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MINIREVIEW

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Do monochorionic dizygotic twins increase after pregnancy by assisted reproductive technology?

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Abstract Although monochorionic (MC) dizygotic twins (DZT) are extremely rare in natural pregnancy, six pairs of such twins have successively been reported in a recent short period. All six cases of MC DZT were the products of pregnancy by assisted reproductive technology (ART). In this overview, we summarize these six cases and discuss possible mechanisms of this twinning and clinical implications of confined blood cell chimerism (CBC). The placental MC membrane was diagnosed ultrasonographically in all cases and pathologically in four. The presence of CBC was confirmed in four cases by haplotyping at polymorphic marker loci in peripheral blood leukocytes, karyotyping of lymphocytes and skin fibroblasts, and/or ABO blood group typing. As CBC is attributable to placental vessel anastomosis between DZT, it may become a risk factor for twin–twin transfusion syndrome (TTTS), mortality, and for other complications in twins. MC DZT may produce psychological trauma, especially in a girl/woman when she grows up and is known to be chimeric for a male karyotype and vice versa, although genital organs are generally normal—unlike freemartin in cattle. In addition, CBC in twins may mislead physicians when genotyping for a disease-susceptibility test is performed in medical practice in the near future. Blood group chimera may also cause confusion if a blood transfusion is necessary. Therefore, sufficient informed consent prior to ART and genetic counseling before/after birth are absolutely necessary for improved quality of life. It is most likely that all six cases are the consequence of fusion between two outer cell masses from two zygotes. The ART used in the six MC DZT included in vitro fertilization-embryonic transfer (IVF-ET) into the uterus, FSH-induced superovulation followed by intrauterine insemination, and/or intracytoplasmic sperm injection (ICSI). The use of an ovulation-inducing agent and implantation of several fertilized eggs at close sites are probably the events common among these cases. Assisted hatching, simultaneous ET, the use of eggs that have developed to the blastcyst stage, and cell culture procedures that lead to changes of the nature of cell surface, all may increase the chance of a cell fusion. This "chance hypothesis" can simply explain why MC DZT are very rare in natural pregnancy. Large-scale research on the prevalence of ART-associated MC DZT and long-term follow-up of the twins are essential.

Keywords Monochorionic dizygotic twins · Twinning · Confined blood cell chimerism · Assisted reproductive technology · Haplotype analysis · Placental vessel anastomosis · Freemartin

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Introduction

In view of the nature of the placenta, monozygotic (MZT) and dizygotic (DZT) twins are classified as dichorionic-diamniotic MZT/DZT, monochorionic (MC)-diamniotic MZT/DZT, and monochorionic-monoamniotic MZT. MC twins comprise 65% of MZT but are extremely rare in DZT (Redline 2003) (Table 1). A general rule exists in the field of obstetrics that MC twins are almost exclusively MZT (Quintero et al. 2003). However, a case of MC DZT, which possibly resulted from pregnancy by assisted reproductive technology (ART), was recently reported (Souter et al. 2003). This struck a note of warning about ART-mediated MC DZT, and in a short period since then, five other cases of MC DZT associated with ART have successively been reported (Nishio et al. 2003; Tsuruta et al. 2003; Yam-

Table 1 Four different placentations and zygosities of twins. *CM* chorionic membrane, *AM* amniotic membrane, *DZT* dizygotic twins, *MZT* monozygotic twins

Placenta	Number of placenta	Zygosity of twins (%)			
		CM	AM	DZT	MZT
Dichorionic-diamniotic					
Separated placenta	2	2	2	60	25
Fused placenta	2	2	2	40	10
Monochorionic-diamniotic	1	1	2	~0[a]	65
Monochorionic-monoaminiotic	1	1	1	–	< 1

[a]Extremely rare, but seen in six recently reported cases

aguchi et al. 2003) or information given to us from Japan (personal communication to Niikawa; Niikawa 2004). As all MC DZT twins are theoretically chimeric for blood cells and pregnancies by ART have increased enormously in recent years (Daniel 2000; Sills et al. 2000; Cohen 2003; Unger et al. 2004), questions arise about (1) how frequently MC DZT occurs through ART, (2) to what clinical problems such twinning and chimerism lead, and (3) what the mechanisms for the occurrence of the twinning are.

Here we review the six cases from genetic and clinical points of view, especially regarding clinical implications and management of MC DZT.

