The incidence of monozygotic twinning following PGD is not increased

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BACKGROUND: Monozygotic (MZ) twin pregnancies are associated with increased perinatal mortality and morbidity, and risk of congenital anomalies. The causes of MZ twinning in humans are unclear but the incidence may increase after PGD, for example, as a result of holes created in the zona pellucida. We compared the incidence of MZ twin pregnancies in ICSI cycles with PGD, versus ICSI cycles without PGD.

METHODS: In this retrospective comparative cohort study, we analysed incidence of twin pregnancies in unselected patients undergoing ICSI and PGD (group A; 1992 cycles) with blastocyst transfer at Day 5, versus a period-matched control population of unselected patients undergoing ICSI and blastocyst transfer at Day 5 without PGD (group B; 2429 cycles) from January 2001 to December 2006.

RESULTS: Clinical pregnancy per embryo transfer was established in 618/1992 (31.0%) and 947/2429 (39.0%) in group A versus B, respectively (P < 0.01). Overall MZ twin rate was 29/4421 (0.7%) per embryo transfer and 29/1565 (1.9%) per established clinical pregnancy. The incidence of MZ twinning per established clinical pregnancy did not differ between groups (1.5 versus 2.1%, group A and B, respectively). In group A, seven MZ twins were born versus 19 MZ twins in group B. In group B, one MZ twin pregnancy resulted in two stillbirths. In group A, two MZ twins had severe congenital malformations versus none in group B.

CONCLUSIONS: The incidence of MZ twinning was not increased in PGD compared with regular ICSI with blastocyst transfer. This information is useful in counselling patients about potential risks of PGD.

Key words: monozygotic / twins / PGD / ICSI / blastocyst transfer

Introduction

Monozygotic (MZ) twinning occurs in 0.42% (Bulmer, 1970) to 0.45% (Derom et al., 1987; Costa, 2001) of spontaneous pregnancies. By definition MZ twins arise from a single zygote splitting into two separate individuals. The incidence is about one-third of spontaneously conceived twin pregnancies. The causes of MZ twinning in humans are not clear (Hershlag et al., 1999; Hall, 2003; Toledo, 2005). The incidence of MZ twin pregnancies in assisted reproduction treatment (ART) conceptions is higher compared with pregnancies after spontaneous conception. Figures of 0.72% in IVF and 0.86% in ICSI (Schachter et al., 2001), to as high as 8.9% (Abusheika et al., 2000) have been reported. The true incidence, however, is unknown owing to the use of proxy estimations (Blickstein, 2005). Factors which appear to influence MZ twinning are ovulation induction (Derom et al., 1987), embryo culture conditions (Edwards et al., 1986) and artificial breach of the zona pellucida, for instance ICSI and assisted hatching (AH) (Hershlag et al., 1999). Other reports describe a higher incidence of twinning, including MZ twinning, in pregnancies after transfer of blastocyst stage embryos, compared with pregnancies achieved after transfer of cleavage stage embryos (Edwards et al., 1986; Hall, 2003, 2005).

Reports on the incidence of MZ twinning after the use of ART in combination with PGD and preimplantation genetic screening (PGS) are lacking. The incidence of MZ twin pregnancies in PGD is potentially increased as a result of different mechanisms, including breaks and intentional holes in the zona pellucida associated with handling, and more specifically with blastomere biopsy (Table I).

