Grade and looseness of the inner cell mass may lead to the development of monochorionic diamniotic twins

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Objective: To examine the relationship between the inner cell mass (ICM) grade and its morphological configuration on the occurrence of monochorionic diamniotic (M-D) twinning.
Design: Retrospective embryo cohort study.
Setting: Private IVF clinic.
Patient(s): Evaluation of frozen-thawed single blastocyst transfers with hormone replacement treatment in 8,435. This cohort included 71 blastocysts and their ICMs observed by time-lapse photography.
Intervention(s): Any changes in configuration of the ICMs observed by time-lapse photography were analyzed retrospectively.
Main Outcome Measure(s): The amount of loosening of blastomeres within the ICM was evaluated by time-lapse observations. The number of cells that were involved in the loosening process was also assessed. Both of these parameters were correlated with the type of monozygotic twinning that eventuated.
Result(s): The M-D twinning incidence resulting from blastocysts with a high grade ICM (grade A) were transferred was 0.38% (3/796), whereas it was significantly higher, 1.38% (34/2,463), when blastocysts with a poorer (B and C) grade ICM were transferred. Among 71 transferred frozen-thawed blastocysts that were studied with time-lapse photography, there were two dichorionic diamniotic and one M-D twins. Careful observations of the embryo that resulted in the one M-D case, revealed that the ICM acquired a looser appearance due to decompaction of at least eight cells. This type of decompaction was not observed in the ICMs of other transferred blastocysts.
Conclusion(s): The occurrence of M-D twinning may be avoided by excluding blastocysts that contain decompacting ICMs. (Fertil Steril® 2016;106:640–4. ©2016 by American Society for Reproductive Medicine.)
Key Words: Monozygotic twinning, inner cell mass, loosening of ICM, frozen-thawed blastocysts, single embryo transfer, time-lapse observations

It is an accepted concept that monozygotic twinning occurs at a significantly higher rate after assisted reproductive technology (ART) procedures when compared with their natural incidence (1–3). Monozygotic twinning has three variations—monochorionic diamniotic (M-D), monochorionic monoamniotic (M-M), and dichorionic diamniotic (D-D) twins. Monochorionic diamniotic twins are at risk of twin-twin transfusion due to their shared placenta, which causes growth discordance and fetal loss (4, 5). Monochorionic monoamniotic twins, which share both placenta and amniotic sac, are rare and occur in 1%–2% of monozygotic twin pregnancies, but are more at risk because of the danger of cord entanglement (6, 7). The risk associated with monozygotic D-D twins has not yet been fully understood, whereas dizygotic D-D twins have a lower obstetric risk compared with monozygotic M-D and M-M twins. Although the mechanisms and factors that contribute to dizygotic D-D twinning are well established, very little is known about the mechanisms involved in monozygotic M-D, M-M, and D-D twinning. Although several factors, involved in ART, that predispose to monozygotic twinning have been proposed, the relative risks associated with D-D, M-D, and M-M individually have yet to be fully established. At present, there are conflicting reports regarding some methods of ART, such as

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intracytoplasmic sperm injection (ICSI), assisted hatching (AH), and extended culture through the blastocyst stage. Although there may be a tacit agreement that monozygotic twinning rates have been lower in recent years (8, 9) due to improvements in culture media, the rate of monozygotic twinning has not been reduced to their incidence in natural conception, which is approximately 0.4% (10).

Although little is known about inner cell mass (ICM) formation and its intercellular adhesion, a previous study reported that calcium-dependent cell-cell adhesion system regulates ICM formation and cell surface polarization in early stage mouse embryo development (11). It should be noted that ethylenediaminetetraacetic acid (EDTA), which chelates heavy minerals including calcium and magnesium is commonly used as a supplement in embryo culture media in the expectance of better embryo development (12). Embryos cultured in vitro may have loose cell adhesion/cohesion in the presence of EDTA supplementation in culture media.

