Incidence and zygosity of twin births following transfers using a single fresh or frozen embryo

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STUDY QUESTION: Are all twin births following single embryo transfer (SET) monozygotic?

SUMMARY ANSWER: Between 1 in 10 and in 1 in 5 twins born after SET are the result of a concurrent natural conception.

WHAT IS KNOWN ALREADY: The twinning rate after SET is higher than following natural conception. Most studies of twins following SET have incorrectly assumed monozygosity or have not been able to assess the zygosity.

STUDY DESIGN, SIZE, DURATION: This study is a retrospective cohort study assessing the gender discordance of all live born twins following fresh or frozen SET.

PARTICIPANTS/MATERIALS, SETTING, METHODS: A total of 4701 patients in a large private IVF unit who gave birth following SET with a fresh or frozen embryo with complete follow-up. Of 137 viable twins at the 7-week ultrasound, 109 were delivered as twins. Gender discordance and Weinberg’s differential rule were used to estimate dizygosity. Twin rates were compared for fresh and frozen transfers by insemination method and transfer day.

MAIN RESULTS AND THE ROLE OF CHANCE: The overall live twin birth rate was 2.3% (109/4701). Based on the 7-week scan, 2 of the twins were monochorionic monoamniotic, 62 were monochorionic diamniotic and 45 were dichorionic diamniotic. There were a total of 12 gender discordant twins (11%), 7 from the Day 2/3 transfers and 5 from Day 5 transfers. Nine of the 12 discordant twins were from natural cycle frozen embryo transfers, the remaining 3 were from fresh cycles.

LIMITATIONS, REASONS FOR CAUTION: To assess gender discordance only live born twins were studied. DNA fingerprinting of twins is a more accurate way to assess zygosity than measuring gender discordance. Same sex twins in this study are not necessarily monozygotic and the dizygotic rate in this study may therefore be higher. This rate was estimated using Weinberg’s differential rule.

WIDER IMPLICATIONS OF THE FINDINGS: As many as 1 in 5 twins born after SET may be the result of a concurrent natural conception. Couples therefore need to be counselled regarding the relative benefits and risks of intercourse in assisted reproduction technology cycles where spontaneous conception is possible.

STUDY FUNDING/COMPETING INTEREST(S): None.

TRIAL REGISTRATION NUMBER: Not applicable.

Key words: zygosity / monozygotic / single embryo transfer / IVF / twin

Introduction

Transferring a single blastocyst has been shown to significantly reduce multiple pregnancy rates (Pandian et al., 2009) and increase the likelihood of delivering at term a single healthy baby (Wang et al., 2010). There is an acceptance that the incidence of monozygotic twin births is higher in the assisted reproduction technology (ART) population compared with naturally conceived births (Yovich et al., 1984; Blickstein, 2005); however,
there are conflicting reports regarding treatment methods in ART and their involvement in increasing the twinning rate. Some reports suggest transfer at the blastocyst stage may be associated with higher monozygotic pregnancies (Da Costa et al., 2001; Tarlatzis et al., 2002; Milki et al., 2003; Kawachiya et al., 2011), whereas others speculate there is an overestimation or underestimation of monozygotic twins due to methodology and acceptable definitions (Vithala et al., 2009). When only single embryo transfers (SETs) are considered together with ultrasound imaging, there is no increase in monozygotic twin rates in blastocyst transfers compared with cleavage stage embryo transfers (Papanikolaou et al., 2006, 2010).

Zona manipulation in the form of assisted hatching or ICSI has been implicated in increasing the monozygotic twinning rate. Some authors report an association with ICSI and increased risk of monozygotic twins (Alikani et al., 2003; Skiadis et al., 2008), with the assumption that monozygosity is also implied when the number of fetuses exceeds the number of embryos transferred. Other reports show no dependence on the insemination method and the rate of monozygotic twins (Milki et al., 2003; Elizur et al., 2004). Sills et al. (2000) showed that ICSI with or without assisted hatching had no impact on the incidence of monozygotic pregnancies in both fresh IVF cycles and frozen embryo transfer (FET) cycles.

The aim of this study therefore is to use gender discordance to estimate the monozygotic and dizygotic twinning rates following the transfer of single fresh or frozen embryos at the cleavage or blastocyst stage.

**Materials and Methods**

The Monash IVF database of patients treated between January 2007 and December 2011 was retrospectively reviewed. The primary outcome measure was the proportion of gender discordant live born twins following SET.