MZ twin pregnancies are associated with a 3–5-fold higher perinatal mortality rate as compared with dizygotic (DZ) twins, and with up to seven times the risk of perinatal mortality as compared with singleton pregnancies (Derom et al., 1987; Malone, 2003; Toledo, 2005). MZ twins are at greater risk of perinatal morbidity associated with premature and preterm birth than DZ twins (Sebire et al., 1997). Up to 30% of monochorionic diamniotic (MCDA) twin pregnancies are associated with twin-to-twin transfusion syndrome (TTTS). TTTS is associated with severe neurological damage and accounts for 15% of the perinatal mortality in MCDA twins (Machin, 2004). In MZ twins, congenital
Table I  Factors associated with MZ twinning

<table>
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Table 1: Factors associated with MZ twinning

Anomalies occur frequently both in live born babies and miscarriages. They may include congenital abnormalities such as cloacal anomalies, neural-tube-defects and congenital heart defects, dislocations such as limb reduction defects and amyoplasia related to shared placental circulation, as well as deformations associated with constraint and intrauterine crowding e.g. clubfeet, dislocated hips and craniosynostosis. The risk of congenital anomalies in MZ twins is 2–3-fold that of singleton neonates, with an estimated incidence of 10% (Hall, 2003).

The aim of this study was to assess the incidence of MZ twin pregnancies in a large cohort of consecutive PGD treatment cycles, in comparison with a period-matched cohort of consecutive ICSI cycles with blastocyst transfer at the same centre.

Materials and Methods

In this retrospective cohort study, we analysed the incidence of MZ twin pregnancies in a cohort of consecutive treatment cycles of ICSI combined with PGD and blastocyst transfer (group A), and compared the results with a control cohort of consecutive cycles of ICSI and blastocyst transfer without PGD (group B). The study was conducted at a large tertiary referral centre for reproductive medicine and PGD, and included data from January 2001 to December 2006.

There were no differences in laboratory conditions or clinical procedures between group A and B, except for the biopsy procedure for PGD in group A. The details of the ICSI procedure have been described previously (Van Landuyt et al., 2005). In group A (PGD/PGS), ICSI was performed rather than classical IVF to prevent contamination with residual sperm DNA in case of PCR-based PGD (Liebaers et al., 1998) and to maximize the fertilization rate in PGS. AH was never used. Fertilization was assessed 16–18 h after ICSI. Further development was evaluated in the morning of Day 2 and again on Day 3, when embryos were evaluated before biopsy. Different blastocyst culture media were used over the years studied [Medicult® Blastassist® system (Jyllinge, Denmark); Vitrolif® G2® and G3® series (Goteborg, Sweden); Cook medical® Sydney IVF® media (Limerick, Ireland)]. There was however, no difference in culture medium used between group A and group B. According to the number of anucleate fragments, the embryos were subdivided into grades A, B, C and D as described previously (Vandervorst et al., 1998). From the 5-cell stage onwards for fluorescence in-situ hybridization (FISH) analysis and from the 6-cell stage onwards biopsy of grades A, B and C embryos were performed on Day 3 of culture. Laser-assisted biopsy was applied consistently as previously described (De Vos and Van Steirteghem, 2001; Sermon, 2002). Overall the aspiration method was used to remove one or two blastomeres from the embryo. For PCR analysis, each blastomere was placed in a solution that lysed the cell and releases the DNA (Sermon et al., 2004). For FISH purposes, a blastomere was spread on a slide using the HCl/Tween 20 method (Coonen et al., 1994).

The PCR procedures were performed as previously described (Sermon et al., 2001; Sermon and De Rycke, 2007). At our centre, multiplex PCR was the standard method of DNA amplification at single cell level, reducing both the risk of undetected contamination and allele drop out by the use of linked markers alone, or linked markers combined with the detection of a specific mutation (Sermon, 2002).

Numerical chromosomal analysis was performed using a FISH procedure, allowing analysis of chromosomes X, Y, 13, 18 and 21, and chromosomes 16 and 22 in a second round of hybridization (Staesens et al., 1999, 2004). For reciprocal translocations, the direct labelled and commercially available probes (from Vysis or CytoCell) consisted of a combination of three probes, one telomeric and two centromeric, or two
telomeric and one centromeric of the chromosomes involved. For Robert-sonian translocations, we used a combination of either two subtelomeric or two locus-specific probes or a combination of a subtelomeric and a locus-specific probe. By this approach, the embryos carrying normal or balanced chromosomes can be differentiated from the embryos carrying unbalanced chromosomes.

