

Evaluation of clinical parameters as predictors of monozygotic twins after single frozen embryo transfer

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Objective: To determine if recent evolutions in laboratory protocols, including the increased use of natural cycles and the use of a hyaluronan-containing transfer medium, affected the rate of monozygotic twin (MZT) pregnancies after single frozen embryo transfer (FET).

Design: Retrospective cohort study.

Setting: Urban university-based fertility center.

Patient(s): Patients who underwent single FET between January 2016 and December 2018 resulting in an intrauterine pregnancy.

Intervention(s): Transition to a transfer protocol with a hyaluronan-containing transfer medium in July 2017.

Main Outcome Measure(s): Number of MZT pregnancies.

Result(s): There were 1,619 cycles that met the inclusion criteria and 31 (1.9%) resulted in MZT pregnancies. A hyaluronan-containing transfer medium was used in 875 (54.1%) cycles. Programmed cycles were used for 1,385 (85.5%) FETs and 234 (14.5%) cycles were natural. The mean age at FET, oocyte age, endometrial echo thickness, inner cell mass grade, trophectoderm grade, expansion, and day of blastocyst vitrification were similar between the groups. The use of a hyaluronan-containing transfer medium resulted in fewer MZTs. After controlling potential confounders with a multivariate regression, the use of the hyaluronan-containing medium still resulted in fewer MZTs. Monozygotic twins were colinear with preimplantation genetic testing (PGT), so PGT was excluded as a variable in our regression. A regression of PGT only cycles showed that the use of the hyaluronan-containing medium was still associated with a reduction in MZT pregnancies.

Conclusion(s): The use of a hyaluronan-containing transfer medium was associated with a lower rate of MZTs. Other clinical parameters, including cycle type, were not associated with changes in the number of MZTs. The use of PGT needs to be further investigated as a risk factor for MZTs. (Fertil Steril Rep® 2021;2:428–32. ©2021 by American Society for Reproductive Medicine.)

Key Words: EmbryoGlue, frozen embryo, monozygotic twins, monozygotic splits, transfer, natural cycle protocols

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Multiple gestation pregnancies result in significant maternal and fetal morbidity. From a maternal health standpoint, twin pregnancies are associated with an increased risk of hyperemesis, gestational diabetes, hypertensive disorders, anemia, postpartum hemorrhage, cesarean delivery, and postpartum depression (1–3). In addition, fetal and

neonatal morbidity are high, with higher rates of preterm birth and consequently higher rates of cerebral palsy and neonatal death (4). Compared with dizygotic twins that result from two distinct embryos, monozygotic twins (MZTs), which arise from the splitting of one embryo, have further increased fetal morbidity with even higher rates of preterm

birth, as well as increased rates of other complications such as growth discordance, twin-twin transfusion syndrome, and higher rates of perinatal mortality (5, 6).

Given the aforementioned risks, multifetal gestations of any kind are considered an adverse event within the realm of assisted reproductive technology (ART). To reduce the rate of twins after ART, elective single embryo transfer (eSET) has become a widely accepted model for care (7). However, MZT pregnancies still remain a possibility with eSETs. Currently, MZT rates after eSET range from 0.97% to 2.35%, which is higher than the rate of 0.4% that is seen with spontaneous conception (6). Given these findings,

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several studies have sought to identify the mechanisms and underlying risk factors that contribute to the increased rates of MZTs during eSET, with the most compelling risk factors identified to date being embryo transfer at the blastocyst stage and younger oocyte age (5, 6, 8–21). Other data suggest that manipulation of the zona pellucida, such as with assisted hatching (AH), may increase the rates of MZTs. The proposed mechanism is that increasing the flexibility of the zona pellucida increases the risk of division of the inner cell mass (ICM) and consequently the development of two fetal poles (6). However, data on whether or not manipulation of the zona pellucida truly increases the risk of MZTs are mixed (6, 8, 9, 16, 19–23).

