Monozygotic twinning following assisted conception: an analysis of 81 consecutive cases

Mina Alikani¹,², Natalie A.Cekleniak¹, Eurof Walters² and Jacques Cohen¹

¹The Institute for Reproductive Medicine and Science of Saint Barnabas Medical Center (IRMS-SBMC), 101 Old Short Hills Road, Suit 501, West Orange, NJ 07052, USA and ²Thorpes, The Grip, Linton, Cambridge CB1 6NR, UK

BACKGROUND: This study attempts to identify risk factors for monozygotic (MZ) twinning following assisted conception. METHODS: Eighty-one MZ twinning pregnancies that occurred over a 7-year period in our IVF/embryo transfer programme were evaluated. These were compared with 4224 non-MZ pregnancies from the same period. RESULTS: The overall incidence of MZ twinning was 1.88% (81/4305) of all clinical pregnancies. A total of 63% (51/81) of the MZ pregnancies included one or more other implantations as well. MZ twinning was unrelated to maternal age, paternal age, gonadotrophin dosage, peak estradiol and progesterone levels, number of oocytes collected, and number of embryos replaced. Patients with MZ twinning had significantly more attempts at assisted reproduction than those with non-MZ multiple pregnancy. A logistic regression analysis suggested a role, albeit not emphatic, for the zona pellucida in MZ twinning. Twenty-four of the 65 MZ cases with known placentation were monoamniotic. This incidence far exceeds that seen following spontaneous conception (P < 0.0001). CONCLUSIONS: The risk of monoamniotic twinning is increased following IVF/embryo transfer. Zona pellucida disruption appears to increase the incidence of MZ twinning. However, the overall micromanipulation data together with the unexpected placentation data suggest that zona-mediated embryo splitting is not the only mechanism of twinning under artificial conditions.

Key words: blastocyst/monoamniotic/multichorionic/monozygotic twinning/ovulation induction

Introduction
Roughly 1.5% of all clinical pregnancies following ovulation induction with gonadotrophins [with or without IVF/embryo transfer] are diagnosed as monozygotic (MZ) multiple gestations (Edwards et al., 1986; Derom et al., 1987; Wenstrom et al., 1993). This incidence is over four times the natural incidence of MZ twins, which has been quoted as three to four per 1000 live births (Bulmer, 1970). Monozygotic multiple pregnancies are generally identified when ultrasound examination around 7 weeks gestation reveals a gestational sac containing more than one fetus, either in one amniotic sac (monoamniotic-multichorionic) or in two sacs separated by a septum (diamniotic-multichorionic). Following IVF/embryo transfer, MZ twinning is also implied when the number of fetuses exceeds the number of embryos replaced in the uterus.

Monoamnioticity, which occurs in two-thirds of spontaneously conceived MZ twins, increases the risk of adverse outcome, including perinatal mortality, preterm birth and birth weight discordance (Machin et al., 1995; Dube et al., 2002). The association between MZ twinning and assisted conception is therefore of great clinical interest.

Apart from ovulation induction per se (Derom, 1987), the debate on the aetiology of MZ twinning in the context of assisted reproduction has mainly concerned the role of the zona pellucida, and in particular, the effects of artificially imposed alterations in its structure during IVF (Edwards et al., 1986). Opinion is rather divided, with some reports proposing a role (Alikani et al., 1994; Slotnick and Ortega, 1996; Saito et al., 2000; Schieve et al., 2000), and others discounting such a role (Blickstein et al., 1999; Sills et al., 2000; Schachter et al., 2001). The high frequency of MZ twinning following blastocyst transfer (Behr et al., 2000; da Costa et al., 2001) complicates matters further, since zona manipulation is rarely involved in such transfers, but zona hardening may result from prolonged culture (Edwards et al., 1986).

Valid as they may be, many of these arguments are based on small numbers of cases, and are put forth without consideration of all potential confounding factors, patient- and embryo-related.

