Blastocyst culture is associated with an elevated incidence of monozygotic twinning after single embryo transfer

In a 7-year (2002–2008) retrospective study of a large IVF program based on minimal ovarian stimulation and single ET (47,841 single ETs), monozygotic twinning occurred in 1.01% of 14,956 clinical pregnancies. Blastocyst culture was associated with a significantly increased monozygotic twinning risk (adjusted odds ratio, 2.04; 95% confidence interval, 1.29–4.48), whereas embryo freezing, type of stimulation protocol used, intracytoplasmic sperm injection fertilization, or zona removal did not influence its incidence. (Fertil Steril® 2011;95:2140–2. ©2011 by American Society for Reproductive Medicine.)

Key Words: Monozygotic twinning, single embryo transfer, blastocyst culture, frozen-thawed embryo transfer, minimal stimulation, in vitro fertilization

Multiple pregnancies, especially high-order multiples, are one of the main complications of assisted reproductive technology and are associated with important maternal and perinatal risks (1). To achieve a significant reduction of multiple gestations, the number of transferred embryos should be reduced; this has been encouraged by professional societies worldwide (2, 3). Elective single embryo transfer has been proposed as a way of obtaining only singleton pregnancies, which has been advocated as the most desirable outcome following IVF (4). Although an exclusive single ET policy is capable of radically reducing twin rates, multiples can still arise due to early splitting of the embryo resulting in two identical twins (5). Monozygotic twinning (MZT) is known to be associated with a higher risk of perinatal mortality and other complications, such as twin-to-twin transfusion syndrome, which occurs in 10%–20% of monochorionic twins (6).

For more than 15 years, Kato Ladies Clinic (KLC) has been pioneering novel ovarian stimulation protocols involving minimal stimulation and natural cycle IVF in conjunction with a high proportion of single ET cycles (7). In such a setting, MZT could have a significant effect on outcome and needs particular attention. Therefore, our aim was to determine the incidence of MZT in a retrospective series, which to our knowledge is the largest single ET cohort ever analyzed, and to search for possible risk factors influencing its occurrence.

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MATERIALS AND METHODS

The MZT rate was retrospectively evaluated in all clinical pregnancies that resulted from all consecutive single ETs performed in a private fertility center (Kato Ladies Clinic, Tokyo, Japan) between January 2002 and December 2008. Institutional review board approval was not required for because of the retrospective nature of the study and the study data were managed to exclude the subjects' identification. The details of the minimal stimulation protocol used were described previously (7). Ovarian stimulation was performed using clomiphene citrate (from cycle day 3 until the day before triggering) in combination with a small amount of hMG or rFSH. The final oocyte maturation was triggered with a nasal GnRH agonist, and the oocytes were retrieved 32–35 hours later. Unstimulated natural cycle IVF was performed in a smaller portion of cases (8). After insemination by conventional IVF or intracytoplasmic sperm injection (ICSI), normally fertilized 2PN zygotes were cultured individually in 20 μl of cleavage-stage medium (SAGE, Pasadena, CA). Blastocyst culture was performed using commercially available media (Quinns Advantage; SAGE) enriched by 10% protein supplement (Serum Protein Substitute; SAGE). All embryos were cultured at 37°C under the gas phase of 5% O_2, 5% CO_2, and 90% N_2 with full humidity in water jacket small multigas incubators (Astec, Fukuoka, Japan). Zona removal was performed for approximately one third of fresh or thawed blastocysts by completely removing the zona pellucida with a laser shot system (Octax Microscience, Bruckberg, Germany). Cryopreservation was performed using the Cryotop vitrification method (Kitazato, Shizuoka, Japan) as described previously (9). The ET procedure was performed under vaginal ultrasound guidance using a specially designed soft silicone inner catheter (Kitazato) by placing a single embryo in a minimal volume to the upper part

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TABLE 1

Number of embryo transfer procedures with corresponding clinical pregnancy, live birth, and MZT rates.

