

Monozygotic twinning associated with assisted reproductive technologies: a review

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Abstract

Twin birth rates have increased markedly in developed countries since the 1970s for two primary reasons: increasing maternal age and the advent and increasing use of fertility treatments. In addition, monozygotic (MZ) twin pregnancies have been reported to occur at a significantly higher rate following assisted reproductive technologies (ART) procedures compared with the natural incidence. Twin pregnancies are of concern due to a dramatically increased risk of associated complications. Monozygotic twin pregnancies carry a 10–20% risk of twin–twin transfusion syndrome, and monoamniotic monochorionic twins are additionally at risk for cord entanglement. While the mechanisms and contributory factors for dizygotic twinning are well established, very little is known about the mechanisms involved in MZ twinning or the factors that contribute to its occurrence. In this review, we will discuss a number of potential mechanisms involved in MZ twinning and explore factors that may be contributing to the increased incidence of ART-associated MZ twins. An improved understanding of the factors that contribute to increased MZ twinning associated with ART will help to elucidate the poorly understood mechanisms involved in the process and will further aid in reducing the overall incidence of multiple pregnancies with their associated risks following ART procedures.

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Introduction

In humans, twins generally result from one of two scenarios. Dizygotic (DZ) twins result from the ovulation and fertilization of two oocytes with the resultant twins sharing the same genetic relationship as common siblings. Monozygotic (MZ) twins, on the other hand, are the result of a single fertilized embryo splitting at some stage of early embryo development to form two separate embryos derived from one zygote. DZ twinning is associated with increased maternal serum follicle-stimulating hormone associated with maternal genetics (White & Wyshak 1964), age (Beemsterboer *et al.* 2006), parity (Rao 1978), and other factors (Hall 2003). The occurrence of DZ twinning varies widely between populations ranging from about 6/1000 in Asia to 10–20/1000 in the United States and Europe and as high as 40/1000 in Africa (Hall 2003).

While the genetic contribution to DZ twinning is well established, genetic associations with the occurrence of MZ twins have been described infrequently, and penetrance for this trait seems to be extremely low (Parisi *et al.* 1983, Steinman 2003, Hamamy *et al.* 2004). Monozygotic twin occurrence is universally about 0.4–0.45% following non-stimulated *in vivo* conception (MacGillivray 1986,

Derom *et al.* 1987). The universally low incidence of MZ twinning independent of environmental factors has been suggested to support the notion of a genetic rather than an environmental contribution (Bortolus *et al.* 1999). Overall, MZ twin pregnancies account for ~30% of all naturally conceived twin pregnancies.

With the relatively recent advent and growing popularity of assisted reproductive technology (ART) procedures, the occurrence of both DZ and MZ twins has increased. Monozygotic twinning following *in vitro* fertilization (IVF) was first reported in 1984 (Yovich *et al.* 1984), and since that time numerous studies have demonstrated an increased occurrence of MZ twins associated with ART procedures. Following 127 977 ART procedures in the United States in 2004, 32% of live birth deliveries were twin or higher order pregnancies (Wright *et al.* 2007). In Europe, summaries of 365 103 ART cycles in 2003 indicated that twin and triplet pregnancies accounted for 23% of all deliveries (Andersen *et al.* 2007).

The United States and European summaries do not include data on the zygosity of twin births, but other published reports indicate that MZ twinning rates following ART procedures are between two and twelve times higher than the natural occurrence of 0.4%

(Edwards *et al.* 1986, Blickstein *et al.* 1999, 2003, Sills *et al.* 2000a, Alikani *et al.* 2003), and several cases of MZ triplets derived from ART procedures have also been reported (Ghulmiyyah *et al.* 2003, Risquez *et al.* 2004, Unger *et al.* 2004, Henne *et al.* 2005, Faraj *et al.* 2007, Yanaihara *et al.* 2007). A population-based study analyzing data provided by the British Human Fertilization and Embryology Authority representing 15 644 cycles involving single embryo transfers and 1104 live births found the rate of multiple pregnancies among live births to be 2.3% (Blickstein *et al.* 2003). In many studies, the rate of MZ twinning is erroneously based only on the number of monochorionic twins detectable by ultrasound, and dichorionic twins are inaccurately labeled as DZ. This undoubtedly results in underrepresentation of the actual rate of MZ twinning in many cases (Wenstrom *et al.* 1993, Blickstein 2005).

Following ART procedures, DZ twin occurrence is higher as a result of ovarian stimulation protocols and multiple embryo transfers. The reasons for increased MZ twinning frequency associated with ART techniques are less well defined; however, ovarian stimulation, zona manipulation including intracytoplasmic sperm injection (ICSI) and assisted hatching (AH), maternal age, and factors associated with *in vitro* embryo culture may contribute as discussed below.