In this report, we present a systematic approach to the identification of any distinguishing characteristics of MZ twinning conceptions following IVF/embryo transfer. Eighty-one MZ twinning cases were thus evaluated and compared with
Materials and methods

Patients

The study was approved by the Internal Review Board of Saint Barnabas Medical Center (IRB Protocol Number 01-20). All patients (or their oocyte donors) underwent ovulation induction, ultrasound-guided oocyte retrieval, IVF, and non-surgical ultrasound-guided intra-uterine transfer of fresh or frozen–thawed embryos. The criterion for inclusion of patients in the study was confirmation of clinical pregnancy by detection of fetal heart activity by 6 weeks gestation. A total of 4305 cycles with singleton and multiple gestations were thus included. Eighty-one cycles involved two or more MZ fetuses. Of these, 64 were regular fresh embryo transfer cycles, eight were oocyte donation cycles and nine were regular or oocyte donation frozen–thawed embryo transfer cycles.

Ovulation induction, IVF/embryo transfer and assessment of pregnancy

All patients undergoing IVF were placed on one of two protocols: (i) mid-luteal (cycle day 21) down-regulation with leuprolide acetate (0.5 mg) (Lupron; Tap Pharmaceuticals Inc., Lake Forest, IL, USA) followed by stimulation with gonadotrophins on day 3 of the subsequent menses; or (ii) a microdose leuprolide acetate (50 μg twice a day) flare protocol utilizing leuprolide on day 2 of an oral contraceptive withdrawal bleed followed by gonadotrophin stimulation just 1 day later (day 3 of menses). Patient diagnosis and previous response were used to determine the optimal protocol. Various urinary gonadotrophins (Metrodin; Serono Laboratories Inc., Randolph, MA, USA; Pergonal; Serono Laboratories Inc.; Humagone; Organon Inc., West Orange, NJ, USA; Repronex; Ferring Pharmaceuticals Inc., Tarrytown, NY, USA) and recombinant gonadotrophins (Gonal F; Serono Laboratories Inc.; Follistim; Organon Inc.) were employed at different times. The daily gonadotrophin dosage varied but was usually between 300 and 450 IU. Ovulation was triggered with either 5000 or 10 000 IU of hCG (Profasi; Serono Laboratories Inc.; Pregnyl; Organon Inc.; Novarel; Ferring Pharmaceuticals Inc.). For transfer of frozen–thawed embryos, either a natural cycle or a cycle medicated with luteal leuprolide acetate and oral estradiol (E2) was used to time the day of thaw and replacement.

Oocyte retrieval was performed 35 h after hCG administration. Insemination was either by standard methods or by ICSI in case of male factor infertility. Fertilization was assessed ~24 h from oocyte retrieval (~18 h from insemination). Thereafter, embryos were assessed daily until the time of replacement. Embryo quality assessment and selection for replacement and cryopreservation have been described in detail elsewhere (Alikani et al., 2002). Embryo replacement was primarily on day 3 after oocyte retrieval. However, 88 pregnancies (including three with MZ twinning) in this study resulted from day 5 blastocyst replacements. Embryos were evaluated on the morning of day 3 of development. Cell number and symmetry, fragmentation, multinucleation and zona morphology were noted. Embryos were selected for assisted hatching on the morning of day 3 of development, before replacement in the uterus in the afternoon of the same day. The criteria for application of assisted hatching and the method of assisted hatching and fragment removal have been described in detail elsewhere (Cohen et al., 1992; Alikani, 2001).

From August 1995 until October 1999, the average numbers of embryos replaced in regular (average age 35.46 years) and oocyte donation cycles were 3.31 and 2.72, respectively. In October 1999, a major organizational overhaul in our centre allowed implementation of a policy that aggressively reduced the number of embryos for replacement. This was despite a significant increase in the average age of the patients treated. The average numbers of embryos replaced in regular (average age 36.57 years) and oocyte donation cycles since then are 2.84 and 2.21, respectively.

