Potential risk of monochorionic dizygotic twin blastocyst formation associated with early laser zona dissection of group cultured embryos

Mitchel C. Schiewe, Ph.D.,[a] John B. Whitney, B.S.,[a] and Robert E. Anderson, M.D.[a,b]

[a] Southern California Institute for Reproductive Sciences and [b] Southern California Center for Reproductive Medicine, Newport Beach, California

Objective: To document the risk of in vitro monochorionic dizygotic twin formation in the implementation of a program of blastocyst biopsy with preimplantation genetic screening (PGS).
Design: Case report.
Setting: Private infertility laboratory.
Patient(s): Prospective PGS patients with intracytoplasmic sperm injection–derived, group-cultured blastocysts over a 3-year period.
Intervention(s): Group culture in Global medium (Life Global) to optimize blastocyst formation of zygotes produced for blastocyst biopsy for PGS ($n \leq 8$ embryos/25 μL droplet), and laser zona dissection (LZD) of all day-3 cleaved embryos to promote pre-expansion trophectodermal extrusion at the blastocyst stage (i.e., premature hatching).
Main Outcome Measure(s): Blastocyst formation and quality grading on days 5 and 6 of in vitro culture for the vitrified embryo transfer of single or dual euploid blastocysts.
Result(s): Over 3,000 blastocysts were produced in vitro. On two separate occasions, complete trophectodermal amalgamation was observed between two hatching blastocysts. Vitrified single-euploid blastocyst transfers efficiently implanted and established clinical pregnancies similar to dual-euploid blastocyst transfers, without the risk of twin formation.
Conclusion(s): The amazing occurrence of monochorionic dizygotic twin formation has now been documented in vitro, supporting the theory that assisted reproductive technology may facilitate this rare perinatal condition. Furthermore, we have provided clinical evidence that the transfer of a single-euploid blastocyst can optimize a patient's pregnancy success while reducing potentially undesirable conditions associated with monochorionic twin pregnancies. (Fertil Steril® 2015;103:417–21. ©2015 by American Society for Reproductive Medicine.)
Key Words: Blastocysts, in vitro derived, monochorionic dizygotic twins

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Monozygotic twins compromise the majority of monochorionic twin pregnancies, and dizygotic twins are extremely rare under natural conditions (1, 2). Monochorionic twin pregnancies are undesirable from the clinical perspective, as they increase the risk of fetal and neonatal complications (3, 4). Additional complications can transpire in dizygotic individuals as they are susceptible to various levels of chimerism (5–9). The establishment of monochorionic dizygotic twining has been proposed to occur more frequently in assisted reproduction technology (ART) cycles (10), as reviewed in six documented cases (11). The latter cases involved multiple-embryo transfers or stimulated intrauterine insemination cycles, thus increasing the risk of embryo-embryo interactions.

Additional monochorionic dizygotic twins have occurred since 2005 with the advent of improvements in blastocyst culture and embryo transfer (ET) success rates. However, the number of fresh/frozen embryos transferred has steadily declined in the United States, from n = 2.9/2.8 in 2003 to n = 2.0/1.8 in 2012 (e.g., in women 35 to

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Reprint requests: Mitchel C. Schiewe, Ph.D., SCIRS, 361 Hospital Road, Suite 433, Newport Beach, California 92663 (E-mail: scirslab@verizon.net).

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37 year old) (12), toward the goal of one embryo per transfer, which would effectively eliminate dizygotic twin pregnancies. In the last 5 to 10 years, vitrification technology has transformed embryo cryopreservation by ensuring a high level of survival and developmental competence equal to fresh embryos. In combination with the preimplantation genetic screening (PGS) of blastocysts, single-embryo transfer (SET) of vitrified, euploid blastocysts can produce repeatable high live-birth rates independent of patient age (<43 year old) (13, 14).

In the establishment of PGS-blastocyst biopsy procedures, programs must decide whether laser zona dissection (LZD) will be performed before blastocyst formation (day 3 or 4) or at the time of blastocyst cavitation (day 5 or 6). We have determined that early rent formation in the zona pellucida of day-3 cleavage embryos produces an ideal premature hatching of trophectoderm (TE) cells to facilitate easy and immediate biopsying of blastocysts, initiating TE extrusion at the full blastocyst stage. However, the group culturing of induced-hatching blastocysts can position TE cells in very close proximity to one another, creating an unknown risk of chimerism.