In the present study we explored the possible risk factors from a new aspect, focusing on the grade of ICMs of expanding and/or expanded blastocysts. We hypothesized that the susceptibility of ICMs to splitting could be related to the grade and looseness of ICM cells. We postulated that such factors could predispose to the formation of two separate ICMs, which may result in M-D twinning (3, 13, 14). Time-lapse photography enabled us to clearly analyze such predisposing factors.

MATERIALS AND METHODS

All treatment cycles involving hormone therapy (HT) and single frozen-thawed ET, resulting in a clinical pregnancy (presence of gestational sac), from January 2011 to December 2014, at a single fertility clinic, were retrospectively reviewed. In addition, the ICMs of blastocysts were observed by time-lapse microscopy (Embryo Scope), from June 2013 to December 2014, were also retrospectively analyzed. Institutional review board approval was not required for this study because of the retrospective nature of the study and because the test subjects were examined anonymously.

Assessment of Monozygosity

Pregnant patients underwent a vaginal ultrasound, at 6–7 weeks and at 8–9 weeks. Monozygotic twinning was confirmed when two fetal poles were observed. These fetal poles are sometimes observed before fetal heartbeats (FHBs) are detected. If a dividing intervening amniotic membrane was visible, the pregnancy was considered to be M-D, whereas when there was lack of dividing membrane, it was considered to be M-M. When two gestational sacs were observed, it was designated D-D.

The presence of a gestational sac defines a clinical pregnancy. In this article we analyzed the ICM morphology both when the gestational sac and FHBs were detected.

IVF and ICSI Procedures

In the oocyte retrieval cycle, patients were stimulated using standard GnRH agonist/FSH protocols or antagonist/FSH protocol. Ovulation induction was triggered when the second leading follicle was >18 mm in diameter. Ultrasound-guided transvaginal oocyte retrieval was performed 35–36 hours later.

The IVF laboratory procedures were as follows. Immediately after retrieval, oocytes were placed in Universal IVF Medium (Origio a/s) overlaid with mineral oil (Irvine Scientific). Oocytes were inseminated 3–5 hours later using conventional insemination procedures or ICSI, depending on semen parameters. After a fertilization check, performed approximately 18 hours after insemination, the resultant zygotes were placed in global medium (LifeGlobal) with 10% human serum albumin (HSA; LifeGlobal) where they remained until day 5. One to four embryos were cultured in groups in a 50μL droplet of culture medium under mineral oil.

We performed stimulation of endometrium ET for most patients with a single blastocyst transfer (~90%) 3 days before a frozen-thawed blastocyst was transferred (15). The embryo culture supernatants of global medium were preserved at -20°C. The blastocysts and the embryo culture supernatant were cryopreserved until the transfer cycle. To evaluate the configuration of blastocysts, we used the blastocyst grading method of Gardner and Schoolcraft (16). The blastocyst grading was assessed by 1 of 17 experienced embryologists, all trained for 2.5 years. The blastocyst grading assessment was completed 1–2 hours before ET, which were performed approximately 2–3 hours after thawing.

HT and ET

In the study cycle, transdermal E_2 (Estraderm M; Kissei) was used in combination with a vaginal P suppository (Ultrogestan; Central apotheke parfumerie) for HT. Preparation of the endometrium was started on day 2 of the HR cycle and achieved in a step-up regime (2.16–4.32 mg). The P suppository (600 mg/d) was started on day 15. One frozen-thawed blastocyst underwent AH and was transferred on day 20 of the HR cycle. Assisted hatching was performed on most blastocysts (>90%), depending on the patient's preference, after careful patient-embryologist consultation. We performed AH for 93.8% of patients. There was no significant difference between embryos with AH (1.06%, 35/3,311) and without AH (0.75%,1/134) related to M-D twinning rate. In most cases AH involved one-quarter of the zona pellucida (ZP) using laser shots (ZILOS-tk Laser, Hamilton Thorne, Inc.), and in a small percentage of cases AH involved one-half of the ZP. In all cases laser was used for the AH. Embryo transfer was performed transcervically using an IVF catheter (Fuji Systems). The embryo culture supernatant, in which the patient's own embryos had been developed, was thawed and injected into the uterine cavity on day 17 of the HT cycle. The supernatant injection was performed transcervically in the uterus using an IVF catheter. The catheter, loaded with 20 μL of the embryo culture supernatant, was inserted into the uterine cavity, and the supernatant was released when the tip of the catheter was approximately 1 cm from the fundus of the uterine cavity.