Six cases of ART-associated MC DZT

Case 1, reported from the United States (Souter et al. 2003), was associated with in vitro fertilization (IVF) followed by embryo transfer (ET) into the uterus (Table 2). The pregnancy initially contained triplets consisting of a nonviable fetus and two viable fetuses. The two infants, a boy and a girl, were both chimeric for sex chromosome constitutions (46,XX/46,XY) in their peripheral blood lymphocytes, but their external genitalia appeared normal. The MC membrane of the placenta was confirmed by both ultrasonography and pathological examinations, and chimerism confined to blood cells [further called "confined blood chimerism" (CBC)] was determined after birth by a comparison of genotypes between peripheral blood leukocytes and skin fibroblasts. Five other cases (cases 2–6), five pairs of a boy and girl with CBC, were found in Japan (Table 2). Case 2 (Nishio et al. 2003) was a pair of twins, but its other conditions were very similar to those of case 1. Case 3 (Tsuruta et al. 2003) seemed unique among the six cases because the twins were associated with FSH-induced superovulation followed by intrauterine insemination, not by IVF. Case 4 (Yamaguchi et al. 2003) was the outcome of pregnancy with ICSI. The pregnancy initially included triplets: one died in uterus, one newborn had polycytemia, and the other was anemic. In case 5 (personal communication to Niikawa; Niikawa 2004), there was not enough information about the details of ART. Case 6 (personal communication to Niikawa; Niikawa 2004) resulted from a pregnancy by ART, and the twins were chimeric for the ABO blood group that was confirmed after birth. Thus, all the six cases of MC DZT were discordant for gender and associated with ART, five with IVF.

Possible mechanism for MC twinning

The general mechanisms for MZT and DZT twinning are pregnancy of one fertilized egg followed by its separation and pregnancy of two fertilized eggs, respectively. Formation of the placenta in MZT depends on the timing of separation of the fertilized egg (Hall 2003). The blastocyst develops from day 4 after fertilization, and the outer cell mass that becomes later the tropho-

Table 2 Six cases of monochorionic dizygotic twins. *ART* assisted reproductive technology, *MCM* monochorionic membrane, *CBC* confined blood cell chimerism, *IVF–ET* in vitro fertilization and embryo transfer, *USG* ultrasonography, *TESE* testicular sperm extraction, *ICSI* intracytoplasmic sperm injection

Cases	ART	Karyotype	Diagnosis of Fetuses		
			MCM	CBC	
1	IVF-ET	chiXX/XY	USG + pathology	Genotyping in leukocytes:chimera(+) /skin:chimera(−)	Initially, triplets with a nonviable fetus; a boy and girl
2	IVF-ET	chiXX/XY	USG + pathology	Genotyping in leukocytes:chimera(+) /skin:chimera(−)	A boy and girl with normal external genitalia;anastomosis
3	Super-ovulation + intrauterine insemination	chiXX/XY	USG +pathology	?	A boy and girl with normal external genitalia
4	TESE-ICSI	chiXX/XY	USG +pathology	Genotyping in leukocytes:chimera(+) /skin:chimera(−)	Triplets initially;one reduced;polycytemia in one; anemia in one; a boy and girl
5	IVF-ET	chiXX/XY	USG	?	A boy and girl
6	IVF-ET	chiXX/XY	USG	chimeric ABO blood group	A boy and girl with chimeric ABO blood group

blast joins with the extraembryonic mesoderm to form the chorion. When separation of one fertilized egg occurs between days 4 and 7, a double inner cell mass (ICM) is formed. As the chorionic membrane has already started to develop by this stage, two embryos share one common chorionic membrane with two amniotic membranes. Separation between days 8 and 14 after the formation of the amnionic membrane but before the establishment of the embryonic axis results in a monochorionic–monoamniotic placenta, the rarest type, occurring in $<1\%$ of MZT pregnancies. On the other hand, two independently fertilized eggs usually form either two separate normal placentas or a fused dichorionic–diamniotic placenta (Ogita et al. 1991) (Fig. 1). If the two fertilized eggs are fused before implantation at their outer cell mass, two ICMs are formed, leading to a monochorionic–diamniotic placenta (Fig. 1). In DZT by natural pregnancy, as the site of fertilization of an egg is usually apart from the site of the other egg, such fusion seldom occurs. Thus, ART may have some influencing effect(s) on cell fusion or adhesion.

The six reported cases reviewed here may have been the consequence of fusion at a late morula stage (before day 4) between two sorts of outer cell mass derived from two zygotes (Souter et al. 2003). The use of an ovulation-inducing agent and the position of fertilized eggs close to each other may be events common among the six cases. Assisted hatching by artificial disrupting of the zona pellucida with various methods, in utero implantation of several fertilized eggs at close sites by simultaneous embryo transfer, the use of fertilized eggs that have developed to the blastcyst stage, and various cell culture procedures or conditions that lead to changes of the nature of cell surface, all may increase the chance of cell fusion. This ''chance hypothesis'' can simply explain why MC DZT is extremely rare in natural pregnancy.