Intramuscular progesterone (50 mg) was first administered 3 days prior to oocyte retrieval and continued until βhCG was assayed 16 days after hCG administration. Progesterone was continued in case of a positive βhCG until the first ultrasound was performed at 5 weeks gestation. At this time, the number of gestational sacs, and if visible, yolk sacs, was noted. At 6 weeks gestation, fetal heartbeat was assessed and crown–rump length (CRL) measurements were taken. At 7 weeks, in addition to measurement of fetal heartbeat, interval growth was assessed with CRL. The final ultrasound prior to patient discharge for obstetric care was performed at 8 weeks and evaluated for growth and presence of a fetal heartbeat within the normal developmental range.

When the number of yolk sacs, fetal poles or fetal heartbeats exceeded the number of embryos replaced, or two or more yolk sacs, fetal poles or heartbeats were observed within a single gestational sac, the patient was evaluated for MZ twinning. In case of the latter, the pregnancy was followed carefully for the presence of a dividing membrane between the fetuses. A diamniotic-monochorionic placentation was diagnosed by visualization of the delicate, thin membrane separating the fetuses. If no membrane was seen, placentation was assessed as monoamniotic-monochorionic.

When the number of gestational sacs exceeded the number of embryos replaced, but the extra sacs appeared without evidence of embryo development, the situation was judged to be ambiguous with regard to zygosity. Although these cases may indeed represent MZ twinning, conclusions cannot be drawn with certainty, since a small fluid collection could potentially be mistaken for a gestational sac. These pregnancies (n = 11) were excluded from the present analysis.

Diamniotic-dichorionic MZ twinning cases were assumed to have occurred when a single embryo replaced gave rise to two or more gestational sacs with fetal poles or fetal hearts, or the number of such gestational sacs exceeded the number of embryos replaced. Other like-sex dichorionic multiple gestations (or births) could only have been identified as MZ using DNA fingerprinting (Machin, 1990), which was not performed for this study. This is a potential source of underestimation of MZ twinning.

Data collection and statistical analysis

Data were collected from a large database (EggCyte™, 1995–2002; ART Institute of New York and New Jersey, Livingston, NJ, USA) containing patient demographics and clinical parameters for each cycle included in the study, as well as individual entries for oocytes and embryos that resulted from each cycle. The database has been described in detail elsewhere (Tomkin and Cohen, 2001).

Continuous random variables such as age and hormone levels were analysed by means of standard analysis of variance (ANOVA) methods. Proportions were analysed by logistic regression. In order to identify the possible association between MZ twinning and zona pellucida drilling for assisted hatching, several potential confounding variables were included in many analyses. However, on some occasions there were limited opportunities to impose this refinement, due to the sparsity of data for certain subgroups. We therefore restricted our investigation to selected subgroups in the database (see below).

Univariate ANOVA was used to identify variables that could distort the main investigation, and such variables were then included in multifactor analyses. The results of analyses are presented as
Table I. Overall incidence of MZ twinning

<table>
<thead>
<tr>
<th>Cycle type</th>
<th>Non-MZ twin pregnancies</th>
<th>MZ twin pregnancies</th>
<th>Total</th>
<th>Incidence of MZ twinning (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular</td>
<td>3185</td>
<td>64</td>
<td>3249</td>
<td>1.97</td>
</tr>
<tr>
<td>Recipient</td>
<td>537</td>
<td>8</td>
<td>545</td>
<td>1.47</td>
</tr>
<tr>
<td>Thaw</td>
<td>502</td>
<td>9</td>
<td>511</td>
<td>1.76</td>
</tr>
<tr>
<td>Total</td>
<td>4224</td>
<td>81</td>
<td>4305</td>
<td>1.88</td>
</tr>
</tbody>
</table>

When data are assembled from patient records within one treatment centre, as is the case in this and many other studies, there is one further matter that demands attention. The frequencies in the various subclasses can be regarded as being representative of the pattern existing at the centre and the data analysed accordingly using a ‘survey’ type model. Alternatively, these frequencies may be regarded as arbitrary, and the analysis may be performed using a ‘fixed effects’ model. The estimated mean values for a particular factor in a fixed effects model are adjusted for all the other factors in the model. This is so that the effect of the factor of interest can be viewed in isolation without skewing by the effect of other factors. When data are very sparse (or indeed completely absent) for some subgroups, the analysis is often erratic and may give anomalous results. For that reason it is prudent to carry out some investigations only on those portions of the database where there is reasonable representation for all subgroups.