**IVF/ICSI process**

Stimulation regimens were used with or without the oral contraceptive pill scheduling. The regimens all used recombinant FSH for ovarian stimulation. Down-regulation was achieved with either a GnRH agonist or a GnRH antagonist.

Oocyte retrievals were performed 38 h post-hCG administration (either recombinant Ovidrel (Merck Serono, Australia) at 250 μg or urinary Pregnyl (MSD, Australia) at 10 000 IU). Oocytes were fertilized using either standard insemination or ICSI and fertilization results assessed between 16 and 20 h post-sperm insemination. Embryos were cultured in a single 10 μl droplet in the COOK culture system (COOK Medical, Australia). Day 3 embryos were frozen at the cleavage stage using a propandiol slow-freezing system, the COOK freeze kit (COOK Medical, Australia), and blastocysts were vitrified. Culture media and culture conditions remained unchanged throughout the study period. A SET, mostly on an elective basis, took place on Day 2, 3 (cleavage stage) or Day 5 (blastocyst stage). Luteal support consisted of either Crinone 8% or twice daily progesterone pessaries 200 mg vaginally (Orion, Australia).

All patients within our programme are provided with an identical patient handbook which contains written information regarding the potential benefits and risks of sexual intercourse during an ART cycle. Only patients scheduled to have pre-implantation genetic screening are instructed not to engage in sexual intercourse but these patients were not included in the study to remove such bias.

**Assessment of clinical pregnancy and chorionicity**

Pregnancy was defined as a viable intrauterine pregnancy (fetal heart observed) on transvaginal ultrasound during the 7-week scan. During this scan, the chorionicity and amnionicity of the twin pregnancy was recorded. The birth outcome of the clinical pregnancy was known in each case.

**Birth outcomes**

In Australia, there is a regulatory requirement to report all IVF cycle outcomes, including births, and as such there was 100% follow-up in this cohort. For this study data were collected on the number and the gender of the babies born.

**Weinberg’s differential rule**

Gender discordance in twins was first used to provide one estimate of the dizygotic twining rate but this calculation excludes the gender concordant dizygotic twins. To provide an estimate of the true number of dizygotic twins in our birth cohort Weinberg’s differential rule was used (Fellman, 2013). It is based on the principle that, unless the sex ratio (male/female) in a population deviates significantly from 1, there should always be a gender-concordant dizygotic twin for every dizygotic gender-discordant twin. Weinberg’s differential rule thus states that the total number of dizygotic twins is double the number of gender-discordant twins. The number of monozygotic twins is then determined by subtracting the calculated number of dizygotic twins from the total number of twins. Fellman and Eriksson (2006) analysed the reliability of Weinberg’s differential rule and found that it was statistically robust. The male/female sex ratio is reported to vary to some extent with the type of ART treatment used. In an Australian and New Zealand birth cohort of 13 368 babies the male/female sex ratio after blastocyst SET with IVF and cleavage-stage SET with ICSI represented the extremes at 1.28 and 0.95, respectively (Dean et al., 2010). Our birth cohort represents a mix of ART procedures with a significantly higher proportion of babies born using a blastocyst with ICSI, which in the study by Dean et al. (2010) was associated with a male/female sex ratio of 1.11. Compared with the application of Weinberg’s differential rule in a fertile population where the male/female sex ratio is estimated to be 1.06 (Grech et al., 2002), it is possible that its use in this birth cohort may slightly overestimate the true dizygotic twin rate.

**Statistical analysis**

Chi-square test was used for the comparison of proportions with a P value of <0.05 considered statistically significant. Data are presented as frequency (%). PASW version 19 (SPSS, Inc., Chicago, IL, USA) was used for data analysis.

**Ethical approval**

This retrospective study was approved by the Human Research and Ethics Committee of the Monash Surgical Private Hospital (P07078, 25 November 2013). Patients gave consent to the use of their data in the Monash IVF general consent form for retrospective data analysis.

**Results**

There were a total of 4701 Monash IVF patients who had a SET during an IVF cycle (both fresh and frozen) and had a delivery between 2007 and 2011. The average patient age at the time of oocyte retrieval was 34.1 years. At the 7-week ultrasound 136 viable twin pregnancies were observed (1.06%). In 109 of these 136 pregnancies 20 resulted in miscarriages with no reported births and 7 with demise of I twin and a resultant singleton delivery. There were 109 SETs...
that resulted in twin births with an overall live twin birth rate of 2.3% (109/4701).