Clinical implications of MC placenta

The MC placenta of all types, irrespective of zygosity, shows an increased risk of fetal and neonatal complications, including congenital anomalies, intrauterine growth retardation (IUGR), twin–twin transfusion syndrome (TTTS), and mortality associated with spontaneous demise of a fetus (Hall 2003). TTTS characterized by overgrowth of a recipient twin and undergrowth of the donor twin is attributed to vascular communication via placenta between twins (Wee and Fisk 2002; Nicolaides 2002; Crombleholme 2003). Polycytemia and anemia seen in case 4 are an example of TTTS, and to our knowledge, only one case of MC DZT with TTTS other than case 4 has been reported in the literature (Quintero et al. 2003). Infants with TTTS are at high risk for congestive heart failure, premature delivery, and perinatal death (Gaziano et al. 2000; Adegbite et al.

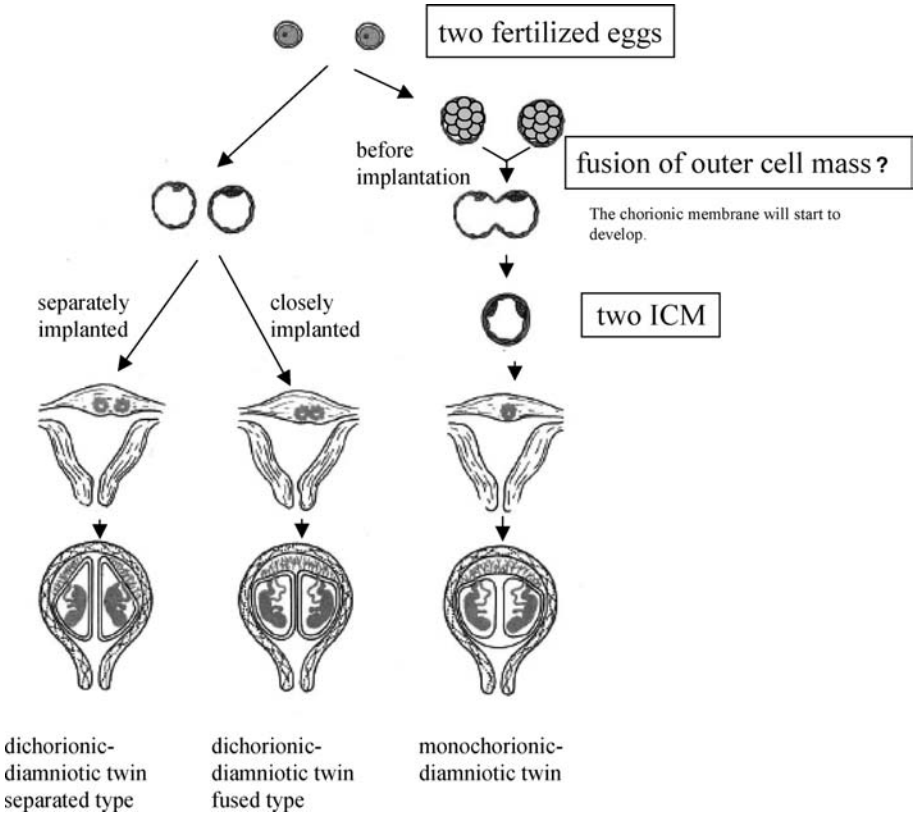

Fig. 1 Schematic presentation of dizygotic (DZT) twinning. This schema was quoted from a textbook, Medical Genetics (Ogita et al. 1991) and modified. Two independently fertilized eggs usually form either two separate normal placentas or one placenta with dichorionic–diamniotic membranes. If two fertilized eggs are fused at their outer cell mass before day 4, a double inner cell mass is formed within a blastcyst, leading to a monochorionic–diamniotic placenta

2004). Therapeutic reduction was done for a fetus in case 4, while the cause of fetal death in case 1 was unknown.

Placental vessel anastomosis in DZT results in CBC, i.e., hematopoietic stem cell chimerism, a similar condition as seen in allogeneic bone marrow transplantation or stem-cell transfusion (Kuhl-Burmeister et al. 2000). As the probability that DZT share a genotype is only one fourth; those with CBC may show unusual genotype as if they have three or four different alleles at a certain locus. If this occurs at the *ABO* locus, such twins have unusual ABO blood groups, as seen in case 6. Thus, CBC may mislead physicians when typing blood groups or HLA or genotyping for disease-susceptible gene(s) at the time of so-called ''personalized medicine'' in the near future (Vabres et al. 2002). Since these twins are generally immunotolerant against any blood cells derived from their counterparts, they continue to have CBC all their life, and it is less likely that they will have a significant problem at blood transfusion or tissue transplantation from a recipient. As far as three of the six cases reviewed above are concerned, it was confirmed by karyotyping and genotyping using DNA polymorphic markers that each twin with CBC had only his or her own karyotype and genotype in somatic tissues other than the blood cell. This may imply that the chimerism was truly confined to the blood cells and will never be transmitted to the next generation. Although the chimerism may not directly lead to clinically significant, harmful effect(s) on the twins, a potential role of microchimerism in the development of autoimmuno diseases in their adulthood has been suggested (Nelson 1999, 2002; Bianchi 2000, 2004; Lambert and Nelson 2003). MC DZT may have a psychological trauma, especially in a girl/woman chimeric for an XY karyotype, and vice versa. Therefore, genetic counseling, providing of further information, and persistent follow-up studies are essential to their future quality of life.

