Human zona pellucida micromanipulation and monozygotic twinning frequency after IVF

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To assess the association of zona pellucida micromanipulation and subsequent development of monozygotic twins, cases of assisted embryo hatching (AH) and intracytoplasmic sperm injection (ICSI) were identified and related to treatment type, implantation and zygosity data. Embryology records from all patients undergoing in-vitro fertilization (IVF) at this centre from January 1995 to March 1998 were reviewed. In this study, 3546 transfer cycles were completed, with clinical pregnancy established in 1911 (54% per transfer) patients undergoing a single IVF cycle. These pregnancies occurred in 1674 (88%) IVF cycles, 120 (6%) donor oocyte cycles (DER), and 117 (6%) frozen embryo transfer (FET) cycles. During the study period, 23 cases of monozygotic (MZ) twins were identified, representing an overall frequency of 1.2%. Chorionicity was determined by transvaginal ultrasound at 7 weeks when the number of embryos transferred was less than the number of fetal heartbeats, or when >1 fetal heartbeat per gestational sac was seen. Zygosity was confirmed by placental evaluation at delivery, and corroborated the antenatal diagnosis in all cases. Among IVF study patients the frequency of MZ twinning was not statistically different between zona manipulated and zona intact subgroups. While this investigation is the largest to date describing the relationship between MZ twins and zona procedures, studies with even greater statistical power are needed to clarify it more precisely, particularly in DER and FET settings. A greater overall frequency of MZ twinning for IVF patients may be a function of the higher number of embryos transferred in IVF, rather than discrete zona manipulations.

Key words: assisted hatching/in-vitro fertilization/monozygotic/twinning

Introduction

Monozygotic (MZ) twinning results from the division of a single fertilized ovum into two genetically identical embryos, and is thought to occur in 0.42% of all deliveries (Bulmer, 1970). Although the identical genotype in MZ twins has been well documented (Azuma et al., 1989), the aetiology of events precipitating the division of the early conceptus has yet to be clarified. Large-scale zygosity testing has shown that genetic differences may exist in MZ twins, as major discordances in birth weight, genetic disease and congenital anomalies have been observed (Machin, 1996).

Experimental studies using animal models have called attention to the zona as an important factor in the twinning equation. Veterinary research established that viable MZ twins could be produced by in-vitro ‘splitting’ of day-8 heifer embryos (Ozil, 1983). Serious developmental malformations in some offspring rendered this microsurgical technique of little value for human applications. Another microsurgical approach subjected sheep embryos to zona ripping, blastomere aspiration and repair of the zona breach by an agar ‘microbandage’ and was successful in producing some MZ twin sheep (Willadsen, 1979).

Unlike dizygotic twinning, the rate of MZ twinning appears to be relatively unaffected by race, age, family history or parity (Bulmer, 1970). Studies of families with very high frequencies of MZ twinning (Harvey et al., 1977; Shapiro et al., 1978) have suggested a heritable component to this process however. A review of outcomes in human assisted reproduction has thus far identified only two phenomena which appear to influence MZ twinning: ovulation induction (Derom et al., 1987) and zona pellucida (ZP) architecture or manipulation (Alikani et al., 1994). Certain IVF culture conditions may also be contributory to twin development. Since intracytoplasmic sperm injection (ICSI) and assisted embryo hatching (AH) are both zona-breaching procedures commonly used in IVF, the subject of MZ twins in the context of ZP micromanipulation has been actively studied (Alikani et al., 1994; Slotnick and Ortega, 1996; Hershlag et al., 1997). However, the role of AH and ICSI (when used either alone or together) in the development of MZ twins has thus far only been fully assessed in small patient groups. To evaluate the potential connection between assisted hatching, ICSI, and the development of MZ twins, the clinical features of 23 IVF cycles resulting in MZ twins were reviewed and compared with a control group of 1888 non-twin pregnancies. Additionally, frozen embryo transfer, donor oocyte and standard IVF cycles were compared with respect to the frequency of MZ twinning.