Results

The incidence of MZ twinning

The overall data are presented in Table I and II. The incidence of MZ twinning among all clinical pregnancies was 1.88% (81/4305). When categorized according to the type of cycle attempted, the incidence was 1.97% (64/3249) for regular fresh embryo transfer cycles, 1.47% (8/545) for oocyte donation fresh embryo transfer cycles, and 1.76% (9/511) for regular and oocyte donation frozen–thawed embryo transfer cycles. The latter group was analysed separately, since the circumstances of these patients differed from those of others.

Overall implantation rate, defined as the number of fetal hearts detected per embryo replaced, was 93% (238/256). Counting MZ fetuses as single implantations, the implantation rate was 59% (153/256). In total, 39% (94/238) of the detected fetuses in MZ pregnancies were spontaneously or selectively aborted, the majority having been MZ pairs, but the actual number of MZ fetuses could not be ascertained. Four pairs of MZ twins were stillborn. The live birth (and ongoing pregnancy) rate was 57% (136/238).

Patient profile and MZ twinning

The results are summarized in Tables III and IV. Oocyte donation cycles were excluded from the analyses in Table III to avoid skewing of data by fertile oocyte donors. Three groups were defined: group A included pregnancies with evidence of MZ twinning; group B included non-MZ multiple pregnancies; and group C included singleton pregnancies. The factors assessed included maternal age, paternal age, the total number of assisted conception attempts, the number of ampoules of drug given, number of days of gonadotrophin administration, peak E2 level at the time of hCG, peak progesterone level at the
time of hCG, number of oocytes retrieved, and number of embryos replaced. The total number of assisted conception attempts was higher for group A than group B ($P < 0.01$). No significant differences were found with respect to any other parameters, although a slight trend toward a difference in the age of the patients in groups A and B was noted ($P = 0.07$), with group A patients being on average older than group B patients.

Table IV contains details of all fresh embryo transfer pregnancy cycles including oocyte donation cycles. Embryo quality was determined by comparing the mean number of cells for the cohort of embryos on the morning of day 3 of development and at the time of replacement, total cytoplasmic volume lost to fragmentation at the time of evaluation, fragmentation at the time of replacement, the mean percentage of embryos with fragments removed before replacement and the mean percentage of embryos with multinucleated blastomeres. No significant differences were found between groups A and B.

The same analyses were repeated for frozen–thawed embryo replacements and no significant trends were seen (data not shown).

The incidence of MZ twinning was assessed for patients whose eggs were treated with ICSI or standard insemination. Monozygotic twinning occurred in 37 of 1537 cycles (2.35%) with ICSI and 35 of 2185 (1.58%) with standard insemination. This difference is not statistically significant. The rate of MZ twinning following zona drilling for blastomere biopsy was unremarkable (1/140; 0.71%).

The relationship between MZ twinning and zona drilling for assisted hatching is presented in Table V. The patients in groups A and B were divided into three subgroups of patients in whom none, some or all of the replaced embryos (two, three or four embryos replaced) had assisted hatching. The proportions are corrected for the number of embryos replaced and whether or not ICSI was applied at the same time. MZ twinning appears to increase with increased hatching. Mean age of the patients in each subgroup was evaluated. The differences among the age of subgroup patients are significant, but the mean age of the patients in groups A and B is very similar.