	Study group A	Study group B	Study group C	Study group D	Total
Transfers, n	20,453	2,402	1,540	23,386	47,841
Clinical pregnancies, n (%)	4,477 (21.9)	587 (24.4)	399 (25.9)	9,493 (40.6)	14,956 (31.3)
Live births, n (%)	3,341 (16.3)	442 (18.4)	302 (19.6)	7,031 (30)	11,129 (23.3)
Monozygotic clinical pregnancies, n (% of CP)	29 (0.65)	2 (0.34)	9 (2.25)	111 (1.17)	151 (1.01)
Monozygotic deliveries, n	24	2	8	85	119

Note: Group A = fresh cleavage-stage; group B = frozen cleavage-stage; group C = fresh blastocyst; group D = frozen blastocyst.

Kawachiya. MZT after single embryo transfer. Fertil Steril 2011.

of the uterine cavity. In FET cycles, embryos were transferred during a natural cycle or after endometrial preparation with exogenous estrogen and P. In natural cycles, cleavage-stage embryos and blastocysts were transferred on days 2 and 5, respectively, after ovulation was confirmed. In hormonal replacement cycles, transdermal estradiol patches were started from cycle day 2 and Duphaston was added from cycle day 11, after which cleavage-stage embryos or blastocysts were transferred 1 or 7 days later, respectively. The association between MZT (detected at first-trimester ultrasound examination) and blastocyst culture (yes or no), maternal age (years), type of stimulation protocol used (unstimulated versus clomiphene-citrate), embryo vitrification (yes or no), ICSI fertilization (yes or no), and zona removal (yes or no) was evaluated by a multiple logistic regression analysis in each subgroup. Backward stepwise elimination was used to generate the most parsimonious model (a $P<0.05$ value was used for inclusion and $P>0.1$ for excluding variables).

RESULTS

A total of 47,841 single ETs were performed during the study period, resulting in 14,956 (31.3%) clinical pregnancies and 11,129 (23.3%) live births. In this cohort, the majority of the transfers were performed at the blastocyst stage (52.2%) and the remaining at the cleavage stage (47.8%). Blastocyst-stage transfers were mostly (93.8%) performed in cryopreserved ET cycles, whereas at cleavage-stage the majority were fresh cycles (89.5%). In our cohort, the highest live birth rates per ET (30%) were achieved by implanting a single cryopreserved blastocyst.

The MZT rate was evaluated in all 14,956 clinical pregnancies that resulted from the single ET procedures. A total of 151 (1.01%) MZT cases have occurred: 31 (0.61%) after single cleavage-stage ET and 120 (1.21%) after single blastocyst transfer. The number of ET procedures, corresponding clinical pregnancy, and live birth and MZT rates are summarized in Table 1. Two triplet pregnancies were also observed in our series: one resulted in spontaneous reduction and one resulted in a twin delivery.

Of the 151 MZT pregnancies observed at the first trimester, 119 (78.8%) continued until term. Spontaneous complete miscarriage occurred in 14 cases, whereas spontaneous reduction of one of the twins ("vanishing twin") was observed in 18 cases. Of the delivered monozygotic twins, 70 (59.8%) and 47 (40.2%) were dichorionic diamniotic and monochorionic diamniotic twins, respectively. No monochorionic monoamniotic cases were observed in our series. The mean ± SD gestational age at delivery was 35.3 ± 2.7 weeks' gestation, with a mean birth weight of 2,172 ± 485 g. Seventy-six (63.8%) preterm deliveries (<37 weeks' gestation) occurred. One-hundred seventy-nine (61%) neonates were low birth weight (<2,500 g). The perinatal mortality rate was 1.25% (3/239), with stillbirth of one member of a twin pair and the postnatal death of a very low birth weight (<1,500 g) twin pair at 24 weeks' gestation. Five of a total of 238 live born neonates (2.1%) had congenital malformations (hypospadiasis, ventricular septum defect, persistent ductus arteriosus, umbilical herniation, and external ear defect).