At birth the twins were assessed in terms of their gender and the type as per Table I. As expected the monochorionic twins were all the same sex. There were a total of 12 discordant twins, 7 from the Day 2/3 transfers and 5 from Day 5 transfers. Nine of the 12 discordant twins were from natural cycle FETs. The remaining three were from fresh cycles. Based purely on the gender discordance at least 11% (12/109) of the twins are dizygotic, with 4.4% (3/68) of the fresh and 22% (9/41) of the FETs being discordant. If we utilize Weinberg’s differential rule (Fellman, 2013) and double the number of gender discordant twins, this gives us a calculated dizygotic rate of 22% (24/109) by the Weinberg method amongst SET twins. The calculated dizygotic rate amongst all births is 0.5% (24/4701). However, when we separately examine the number of twins following SET with fresh and frozen embryos with Weinberg’s differential rule the calculated dizygotic rate is 5-fold lower in the fresh transfer group (0.2%) compared with the frozen group (1%).

Our overall dichorionic diamniotic birth rate is 1.0% (45/4701), suggesting that approximately half of these are the result of embryo splitting and the other half the result of a spontaneous conception. In this birth cohort, the frequency of dichorionic diamniotic pregnancies after Day 2/3 transfers was 1.0% and for blastocyst Day 5 transfers 0.9% (NS). It must be noted there is no record of which patients did engage in intercourse during their ART treatment. However, all women with a discordant twin were in a heterosexual relationship, had patent tubes and a partner with sperm present in the ejaculate.

There were 3504 patients who had a Day 5 embryo transfer and 1197 who had a transfer at the cleavage stage, Day 2 or 3 post-oocyte retrieval. When we examined the twinning rate according to the day of transfer (Table II), there was no significant difference between cleavage stage Day 2/3 embryos and Day 5 blastocyst stage embryos. Table II shows the number of twins that have resulted from fresh stimulated oocytes retrieval cycles (fresh cycles) and FET cycles, again indicating that the twinning rate is not influenced by the day of transfer nor by the type of transfer. When examining the method of insemination, again this did not influence the twinning rate between standard IVF insemination and with ICSI. The overall twinning rate for standard IVF was 2.0% and for ICSI 2.5%, however, there was no statistically significant difference.

Table III shows the zygosity of twins as determined by the 7-week ultrasound scan stratified by day of embryo transfer or insemination method. Of the 109 twins identified in the study, 2 were monochorionic monoamniotic (mono/mono), 1 from a cleavage stage and 1 from a

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**Table I** Sex of twins according to day of transfer and type of twin.

<table>
<thead>
<tr>
<th>Day 2/3 transfer</th>
<th>Female/female</th>
<th>Mono/Mono</th>
<th>Mono/Di</th>
<th>Di/Di</th>
<th>Male/female</th>
<th>Mono/Mono</th>
<th>Mono/Di</th>
<th>Di/Di</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mono/Mono</td>
<td>Mono/Di</td>
<td>Di/Di</td>
<td>Mono/Mono</td>
<td>Mono/Di</td>
<td>Di/Di</td>
<td></td>
</tr>
<tr>
<td>Fresh cycle (n)</td>
<td>866</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>FET cycle (n)</td>
<td>331</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table II** Twinning birth outcomes from SET by day of transfer and type of cycle.

<table>
<thead>
<tr>
<th>Day 2/3 transfer</th>
<th>Day 5 transfer</th>
<th>P-value (P &lt; 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>24/1197 (2.0%)</td>
<td>85/3504 (2.4%)</td>
</tr>
<tr>
<td>Fresh cycles only</td>
<td>12/866 (1.4%)</td>
<td>56/2164 (2.6%)</td>
</tr>
<tr>
<td>FET cycles only</td>
<td>12/331 (3.6%)</td>
<td>29/1340 (2.2%)</td>
</tr>
<tr>
<td>IVF cycles (Fresh + FET)</td>
<td>10/416 (2.4%)</td>
<td>22/1172 (1.9%)</td>
</tr>
<tr>
<td>ICSI cycles (Fresh + FET)</td>
<td>14/781 (1.8%)</td>
<td>63/2332 (2.7%)</td>
</tr>
</tbody>
</table>