It is important to note that protocols in ART and within the embryology laboratory have evolved over time. Changes can include incubator type, culture media, oxygen tension, and medications for ART protocols, to name a few. We hypothesized that the evolution of some of these laboratory protocols during frozen embryo transfer (FET) could affect the rate of MZTs. At our institution, two important and recent changes include the increasing use of natural FET protocols and the use of a hyaluronan-enriched transfer medium after embryo thaw. Therefore, our objective was to elucidate if the aforementioned changes in our laboratory protocols impacted the rate of MZT gestations.

MATERIALS AND METHODS

Design

We performed a retrospective cohort study of all FETs resulting in documented intrauterine pregnancies from January 2016 to December 2018 at the NYU Langone Fertility Center. The study was performed with NYU IRB approval (#s13-0039). On July 17, 2017, all FET protocols were changed to include a hyaluronan-containing transfer medium, designed to augment implantation rates.

Subjects/Setting

All patients who underwent FET in the study time period were reviewed. A complete sampling method was used. We excluded patients who had double embryo transfers or who did not have a documented intrauterine pregnancy (e.g., patients with a negative pregnancy test or an ectopic pregnancy).

Variables and Data Collection

All demographics and outcome variables were collected from the electronic medical records. The demographics collected included the age at the time of oocyte aspiration in years (“oocyte age”), age at FET in years, endometrial echo thickness (EE) in millimeters, the use of donor oocytes, the use of a hyaluronan-enriched transfer medium, the use of intracytoplasmic sperm injection (ICSI), and the use of preimplantation genetic testing (PGT). To evaluate the effect of the oocyte age, the patients were categorized into the Society for Assisted Reproductive Technology age groups, with the addition of a youngest category for those <30 years old. The age groups were as follows: <30, 31–34, 35–37, 38–40, 41–42, and

>42 years. The primary outcome was the number of MZT pregnancies, which was defined as the presence of two fetal heartbeats on luteal transvaginal ultrasound with a thin dividing membrane (“T sign”) for monochorionic/diamniotic pregnancies or no dividing membrane for monochorionic/monoamniotic pregnancies. The primary independent variables, chosen for their increased use in the laboratory over our study time period, were FET protocol type (programmed or natural cycle) and use of a hyaluronan-containing transfer medium. The covariates collected from the medical records and evaluated as possible clinical parameters or predictors included the day of blastocyst biopsy or vitrification, the use of PGT, the use of ICSI, the use of donor oocytes, extent of embryo expansion, ICM and trophoctoderm (TE) grades, patient EE, oocyte age, and patient age at the time of FET.

Embryo Warming Protocol

Blastocysts of patients who returned for FET underwent standard embryo warming in our laboratory as previously described (24). Of note, AH is performed on all embryos that are planned for TE biopsy for PGT on day 4 after fertilization at our center. In addition, AH is performed, per our standard protocol, for all embryos at the time of embryo thaw unless the embryo has hatched from the zona. As mentioned, in July 2017, a bicarbonate-buffered transfer medium enriched with hyaluronan and recombinant human albumin sold under the trade name EmbryoGlue (Vitrolife, Gothenburg, Sweden) was introduced into the transfer protocol for FET. All blastocysts underwent standard warming with the use of the IrvineScientific kit. All blastocysts after July 2017 remained in the hyaluronan-enriched medium for 30–180 minutes before transfer.

Frozen Embryo Transfer Protocols

Patients underwent a programmed, or hormone-replaced, protocol or a natural cycle protocol for frozen embryo as deemed appropriate by their physician. For a programmed or hormone-replaced embryo transfer protocol, the patients were administered oral estradiol up-titrated from 2 mg/day to 6 mg/day for at least 10 days or until the endometrium measured ≥ 7 mm in diameter. Progesterone in oil was initiated and embryo transfer planned appropriately, often with a day-5 embryo transfer on the sixth day of progesterone. Natural cycles involved transvaginal monitoring of follicular growth and serum estradiol level until a dominant follicle reached 18 mm and ovulation was confirmed. Progesterone supplementation via vaginal suppository was given after ovulation, and embryo transfer was performed on the sixth day of progesterone supplementation. All embryo transfers were direct embryo transfers under ultrasound guidance. The patients were instructed to avoid penetrative intercourse during the entirety of their FET cycle.