MATERIALS AND METHODS

Between 2011 and 2013, oocytes were recovered and intracytoplasmic sperm injection (ICSI) was performed on over 500 perspective PGS cases. All patients were fully informed and consented to the potential risks of blastocyst biopsying and PGS procedures. Patients signed the consent knowing their data could be reviewed, summarized, and published in a deidentified manner as required by the professional organization, the institutional review board, and federal standards. The findings we report were generated in the process of performing standard laboratory ART procedures, including TE biopsies and vitrification, which were developed in conjunction with an approved institutional review board protocol.

Using MCO-5M mini Sanyo/Panasonic tri-gas incubators (5% O_2/5.3–6.0% CO_2), we group-cultured up to eight embryos per 25-μL droplet of LG medium (Global; Life Global) supplemented with 7.5% synthetic protein supplement under Ovoil (Vitrolife) until blastocyst biopsy. Embryos were initially evaluated on day 3, at which time LZD was performed using a 1,410-nm diode laser (Zylos; Hamilton Throne). The laser was set at 600 mHz with a 250-ms pulse duration to deliver two to three sequential ablations to breech the zona pellucida (ZP) with a 10–12-μm furrow. Early rent formation in the ZP (35–50 μm long) was performed to promote early TE herniation, beginning at the full blastocyst stage. The same laser settings were used for biopsying the TE using a combination of LZD (2–10 pulses) and mechanical separation (refer to our 2012 video from the American Society for Reproductive Medicine annual meeting) (15).

Evaluation of blastocyst formation and morphologic grading of blastocysts was performed on days 5 and 6, before biopsy by use of Gardner classifications (16). The Gardner grading of blastocysts was modified to account for premature hatching: 3 = 10% or less TE herniation (full blastocyst), 4 = 10%–50% TE extrusion (expanded blastocyst), and 5 $\geq$ 50% TE hatching (hatching blastocyst). In year 1, the orientation of blastocyst herniation was recorded to assess the location of the inner cell mass (ICM): fully extruded (hatching ICM first), partially extruded (ICM at the ZP opening), or not extruded (ZP enclosed). After blastocyst biopsying, most blastocysts were individually cryopreserved using microSecure vitrification (μS-VTF) (17) with non–dimethyl sulfoxide containing VTF solutions (Innovative Cryo Enterprises LLC), before subsequent transfer of one or two euploid blastocysts in a vitrified embryo transfer (VFET) cycle. Differences in pregnancy outcomes were statistically determined by chi-square analysis, and $P<.05$ was considered statistically significant.

Based on blastocyst assessments and other known averaged occurrences (e.g., fertilization rates and blastocyst production rates), statistical modeling was performed to predict the risk of monochorionic dizygotic twin or conjoined twin formation under natural ovulation or ET conditions.

RESULTS

In a preliminary investigation of more than 300 hatching blastocysts, the orientation of the ICM with respect to TE herniation revealed 14% herniating all (8%) or part (6%) of their ICM first, while 86% retained their ICM within the ZP upon hatching. There were no observed problems associated with LZD-induced blastocyst trapping, as fully hatched blastocysts are commonly observed on day 6, and high implantation and live-birth rates of single or dual VFET of euploid blastocysts has occurred (see Table 1). The implantation of twin fetuses was significant after dual ET (DET), with no monozygotic twin formation after SET. Although one of the DET twins was initially diagnosed as sharing a single yolk sac (at 6 to 8 weeks), by 13 weeks a detailed ultrasound confirmed it was dichorionic dizygotic. It is interesting that implantation success was more efficient after SET, at 17.3% higher ($P<.05$), with no differences observed in clinical pregnancy outcomes.