**Table III** Type of twin from SET.

<table>
<thead>
<tr>
<th>Day 2/3 Transfer</th>
<th>Day 5 Transfer</th>
<th>P-value (P &lt; 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twinning rate</td>
<td>24/1197 (2.0%)</td>
<td>85/3504 (2.4%)</td>
</tr>
<tr>
<td>Mono/Mono</td>
<td>1/1197 (0.08%)</td>
<td>1/3504 (0.02%)</td>
</tr>
<tr>
<td>Mono/Di</td>
<td>11/1197 (0.9%)</td>
<td>51/3504 (1.5%)</td>
</tr>
<tr>
<td>Di/Di</td>
<td>12/1197 (1.0%)</td>
<td>33/3504 (0.9%)</td>
</tr>
<tr>
<td>ICSI</td>
<td></td>
<td>IVF</td>
</tr>
<tr>
<td>TWINNING RATE</td>
<td>77/3113 (2.5%)</td>
<td>32/1588 (2.0%)</td>
</tr>
<tr>
<td>Mono/Mono</td>
<td>1/3113 (0.03%)</td>
<td>1/1588 (0.06%)</td>
</tr>
<tr>
<td>Mono/Di</td>
<td>48/3113 (1.5%)</td>
<td>14/1588 (0.9%)</td>
</tr>
<tr>
<td>Di/Di</td>
<td>28/3113 (0.9%)</td>
<td>17/1588 (1.0%)</td>
</tr>
</tbody>
</table>

Mono/mono, monochorionic/monoamniotic; Mono/Di, monochorionic/diamniotic; Di/Di, dichorionic/diamniotic; FET, frozen embryo transfer.
blastocyst stage transfer. There was no difference in the type of twin between different days of transfer, between ICSI and standard IVF and frozen versus fresh transfer (data not shown).

Discussion

To the best of our knowledge this study is the first to estimate the dizygotic rate in live born twins following SET. Our results indicate that at least 1 in 10 of twin births following SET were likely the result of a concurrent natural conception when we only consider gender discordant twins from SETs. Taking into consideration the limitations of this method used to estimate zygosity of twinning rates (Fellman, 2013) we can calculate with Weinberg’s differential rule that the true dizygotic rate following SET in our ART population is closer to 1 in 5 twin births. SETs account for 75% of the discordant twins in our birth cohort following SET.

Worldwide there is an imperative to reduce the multiple pregnancy outcomes by electively performing SET. However, even with SET there remains the possibility of a multiple pregnancy. It is widely accepted that the incidence of monozygotic twinning is several fold higher in the IVF population, but there is significant disagreement on what the actual monozygotic twinning rate is with publications quoting from 0.7 to 3.1% compared with 0.4% for natural conceptions (Bulmer, 1970). Furthermore, the assumption of monozygosity has often been made based on the fact that twins resulted from an SET (Blickstein et al., 1999; Kawachiya et al., 2011). However, there are some case reports that clearly show that twins following SET can also be the result of a natural conception occurring during the embryo transfer cycle (Kyono et al., 2009; van der Hoorn et al., 2011).

In our study, twin birth rates were examined to determine whether there was a higher incidence of twins from SET associated with the developmental stage of the embryo, the type of insemination and the use of frozen embryos. None of these parameters affected the twinning rate in this study. Similarly, when the twin live birth rate was further investigated examining the amnionicity and chorionicity and the zygosity of the twins, this did not show a significant difference relative to the parameters above.

Some studies have shown that manipulating the zona pellucida, either through ICSI or assisted hatching increases the monozygotic twinning rate of IVF embryos (Tarlatzis et al., 2002; Skidiats et al., 2008; Vitthala et al., 2009). It is speculated that the mechanical compression of the inner cell mass as it herniates through the compromised zona pellucida results in the splitting of the inner cell mass. Other studies have not substantiated these findings with no differences in monozygotic twinning rates in ICSI compared with standard IVF insemination (Sills et al., 2000; Milki et al., 2003; Elzur et al., 2004). The latter findings are supported by the present study, showing no association with insemination method and monozygosity, with both ICSI and IVF showing similar twinning rates.

There have also been reports that the monozygotic twinning rate increases in embryos transferred at the blastocyst stage (Da Costa et al., 2001; Sheiner et al., 2001). Milki et al. (2003) showed a 5.6% monozygotic twinning pregnancy rate in the blastocyst transfer group compared with 2% in the cleavage stage embryo transfers. The authors concluded there was a significant increase in monozygotic twinning in blastocyst stage embryo transfers. The same group published follow-up data in 2007 indicating their blastocyst monozygotic twinning rate decreased to 2.3%, not statistically significant compared with their cleavage embryos of 1.8% (Moayeri et al., 2007). This was attributed to improved experience and changed blastocyst culture conditions. In agreement with the findings of Moayeri et al. (2007), our own study demonstrated no increase in twin births relative to the developmental stage of the embryo in any of the twin groups examined.