Analysis

The continuous variables were first assessed for normality using the Kolmogorov-Smirnov test and determined to be nonparametric, and thus the Mann-Whitney *U* or

Kruskal-Wallis tests were used for the comparison of the medians. The categorical variables were analyzed using Fisher's exact or chi-squared tests where appropriate. A stepwise multiple logistic regression was used for modeling and adjustment of covariates for the entire dataset and in addition for a subgroup analysis of only embryos that underwent TE biopsy for PGT. An alpha error of <0.05 was considered statistically significant. Statistical analyses were performed in SPSS (v25.0).

RESULTS

A total of 1,619 cycles resulted in clinical intrauterine pregnancies after single FET and were included for analysis. Within this cohort, there were a total of 31 MZT pregnancies identified, for an overall MZT rate of 1.9%. When comparing the MZT and no-MZT groups, the two groups were similar with regards to mean (\pm SD) patient age (37.5 ± 4.7 years MZT vs. 37.2 ± 4.5 years no MZT, $P = .90$), endometrial thickness (9.3 ± 1.8 mm MZT vs. 9.1 ± 2.9 mm no MZT, $P = .60$), and oocyte age distribution by Society for Assisted Reproductive Technology age group ($P = .72$).

In total, the hyaluronan-containing transfer medium was used in 875 (54.1%) cycles. Monozygotic twin pregnancies were noted to be significantly less frequent in the group exposed to the hyaluronan-enriched media (11/875 [1.3%] MZTs in the hyaluronan-media group vs. 20/744 [2.7%] in the no hyaluronan-enriched media group, $P < .04$).

With regards to the cycle type, 1,385 (85.5%) cycles were programmed and 234 (14.5%) were natural. When comparing natural cycle protocols with programmed cycles, there was no statistically significant difference in the MZT rate between these groups (25/1,385 [1.8%] MZTs in programmed vs. 6/234 [2.6%] MZTs in natural cycles, $P = .44$).

Preimplantation genetic testing and ICSI were used in 1,423 (87.9%) and 579 (35.7%) cycles, respectively. All MZT embryos underwent PGT, which was statistically significant (31/1,423 [2.2%] MZTs in the PGT group vs. 0/196 [0] MZTs in the no-PGT group, $P < .03$). Unlike PGT, the use of ICSI was not associated with a higher rate of MZTs (12/579 [2.1%] MZTs in the ICSI group vs. 19/1040 [1.8%] MZTs in the no ICSI group, $P = .71$).

Donor eggs were used in 136 (8.4%) FETs. There was no difference in the number of MZTs in patients who used donor eggs compared with the number in those who used autologous

oocytes (4/136 [2.9%] MZTs in the donor egg group vs. 27/1483 [1.8%] in the non-donor egg group, $P = .33$).

In addition, embryo grading was evaluated. The MZT and non-MZT embryos were similar with regards to their ICM grade ($P = .93$), TE grade ($P = .56$), and expansion ($P = .74$). Table 1 summarizes the MZT rate by many of the previously mentioned clinical parameters.

Assisted hatching was performed at least once on all but 10 non-PGT embryos in the entire study cohort. All embryos that resulted in MZTs underwent AH, although this was not statistically significant (31/1,609 [1.9%] MZTs in the AH group vs. 0/10 [0] MZTs in the no-AH group, $P = .99$).

To control for potential confounders, a multivariate logistic regression was performed (Table 2). An initial analysis showed that MZTs was collinear with PGT; therefore, this variable was excluded as an independent variable in the model. The independent variables included in the regression model were oocyte age and age at FET in years, EE thickness in millimeters, cycle type (programmed or natural), the use of ICSI, and the use of a hyaluronan-enriched transfer medium (Table 2). In this analysis, the use of the hyaluronan-containing medium still resulted in significantly fewer MZT pregnancies (B -1.1 , $P < .02$, Hosmer-Lemeshow $P = .04$). To exclude the collinearity with PGT, a subsequent regression using the same parameters with PGT only cycles was performed, which showed that the use of the hyaluronan-enriched media continued to be associated with a reduction in MZTs (B -1.1 , $P < .01$, Hosmer-Lemeshow $P = .07$), independent of all other parameters (Table 3).