Complications in twin pregnancies

The occurrence of MZ twins is of clinical importance because of the increased complications associated with twin pregnancies in general as well as MZ twins specifically. Twin pregnancies are characterized by an increased incidence of both fetal and maternal complications. Fetal complications include restricted growth as well as increased incidence of fetal loss, pre-term delivery, and perinatal loss. Mothers carrying twins more frequently suffer pregnancy-associated nausea and vomiting, gestational hypertension, and pre-eclampsia than those carrying singletons (Rao *et al.* 2004).

Placental arrangement is an important factor in outcome of MZ pregnancies, as occurrence of complications depends largely on the degree of placental sharing. The placental architecture of MZ twins, which can vary from completely separate amniotic and chorionic sacs to a single amniotic and chorionic sac shared by both twins, indicates the embryonic stage at which the embryo splits. If the embryo divides prior to embryonic cell differentiation (within the first 4 days following fertilization), the resultant fetuses will be dichorionic diamniotic (DCDA). If the inner cell mass (ICM) divides during the pre-implantation blastocyst stage, the twins will be monochorionic diamniotic (MCDA), and if the division occurs in the post-implantation blastocyst period, monochorionic monoamniotic (MCMA) twins result (Hall 2003). DCDA conformation accounts for ~25–30% of MZ

twin pregnancies, MCDA occurs in 70–75% of MZ twin pregnancies, and MCMA in 1–2% of liveborn MZ twins (Hall 2003). The great majority of DZ twins display a DCDA placental arrangement; however, it should be noted that an MCDA arrangement has been observed in DZ twins (Souter *et al.* 2003).

The incidence of fetal complications is similar between DZ twins and dichorionic MZ twins; however, monochorionic twins are susceptible to additional complications. The majority of monochorionic twins have vascular anastomoses, and this shared blood supply can result in twin–twin transfusion syndrome, a condition characterized by unequal sharing of the maternal blood supply, which results in asymmetrical fetal growth and fetal mortality in 80% or more of untreated cases (Fieni *et al.* 2004). The syndrome is estimated to occur in 10–20% of monochorionic twin pregnancies (Harkness & Crombleholme 2005). MCMA twin pregnancies carry even greater risk due to the potential for umbilical cord entanglement (Dickinson 2005).

A study of the natural course of MZ twin pregnancies evaluated pregnancy outcomes of MZ twins in a historical cohort of 651 twin pairs born between 1907 and 1938. In this group, perinatal mortality was 27.7% for monochorionic twins and 15.8% for dichorionic twins, and the risk of perinatal mortality was associated with low birth weight and pre-term delivery (Hack *et al.* 2006). Owing to the scarcity of data reporting chorionicity of ART-derived MZ twins, the relative occurrence of each type is unclear, but a recent report of 74 ART-derived MZ pregnancies of known chorionicity indicated ~10% were DCDA, 53% were MCDA, and an alarming 37% were MCMA (compared with 1–2% of *in vivo* produced MZ twins; Alikani *et al.* 2003). Derom *et al.* (1987) reviewed a much smaller number of MZ twins and found only 46% of MZ twins resulting from ovarian stimulation were monochorionic compared with 63% of controls, but the difference was not significant. The same group later reported an increased incidence of monochorionic MZ twins following ovarian stimulation of 80% compared with 64% in controls (Derom *et al.* 2006). One study has been cited as reporting a significantly lower incidence of monochorionic twins following ovarian stimulation and IVF compared with spontaneously conceived twins, but zygosity of dichorionic twins was not characterized in this study (Putterman *et al.* 2003) so the relative reduction in monochorionic twins is most likely the result of a disproportionate increase in DZ twin pregnancies.

Proposed mechanisms for MZ twinning

Surprisingly little is known about the mechanisms or risk factors involved in early embryonic development that give rise to MZ twins; however, a number of theories have been proposed. It is clear based on the various placental

configurations observed in MZ twins that timing of the twinning event as well as the mechanism involved can vary (Scott 2002). A major limitation in understanding MZ twinning mechanisms is the lack of animal models for study. The only naturally occurring animal model to produce MZ offspring on a regular basis is the armadillo, which produces identical quadruplets resulting from two binary fissions of a single fertilized oocyte (Blickstein & Keith 2007). It has been proposed that these fission events are the result of delayed post-fertilization implantation in the armadillo. Following breeding, the embryo develops to hatching blastocyst and then undergoes a period of developmental arrest. After several months of arrested development, the embryo resumes growth, the epiblast undergoes the two binary fissions, and implantation occurs (Enders 1962, 2002a).