Time-lapse Recordings

Time-lapse recordings were performed using EmbryoScope Time-lapse system (Vitrolife) from June 2013 to December

TABLE 1

Incidence of twins and triplet after transfers of single frozen-thawed blastocyst in women given hormone therapy.

Outcome	No.	%	*P* value	Female/female	Male/male	Female/male	Unknown	Aborted
Transferred embryos	8,435							
Chemical pregnancy	4,945	58.6 (4,945/8,435)						
Clinical pregnancy	3,445	40.8 (3,445/8,435)						
FHB (+)	3,050	36.2 (3,050/8,435)						
D-D clinical pregnancy	39	1.13 (39/3,445)						
D-D double FHBs	18	46.2 (18/39)	[a]					
D-D single FHB	16	41.0 (16/39)						
D-D double live births	17	43.6 (17/39)	[c]	11	4	2		
D-D single live birth	13	33.3 (13/39)		9	3		1	
M-D clinical pregnancy	36	1.04 (36/3,445)						
M-D double FHBs	33	91.7 (33/36)	[b]					
M-D single FHB	0	0 (0/36)						
M-D double live births	26	72.2 (26/36)	[d]	9	16		1	1
M-D single live birth	2	5.6 (2/36)					2	
M-M clinical pregnancy	3	0.09 (3/3,445)						
M-M double FHBs	1	33.3 (1/3)						
M-M single FHB	2	66.7 (2/3)						
M-M double live births	0	0 (0/3)						
M-M single live birth	2	66.7 (2/3)		1	1			
Monochorionic triplet	2	0.06 (2/3,445)						
M-T double FHBs[e]	1	50.0 (1/2)						
M-T double live births	1	50.0 (1/2)			1			
Total of twin and triplet	80	2.32 (80/3,445)						

Note: D-D = dichorionic diamniotic; FHB = fetal heartbeat; IUFD = intrauterine fetal death; M-D = monochorionic diamniotic; M-M = monochorionic monoamniotic; M-T = monochorionic triplet.
[a,b] $P<.001$.
[c,d] $P=.0017$.
[e] One of three fetuses resulted in IUFD.

Otsuki. Monochorionic diamniotic twinning. Fertil Steril 2016.

2014. The time-lapse images were captured automatically every 15 minutes, at seven focal planes. Any changes in the configuration of the ICM were retrospectively analyzed by evaluating the ICM morphology at all seven focal planes. During the course of this study, the recordings were restricted to patients aged between 30 and 40 years, when each yielded more than four oocytes, to enable the evaluation of possible effects of the time-lapse system. Loosening of the ICM was defined as the presence of more than five cells that were loosely arranged in an ICM, which previously contained tightly grouped cells observed by the time-lapse system.

Statistical Analysis

A χ^2 test was used for the comparison of proportions. A $P<.05$ was considered statistically significant.

RESULTS

There were a total of 8,435 frozen-thawed single blastocyst transfers with HT during the study period, resulting in 3,445 (40.8%) clinical pregnancies. Of all these clinical pregnancies, a total of 80 monozygotic twinnings (2.32%) were identified. The numbers of M-D, M-M, and D-D twins were 36 (1.04%), 3 (0.09%), and 39 (1.13%), respectively. Monochorionic triplets were also confirmed in two cases (0.06%) (Table 1). Gender discordance in D-D twins was found in two D-D cases in this study, assuming that it was a combination of single embryo transfer and natural conception during the HT cycle for frozen-thawed single blastocyst transfer.