A unique observation was made, however, after the first year: two prematurely hatching day-5 blastocysts had merged together (Fig. 1A, case A). These blastocysts had completely fused (i.e., amalgamation) to form a single blastocoel cavity

TABLE 1

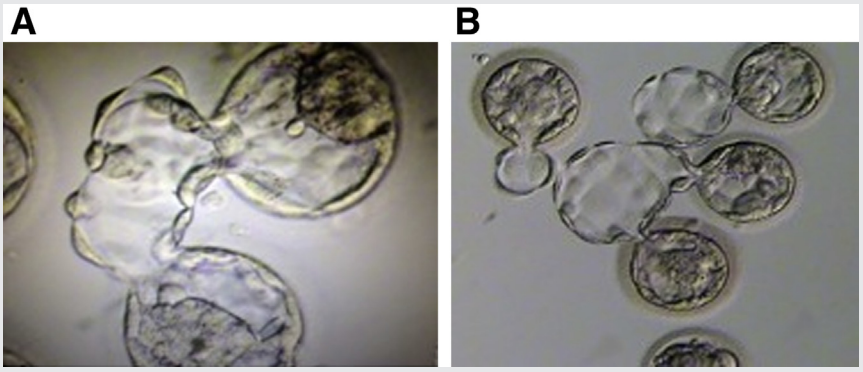
Efficiency of applying day-5 and day-6 blastocyst biopsy with preimplantation genetic screening technology in VFET cycles implementing single-embryo transfer (SET) or dual-embryo transfer (DET) of euploid blastocysts.

Parameter	Euploid SET	Euploid DET
No. of VFET cycles	42	36
Mean age	36.6	36.5
No. of embryo ET	42	72
Clinical pregnancies (%)	88.1	88.8%
Live-birth rate (%)	71.4	86.1
Implantation (%)	88.1	70.8[a]
Twin pregnancies (%)	0.0	44.4[a]

Note: ET = embryo transfer; VFET = vitrified embryo transfer.
[a] Column values within rows are different ($P<.05$).

Schiewe. Monochorionic dizygotic blastocysts. Fertil Steril 2015.

FIGURE 1



(**A**) Monochorionic dizygotic blastocyst formation in vitro is distinctly recognized by the complete amalgamation of fused TE layers, creating a shared blastcoelic cavity and separate fraternal inner cell masses (objective magnification: ×200). (**B**) Blastocysts grown in group culture often exhibit closely opposed hatching TE, but rarely experience cellular fusion (objective magnification: ×100).

Schiewe. Monochorionic dizygotic blastocysts. Fertil Steril 2015.

with two distinct ICM. This phenomenon was repeated 1.5 years later (see Fig. 1B, case B). Therefore, a formation of monochorionic dizygotic twins occurred twice over a 3-year period with over 3,000 embryos cultured for biopsy (1 of 1,500; <0.0007% occurrence). In these two cases, embryos had been cultured in groups of eight and six embryos per 25-μL droplet, which is within the optimal range of 2.5–5.0 μL per embryo, as was previously determined to enhance mouse blastocyst development (18).

These dizygotic twin blastocysts were successfully biopsied by first separating them in half, then rotating each collapsed blastocyst within their perivitelline space of the ZP. Subsequently, we biopsied the reannealed TE at an alternative site on each embryo, followed by vitrification but no transfer. In case A (biopsy ID 15), the patient had previously opted to not biopsy more than 15 blastocysts, so PGS was only performed on number 15A, which was determined to be a 46,XX euploid embryo. In case B (biopsy ID 6 and 7), the testing results revealed euploid 46,XX and aneuploid 45,XY; monosomy 8 blastocysts, respectively. Healthy twins and a singleton were produced upon VFET of dual (patient A) or single (patient B) blastocysts, respectively.

DISCUSSION

In the process of successfully applying day-5 or day-6 PGS technology, we have not only learned how resilient micromanipulated blastocysts are to cellular loss and damage but also how dynamic and aggressive cellular growth and migration of the TE can be before blastocyst biopsying. This is the first known documentation of unintentional, monochorionic dizygotic twin formation in vitro. Although experimental fusion of earlier embryo stages (≤morulae) has been performed to create chimeric individuals (19) or giant blastocysts (20) for interspecific embryo transfer and reconstruction studies, our amalgamated blastocysts pose a unique situation in terms of PGS analysis, with the mixing of TE cells. Our modified biopsying approach proved effective to ensure the accuracy of the euploid determination. The situation was brought to the attention of the physician and patient at the time of reporting the final cryopreservation outcome, along with a recommendation to delay the potential use of the blastocysts (i.e., if euploid) as a final option because of the risk of chimerism.