The hypothesis that embryo fission occurs as a result of delayed implantation does not reconcile the absence of MZ twin formation in other species that undergo embryonic diapause; however, there may be some conserved mechanisms common to armadillo and

some cases of human MZ twinning. The possibility of some human MZ twins arising from a late splitting event similar to the armadillo is supported by the observation of a partially split embryonic shield in a day 15 rhesus monkey embryo (Enders 2002b).

Monozygotic twinning has been induced experimentally in a number of species by a variety of mechanisms. Early in the twentieth century, experiments in fish demonstrated slowing of development in early fertilized embryos by either lowering incubation temperature or reducing available oxygen resulted in increased incidence of MZ twins (Stockard 1921). Likewise, hypoxic embryo culture in mice and delayed fertilization in rabbits has been shown to induce MZ twinning (Hall 2003). In these cases, twinning may be induced by disruption of communication between blastomeres at various stages of development (Fig. 1A-1, B-1 and C-1). With the improvement in microscopic and micromanipulator technology, mechanical induction of MZ twinning by bisection of pre-implantation embryos, generally morulae and blastocysts, has proven effective in a

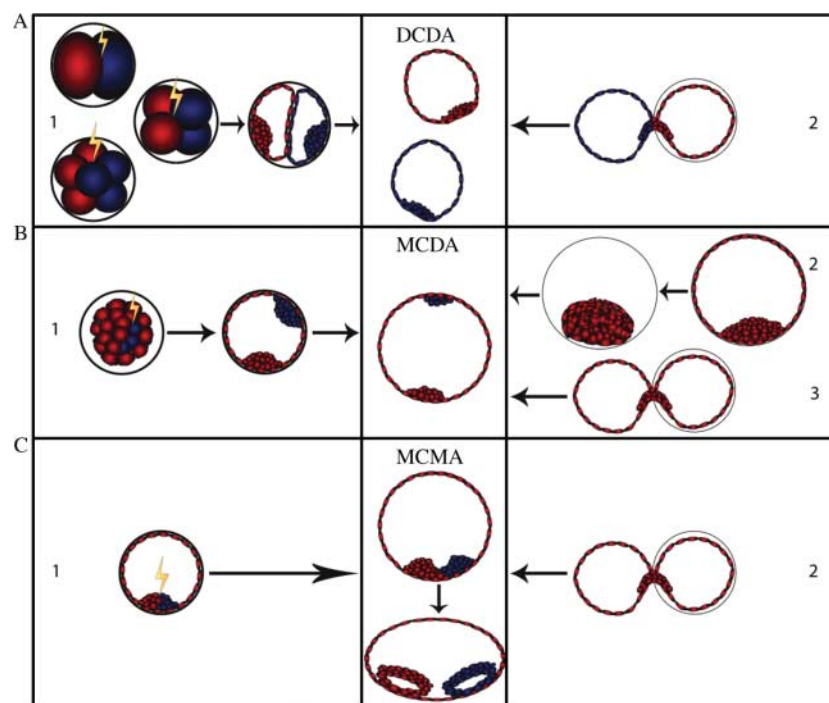


Figure 1 Potential mechanisms by which MZ twins might arise following ART. (A) DCDA MZ twins might result from: 1) insult to the early embryo by factors such as sub-optimal culture conditions or temperature fluctuations resulting in disruption of communication between blastomeres and the independent formation of two separate blastocysts. 2) Breach in the zona by ICSI or AH, or hardening of the zona by ovarian stimulation or sub-optimal culture conditions resulting in abnormal blastocyst hatching. If hatching results in bisection of the trophoctoderm and ICM, two separate blastocysts can form. (B) MCDA MZ twins might result from: 1) insult to the morula resulting in disruption of communication between inner blastomeres and the independent formation of two separate ICMs within the same blastocyst. 2) Blastocyst collapse, adhesion of ICM cells to another point within the trophoctoderm, and the formation and growth of a second ICM. 3) Breach in or hardening of the zona resulting in abnormal blastocyst hatching. If hatching results in adhesion of ICM cells to another point within the trophoctoderm, the formation and growth of a second ICM can result. (C) MCMA MZ twins might result from: 1) insult to the early blastocyst resulting in disruption of communication between early ICM blastomeres and the formation of two separate embryonic disks within the same blastocyst. 2) Breach in or hardening of the zona resulting in abnormal blastocyst hatching. If hatching results in disruption of communication between ICM cells (e.g. by apoptosis of some ICM cells), the formation of two separate embryonic disks within the same blastocyst can result.

number of mammalian species, including cattle (Ozil *et al.* 1982), mice (Nagashima *et al.* 1984), sheep (Chesne *et al.* 1987), pigs (Reichelt & Niemann 1994), and rhesus monkeys (Mitalipov *et al.* 2002).