The incidence of two FHBs from a frozen-thawed single blastocyst transfer with HT was significantly higher in M-D twinning than in D-D twinning ($P<.001$). The incidence of dual live births was also significantly higher in M-D compared with D-D twinning ($P=.0017$) (Table 1).

The grades of blastocysts were evaluated using the blastocyst grading method of Gardner and Schoolcraft (16) and M-D twinning rates were analyzed. The M-D twinning incidence with high quality blastocysts (≥3BB) was 0.87% (17/1,958), whereas it was 1.48% (20/1,349) when low quality blastocysts (<3BB) were transferred ($P=.11$). The M-D twinning incidence when blastocysts with high grade ICM (grade A) were transferred was 0.38% (3/796), whereas it

TABLE 2

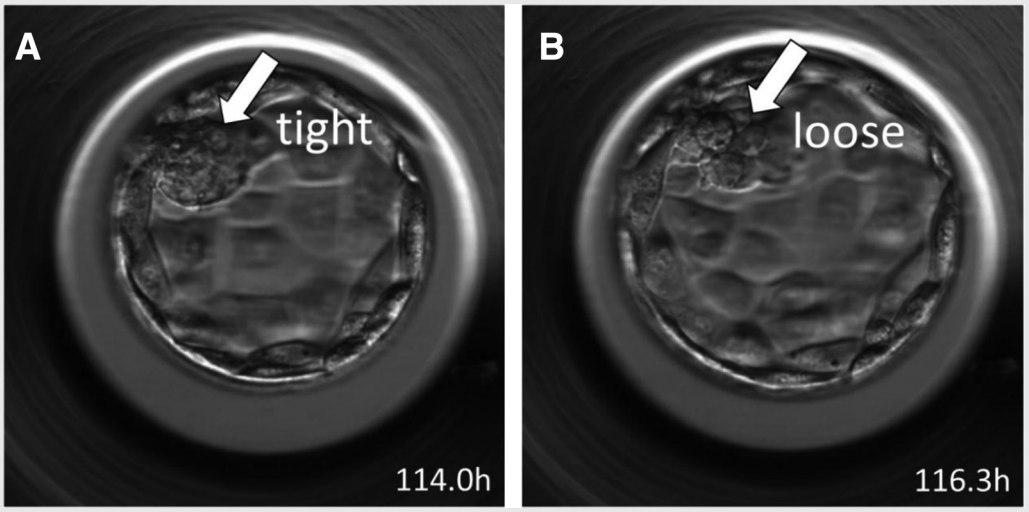
Blastocyst grade and monochorionic diamniotic twinning incidence after transfers of single frozen-thawed blastocysts in women given hormone therapy.

Grade	No. of clinical pregnancy	No. of M-D twinning	%	*P* value
High quality blastocyst (≥3BB)	1,958	17	0.87	
Low quality blastocyst (<3BB)	1,349	20	1.48	
ICM grade A (AA, AB, AC)	796	3	0.38	[a]
ICM grade B and C	2,463	34	1.38	[b]
Total	3,307	37	1.12	

Note: ICM = inner cell mass; M-D = monochorionic diamniotic.
[a,b] $P=.033$.

Otsuki. Monochorionic diamniotic twinning. Fertil Steril 2016.

FIGURE 1



Careful observation of the blastocyst that resulted in the birth of a monochorionic diamniotic twin revealed that eight cells in its inner cell mass (ICM) became decompacted and the cell mass developed a looser appearance. The timing of these changes was as follows: (**A**) Tight junctions formed in the ICM at 114.0 hours after insemination. (**B**) These junctions in the ICM loosened at 116.3 hours after insemination.

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was 1.38% (34/2,463) with blastocysts that contained a lower grade ICM (grades B and C) (P=.033) (Table 2).

