of re-expansion (Fig. 4), and pregnancy outcomes were evaluated.

In the logistic regression analysis, significant correlations were found between pregnancy outcomes and blastocyst morphology, blastocoele expansion degree, the presence of NF or SB and day of biopsy in the euploid embryos (Table 2). TQ and GQ embryo grades showed a higher ongoing pregnancy rate and lower miscarriage rate than MQ and PQ embryos (ongoing pregnancy rates; TQ: 64.3%, GQ: 55.1%, MQ: 36.6%, PQ: 33.3%, total miscarriage rates; TQ: 18.3%, GQ: 21.5%, MQ: 32.9%, PQ: 36.6%). When pregnancy outcomes were compared according to blastocele expansion grade, ongoing pregnancy rates increased in embryo transfers with larger expansion grade (6: 61.4%, 5: 59.8%, 4: 59.3%, 3:38.3%, 2:

Table 1. Patient demographics and characteristics

n = 2758	Mean ± standard deviation	Minimum-maximum
Age (year)	36.85 ± 4.12	26.0-45.0
BMI (kg/m^2)	24.36 ± 4.34	13.8-42.3
AMH (ng/mL)	2.88 ± 2.40	0.01-22.0
Basal FSH	8.12-2.81	0.10-18.0
Infertility duration (year)	4.68 ± 3.95	2-25
Previous IVF cycles	4.84-2.81	2.0-26.0
Duration of stimulation (day)	9.07 ± 1.64	4.0-18.0
Daily gonadotropin doses	247.41 ± 70.95	87.50-725.0
Total gonadotropin doses	2246.61 ± 852.94	150.0-9450.0
Total oocyte	12.78 ± 8.14	1.0-58.0
MII oocyte (maturation%)	11.19 ± 7.03 (87.5%)	1.0-54.0
2PN (fertization %)	9.26 ± 6.02 (82.75%)	1.0-46.0
Sperm count (mill/mL)	14.18 ± 14.69	0.001-110.0
Sperm source for ICSI (%)		
Semen	2581 (93.5%)	
TESA	65 (2.4%)	
TESE	110 (4%)	
Embryo biopsy/ transfer day (%)		
Day 5	2561 (92.8%)	
Day 6	197 (7.1%)	
Embryo quality (%):		
TQ	1650 (59.8%)	
GQ	923 (33.4)	
MQ	134 (4.9%)	
PQ	51 (1.8%)	

Data are shown as mean ± standard deviation or number (percentage) or minimum-maximum. BMI = body mass index, AMH = anti-mullerian hormone, FSH = folliclestimulating hormone, MII = metaphase II, PN = pronucleus, TESA = testicular sperm aspiration, TESE = testicular sperm extraction, TQ = top-quality, GQ = good-quality, MQ = moderate-quality, PQ = poor-quality

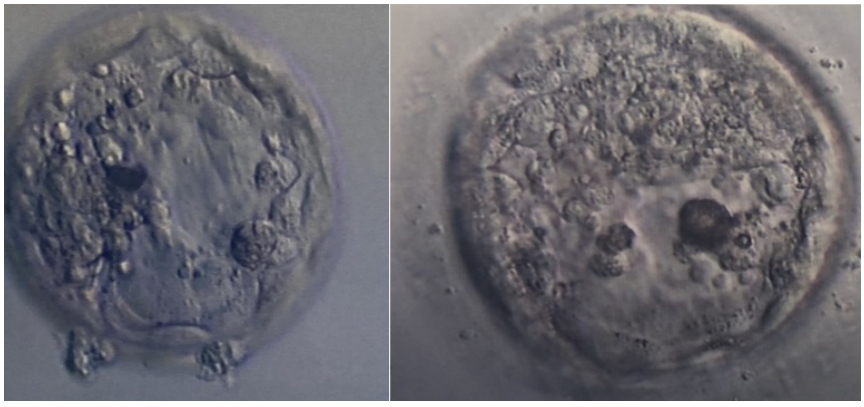

Fig. 1. Embryo containing necrotic foci.