Though MZ twinning occurs rarely if ever in mice (McLaren *et al.* 1995), it has been demonstrated that *in vitro* culture of mouse embryos can induce MZ twin formation in ~1% of embryos cultured (Hsu & Gonda 1980). The twinning was induced when hatched blastocysts attached to the culture dish antipolar to the ICM, and embryonic growth coupled with physical growth restrictions resulted in subdivision of the ICM. Another study reported the occurrence of double ICMs at a rate of 0.6 and 3.1% respectively for *in vivo* and *in vitro* fertilized mouse embryos prior to hatching (Chida 1990).

Two cases from human IVF suggest that double ICMs account for some MZ pregnancies. One report exists on the day 5 transfer of two blastocysts, including one with a double ICM, resulting in a triplet pregnancy (Meintjes *et al.* 2001). Unexpectedly, the MZ twins were DCDA, indicating the trophoctoderm was presumably split in the embryo. In another IVF laboratory, time-lapse photography of developing human IVF embryos documented the formation of two distinct ICMs in two of twenty-six embryos analyzed. The formation of double ICMs was believed to result from ectopic adhesion and subsequent proliferation of ICM cells on the opposing trophoctoderm wall following blastocoel collapse and re-expansion (Fig. 1B-2; Payne *et al.* 2007).

The role of the zona on induction of MZ twinning by mechanical splitting or ICM disruption of the hatching blastocyst has also been proposed (Edwards *et al.* 1986; Figs 1A-2, B-3, C-2 and 2). Factors such as thickening or hardening of the zona have been reported to contribute to twinning (Edwards *et al.* 1986, Tarlatzis *et al.* 2002).

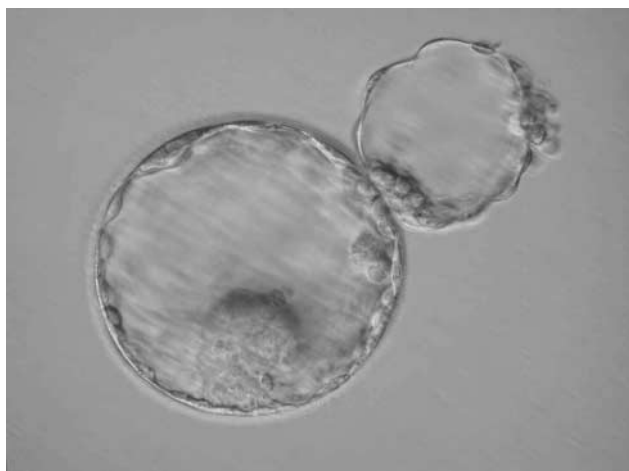


Figure 2 Abnormally hatching blastocyst following ICSI. Blastocyst hatching through a small hole in the zona as a result of zona hardening or zona manipulation may result in MZ twin formation by either complete embryo bisection or disruption of ICM cells.

While the zona may be involved in some cases of MZ twinning, it is certainly not the exclusive mechanism as MZ twinning has been demonstrated to occur following transfer of zona-free blastocysts (Frankfurter *et al.* 2001).

Together, the multiple mechanisms of MZ twinning observed and induced experimentally along with the occurrence of multiple placental arrangements in ART-associated MZ pregnancies suggest that MZ twinning is likely associated with a variety of factors that may include delay in fertilization, embryo development, or implantation, mechanical disruption of the early embryo, and alterations in culture conditions. Indeed, a combination of factors is likely involved.

Association of MZ twinning with ART

Over two decades ago, it was observed that MZ twinning frequency was greater following ART procedures (Edwards *et al.* 1986) and artificial induction of ovulation (Derom *et al.* 1987). These early reports have been followed by numerous additional corroborating studies cited below; however, a large Danish cohort study recently published compared MZ and DZ twin rates between natural conceptions and conceptions following infertility treatment and found no association between fertility treatment and MZ twin deliveries (Zhu *et al.* 2007). Owing to the relatively limited number of individuals in the study undergoing infertility treatment and the small number of MZ twins in the group, the authors were not able to analyze MZ twinning rates based on treatment type. As a result, ICSI, IVF, intrauterine insemination, and hormone treatment cases were all analyzed together.

In the context of ART, there exists a great deal of debate over the factors that contribute to increased MZ twinning as well as the mechanisms involved. Maternal age, ovarian stimulation, zona manipulation, temperature effects, and *in vitro* embryo culture have all been suggested as contributory factors in ART-associated MZ twinning. Table 1 summarizes reports investigating the impact of some of these factors on MZ twinning rates.