Frozen-thawed blastocysts (71) were transferred after observations using time-lapse microscopy. The hCG, GC, and FHB positive rates were 74.2% (52/71), 63.4% (45/71), and 57.7% (41/71), respectively. Among the 41 cycles with positive FHB, 36 cases were delivered, 4 cases miscarried, and the clinic lost contact with 1 case. Also two D-D twins and one M-D were recorded. In addition, a M-D twin was born in the group of blastocysts that were carefully monitored by time-lapse microscopy. The ICM of the blastocyst that produced this twin developed at least eight decompacted cells (Fig. 1). Such decompaction was not observed in the ICMs of the other 66 blastocysts. In the remaining four transferred blastocysts, the ICMs were unclear and cell loosening within their ICMs could not be detected.

DISCUSSION

In the present study, it was found that blastocysts containing ICMs classified as high grade produced a low incidence of M-D twinning. However, this finding is not in agreement with other publications (17–19) suggesting that younger age groups are at risk of M-D twinning as blastocyst grade is generally higher in younger than in older patients (20). This could be related to findings indicating that M-D embryos of older patients have more chromosomal abnormalities and result in miscarriage before reaching clinical pregnancy.

Our time-lapse data indicated that "loosening of the ICM" could be one of the risk factors that may predispose to splitting of the ICM, which could lead to M-D twinning. The time-lapse data are consistent with the ICM grade data, as high quality ICMs are generally larger and tighter. The incidence of M-D twinning could be reduced by avoiding the transfer of embryos that contain loosened ICMs, as confirmed by time-lapse observations. In addition such observations would detect splitting of single ICMs into two or three ICMs in an expanded blastocyst. However, as the incidence of M-D twinning is around 1% and availability of time-lapse data are limited, multicenter studies would be necessary to confirm these findings.

The cause of each M-M, M-D, and monozygotic D-D twinning may be different and we need to investigate the predisposing causes and risk factors individually. Our data showed higher birth rates of monozygotic M-D twins than of monozygotic D-D twins without a reduced number of fetuses. This could be due to the proportion of ICMs that become split. A loosened ICM may split into two ICMs of nearly the same size, alternatively the cell mass may divide into two ICMs of different size during the hatching process (3), which may occur more frequently when AH is performed. It is also possible that the division of the entire blastocyst occurs during the freezing and thawing process, as previously reported (21). Further studies will be needed on AH methods as a possible cause of monozygotic D-D twinning. It should also be noted that in cases of D-D twinning after transferring frozen-thawed single blastocysts, it may be initially difficult to distinguish whether the twins are dizygotic (e.g., the implantation of a frozen-thawed blastocyst and a naturally conceived embryo, when the sex of each twin is the same). Therefore our data dealing with D-D twinning has a limitation and further studies are needed. Interestingly, the percentage of male-male M-D twins was greater than female-female twins and this was consistent with a previous report by Knopman et al. (17). In contrast to M-D twins, the percentage of male-male monozygotic D-D twins was lower than female-female twins in our study of frozen-thawed single blastocyst transfers combined with HT.

Monochorionic monoamniotic twinning is unlikely to be related to mechanical zona manipulation, such as AH and ICSI, as the condition occurs after implantation. However, a susceptibility to splitting of the ICM in ART should continue to be a concern as the outcome could be M-D twinning.

To our knowledge, the present report contains the largest number of subjects treated with frozen-thawed single blastocyst transfers combined with HT. Because single ETs were recommended by the Japanese Academic Society of Obstetrics and Gynecology in 2007 and blastocyst vitrification is well established with a high survival rate, frozen-thawed single blastocyst transfer has become a major practice that is likely to continue in the future of ART in Japan. Looking forward, it will be important to focus on further improving cryopreservation technology, AH methods, as well as refinements of culture methods, to reduce the occurrence of monozygotic twinning.

In conclusion, the occurrence of M-D twinning may be reduced by avoiding the transfer of blastocysts that show loosening of their ICM. As such morphological changes can be readily confirmed by time-lapse observations, further prospective studies are required to support this hypothesis. Accurate microscopy could also detect the splitting of ICMs into two or three ICMs in an expanded blastocyst.

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