### Table IV. Embryology profiles of all fresh embryo transfer pregnancies [values are mean (± SEM)]

<table>
<thead>
<tr>
<th>Pregnancy group</th>
<th>No. patients</th>
<th>Day 3 cell number</th>
<th>Cell number at ER</th>
<th>Day 3 fragmentation volume (%)</th>
<th>Percentage of embryos with FR</th>
<th>Fragmentation volume at replacement</th>
<th>Percentage of embryos with MNB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>72</td>
<td>6.50 (0.14)</td>
<td>7.80 (0.12)</td>
<td>15.9 (1.17)</td>
<td>64.4 ± 4.00</td>
<td>9.00 (0.75)</td>
<td>13.5 (1.67)</td>
</tr>
<tr>
<td>Group B</td>
<td>1634</td>
<td>6.55 (0.10)</td>
<td>7.69 (0.02)</td>
<td>15.8 (0.23)</td>
<td>64.3 ± 0.88</td>
<td>8.92 (0.16)</td>
<td>11.2 (0.30)</td>
</tr>
<tr>
<td>Group C</td>
<td>2085</td>
<td>6.31 (0.03)</td>
<td>7.48 (0.03)</td>
<td>17.2 (0.25)</td>
<td>70.8 ± 0.77</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>A versus B</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

ER = embryos replaced, values are mean cell numbers at the time of embryo replacement in the afternoon of day 3; FR = fragment removal, values are percentage of replaced embryos with cytoplasmic fragments removed; MNB = multinucleated blastomer; NS = not significant.

*Group A includes monozygotic twin multiple pregnancies; group B includes non-MZ twin multiple pregnancies; and group C includes singleton pregnancies.

*The statistical comparisons of groups were derived from a one-way ANOVA.

### Table V. The impact of assisted hatching on the incidence of MZ twinning

<table>
<thead>
<tr>
<th>Assisted hatching group</th>
<th>Extent of assisted hatching</th>
<th>Incidence of MZ twinning $^a$</th>
<th>Group A, mean age (years)</th>
<th>Group B, mean age (years)</th>
<th>All, mean age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>0.004 ± 0.004</td>
<td>32.0 ± 0.26</td>
<td>31.5 ± 0.21</td>
<td>31.8</td>
</tr>
<tr>
<td>2</td>
<td>Some</td>
<td>0.040 ± 0.010</td>
<td>33.2 ± 0.19</td>
<td>33.0 ± 0.16</td>
<td>33.1</td>
</tr>
<tr>
<td>3</td>
<td>All</td>
<td>0.055 ± 0.011</td>
<td>35.6 ± 0.14</td>
<td>34.9 ± 0.13</td>
<td>35.3</td>
</tr>
</tbody>
</table>

$^a$Incidence are proportions (± SEM) of MZ pregnancies of all multiple pregnancies after two, three or four fresh embryos were replaced.

$^b$Mean age in years, A versus B (33.6 versus 33.1 years).

$^c$Mean age in years, group 1 versus 2 versus 3.

### Table VI. Placental configuration and sex of fetuses in monochorionic pregnancies

<table>
<thead>
<tr>
<th>Placental configuration</th>
<th>Total no.</th>
<th>No. MZ pairs of known sex</th>
<th>Male (no. pairs)</th>
<th>Female (no. pairs)</th>
<th>Proportion males</th>
<th>Proportion females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoamniotic</td>
<td>24</td>
<td>13</td>
<td>6</td>
<td>7</td>
<td>0.462</td>
<td></td>
</tr>
<tr>
<td>Diamniotic</td>
<td>41</td>
<td>23</td>
<td>12</td>
<td>11</td>
<td>0.522</td>
<td></td>
</tr>
<tr>
<td>Unknown amnion</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>40</td>
<td>18</td>
<td>22</td>
<td>0.450$^a$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$The proportion for dizygotic twins and singletons is 0.514.

The impact of zona pellucida manipulation on MZ twinning

The incidence of MZ twinning was assessed for patients whose eggs were treated with ICSI or standard insemination. Monozygotic twinning occurred in 37 of 1537 cycles (2.35%) with ICSI and 35 of 2185 (1.58%) with standard insemination. This difference is not statistically significant. The rate of MZ twinning following zona drilling for blastomere biopsy was unremarkable (1/140; 0.71%).

The relationship between MZ twinning and zona drilling for assisted hatching is presented in Table V. The patients in groups A and B were divided into three subgroups of patients in whom none, some or all of the replaced embryos (two, three or four embryos replaced) had assisted hatching. The proportions are corrected for the number of embryos replaced and whether or not ICSI was applied at the same time. MZ twinning appears to increase with increased hatching. Mean age of the patients in each subgroup was evaluated. The differences among the age of subgroup patients are significant, but the mean age of the patients in groups A and B is very similar.