DISCUSSION

We found a 1.9% MZT rate in this cohort of FETs, which was consistent with prior studies demonstrating an elevated risk of MZTs with ART (6). As previously mentioned, during the study time period our laboratory protocols evolved to include a hyaluronan-enriched transfer medium. We found that the use of this transfer medium reduced the rate of MZTs in our cohort. This remained true even after controlling for potential confounders using a multivariate regression model and after performing a subgroup analysis that evaluated euploid embryos only. Although the mechanisms behind these findings are unknown, they may be related to the important role that hyaluronic acid plays in providing structural support and

TABLE 1

Monozygotic twin after frozen embryo transfer by clinical parameter.

	No MZT (n = 1,588)	MZT (n = 31)	P value
Hyaluronan-enriched media	864 (54.5%)	11 (35.5%)	.04
No hyaluronan-enriched media	724 (45.5%)	20 (64.5%)	
Programed cycle	1360 (85.6%)	25 (80.6%)	.44
Natural cycle	228 (14.3%)	6 (19.4%)	
ICSI	567 (35.7%)	12 (38.7%)	.71
No ICSI	1021 (64.3%)	19 (61.3%)	
PGT	1392 (87.7%)	31 (100%)	.03
No PGT	196 (12.3%)	0 (0%)	

Note: ICSI = intracytoplasmic sperm injection; MZT = monozygotic twin; PGT = preimplantation genetic testing.

Kelly. Predictors of FET monozygotic twins. *Fertil Steril Rep* 2021.

TABLE 2

Multivariate logistic regression on clinical parameters associated with monozygotic splitting.

Variable	B ^a	Standard error	Adjusted odds ratio	Confidence interval	P value
Constant ^b	-6.18	1.73	0.00		<.01
Endometrial thickness (mm)	0.01	0.04	1.01	0.94–1.08	.82
Cycle type (programmed or natural)	-0.30	0.46	0.74	0.30–1.84	.52
Hyaluronan-enriched media	-1.04	0.43	0.35	0.15–0.82	.02
Age at FET (years)	0.08	0.05	1.08	0.99–1.19	.09
Age group at oocyte retrieval	-0.06	0.16	0.95	0.69–1.30	.95
ICSI	0.21	0.38	1.23	0.59–2.57	.59

Note: FET = frozen embryo transfer; ICSI = intracytoplasmic sperm injection.

^a B = the regression coefficient; it represents the slope of the line between the predictor variable and the dependent variable.

^b The constant represents the value at which the regression line crosses the y-axis.

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compressive strength to cells (25). Hyaluronic acid is a glycosaminoglycan that interacts and binds to various components of the extracellular matrix. It is negatively charged and therefore hydrophilic. This, combined with its long polymer length, results in large amounts of water being bound to the extracellular matrix, thereby creating hydrated space around cells and improving the compressive strength of cells (25). By increasing the compressive strength of cells and creating more cell-to-cell adhesions through hydrogen bonding, hyaluronic acid may play a critical role in keeping the ICM together and preventing splitting (26). Additionally, the high viscosity of the hyaluronan-containing medium may also lower shear forces during embryo transfer, which may help to prevent splitting.

The other major change in our FET protocols was the increased use of natural cycles as opposed to programmed cycles. The effect of natural cycles on MZT rates has not previously been studied. Our data suggested that there was no difference in the rate of MZTs regardless of the cycle type. Therefore, patients who prefer to undergo an FET with a natural protocol can be reassured that our institutional experience supports this as a practice that will not alter their risk of having an MZT pregnancy.