Materials and methods

Monozygotic twins were identified by transvaginal sonography on day 49 when the number of fetal heartbeats exceeded the number of...
gestational sacs, or when two heartbeats were seen within a single gestational sac. Clinical pregnancy, implantation and embryology data were analysed for all IVF patients treated at The Center for Reproductive Medicine & Infertility, New York Presbyterian Hospital–Weill Medical College of Cornell University for a 39 month period (January 1995–March 1998 inclusive).

**Ovarian stimulation and monitoring**

Pituitary down-regulation was achieved with leuprolide acetate (1 or 0.5 mg/day, s.c.) commencing 8 days after ovulation in the cycle preceding gonadotrophin treatment. The gonadotrophin releasing hormone (GnRH) agonist dose was reduced by half on the first day of gonadotrophin therapy, and maintained at that level until the day of human chorionic gonadotrophin (HCG) administration. Ovulation induction was achieved with 2–6 ampoules of gonadotrophin. Daily gonadotrophin dose was adjusted in response to follicular growth and serum oestradiol concentrations (Davis and Rosenwaks, 1992). In general, a ‘step-down’ decremental pattern was followed (Damario et al., 1995). Up to 10 000 IU HCG was given i.m. when at least two follicles were ≥17 mm diameter (Sills et al., 1998).

**Oocyte retrieval**

Transvaginal ultrasound-guided needle aspiration of oocytes was performed 34–35 h after HCG administration. Propofol (1 mg/kg) with fentanyl citrate (100 µg) was given i.v. for analgesia in all cases. Immediately following retrieval, oocytes were washed in HEPES-buffered human tubal fluid (HTF) prepared in our laboratory (L.L.V.) and placed into droplets of HTF + 10% maternal serum under mineral oil (Squibb, Princeton, NJ, USA). Cells were incubated in a humidified 5% CO₂ atmosphere at 37°C.

Oocytes were graded for maturity using cumulus expansion criteria (Veeck, 1988). After 5 h, processed spermatozoa (final concentration = 1.5×10⁵/ml) were added to droplets of medium each containing one oocyte. In cases of male factor infertility, ICSI was performed (Palermo et al., 1996). Fertilization was considered successful after noting the presence of two pronuclei and the second polar body. After 12–18 h, oocytes were transferred to droplets of fresh media with 15% maternal serum for continued culture.

**Assisted embryo hatching**

For patients receiving AH, the zona pellucida was artificially opened using acidic Tyrode’s solution (pH 2.35). Clinical indications for use of AH included maternal age, embryo fragmentation and zona thickness (Zaninovic, 1998).

Holding micropipettes were cut and fire-polished on a microforge (Narishige, Tokyo, Japan) to a final outer diameter of 60–80 µm and inner diameter of 20 µm. A similar approach was used to fashion the hatching micropipette, although its outer diameter was ~10 µm. The embryo was positioned on the holding pipette so that the AH micropipette tip would be targeted either at a zona region overlying vacant perivitelline space between blastomeres, or an area adjacent to cytoplasmic fragments. The hatching site was consistently oriented at the 3 o’clock position. Acidic Tyrode’s was gently expelled over a small area of 20–30 µm, which corresponded to the dimensions of the hatched site for all study patients.

**Embryo transfer**

Embryos were transferred at 72 h following oocyte retrieval, delivered ~1 cm inferior to the uterine fundus. Embryos were suspended in 20–30 µl 75% maternal serum in HTF contained within a Wallace (Marlow Surgical Technology, Willoughby, OH, USA) or TomCat (Sherwood Medical, St Louis, MO, USA) catheter. Non-transferred embryos suitable for freezing were cryopreserved if requested by the patient.

**Luteal support and pregnancy determination**

All patients received i.m. progesterone (50 or 100 mg/day progesterone in oil) following fertilization. Progesterone was maintained at this dose either until a negative pregnancy test was obtained (βHCG <5 IU) 2 weeks post-transfer, until fetal cardiac activity was seen via sonogram at cycle day 49, or until pregnancy failure or demise was diagnosed. All pregnancy sonograms were performed by physicians using a 5.0 MHz transvaginal probe (Logiq 400, GE Medical Systems, Milwaukee, WI, USA). Postpartum placental examination confirmed the antenatal zygosity diagnosis for all deliveries.