Placentaion and sex ratio

Seventy-four pregnancies were diagnosed as monochorionic (a single gestational sac containing more than one fetus), while eight were judged to be dichorionic (two gestational sacs and two amniotic sacs, as seen in dizygotic twins). The status of the amnion was known in 65 of the monochorionic cases; nearly 37% (24/65) were monoamniotic. Assuming the upper value of the generally accepted monamniotic rate in natural conception (3%), the probability of as many as 24 pairs of monoamniotic twins in a random sample of 65 cases is infinitesimal ($P < 0.01$).
0.00001). The proportion of males among monochorionic twins was lower than expected (0.450; 18/40). However, these data are not conclusive. Placentation and sex ratio data are presented in Table VI.

Discussion
The overall incidence of MZ twin conception in this study approached 2% of all clinical pregnancies after fresh and frozen–thawed embryo transfers. As is the case with spontaneous conceptions, this incidence is likely to be understated since diamniotic-dichorionic MZ gestations are rarely identified (Machin et al., 1995). We have reported eight presumably dichorionic cases in which the number of gestational sacs as well as the fetuses exceeded the number of embryos replaced. This represents 10% of the total number of MZ pregnancies in the study; the expected frequency is ~30% (Boklage, 1981). To understand the underlying mechanisms of MZ twinning and to obtain a better estimation of its incidence, it is necessary and worthwhile to initiate a study in which the zygosity of all like-sex twins conceived following multiple embryo transfer is determined at birth.

The figure of 2% reported here is in general agreement with those of others in the field of assisted reproduction, but represents a significant increase over the reported MZ twin births after spontaneous conception (three to four per 1000 births). In this context, it must be noted that the latter figure is based on live birth data (Bulmer, 1970), but the MZ twinning rate reported after assisted conception is based on detection of cardiac activity between 6 and 9 weeks gestation.

More reliable data would be provided either by comparing the current natural rate estimates to the live MZ twin birth rate for the assisted conception pregnancies or by accounting for the loss rate of MZ twins after spontaneous conception. Live birth rates of MZ twins after assisted conception are seriously obscured by (i) occurrence in one and the same pregnancy of MZ and non-MZ fetuses, and (ii) elective termination of MZ in favour of the non-MZ fetuses. On the other hand, in a study of spontaneously conceived and aborted complete fetuses and embryos, Livingston and Poland (1980) found 1.8% (35/1939) to be MZ twins based on placental morphology, suggesting that MZ twinning occurs far more frequently than it is maintained.

Our analyses failed to show any distinguishing characteristic of MZ twin conceptions with respect to maternal age, paternal age, number of days of gonadotrophin, gonadotrophin dosage, peak E2 and progesterone levels at the time of hCG, number of oocytes collected, and number of embryos replaced. However, the total number of attempts at assisted conception, i.e. attempts prior to and following the MZ twin conception, was higher in the MZ twinning group, suggesting that a complex reproductive history may be a potential risk factor for MZ twinning.

It was suggested recently that changes in the configuration of the inner cell mass (ICM) of in-vitro-grown blastocysts, induced by apoptosis and resulting from certain culture conditions, may cause ICM splitting and MZ twinning (Ménézo and Sakkas, 2002). Likewise, we hypothesized that twinning may result from poor embryo quality, manifested by aberrant cleavage patterns in early embryos. However, our investigation of the cleavage profiles of embryos in the MZ and non-MZ groups did not reveal any significant trends with respect to cleavage rate, fragmentation or multinucleation among replaced embryos, nor did the complete cohort of embryos for the two groups appear to differ in any significant way. These observations suggest that even if there are differences among the embryos that split and those that do not, these differences are not morphologically apparent or quantifiable at the cleavage stages. Nevertheless, in the absence of any other identifying characteristics and in view of the placentation data, we suggest that a change in the formation/shape of the ICM, possibly as a result of suboptimal culture conditions, may contribute to the twinning process.