There have been suggestions that blastocysts exposed to poor culture conditions, potentially due in part to culture media composition, may increase the incidence of monozygotic pregnancies (Behr et al., 2000; Cassuto et al., 2003). Menezo and Sakkas (2002) published on 800 births from blastocyst transfers with no monozygotic twins. A co-culture system was used to culture the embryos to the blastocyst stage with less glucose required in the culture media compared with sequential culture systems with no co-culture. The authors speculated that the elevated glucose concentrations in sequential culture media may induce apoptotic events due to increased free radicals causing the inner cell mass to split. Both Moayeri et al. (2007) and Jain et al. (2004) suggest the operator experience along with optimized culture conditions has decreased the monozygotic twinning rates in their blastocyst transfers. Taken together with Menezo and Sakkas’ (2002) suggestions of free radical scavengers affecting the inner cell mass, the possible increase of antioxidants in more recent sequential culture media and optimized conditions may thus account for more recent reports, including ours, of lower rates of monozygotic twins with blastocyst stage embryo transfers.

In our study there was no significant difference in twinning rates when ICSI and blastocyst embryos were transferred, compared with IVF and cleavage stage transfer. Our results do contradict the meta-analysis performed by Vitthala et al. (2009), which concluded that ICSI and blastocyst transfers appeared to increase the risk of monozygotic twins. However, as acknowledged by Kawachiya et al. (2011) it is difficult to get accurate estimates of relative risks of monozygotic twins in different subgroups because of the overall low incidence of monozygotic twins.

The largest retrospective analysis to date by Kawachiya et al. (2011) reported a significant increase in monozygotic twin births from single blastocyst transfers compared with cleavage stage transfers in 47 841 cycles. However, the analysis was flawed as the monozygosity of all twins was assumed on the basis that a single embryo was transferred, despite the fact that 60% of the twins were dichorionic diamniotic. Alternative explanations, such as concurrent natural conception, were not discussed. At least in the study by Wright et al. (2004) the authors acknowledge that their study design did not enable them to distinguish between mono- and dizygotic twins. Vitthala et al. (2009) also commented that their meta-analysis included few studies that looked exclusively at SETs or used either DNA testing or ultrasound as a determinant of zygosity.

To successfully and meaningfully compare results there is thus a clear need to further differentiate between the incidence of monozygotic twins and the overall incidence of twinning resulting from an SET. The possibility of dizygotic births resulting from natural conception during an IVF cycle cannot be dismissed. Ideally, this is performed using DNA fingerprinting but this is costly and obtaining consent from the parents is not always straightforward. Measuring gender discordance, as in our study, is an alternative option and although it does not reflect the true incidence of dizygotic births following SET, it at least allows for a more accurate analysis of the true monozygosity and the various factors that potentially influence it.

In our study of twins following SET there were 12 gender-discordant pairs. Using Weinberg’s differential method of estimating dizygosity,
we can estimate that the dizygotic birth rate in our study was ~0.5%. Of the 12 discordant twins in this study, 9 were from natural FET cycles (9/1178; 0.8%) and 3 from fresh stimulated cycles (3/3030; 0.1%). Interestingly, there were no discordant twins reported from FET cycles with hormone replacement treatment. This is consistent with the fact that high-dose hormone replacement suppresses spontaneous ovulation. Seven of the twins were from cleavage stage embryo transfers and five from blastocyst stage transfers.

These couples with gender-discordant twins obviously engaged in coitus at the time of their IVF cycle, resulting both in an artificially and a naturally conceived baby. There is evidence to suggest that sexual intercourse potentially assists the implantation process due to the prostaglandins and growth factors released in the uterine environment (Tremellen et al., 2000). However, the benefits of intercourse during ART must be weighed against the increased risk of multiple pregnancies and couples should be counselled appropriately.

In conclusion, IVF-related procedures did not result in an increase in the incidence of twinning in this study. The cycle type (fresh or frozen), the method of insemination (standard IVF or ICSI) nor the day of transfer significantly affected twin birth rates. A significant finding of this study is that where there is an appreciable risk of a natural pregnancy patients sexual intercourse during ART treatment. Clearly, our findings indicate on the recommendations given by clinics or IVF specialists regarding current natural conception, with the majority of these occurring in

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**Authors’ roles**

T.O.: Concept, design, data analysis and interpretation, drafting article and final approval. L.R.: Design, interpretation of data, draft and final approval. M.G.: Collection of data, revising, final approval. C.M.: Data analysis, revising, final approval. B.V.: concept, design, revising and final approval.

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**Conflict of interest**

L.R. and B.V. are minor shareholders in Monash IVF Pty Ltd. There are no further conflicts of interest to declare.

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