It should be noted that multiple blastocysts frequently hatch in close opposition, but they rarely experience cellular fusion and transmigration. Even our second amalgamated blastocyst had an additional 3AA and 4AA blastocyst contacting its TE without fusion (see Fig. 1B). We regard TE amalgamation as an uncommon, low-risk event that does not warrant any changes in our embryo culture/LZD procedure protocols. We adhere to the belief that the group culturing of embryos in microdroplets has a beneficial effect on blastocyst formation (21, 22), although the recent development of time-lapse imaging culture dishes with interconnected micro-drops/wells provides a viable alternative (23–26).

From our experience, we could envision how the rare formation of monochorionic dizygotic twins could occur in vivo, confirming the theory proposed by Miura and Niikawa (11). In predicting such a rare phenomenon, we have estimated the following statistical risk factors: twin ovulations (1/100 = 0.01), normal fertilization (85% = 0.85), high-quality blastocyst formation (50% = 0.5), both euploid blastocysts (assuming patient is 35–37 year old = 0.5^2 = 0.25), and the incidence of TE amalgamation (0.0007). Therefore, one could estimate the possibility of a monochorionic dizygotic twin forming in utero to be 1 in every 74 million ovulation cycles ($0.001 \times 0.85 \times 0.5 \times 0.25 \times 0.0007$). Additionally, factoring in the risk of amalgamation of both blastocysts herniating ICM first (8%; $P=\{0.08\}^2=.0064$), one could predict dizygotic conjoined twins occurring less than 1 in every 4.7 billion ovulation cycles. Of course, in utero twin blastocysts would not be likely to coexist in the confines of a 25-μL droplet of medium, thus explaining why such an in vivo physiologic condition has been rarely documented (27). Another possibility is the formation of a chimeric individual, which has been more frequently observed (8–10, 28).

Our report provides unique evidence of TE plasticity, which is not so surprising considering the programmed nature of aggressive TE transmigration of endometrial tissue after hatching. But what about ICM blastomeres—are they equally capable of such cellular chimeric mixing? Indeed they are, as evidenced by blastocyst reconstitution studies where injected ICM were capable of producing live chimeric offspring in rabbits (29), mice (30), and sheep/goats (18, 31, 32).

Monozygotic twin pregnancies are known to occur more frequently among in vitro-derived embryos (estimated 2%–3% occurrence) (33, 34). Such pregnancies progress with an increased risk of congenital malformations and a 25% risk of twin-twin transfusion syndrome and blood chimerism (4, 35). Our observations offer the possibility that some previously diagnosed monozygotic twins may have actually been same-sex, monochorionic dizygotic twins. The production of a dizygotic chimera or possibly a conjoined fraternal twin would be more likely to occur after the ET of two high-quality blastocysts in utero. In fact, several ART-produced monochorionic dizygotic twins have been documented (10, 11, 35). Such a statistical occurrence could be as low as 1 in every 56,000 transfers (assuming euploid blastocysts with an implantation rate of 0.80 × risk of TE amalgamation = 0.0007), with ICM-ICM chimerism occurring at least once in every 201,800 DETs. In non-PGS ET, the predictive models do not apply and would be significantly reduced because of lower live-birth rates and possible nonviable fusions with aneuploid embryos, which could still pose a chimeric risk.

Overall, this study provides factual evidence for the formation of monochorionic dizygotic twins, where possible conjoined or chimeric cellular fusion could materialize. The solution to averting such potentially undesirable situations while optimizing embryo implantation would be to adhere to a strict policy of euploid blastocyst SET, with the goal being "one embryo, one baby." It is interesting that VFET of a single-euploid blastocyst may increase the efficacy of implantation compared with DET, although live-birth rates are reduced due to other pregnancy-complicating factors. Inarguably, elective SET, with or without PGS, is the only way to prevent rare monochorionic dizygotic twin formations while minimizing the common complications associated with twin pregnancies.

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