Only one case of MZ twinning occurred among 235 patients in whom none of the replaced embryos had assisted hatching. The upper 95% confidence limit for a proportion when there is only a single case in 235 patients is 0.020, which is well below the value observed when there was some assisted hatching involved. These observations suggest that assisted hatching is a potential risk factor for MZ twinning. However, what is uncertain is whether this increased risk is due to the artificial alteration of the zona pellucida per se, or embryo/patient conditions that made this procedure necessary in the first place. In our centre, assisted hatching is reserved for patients with an overall poor prognosis for pregnancy, due to advanced maternal age, repeated implantation failure or relatively poor embryo quality.

It must be conceded that the positive findings regarding risk factors for MZ twinning emerging from this very substantial study might well appear rather disappointing. Even so, ‘negative’ findings from a large study suggest that the ‘effects’ of interest may be small in relation to other sources of variation, both systematic and random. Moreover, they demonstrate the immense effort that is required in the investigation of rare events. Here, the total patient number of >4000 is less relevant to the sensitivity of this study than the 81 total MZ twin cases, especially after the latter is divided into various subclasses to allow for potential confounders. Therefore, despite this relatively large database our findings on risk factors must be regarded as tentative rather than conclusive. On the other hand, since it is highly improbable that a designed experiment of adequate size will be carried out to investigate the phenomenon of MZ twinning, the expansion of knowledge in this area will inevitably depend on investigations of the sort presented here.

The important question of placentation of MZ twins has only been marginally addressed so far (Slotnick and Ortega, 1996). Such knowledge is crucial to the understanding of twinning under artificial conditions and to the proper management of MZ twin pregnancies. In this respect, perhaps the most significant finding in this study was the unexpected increase in the incidence of monoamniotic twinning. This is a condition in which two fetuses share a single amniotic sac as well as a single placenta, and is seen in 1–3% of spontaneously conceived MZ twins that are born (Su, 2002). Even accounting for the high antenatal mortality rate among these twins (reviewed by Allen et al., 2001; Su, 2002), as well the possibility of false-positive
diagnosis of monoamnionicity (Strobahn and Dattel, 1995), the increased proportion of monoamniotic twins seen in this study is highly significant. It is also difficult to explain. Nonetheless, it suggests that whatever the underlying cause of MZ twinning after assisted conception, its consequences manifest themselves relatively late in embryogenesis, and frequently after implantation of the blastocyst and formation of the amnion. This would necessarily exclude blastocyst bicsection (though not necessarily ICM bisection), e.g. during hatching, as the only mechanism of twinning under these conditions. The late onset of monoamniotic MZ twinning in the human has already been suspected based on X-inactivation patterns in these twins. Since discordance of the patterns in such twin pairs is rare, it is more than likely that twinning occurs many cell divisions after the X-inactivation event (Chitnis et al., 1999). In this context, it is noteworthy that the proportion of males among spontaneous MZ fetuses (particularly the monoamniotic pairs) is lower than expected (Derom et al., 1988). It will be interesting to determine whether the same is true following assisted reproduction.

If duplication is any later and is incomplete, conjoined twins are formed; a handful of such cases have been reported in literature. Certainly the monoamniotic pairs is lower than expected (Derom et al., 1996) and two following ICSI (Goldberg et al., 1994) Monozygotic embryos with assisted hatching (Skupski et al., 1992), two following conventional IVF (Boulot et al., 1992; Ho et al., 1996) and two following ICSI (Goldberg et al., 2000; Ericson and Källén, 2001). The apparent rarity of conjoined twin formation despite a considerable increase in the number of monoamniotic twins is at least suggestive of a stable, though as yet unclear, mechanism of twinning of in-vitro-grown embryos.

In this study, all three pairs of MZ twins after blastocyst transfer were monoamniotic, but in two other studies, six of the seven reported cases were diamniotic (Peramo et al., 1999; da Costa et al., 2001). A close examination of the incidence and the types of placentation in MZ twins following blastocyst transfer should shed more light on the mechanism and timing of MZ twinning following assisted conception.

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References


Monozygotic twinning and assisted conception


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