**Measured variables and statistical analysis**

The following variables were evaluated: (i) the general frequency of MZ twins in the total IVF patient population, (ii) MZ twin frequency among patients receiving either AH, ICSI, or both, and (iii) MZ twin frequency among patients not receiving these treatments.

For comparisons in MZ twin frequency for a function of a single variable (i.e. AH versus non-AH), Pearson’s χ²-test without Yates’ continuity correction was used.

A computer-assisted medical record review was utilized for data collection (Windows NT, Microsoft, Redmond, WA, USA; Paradox for Windows NT, Borland/Inprise, Scotts Valley, CA, USA). Data and power analysis of measured variables was performed by computerized data program (EpInfo 6.04b, CDCP/WHO, Atlanta, Georgia USA, Geneva, Switzerland, and S-plus Solaris 5.0, Mathsoft Inc., Seattle, WA, USA). P < 0.05 was considered statistically significant for all comparisons.

**Results**

A total of 4493 IVF cycles was initiated from January 1995 to March 1998, 3546 (79%) of which proceeded to transfer. Cycles were grouped into one of three categories for analysis: (i) standard IVF–embryo transfer, (ii) donor oocyte recipient (DER), and (iii) frozen–thawed embryo transfer (FET). There were 121 DER and 543 FET cycles assessed in the study period. The number of twin sets (all types) delivered from all cycles included 121 DER and 543 FET cycles assessed in the study period. The number of twin sets (all types) delivered from all cycles including 121 DER and 543 FET cycles assessed in the study period. The number of twin sets (all types) delivered from all cycles was 512.

Mean patient age (± SD) for all groups during this period was 35.0 ± 4 years (Table I). Clinical pregnancy (defined as

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### Table I. Summary of IVF cycles resulting in a clinical pregnancy showing how many monozygotic twins for each treatment type

<table>
<thead>
<tr>
<th>Clinical pregnancy cycles</th>
<th>Patients (%)</th>
<th>Age (years)ᵃ</th>
<th>ETᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>1911</td>
<td>35.0 ± 4</td>
<td>3.6</td>
</tr>
<tr>
<td>IVF transfers,</td>
<td>1674</td>
<td>35.0 ± 4.3</td>
<td>3.6</td>
</tr>
<tr>
<td>of which, MZ twins</td>
<td>19 (1.1)</td>
<td>35.2 ± 4</td>
<td>3.2</td>
</tr>
<tr>
<td>DER transfers,</td>
<td>120</td>
<td>28 ± 3.0⁷</td>
<td>4.3</td>
</tr>
<tr>
<td>of which, MZ twins</td>
<td>3 (2.5)</td>
<td>32.0 ± 8</td>
<td>3.0</td>
</tr>
<tr>
<td>FET transfers,</td>
<td>117</td>
<td>33.7 ± 4.4</td>
<td>3.2</td>
</tr>
<tr>
<td>of which, MZ twins</td>
<td>1 (0.9)</td>
<td>28</td>
<td>3.3</td>
</tr>
</tbody>
</table>

ᵃMean ± SD.
bComparison of transferred embryo number P > 0.05 for all categories, by one-way analysis of variance.
³Age of oocyte donor.
ET = average no. embryos transferred; MZ = monozygotic twins; DER = donor egg recipient; FET = frozen-thawed embryo transfer.
Table II. Monozygotic twin distribution among IVF, DER and FET study patients (n = 1911)