One or more unaffected embryos were transferred into the uterus on Day 5 post insemination, in group A as well as in group B subject to availability and (legal) restrictions on the number of embryos for transfer. As in regular IVF cycles, the age of the female partner, the rank of trial and embryo quality determined the number of embryos transferred. For Belgian patients, the number of embryos for transfer was restricted according to age and rules laid out by federal law from July 2003 onwards (Van Landuyt et al., 2006). Supernumerary unaffected embryos were cryopreserved subject to consent by the couple.

A clinical pregnancy was defined as one or more gestational sacs seen at transvaginal ultrasound scan at least 5 weeks after embryo transfer. MZ twins were identified at first ultrasound when the number of fetal heartbeats exceeded the number of gestational sacs, or when the number of sacs exceeded the number of embryos transferred. Chorionicity of the twin pregnancy was routinely determined by transvaginal ultrasound at 7 weeks of gestation, and was confirmed by a second ultrasound scan between 2 and 5 weeks following the initial ultrasound. The monocoorionicity at ultrasound was used as a confirmation of the monozygotic status of the pregnancy following blastocyst transfer. Evidence of division of inner cell mass (ICM) on blastocyst examination on Day 5 was not observed and not used as a criterion to diagnose a MZ pregnancy. The data on treatment characteristics and outcome were recorded by a dedicated team of embryologists, nurses and physicians.

Student’s unpaired t-test and $\chi^2$-test were used to assess differences in continuous and categorical variables, respectively. P-values of <0.05 were considered statistically significant.

Results

The mean patient age at conception was 33.9 (SD ± 4.7) years in group A versus 31.9 (SD ± 4.7) years in group B (P < 0.01). In group A, 1297 patients underwent 1992 PGD blastocyst transfers while in group B 1786 patients underwent 2429 blastocyst transfers. The mean number of embryos transferred in both groups was 1.6. Clinical pregnancy per embryo transfer was established in 618/1992 (31.0%) in group A and in 947/2429 (39.0%) in group B (P < 0.01) (Table II). The overall (group A + B) MZ twin pregnancy rate was 29/4421 (0.7%) per embryo transfer and 29/1565 (1.9%) per established clinical pregnancy. In group A, four MZ twin pregnancies were identified solely by determination of chorionicity at ultrasound on the basis that the number of blastocysts transferred was equal to or higher than the number of gestational sacs observed; five MZ twin pregnancies were identified on the basis of gestational sacs at ultrasound being supernumerary to the number of embryos transferred. In group B 10 MZ twin pregnancies were identified solely by determination of chorionicity at ultrasound, versus 10 on the basis of supernumerary gestational sacs. Despite the secular trends in use of culture media, no culture media-related or time-related changes in incidence of MZ twinning were observed during the time period studied.

Per established clinical pregnancy in group A, nine MZ twin pregnancies were observed (1.5%). Of these, three pregnancies ended in miscarriage, one in selective termination of the twin pregnancy (triplet pregnancy reduced to singleton) and in three pregnancies one twin showed arrested development in the first trimester of pregnancy (vanished twin). In group B, 20 MZ twin pregnancies were observed (2.1%) (versus 1.5% in group A, not significant). Of these, four pregnancies resulted in a miscarriage, six in selective termination of the twin pregnancy (triplet pregnancy reduced to singleton) and one pregnancy showed a vanished twin. One set of MZ twins (11%) was identified as monochorionic monoamniotic (MCMA) in group A, versus four MCMA twins (20%) in group B.