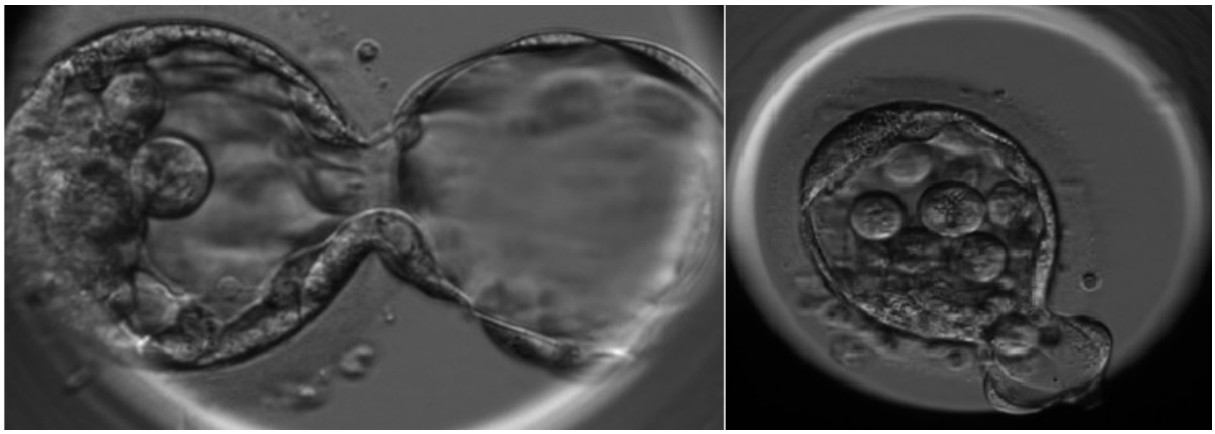

Fig. 2. **Embryo containing separated blastomeres.**

39.5%), while miscarriage rates increased in smaller expansion grades (6: 18.4%, 5: 21.2%, 4:21.0%, 3: 33.3%, 2: 35.0%). Embryos with expansion grades of ≤ 3 have lower ongoing pregnancy rates and higher miscarriage rates than embryos with expansion grades > 3.

The euploid embryos with the NF in the ICM or TE showed lower ongoing pregnancy rates than embryos without NF (negative vs. positive: 60.0% vs 41.0%, 59.9% vs 34.0%, respectively). The presence of SB in blastocysts were found to be associated with decreased ongoing pregnancy rates and increased miscarriage rates (negative vs. positive: 61.8% vs 50.3%, 18.8% vs 25.3%). Furthermore, a correlation was observed between the biopsy day and the rates of ongoing pregnancy and miscarriage. Consequently, euploid embryos that underwent biopsy and vitrification on Day 5 showed ongoing pregnancy rates and reduced miscarriage rates compared to those that underwent biopsy and vitrification on Day 6 (ongoing pregnancy rates for Day 5 vs. 6; 60.7% vs. 43.4%, miscarriage rates for Day 5 vs. 6; 19.6% vs. 25.9%) (Table 2).

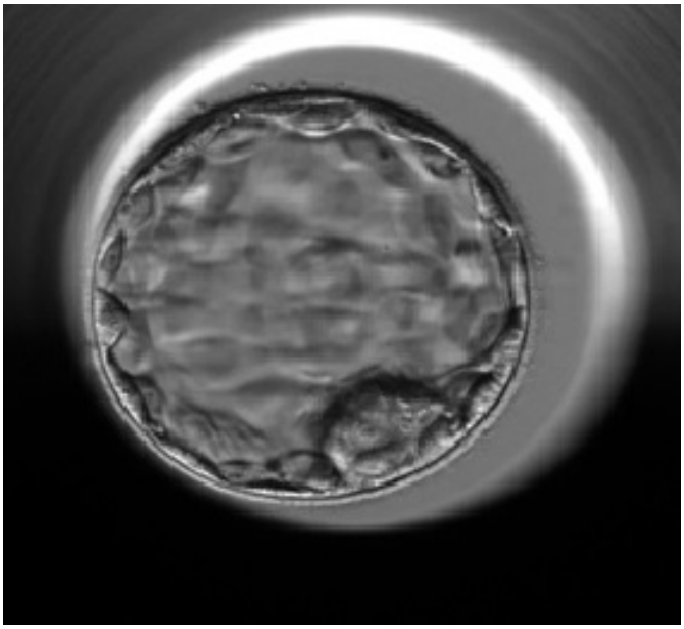

Fig. 3. **Embryo without necrotic foci or separate blastomeres.**

DISCUSSION

Even when a euploid embryo is chosen for transfer and other risk factors are eliminated, there can still be occurrences of implantation failure and pregnancy losses. In our study, we found that in addition to embryo morphology, the day of TB and the presence of separate blastomeres or necrotic foci in the embryo were effective on pregnancy outcomes. We have shown that embryos undergoing TB on day 6 have a lower continuing pregnancy rate and a higher abortion rate than embryos undergoing TB on day 5. Only a few publications in the literature claim the opposite of our study [15, 16]. Capalbo *et al*. [15] revealed that implantation rates did not differ between the group biopsied on day 5 and the group biopsied on day 6 (51.2% on day 5 vs. 48.8% on day 6). Similarly, in a smaller sample, Gonzalez *et al*. [16] showed no difference between the implantation rates of embryos undergoing TB on day 5 and day 6.