Maternal age

While there is some disagreement as to the impact of maternal age on MZ twinning rate (Steinman 2001), a 12–22% increase in MZ twinning has been reported in women over the age of 35 compared with women younger than 25, while parity has been shown to have little or no effect (Bulmer 1970). On the other hand, an epidemiological review of a number of studies concluded that the rate of MZ twinning is not affected by maternal age (Bortolus *et al.* 1999). More recent work in the context of IVF/ICSI treatment reported an increased risk of MZ twinning in women over the age of 35 (Abusheikha *et al.* 2000), although this conclusion was

Table 1 Review of ART-associated MZ twinning publications.

Reference	Type of ART	Total transfers	Total pregnancies	Number of MZ twin pregnancies	MZ twin pregnancies/total pregnancies (%)
Control					
Derom <i>et al.</i> (1987)	None		99 591	447	0.4
Ovulation induction					
Derom <i>et al.</i> (1987)	AOI		1552	18	1.2
Kallen <i>et al.</i> (2002)	AOI		4029	31	0.8
Schachter <i>et al.</i> (2001)	AOI		129	2	1.6
Zona manipulation					
Alikani <i>et al.</i> (2003)	IVF		2185	35	1.6
	ICSI		1537	37	2.4
Elizur <i>et al.</i> (2004)	IVF		389	4	1.0
	ICSI and/or AH		667	6	0.9
Hershlag <i>et al.</i> (1999)	IVF	559	141	0	0.0
	AH	674	250	8	3.2
Meldrum <i>et al.</i> (1998)	IVF		320	3	0.9
	AH		136	1	0.7
Milki <i>et al.</i> (2003)	IVF		267	10	3.7
	ICSI and/or AH		287	8	2.8
Saito <i>et al.</i> (2000)	IVF	3105	196	3	1.5
	ICSI, SUZI, or PZD	1044	83	6	7.2
Schachter <i>et al.</i> (2001)	IVF	475	139	1	0.7
	ICSI and/or AH	1438	463	4	0.9
Schieve <i>et al.</i> (2000)	IVF	21 490	7163	9	0.1
	Mix IVF+AH	3310	1327	2	0.2
	AH	10 703	3650	12	0.3
Sills <i>et al.</i> (2000b)	IVF		121	3	2.5
	ICSI		151	1	0.7
	AH		589	8	1.4
	ICSI and AH		813	7	0.9
Tarlatzis <i>et al.</i> (2002)	IVF	48	15	0	0.0
	ICSI	79	33	6	18.2
Embryo freezing					
Alikani <i>et al.</i> (2003)	Fresh ET		3250	72	2.2
	Frozen-thawed ET		511	9	1.8
Blastocyst transfer					
Behr <i>et al.</i> (2000)	D5 transfer		199	10	5.0
da Costa <i>et al.</i> (2001)	D3 transfer	2584	814	6	0.7
	D5 transfer	335	186	5	2.7
Jain <i>et al.</i> (2004)	D3 transfer	90	47	1	2.1
	D5 transfer	75	38	5	13.2
Milki <i>et al.</i> (2003)	D3 transfer		357	7	2.0
	D5 transfer		197	11	5.6
Moayeri <i>et al.</i> (2007)	D3 transfer		547	10	1.8
	D5 transfer		385	9	2.3
Papanikolaou <i>et al.</i> (2006)	D2 transfer	176	41	2	4.9
	D5 transfer	175	58	0	0.0
Rijnders <i>et al.</i> (1998)	D2 transfer		960	5	0.5
	D3 transfer		735	5	0.7
	D5 transfer		111	3	2.7
Sheiner <i>et al.</i> (2001)	D3 transfer		204	3	1.5
	D5 transfer		9	2	22.2
Wright <i>et al.</i> (2004)	D2 transfer	6679	2337	5	0.2
	D3 transfer	77 256	29 144	117	0.4
	D4 transfer	3169	1010	4	0.4
	D5 transfer	17 628	7921	119	1.5
	D6 transfer	3604	1122	10	0.9

AOI, artificial ovulation induction; AH, assisted hatching; ART, assisted reproductive technologies; D, day; ICSI, intracytoplasmic sperm injection; IVF, conventional *in vitro* fertilization; MZ, monozygotic; PZD, partial zona dissection; SUZI, sub-zonal insemination.

based on trends rather than statistical significance, and the numbers of MZ twins in the study were small. Likewise, Alikani *et al.* (2003) reported a trend ($P=0.07$) toward increasing MZ twin rates following IVF associated with advanced maternal age, while a more recent report that evaluated monochorionic MZ

twins exclusively found no association between maternal age and monochorionic twin occurrence (Skiadas *et al.* 2008). These limited data indicate that increasing maternal age may contribute to an increased incidence of MZ twins, but the contribution is likely minimal in ART cases.