In group A seven MZ twins have been born at a mean gestational age of 35.5 weeks (SD ± 2.5) and a mean birthweight of 2628 g (SD ± 487) versus 19 MZ twins in group B with a mean gestational age of 35.3 weeks (SD ± 3.4) and mean birthweight of 2301 g (SD ± 691). In group B one MZ twin pregnancy resulted in stillbirth of both siblings secondary to premature prelabour rupture of membranes at 20 weeks and 6 days of gestation. In group A two MZ twins were born with severe congenital malformations versus none in group B. The severe congenital abnormalities consisted of unilateral left-sided testicular atrophy in sibling twins born at a gestation of 34 weeks and 5 days (Table II).

Discussion

This study shows that the incidence of MZ twin pregnancies is not increased as a result of the embryo biopsy procedure for PGD. Despite weaknesses in terms of limited sample size and retrospective analysis, the data presented in this study will inevitably contribute to the knowledge on this topic, as reports on the incidence of MZ twinning in PGD are lacking.

The incidence of predominantly dichorionic twins in ART is evidently increased as a result of the transfer of multiple embryos in a lot of cases. The incidence of MZ twins, however, has also been reported to be increased 2–4-fold in ART (Cohen et al., 1992; Costa et al., 2001). MZ twinning has previously been shown to be

| Table II Analysis of twin pregnancies in patients undergoing ICSI and PGD (group A) with blastocyst transfer at Day 5, versus controls undergoing ICSI and blastocyst transfer at Day 5 without PGD (group B) |
|-----------------------------------------------|-----------------|---------------|-----------------|-----------------|---------------|
| # patients | Group A | 1297 | Group B | 1786 | P < 0.01 |
| Mean age (years) | 33.9 | 31.9 | 3.4 | 31.9 | 1.6 |
| Mean # embryos transferred | 1.6 | 1.6 | 1.6 | 1.6 | NS |
| Mean gestational age (weeks) | 35.5 | 35.3 | | | |
| Mean birthweight (g) | 2628 | 2301 | | | |
| Stillbirth | 0 | 2 Siblings | | | |
| # offspring with congenital anomalies | 2 | 0 | | | |
unrelated to maternal age, paternal age, gonadotrophin dosage, peak estradiol and progesterone levels, number of oocytes collected and number of embryos transferred (Alikani et al., 2003); whereas some have argued a causal relationship between the higher number of embryos transferred and MZ twinning (Scott Sills et al., 2000).

In general, DNA analysis is the Gold Standard for determining MZ status but was not performed routinely in our cohort of MZ twins, as is the case in most studies on MZ twins (Blickstein, 2005). The most frequently used estimation of monozygosity counts the number of monochorionic twins, either by antenatal sonography or by post-partum placental examination. However, several limitations must be taken into consideration. Despite the current developments in ultrasonographic equipment that generally allow very accurate determination of chorionicity and amniotic types in multifetal pregnancies at first trimester ultrasound (Monteagudo et al., 1994), limited access to this equipment or inappropriate or untimely application may lead to inaccuracies. First, the incidence of MZ twins may generally be overreported if implantation of the placentas was so close that no distinction could be made. In association with same sex and lack of documentation of the intertwin membrane, which helps in identifying the chorionicity, zygosity [MZ or DZ] may not be determined (Bulmer, 1970). Second, zygosity remains unknown in all like-sex dichorionic twins: estimates of MZ twinning based on chorionicity and fetal sex will miss the MZ—dichorionic twins and therefore underestimate the true incidence of MZ twins. And last, also the number of gestational sacs in excess of or equal to the number of transferred embryos may underestimate the frequency of MZ twins because of the inability to differentiate like-sex dichorionic—DZ from dichorionic—MZ twins.

One of the strengths of this study was to only include cycles with blastocyst transfer at Day 5. In the understanding that MZ twins arising in the first 4 days of embryonic development are dichorionic diamniotic and that these would have been picked up on embryological analysis in the lab prior to blastocyst transfer at Day 5, all monochorionic twin pregnancies in our study are to be defined as MZ, thereby avoiding the aforementioned potential biases in MZ twin identification (Tarlatzis et al., 2002; Blickstein, 2005). On the other hand, the true incidence of MZ twins is underestimated in our study owing to the inability to differentiate like-sex dichorionic—DZ twins from dichorionic—MZ twins following arrestment development of one of two transferred embryos (Scott Sills et al., 2000; Blickstein, 2005) and because there is no confirmation by DNA analysis of monozygosity. Most published reports have used the same criteria for identifying MZ twin pregnancies, i.e. without post-natal DNA analysis being available (Frankfurter et al., 2004; Wright et al., 2004).