<table>
<thead>
<tr>
<th>Clinical pregnancy cycles</th>
<th>AH only (%)</th>
<th>ICSI only (%)</th>
<th>AH + ICSI (%)</th>
<th>Neither AH nor ICSI (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>645</td>
<td>177</td>
<td>876</td>
<td>213</td>
<td></td>
</tr>
<tr>
<td>IVF transfers</td>
<td>589</td>
<td>151</td>
<td>813</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>of which, MZ twins</td>
<td>8 (1.4)</td>
<td>1 (0.7)</td>
<td>7 (0.9)</td>
<td>3 (2.5)</td>
<td>0.4a</td>
</tr>
<tr>
<td>DER transfers</td>
<td>38</td>
<td>9</td>
<td>37</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>of which, MZ twins</td>
<td>1 (2.6)</td>
<td>0</td>
<td>2 (5.4)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>FET transfers</td>
<td>18</td>
<td>17</td>
<td>26</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>of which, MZ twins</td>
<td>0</td>
<td>1 (5.9)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

aBy Pearson’s χ²-test (without Yates’ continuity correction).
bSample size insufficient for analysis.

AH = assisted hatching; MZ = monozygotic twins; FET = frozen–thawed embryo transfer; DER = donor egg recipient.

Figure 1. Ultrasound image of monozygotic (MZ) twins at cycle day 49, showing two embryos (A and B) partitioned by fused amniotic membranes (white arrow) and surrounded by a common chorion (dark arrows). Diamnionic–monochorionic MZ twins are thought to result from embryonic ‘splitting’ between the fourth and eighth day after fertilization.

an intrauterine pregnancy with fetal cardiac activity) was established in 1911 patients (or 54% of transfers). Each patient underwent a single IVF treatment in this investigation. AH was undertaken (either alone or with ICSI) in 1521 (80%) patients. The combined techniques of ICSI and AH were done in 46% (n = 876) of 1911 cases (Table II).

Transvaginal sonography on cycle day 49 revealed MZ twins in 23 of 1911 clinically pregnant patients (Figure 1). This corresponded to an overall MZ twinning rate of 1.2% at this centre. Of all MZ twin pregnancies, 19 (83%) developed in IVF patients, three (13%) were in DER, and one (4%) followed FET. The average number of embryos transferred in MZ twin cases was lower than for non-twin cycles for all treatment categories except FET. The number of embryos transferred in twin and non-twin cycles was not significantly different. For all study patients, the average number of transferred embryos was 3.6.

Among IVF patients with MZ twins (n = 19), the mean patient age (± SD) was 35.2 ± 4 years. In this subgroup, AH was performed in 79% (n = 15); three (13%) of the MZ twin sets did not receive any zona treatment (neither AH nor ICSI) prior to embryo transfer. Of 15 cases of MZ twins that received AH, seven (47%) received this treatment with ICSI and eight (53%) received AH alone.

In 543 FET cycles, 117 (22%) resulted in clinical pregnancy during the study period. Although all patients received embryo thaw and transfer during the study period, cryopreservation of embryos in some cases was performed as early as April 1993. In the FET subgroup, only one case of MZ twins occurred, corresponding to an overall MZ twinning rate of 0.8%. Of all clinical pregnancies following FET therapy, 56 (47%) received no zona treatment before or after freezing.

For DER cycles in the study period, mean anonymous oocyte-donor age was 28.0 ± 3.0 years. For the three donor oocyte recipients with MZ twins, the ages of their donors were somewhat higher (32.0 ± 8.0 years), but not significantly
different ($P > 0.05$). Two recipients developing MZ twins received oocytes from two anonymous donors participating in shared oocyte donation (i.e. one donor matched to two recipients). One recipient with MZ twins received donated oocytes from her sister.

When MZ twinning frequency as a function of zona micro-manipulation was compared among patients in the largest study group (IVF only), no significant difference was identified ($P = 0.4$). Similarly, differences in MZ twinning rates according to zona status among DER and FET subgroups were not observed. These latter findings must be interpreted with caution, however, because the low natural frequency of MZ twinning necessitates a very large sample ($>10,000$ cases) to achieve satisfactory statistical power.

**Discussion**

In humans, MZ (‘identical’) twins have long been considered a rarity, and depictions of such remarkable births have been recovered among the artefacts of ancient civilizations (Margalith, 1994). Modern scientific advances have provided greatly increased understanding of the physiology, classification, and management of twin gestation, but the cause of MZ twinning remains unclear. This study is the largest series of MZ twins following zona manipulation yet reported in an IVF setting; it aimed to elucidate the putative effect that such zona treatments may have on identical twinning.