Numerous studies in the literature indicate similar results to our study on the effect of biopsy day on pregnancy rates [5, 17-20]. In a recently published

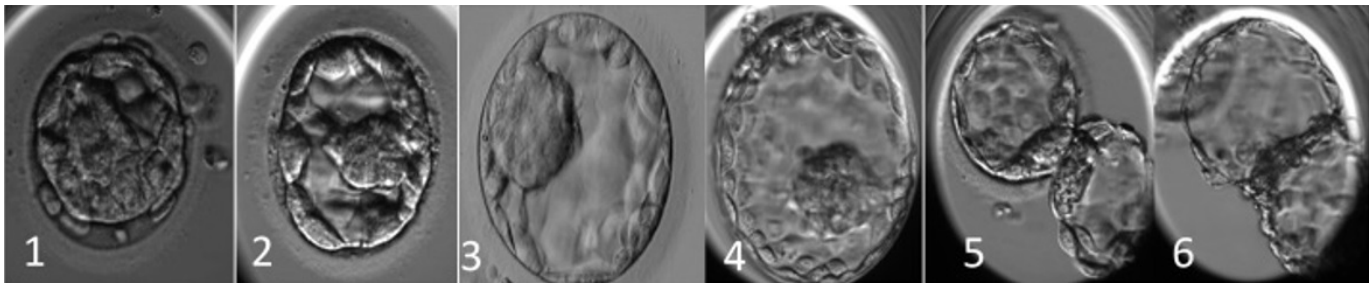

Fig. 4. **Embryos by grade of expansion.**

Table 2. **Logistic regression analysis of risk assessment of ongoing pregnancy and total miscarriage rates in euploid blastocyst transfer cycles**

Morphological features of blastocysts	No. of cycles (n = 2758)	Ongoing Pregnancy (%)	*p* value	Total Miscarriage (%)	*p* value
Blastocyst morphology					
TQ	1650	64.3%	**< 0.001**	18.3%	Ref.
GQ	923	55.1%	**< 0.001**	21.5%	0.10
MQ	134	39.6%	0.43	32.9%	**0.02**
PQ	51	33.3%	Ref.	36.1%	**0.01**
Day of biopsy					
Day 5	2561	60.7%	**< 0.001**	19.6%	Ref.
Day 6	197	43.4 %	Ref.	25.9 %	**0.004**
Presence of NF in ICM					
negative	2680	60.0%	**0.001**	19.8%	Ref.
positive	78	41.0%	Ref.	27.3%	**0.05**
Presence of NF in TE					
negative	2711	59.9%	**0.001**	19.7%	Ref.
positive	47	34%	Ref.	38.5%	**0.02**
Presence of SB					
negative	2201	61.8%	**< 0.001**	18.8%	Ref.
positive	557	50.3%	Ref.	25.3%	**0.004**
Degree of blastocoele expansion					
6	1415	61.4%	**0.005**	18.4%	Ref.
5	520	59.8%	**0.01**	21.2%	0.50
4	696	59.3%	**0.01**	21.0%	0.57
3	94	38.3%	0.89	33.3%	**0.01**
2	43	39.5%	Ref.	35.0%	**0.01**

TQ = top-quality, GQ = good-quality, MQ = moderate-quality, PQ = poor-quality, ICM = inner-cell mass, TE = trophectoderm, NF = necrotic foci, SB = separate blastomere

study, Li *et al*. [19] demonstrated that the live birth rate in embryo transfer cycles with biopsy on day 5 was considerably higher than in embryo transfer cycles with biopsy on day 6 (57.75% vs. 41.67%). Irani *et al*. [17] also showed that the live birth rate was 44.8% in embryo transfer cycles in which the biopsy was performed on day 6 and 60.4% in embryo transfer cycles in which the biopsy was performed on day 5. It is believed that aberrant gene expression in embryos or inappropriate oocyte cytoplasmic maturation contributes to the lower live birth rates of day 6 embryo transfers compared to day 5 embryos, which reach the blastocyst stage more slowly [21]. According to the findings of these studies and our own, if a patient has more than one euploid embryo, we recommend transferring the same-quality embryo biopsied on day 5.