The female cattle chimeric for sex chromosomes (chi60,XX/60,XY) as a consequence of placental anastomosis between DZT are known as ''freemartin''. Freemartin cattle have congenital abnormalities of the genital organ, such as modified ovaries or structures resembling testes, causing sterility (Hinrichs et al. 1999; Rejduch et al. 2000). Pathogenesis is either hormonal imbalance due to male hormones in the female circulation or cellular chimerism (Cavalieri and Farin 1999; Meinecke et al. 2003; Imakawa 2004). However, no freemartin-like abnormalities associated with CBC showing a 46,XX/46,XY karyotype have been reported in human MC DZT. This suggests that pathogenetic effects of vascular anastomosis is different between human and cattle sex-discordant DZT (Jankowski and Ildstad 1997).

Diagnosis of MC DZT

MC-diamniotic placenta is diagnosed pathologically by the absence of two double-membrane layers at the junction site of amniotic cavities. The ''T-sign'' of the membrane as an ultrasonographic finding is also used for clinical diagnosis of MC-diamniotic placenta whereas the lambda-shaped insertion (lambda sign) is characteristic for dichorionic placenta (Carroll et al. 2002). As PCR-based DNA testing using leukocytes is not reliable for the diagnosis of MC DZT because of their mixing genotypes due to CBC, as mentioned above (van Dijk et al. 2002; Phelan et al. 1998; Olsson et al. 2001) . Instead, the testing of other somatic tissues/cells, such as skin biopsy specimen, umbilical cord tissue, and/or buccal cells, using highly polymorphic DNA makers is recommended (Dauber et al. 1999). For sex-discordant DZT, chromosome analysis as well as quantitative PCR analysis or fluorescent in situ hybridization (FISH) using X and Y chromosome-specific probes identifies their CBC and reveals the proportion of nucleated male and female cells (Phelan et al. 1998; Masuzaki et al. 2004).

Future perspectives

Although recently developed ART successfully gives infertile couples the opportunity to have babies, it still has some significant problems. ART, e.g., IVF, assisted hatching by breaching of the zona pellucida, and/or ET at the blastocyst stage, has been noted to increase the rate of multiple pregnancy (twins, triplets, and even quintuplets) and MZT twinning by embryo splitting (Behr et al. 2000; da Costa et al. 2001; Tarlatzis et al. 2002; Milki et al. 2003; Blickstein et al. 2003; Ghulmiyyah et al. 2003; Edi-Osagie et al. 2003). Also, ART is sometimes associated with congenital anomalies, IUGR and TTTS in a fetus, and/or fetal death (Giltay et al. 1998; Strain et al. 1998; Sills et al. 2000; Daniel et al. 2000; Cohen 2003; Unger et al. 2004) . MC DZT may be another problem associated with ART.

Five of the six cases reviewed above were from Japan. This figure may reflect the fact that Japan has the most ART-dealing clinics/hospitals in the world. There must be many more cases of MC DZT than the six cases reviewed. As all six cases were found according to their discordant sexes (XX/XY), at least the same number (1 XX/XX: 2 XX/XY: 1 XY/XY) of twins with concordant sexes may have been overlooked. Therefore, we are probably seeing only the tip of an iceberg. MC DZT cannot totally be ruled out in some previously reported DZT who were the outcome of pregnancy by IVF because not enough information for their placental nature was given (Behr and Milki 2003; Steinman et al. 2003; Alikani et al. 2003) . Furthermore, some twins collected in a large survey for ART-associated MZT might be the case, as MZT twinning has been observed in such a series for more than one fetus in the same gestational (MC) sac (Milki et al. 2003).

In summary, a review of the six cases suggests that an association of MC DZT with ART is not rare, and various methods of ART increase a chance of fusion between the outer cell mass derived from two fertilized eggs. It also indicates that, as far as the six cases re-

viewed are concerned, they all had CBC without obviously harmful effect(s), except for TTTS in case 4. However, they may have a psychological trauma because of their XX/XY CBC. Sufficient informed consent prior to ART, genetic counseling before/after regarding CBC, and long-term follow-up of the twins are thus necessary for their improved quality of life. It remains to be investigated whether ART increases MC DZT. Large-scale government-supported retrospective and/or prospective research on the prevalence of MC DZT after ART-based pregnancy is strongly recommended.

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