While MZ twins are thought to result from the division of a single fertilized egg to form two genetically identical embryos, the precise mechanism(s) responsible for this division are not known. However, several observations have yielded theoretical explanations of the process. For example, the occurrence of inner cell mass ‘splitting’ might cause duplication of the embryo at an early developmental stage (Malter and Cohen, 1989). This splitting hypothesis was supported by animal studies showing that mechanical cleavage of mammalian embryos in vitro could produce MZ or ‘identical’ twins (Willadsen, 1979; Ozil, 1983). While these microsurgical approaches proved effective in the laboratory, the de-novo embryonic fission apparently required for spontaneous MZ twinning has never been directly observed.

Talansky and Gordon (1988) proposed that zona drilling facilitated twinning by inducing a conformational change in some mouse blastocysts. Specifically, some blastocoeles associated with micromanipulation assumed a ‘figure of eight’ shape as the cells attempted to squeeze through the artificially created zona aperture. Some investigators found complete murine blastocoele expansion unimpeded by the zona constriction after hatching in most cases (Malter and Cohen, 1989), but ‘trapping’ was noted in some embryos that received zona drilling (59/132, or 45%) when observed 5 days post-manipulation. One human blastocyst experimentally treated with partial zona dissection ‘hatched partially’ on day 8 and was noted to ‘fold double and split’ resulting in two distinctly separate but grossly unequal blastocoeles (Malter and Cohen, 1989). The connection between assisted embryo hatching and MZ twins remains highly speculative, as blastocoeles have never been observed to divide evenly and completely after passing through an artificial zona opening, either in humans or in any animal model.

A review of six cases of MZ twins with either naturally thin zonae or where zona micromanipulation was performed suggested that zona architecture plays an important role in the development of MZ twins (Alikani et al., 1994). Other investigators (Slotnick and Ortega, 1996) reported five cases of MZ twins after AH from 142 pregnancies and concluded that mono-amniotic multiple gestations were increased in zona-manipulated cycles. More recently, a study of nine MZ twin pregnancies proposed an association between assisted hatching and MZ twinning (Hershlag et al., 1997).

The relevance of MZ twinning in view of assisted conception outcomes is based on the markedly increased hazards attendant to pregnancies of this type. Twin–twin transfusion syndrome (Talbert et al., 1996), fetal entanglement and umbilical cord accidents (Nyberg et al., 1984), caudal regression syndrome (Pfeiffer et al., 1991), and other developmental anomalies (Schnizel et al., 1979) are much more common among MZ twin gestations than in singleton pregnancies (Powers, 1973). Since the potential for such serious pregnancy complications or demise in MZ twins was recognized as high, this investigation did not rely on delivery data or placental morphometry for documentation of twinning; early first trimester sonography was used instead. The accuracy of this method in the determination of MZ twins has been reported by others (Mahony et al., 1984; Barss et al., 1985; Finberg, 1992; Montaegudo et al., 1994; Skupski et al., 1995).

The subjects of MZ twinning and infertility treatments converged soon after the realization that IVF could be associated with an increased frequency of MZ twinning (Edwards et al., 1986). This observation was refined by a study of more than 2500 multiple births (Derom et al., 1987), which postulated that ovulation induction itself and not IVF embryo culture conditions was responsible for the markedly higher rate of MZ twinning following infertility treatments. Increased numbers of mono-amniotic multiple gestations following zona-manipulated cycles have been reported (Slotnick and Ortega, 1996), but this was based on self-reported, anonymous data supplied by 42 IVF centres in the USA. More recently, a 12-fold increase in MZ twins (chorionicity not specified) was identified in a series of >600 patients who received single embryo transfer (Blickstein et al., 1999). An explanation for a high rate of monoamniotic gestations after zona tampering has not yet been articulated.