Numerous studies in the academic literature investigate the correlation between embryo morphology and pregnancy outcomes. We have shown that TQ and GQ embryo transfers have higher ongoing pregnancy rates than MQ and PQ embryo transfers. Contrary to our findings, Capolbo *et al*. [15] concluded in a study involving 956 blastocysts that morphology did not influence pregnancy outcomes in euploid embryo transfer cycles. However, the study was limited by only including 13 embryo transfers of poor quality [15]. In a subsequent study with a larger study group comprising 103 PQ embryo transfers, they demonstrated that TQ and GQ blastocyst transfers had a twofold higher pregnancy rate and a twenty-fivefold lower miscarriage rate compared to PQ euploid embryo transfers and underscored the effectiveness of ICM in predicting pregnancy outcomes [17]. Similarly, in the study by Zhang *et al*. [22], it was reported that pregnancy rates were low, especially in the group with ICM/C. In another study involving 914 single euploid embryo transfers, it was shown that embryo morphology affected pregnancy outcomes, but ICM and TB morphology had similar effects. [23]. In contrast to these studies, in the study examining the results of 660 FET cycles with RPL history, it was shown that ICM in C did not affect the live birth and abortion rates, but, especially, TE C negatively affected the live birth rates.

The results of our study demonstrated that euploid embryos containing separate blastomeres exhibit lower ongoing pregnancy rates and higher abortion rates than those without separate blastomeres. A study by Shenoy *et al*. [24] showed that embryos with separate blastomeres at early or late stages of embryo development had higher aneuploidy rates (68% vs. 53%; $p < 0.001$. Coticchio *et al*. [10] showed that forming separate blastomeres before or after the morula stage in embryos without preimplantation genetic testing (PGT) negatively affected IVF success rates. In particular, they showed that an increase in the number of separate blastomeres leads to loss of embryo material, affecting the number of cells in the TE and ICM and ultimately affecting embryo viability and adversely affecting pregnancy outcomes. They also suggested that these findings should be considered when the embryos without PGT are selected using time-lapse culture systems [10]. Lagalla *et al*. [9] analyzed the results of 1271 PGT/A embryos, showing that separate blastomeres in embryos in the full and partial compaction stages did not affect aneuploidy and implantation rates. They stated that it is associated with abnormal division and represents a self-correction mechanism to eliminate aneuploid cells [9]. The hypothesis suggesting that separate blastomeres act as a self-correction mechanism to diminish or remove the impact of mosaicism presents potential opportunities for future research.

To the best of our knowledge, our study is the first examination of the influence of necrotic foci on pregnancy outcomes in euploid embryos. Necrosis and apoptosis are two distinct cell death processes with distinct morphological characteristics and biological significance. Following irreversible injury, necrosis involves swelling of cells and membrane rupture. The presence of necrotic foci in a blastocyst can be a concerning sign, indicating that some regions of the developing embryo have not received sufficient oxygen and nutrients, leading to cell damage. Few studies in the literature have examined the impact of necrotic foci on pregnancy outcomes in embryo transfer without PGT. Kovacic *et al*. [25] reported that necrotic foci negatively affect pregnancy outcomes, showing that the live birth rate was 45% in good-quality blastocysts without PGT and 32.8% in embryos with necrotic foci on ICM or TB. In contrast to this study, Ebner *et al*. [26] showed that the presence of necrotic foci had no effect on pregnancy outcomes in a small study group of 129 blastocysts without PGT. Our study differs from these studies in that it included only euploid embryos. In our study, we observed a decreased ongoing pregnancy rate (59% vs. 34%) and an increased mis-

carriage rate, especially in necrotic foci in trophectoderm cells. A systematic review by Wang *et al*. [27] supports our results, showing that the number and growth of viable blastomeres in freeze-thawed embryos are important indicators for the potential development of embryos and for predicting clinical outcomes related to implantation.

Limitations

The limitation of this study is its retrospective nature. We believe that our study will lead to further research and novel suggestions on this subject and more detailed studies on this subject should be conducted.

CONCLUSION

These results may provide important guidance on optimizing euploid embryo selection and transfer procedures and identifying better candidates to improve pregnancy success. When selecting the optimal embryo to be transferred in cases, it is important to consider both the embryo's morphological grading and the presence of NF and SB. Our findings also suggest that TB and cryopreservation on Day 5 are important for higher pregnancy outcomes.

Authors' Contribution

Study Conception: GÖzer; Study Design: GÖzer; Supervision: GÖzer; Funding: N/A; Materials: GÖzer; Data Collection and Processing: GÖzer; Statistical Analysis and Data Interpretation: GÖzer; Literature Review: GÖzer; Manuscript Preparation: GÖzer, GÖzkara and Critical Review: GÖzer, GÖzkara.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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