After eliminating four potential confounders, the final logistic regression model showed that blastocyst culture (adjusted odds ratio [OR], 2.04; 95% confidence interval [CI], 1.29–4.48; $P=0.006$) was associated with an increased probability of MZT. Conversely, every year increase in maternal age (OR, 0.94; 95% CI, 0.88–0.99; $P=0.029$) was associated with a lower risk of MZT.

DISCUSSION

In our retrospective study examining a large cohort of single ETs, we have found that MZT occurred at a 1.01% rate per clinical pregnancy. Prolonged embryo culture to the blastocyst stage was associated with an increased risk of MZT.

The exact pathogenesis leading to embryo splitting is unknown, but available evidence suggests that multiple factors might be responsible, including maternal age, ovarian stimulation, prolonged embryo culture, altered in vitro culture conditions, and zona pellucida manipulation (10). Blastocyst culture appears to be one of the important factors that contributes to MZT, as suggested by a recent metaanalysis based on nine included studies regrouping more than 40,000 cycles (11). More recent studies (5, 12, 13) reported concordant MZT rates (1.9%–2.2%), which were considerably lower than the much higher incidence reported in earlier retrospective series. It was suggested that, with improving culture conditions and a larger experience with blastocyst culture, MZT rates would decline gradually (14).

Studies of the incidence of MZT after conception by assisted reproductive technology fertilization are hampered by the fact that, with the exception of postpartum DNA fingerprinting of same-sex twins, no other method (e.g., detection of fetuses or gestational sacs in excess of transferred embryos, chorionicity determination by first-trimester ultrasound) is completely reliable for the determination of zygosity. This problem might lead to an underestimation of the true incidence of MZT. However, single ET cohorts are more likely to give a correct estimation, because dichorionic

monozygotic twins are included (15). The study by Papanikolaou et al. (5) is of particular interest because of its methodological quality and, similar to our series, it has examined a large single ET cohort. However, in contrast to our findings, the study concluded that blastocyst culture does not affect the incidence of MZT following single ET, which might be related to a smaller sample size. In fact, all retrospective series on MZT are limited by the fact that the overall low incidence requires a large sample to detect a significant difference.

Data are still controversial regarding the influence of zona pellucida manipulation (ICSI, assisted hatching, or preimplantation genetic diagnosis) on MZT (10). In our series, ICSI or zona removal was not associated to its occurrence. Finally, in agreement with previous studies (16), the obstetric outcome of MZT pregnancies in our series was less favorable compared with singleton ones because of a high rate of prematurity, low birth weight, and perinatal mortality.

Following minimal stimulation and the retrieval of only one to two oocytes, the resulting embryos were preferentially cultured to blastocyst stage and vitrified. Owing to the highly efficient vitrification method used (95% survival for blastocysts), the cancellation rate for frozen-thawed blastocyst transfer cycles was low, resulting in a substantial number of embryos for transfer in this study. In most published studies, the implantation rate from blastocyst transfer is significantly higher than that measured with cleavage-stage embryos. In our study, the majority of minimal stimulation cycles were performed with the use of an extended clomiphene citrate regimen (7). The antiestrogenic effect of clomiphene citrate on endometrial receptivity in fresh cycles may explain lower-than-expected implantation rates for blastocysts. Furthermore, during FET cycles devitrified blastocysts were transferred in the presumably more favorable endometrial environment of natural or hormonal replacement therapy prepared cycles.

In separating the potential effects of culture versus the transfer of blastocysts on MZT, it may be that blastocysts are easier to damage upon transfer than are cleavage-stage embryos, and culture per se is not related to the incidence of MZT. Because no changes were implemented in the embryo transfer technique used (e.g., catheter type) in the center during the study period, we have assumed that cleavage- and blastocyst-stage embryos were transferred in similar manner. This study cannot exclude the possibility that blastocysts are vulnerable to external mechanical factors that could cause embryo splitting.

In conclusion, our findings suggest that MZT occurs at a significant but relatively low rate in a single ET program. Therefore, patients should be adequately counseled about a less than 1% risk of MZT delivery following single ET, which is more likely after blastocyst culture.

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