In any case, the matter of MZ twinning as a sequela of assisted embryo hatching rarely overshadowed the larger debate regarding the efficacy of, and selection criteria for, this procedure. For example, some investigators have reported zona pellucida thickness to be inversely correlated with oocyte fertilization rate in conventional (non-ICSI) IVF (Bertrand et al., 1995). While some authors report improved reproductive outcomes after assisted embryo hatching (Stein et al., 1995; Check et al., 1996; Hu et al., 1996; Parikh et al., 1996), others have found the treatment to be of questionable benefit (Hellebaut et al., 1996; Lanzendorf et al., 1996; Tucker et al., 1996; Hurst et al., 1998). It was not the aim of our report to...
offer a position in these debates, but rather to revisit the issue of whether such zona treatments influence MZ twinning.

While both AH and ICSI involve an artificial breach of the zona pellucida, they differ markedly in the size of the associated defect. Zona openings formed by AH in this investigation were ~25–30 µm in diameter, while the puncture sites following ICSI were much smaller (~7–8 µm). In contrast to AH, ICSI involves no overall loss of zona material. Indeed, the zona defect following ICSI is thought to be similar in size to that link between MZ twinning and assisted embryo hatching, given the size and number of zona openings has been shown to influence hatching and trophoblast outgrowth in mouse embryos (Cohen and Feldberg, 1991). The rate of MZ twinning among ICSI-only cycles in our study, however, was not unusually high.

An analysis of factors affecting zona characteristics is appropriate since the zona pellucida is central to several theories regarding MZ twinning. A synthesis of findings from earlier studies (Edwards et al., 1986; Derom et al., 1987; Alikani et al., 1994) suggests at least three factors are influential in MZ twinning among patients receiving infertility treatments: ovulation induction per se, certain IVF culture conditions, or zona architecture/micromanipulation. With these three variables occurring together so often in modern clinical infertility practice, multiple regression analysis to determine the specific contribution of each intervention has proven difficult to perform. If the ‘natural’ rate of MZ twinning (0.42%) may be considered valid in settings of spontaneous conception and single embryo implantation, then the ~3-fold increase in the observed MZ twinning rate reported here for IVF patients may be partially explained by the increased number of implantations.

Of note, multiplication of the accepted ‘natural’ MZ twinning frequency (0.42%) by the average number of embryos transferred in our MZ twin subgroup (3.2) yields 1.3%, a value very near to the rate of MZ twinning observed in our population (1.2%). Intermediate between our findings in IVF and the ‘natural’ MZ twinning rates given by Bulmer (1970), are the results following ovulation induction published by Derom et al. (1987), where presumably the number of implantations was higher than in spontaneous conception but lower than in IVF.

Importantly, the clinical rarity of MZ twinning challenges the study of this phenomenon in the context of IVF, as relevant investigations require large samples (>10 000 cases) to detect meaningful differences with suitable statistical power. In our analysis of MZ twin frequency in IVF patients as a function of zona treatment type versus no ZP treatment, the statistical power was limited (0.50) due to sample dimensions. Likewise, even though this series embraced over 1500 AH cycles, a comprehensive assessment of zona characteristics means that this sample must be partitioned according to whether AH occurs alone or in tandem with ICSI, and as a function of DER or FET treatment status as well. Sufficient (0.80) statistical power for such a matrix would depend upon the relative frequencies of these treatment variables, but could require as many as 20 000 cases to detect differences at the P = 0.05 significance level. As the dimensions of the current MZ twins/zona micromanipulation series exceed those of previous reports, earlier conclusions based on even smaller and less persuasive sampling cannot be definitive and merit re-evaluation.

The physiology of both MZ twin evolution and natural blastocyst hatching in the human remain incompletely characterized, and what is known has largely been extrapolated from animal models. It may therefore be premature to attempt a link between MZ twinning and assisted embryo hatching, given the current limited knowledge regarding both phenomena. Although this report finds the part played by AH and ICSI in MZ twinning negligible, the exact roles of these zona treatments remain incompletely defined in the MZ twinning process; continued large-scale, clinical studies of sufficient statistical power will therefore be needed.

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