Ovarian stimulation

The earliest report of increased MZ twinning following ovarian stimulation was based on data collected from the East Flanders Prospective Twin Study (EFPTS), an ongoing population-based study of the East Flanders Province in Belgium, which was initiated in 1964. Evaluation of zygosity of 2648 multiple births indicated an MZ twinning rate of 1.2% following ovarian stimulation, approximately three times higher than the incidence in spontaneously conceived pregnancies (Derom *et al.* 1987). More recently, Kallen *et al.* (2002) indicated that women undergoing ovarian stimulation delivered MZ twins at nearly twice the rate of controls. A later EFPTS analysis indicated that a higher proportion of twins conceived following ovarian stimulation with clomiphene citrate were MZ compared with iatrogenic cases where no clomiphene citrate was used (Derom *et al.* 2006). Another study concluded MZ frequency was more closely associated with controlled ovarian stimulation and ovulation induction by gonadotropin therapy than other ART procedures with an overall MZ rate of 0.95% for all ART procedures and a rate of 1.5% following ovulation induction or ovarian stimulation alone (Schachter *et al.* 2001). It has been suggested that this increased incidence of MZ twins may be a result of either a delay in implantation (Van der Auwera & D'Hooghe 2001, Steinman 2001) or hardening of the zona (Longo 1981, Edwards *et al.* 1986, Derom *et al.* 1987) induced by ovulatory hormones.

Zona pellucida manipulation

Two primary zona manipulation techniques are commonly utilized in assisted reproduction to improve fertilization and implantation rates. ICSI involves direct injection of a spermatozoon into the oocyte cytoplasm. The technique greatly reduces the number of sperm needed for IVF and allows the use of sub- or non-motile sperm. The first ICSI pregnancy was achieved in 1991 (Palermo *et al.* 1992). Since that time, ICSI has become a common procedure for some forms of male factor infertility.

The other zona manipulation technique, assisted hatching, involves chemical, mechanical, or laser manipulation of the zona pellucida of a fertilized embryo in an effort to facilitate embryo hatching and enhance the probability of implantation. Since the initial report in 1990 demonstrating its effectiveness in improving implantation in some patients (Cohen *et al.* 1990), AH has also become a common practice in ART.

It has been proposed that an artificial breach in the zona by ICSI or AH and subsequent herniation of blastomeres through the hole in the zona during blastocyst expansion might result in mechanical splitting of the blastocyst and formation of MZ twins (Talansky & Gordon 1988). This phenomenon has been recorded by

time-lapse photography in bovine embryos (Van Langendonck *et al.* 2000).

There are a number of reports indicating zona manipulation increases the risk for MZ twinning (Hershlag *et al.* 1999, Abusheikha *et al.* 2000, Saito *et al.* 2000, Schieve *et al.* 2000, Tarlatzis *et al.* 2002, Skiadas *et al.* 2008); conversely, there are several studies that demonstrate no association between zona manipulation and increased MZ twinning rates (Meldrum *et al.* 1998, Behr *et al.* 2000, Sills *et al.* 2000b, Schachter *et al.* 2001, Milki *et al.* 2003, Elizur *et al.* 2004, Yanaihara *et al.* 2007).

An early report of six cases of MZ twins observed that each of the six cases was associated either with naturally thin zonae or with some sort of zona manipulation. In the same retrospective study, no MZ twins resulted from transfers of embryos with normal zona thickness or embryos with intact zonae (Alikani *et al.* 1994). Hershlag *et al.* reported no MZ twins occurred out of 559 unhatched embryo transfers and one MZ triplet and seven MZ twin pregnancies resulted from 674 hatched embryo transfers (1.2%; $P < 0.01$). However, the unhatched embryos were transferred between 1990 and 1993, and the hatched embryos were transferred between 1994 and 1996, and there is no indication of the stages of embryos transferred (Hershlag *et al.* 1999).

Analysis of data collected through a national ART survey in Japan between 1994 and 1995 representing nine MZ twin pregnancies out of 279 (3.2%) total pregnancies also revealed a trend toward increased incidence of MZ twins following zona manipulation techniques with an apparent increased risk associated with larger breaches in the zona (Saito *et al.* 2000). In another case, six MZ pregnancies resulted from 102 cycles following ICSI (5.9%), and no MZ pregnancies were detected in 79 cycles following conventional IVF ($P = 0.033$; Tarlatzis *et al.* 2002). Retrospective analysis by Alikani *et al.* (2003) of 81 cycles resulting in MZ twins in one clinic indicated an association between zona manipulation and MZ twinning with 2.4% of MZ twins occurring following ICSI cycles and 1.6% following standard insemination, but the difference was not significant. Two other reports also suggest a possible correlation between zona manipulation and MZ twinning rate (Abusheikha *et al.* 2000, Schieve *et al.* 2000). A recent study evaluated risk factors specifically for monozygotic twinning and found a significant association between ICSI and monozygotic twins as well as an association approaching significance between AH and monozygotic twins (Skiadas *et al.* 2008).