Prior data suggested that decreasing oocyte age and extended embryo culture to blastocysts may be risk factors for MZTs (5, 6, 8–21). However, in a systemic meta-analysis

that included 40 studies to evaluate risk factors for MZTs, Busnelli et al. (6) described only a weak association between younger oocyte age and MZTs. They further noted that embryos derived from younger oocytes were more likely to be transferred at the blastocyst stage, and therefore decreasing oocyte age as a risk factor for MZTs may in fact be confounded by higher rates of extended embryo culture and blastocyst transfer in this group (6). In our multivariate analysis, we found no association between the oocyte age and the rate of MZTs. Furthermore, given that all of the embryos were vitrified or biopsied at the blastocyst stage; our study did not investigate if embryo transfer at the cleavage stage may reduce the risk of MZTs.

Previously, PGT, ICSI, and AH were examined as potential risk factors for MZTs, and the data to date are mixed (6, 8, 9, 16, 19–23). In the aforementioned meta-analysis performed by Busnelli et al. (6), they found that AH was associated with higher rates of MZTs and that there was a lack of an association between PGT and MZTs. In addition, they reported that ICSI may be protective against MZTs. Our analysis showed MZTs were collinear with PGT and supports that PGT needs to be further investigated in larger studies as a potential risk factor. Our data showed no association between ICSI or AH and MZTs.

Only one prior study examined the effects of embryo grading as a risk factor for MZTs. In a 2016 retrospective

TABLE 3

Multivariate logistic regression on clinical parameters associated with monozygotic splitting among preimplantation genetic testing-only cycles.

Variable	B ^a	Standard error	Adjusted odds ratio	Confidence interval	P value
Constant ^b	-7.09	1.99	<0.01		.00
Endometrial thickness (mm)	0.10	0.11	1.11	0.90–1.37	.34
Cycle type (programmed or natural)	-0.29	0.47	0.75	0.30–1.37	.75
Hyaluronan-enriched media	-1.08	0.43	0.34	0.15–0.80	.01
Age at FET (years)	0.09	0.05	1.10	1.00–1.20	.05
Age group at oocyte retrieval	-0.14	0.17	0.66	0.55–2.40	.87
ICSI	0.14	0.38	0.13	0.55–2.40	.72

Note: FET = frozen embryo transfer; ICSI = intracytoplasmic sperm injection.

^a B = the regression coefficient; it represents the slope of the line between the predictor variable and the dependent variable.

^b The constant represents the value at which the regression line crosses the y-axis.

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embryo cohort study, Otsuki et al. (27) found that a low-grade ICM score resulted in a higher risk of MZTs, specifically monochorionic dizygotic MZTs. Here, when evaluating the effects of embryo expansion, TE grade, and ICM grade, we saw no association between embryo quality and the risk of MZTs.

To our knowledge, this was the first study to examine the effect of a hyaluronan-enriched transfer medium and to investigate whether natural cycle protocols affect the risk of MZTs. The strengths of our study included the large cohort size and the ability to control for confounders with a multivariate regression model. However, our study was limited by its retrospective nature and by the fact that monozygosity was determined by ultrasound only and not confirmed with genetic testing for the patients who used natural cycle protocols. Another limitation was that the effect of paternal age on MZTs was not investigated in this analysis, and this variable should be examined in future studies. A post hoc power analysis demonstrated that this study had 55.1% power for evaluating the effects of the hyaluronan-enriched media and 14.6% power for investigation of the effects of protocol type on the rate of MZTs. Given that MZT gestations are rare events, larger prospective studies are needed to confirm whether a hyaluronan-enriched transfer medium is in fact protective against MZT pregnancies after ART. In addition, given the lack of data available on the effects of cycle type on MZT rates, additional studies examining this as well as other potential modifiable risk factors, like the use of PGT, are warranted.

CONCLUSION

In this retrospective cohort study, we observed a statistically significant decrease in MZT pregnancies after FET with the use of a hyaluronan-enriched transfer medium. The use of natural cycle FET protocols did not affect the rate of MZTs. Our analysis showed that MZTs was multi-colinear with PGT, thus PGT should be further examined as a potential risk factor. Given that MZT pregnancies are a rare event, more data, including larger prospective studies, are needed to confirm our findings.

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