Potential mechanisms that may increase the incidence of MZ twins both in IVF/ICSI and PGD are given in Table I. They include breaks in the zona pellucida associated with handling, and especially the creation of intentional holes e.g. by ICSI or embryo biopsy for PGD. The technique for breaching the zona pellucida by laser in order to perform embryo biopsy for PGD is to some degree analogous with that used for AH. This technique has been advocated in patients with poor embryonic characteristics or implantation failure, and is aimed at improving implantation by facilitating the hatching process by creating a hole in the zona pellucida or by zona thinning (Alikani et al., 2003). The opinion about the role of hatching in the etiology of MZ twins in assisted reproduction is divided. By performing AH, the overall multiple pregnancy rates per embryo transfer increases from 6.8 to 13.1% (Hershlag et al., 1999). In one study, the rate of MZ twinning was significantly increased in the hatched series to 1.2% per embryo transfer. A possible explanation is that a zona opening, sometimes too narrow, could lead to splitting of the ICM (Hershlag et al., 1999). In another study AH, in combination with patient and embryo conditions that justified embryo hatching, was shown to increase the risk for MZ twinning significantly (Alikani et al., 2003). Scott Sills et al. (2000) on the other hand suggest that the causal relationship between AH and MZ twinning is highly speculative: among IVF study patients they found that the frequency of MZ twinning was not significantly different between zona manipulated and zona intact subgroups, and they think it may be premature to link MZ twinning and AH. The greater overall frequency of MZ twinning for IVF patients could result from the higher number of embryos transferred in IVF, rather than zona manipulations. They also argue that blastocoelae have never been observed to divide after passing through an artificial zona opening. Also, MZ twin evolution as well as natural blastocyst hatching in humans is incompletely characterized; what is known has been extrapolated from animal models (Scott Sills et al., 2000). Other studies have not been able to establish a relationship between zona breaching techniques and MZ twinning either (Blickstein et al., 1999; Schachter et al., 2001; Milki et al., 2003).

An inducing effect of prolonged embryo culture on the incidence of MZ twinning was suggested before by Edwards et al. (1986) and was later proposed by other authors (Behr et al., 2000). Splitting of the ICM may be induced by perturbations of cell-to-cell adhesion. Also the use of specific culture media can be associated with the appearance of MZ twinning (Behr et al., 2000; Menezo and Sakkas, 2002). A number of hypotheses have been proposed. In vitro related zona hardening as a result of prolonged culture may induce division of embryos into two during (assisted) hatching (Alikani et al., 1994). Another hypothesis presumes a possible anomaly in the distribution of the ICM cells as a consequence of sensitivity to culture media containing an excessive glucose level. The ICM cells appear more sensitive to apoptosis than trophoblastic cells (Menezo and Sakkas, 2002).

The presence of congenital anomalies in one set of sibling PGD twins in this study is to be seen as incidental as the study group is too small to allow statistical analysis. An increased incidence of congenital anomalies in MZ twins, however, has been reported (Hall, 2003). It is clear that MZ twins are at high risk of perinatal morbidity and mortality in view of the low mean birthweight and low gestational age at birth, also observed in this study. A correlation between amnioncysis and congenital anomalies or perinatal complications could not be established in this study.

**Conclusion**

Embryo biopsy for PGD does not cause an increase in the incidence of MZ twins. This information is useful in counselling patients undergoing PGD about the potential risks of the technique. Larger sample sizes are required to provide higher statistical power.

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