A major limitation of studies evaluating the effect of zona manipulation on MZ twinning rate is the extremely low incidence of MZ twinning even following ART procedures. With that limitation in mind, it is certainly easier to demonstrate a lack of significance than to show significance; however, it is clear that additional studies with greater statistical power will be necessary in order

to more fully address the question of whether zona manipulation results in an overall increased incidence of MZ twinning.

Temperature effects

As it has been demonstrated that temperature fluctuations in developing fish embryos result in increased MZ twinning (Stockard 1921), it has been proposed that there may be a temperature effect in ART-associated MZ twinning. The only association with temperature changes and MZ twinning that have been addressed is the incidence of MZ twinning following the transfer of frozen-thawed embryos. There has been brief mention of the potential for such an association (Toledo 2005, Faraj *et al.* 2007), but other reports indicate no relationship between the transfer of frozen-thawed embryos and MZ twinning rate (Alihani *et al.* 2003, Blickstein *et al.* 2003). If embryo cryopreservation results in increased MZ twinning, the increase is likely associated with zona hardening rather than a direct temperature effect. The involvement of minor temperature fluctuations on MZ twinning in manipulated and *in vitro* cultured embryos has not been addressed but may warrant investigation as embryos undergoing these procedures are subject to temperature changes when removed from the incubator for any period of time.

Blastocyst transfer

The first report of MZ twins resulting from the day 5 blastocyst transfer without zona manipulation was in 1999 when one case of two-blastocyst transfer resulted in an MCDA set of twins and another resulted in a singleton and an MCMA set of twins (Peramo *et al.* 1999). Blastocyst embryo transfer generally results in improved pregnancy rates compared with cleavage-stage embryo transfers (Wilson *et al.* 2002, Papanikolaou *et al.* 2006); however, a number of reports have suggested that blastocyst transfer may result in increased MZ twinning rate.

An early retrospective study by Behr *et al.* (2000) collected data from multiple IVF laboratories to analyze MZ pregnancy rates following blastocyst transfer. In all, ten MZ twin pregnancies resulted, yielding an overall incidence of 5.0%. Several other smaller studies have reported increased MZ twinning associated with blastocyst transfer (Rijnders *et al.* 1998, Sheiner *et al.* 2001). Analysis of data reported by 93% of all US ART laboratories to the Centers for Disease Control and Prevention for embryo transfer procedures performed in 1999 and 2000 also demonstrated a link between blastocyst transfer and increased MZ twinning rates. Pregnancies for which the number of ultrasound-detectable fetal heartbeats exceeded the number of transferred embryos were considered to contain at least

one set of MZ twins. Out of 39 198 pregnancies, 226 MZ twins were detected (0.6%). A significantly greater proportion of MZ pregnancies were derived from the day 5 or 6 embryo transfers compared with the cleavage-stage transfers (Wright *et al.* 2004).

Another retrospective study of ICSI embryos found MZ twin occurrence in 5/129 (3.9%) blastocyst transfer pregnancies occurring between 1998 and 1999 compared with only 6/814 (0.7%) following four- to eight-cell transfers performed from 1996 to 1999 ($P < 0.001$; da Costa *et al.* 2001). Similarly Jain *et al.* compared the MZ twinning rate of 96 day 3 embryo transfers performed between 1997 and 1998 with 75 blastocyst transfers occurring between 1999 and 2000. They found that 1/47 (2.1%) day 3 embryo transfer pregnancies were MZ twins compared with 5/32 (13.2%) following blastocyst transfer (Jain *et al.* 2004). A somewhat larger retrospective study by Milki *et al.* (2003) analyzing pregnancy data generated concurrently from cleavage-stage and blastocyst transfers also reported significantly higher incidence of MZ twins following blastocyst transfer (5.6%) compared with cleavage-stage embryo transfer (2.0%; $P < 0.03$). Four years later, this same group performed a similar analysis to determine whether increased experience with blastocyst transfer impacted MZ twinning rates. Results of the more recent study indicated that MZ twinning rates associated with blastocyst transfer had declined over time so that recent MZ twinning rates following blastocyst transfer (2.3%) were not significantly different from rates in the cleavage-stage embryo transfer group (1.8%; Moayeri *et al.* 2007). Their conclusion was that improved culture conditions as well as increased experience with blastocyst embryo culture and transfer contributed to the decline in MZ twinning. The recent study by Skiadas *et al.* (2008) that reported an association between zona manipulation and the incidence of monozygotic twins also found a significantly higher incidence of monozygotic twins following day 5 embryo transfer. Further analysis by the same group found that the combination of zona manipulation and day 5 embryo transfer had a compounding effect on monozygotic twinning rates as an increased incidence of monozygotic twins occurred following day 5 transfer of ICSI embryos compared with either day 3 transfer of ICSI embryos or day 5 transfer of non-ICSI embryos (Skiadas *et al.* 2008). The phenomenon of a synergistic effect of multiple factors on MZ twinning rates had not been demonstrated in previous studies.

A recent randomized trial of women under the age of 36 undergoing single embryo transfer with day 3 or day 5 embryos found significantly higher clinical pregnancy and delivery rates following blastocyst transfer. In this group, there were no MZ pregnancies out of 73 pregnancies derived from blastocyst transfer, and there were two MZ twin pregnancies out of 59 day 3 embryo transfer pregnancies (3.4%; Papanikolaou *et al.* 2006).

As is the case with reports linking zona manipulation with MZ twinning, there is not complete consensus that blastocyst transfer inherently results in increased MZ twinning rates. However, the majority of reports, particularly larger studies, seem to suggest an association between blastocyst transfer and MZ twinning rates. Based on available data, it seems likely that blastocyst embryo transfer contributes to the increased incidence of MZ twinning, but it is certainly not the sole factor.

Sub-optimal culture conditions

Hardening of the zona following prolonged embryo culture (Behr *et al.* 2000) or sub-optimal culture conditions leading to splitting of the ICM by selective apoptosis (Menezo & Sakkas 2002) or blastocoel collapse (Payne *et al.* 2007) in embryos grown *in vitro* have been proposed as mechanisms contributing to increased MZ twin rates in blastocyst transfer pregnancies. Interestingly, two groups have noted the absence of MZ twins following embryo transfer of blastocysts grown with co-culture (Behr *et al.* 2000, Menezo & Sakkas 2002), lending credence to the contribution of extended culture under sub-optimal conditions to the increased incidence of MZ twinning. Menezo's group later reported the effect of prolonged embryo culture on MZ twinning rates was dependent on the culture media, suggesting that improved culture media may reduce the incidence of MZ twins following IVF (Cassuto *et al.* 2003). In addition, the two studies by Milki *et al.* discussed previously suggest that increased experience in embryo culture or improved embryo culture conditions resulted in decreased rates of MZ twinning (Milki *et al.* 2003, Moayeri *et al.* 2007).

Conclusions

There are several factors that confound the characterization of elements contributing to ART-associated MZ twinning. A primary limitation is the lack of a basic understanding of the mechanisms involved in spontaneous MZ twinning. In addition, the extremely low occurrence of spontaneous and ART-associated MZ twins greatly increases the number of study cases necessary to establish appropriate statistical power. By necessity, studies evaluating MZ twin occurrence are retrospective, and the majority contain mixed treatments and/or potentially confounding variables, which further lessen their statistical power. Monozygosity can be diagnosed by ultrasound based on membrane arrangement or *post partum* based on membrane arrangement, physical similarity or genetic testing, the latter being the most reliable. Following IVF, in many cases, more than one embryo is transferred so only monochorionic twins can be diagnosed by ultrasound to be MZ thus missing DCDA MZ twins, which in spontaneous MZ twins can account for a quarter or more of the cases (Hall 2003).

Additionally, postpartum assessment of zygosity following multiple embryo transfer fails to include vanishing twins or complete miscarriages that have been reported to occur in 12.1 and 5.0% of IVF/ICSI twin pregnancies respectively. In light of these facts, the true incidence of MZ twins is likely greater than the reported incidence in many cases.

Routine determination of both zygosity and chorionicity of all multiple births will greatly aid in understanding the timing and associated mechanisms involved in MZ twinning. The Society for Assisted Reproductive Technologies, ESHRE and other reporting agencies could add this to the information collected from reporting clinics. Further, the increasing use of single embryo transfer will shed light on the true incidence of MZ twinning.

While the data regarding chorionicity are too sparse to determine the overall occurrence of each type of placentation, the fact that all three configurations of MZ placentation have been observed following ART indicates that MZ twinning occurs at various times during embryo development and by a variety of mechanisms.

Based on the reports reviewed here, it is evident that a number of factors likely contribute to increased MZ twinning associated with ART; however, some factors are probably more important than others. Among the larger studies, the factors that seem to make the most significant contributions to MZ twinning are ovarian stimulation and extended embryo culture coupled with blastocyst transfer. Additional, larger, and more detailed studies will allow for better assessment of the relative risks of each of the potentially contributory factors to MZ twinning. By increasing our understanding of the incidence as well as the causal factors of MZ twinning in ART, we will be better equipped to reduce its incidence and thus avoid the associated complications to fetus and mother.

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