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Monozygotic twins and transfer at the blastocyst stage after ICSI

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The incidence of monozygotic twinning (MZT) is higher in pregnancies conceived after assisted reproduction than after natural conception. Alterations, produced by ovarian stimulation, in-vitro culture conditions and specifically alterations of zona pellucida are mentioned as possible causes of this phenomenon. A retrospective review was performed of the incidence of MZT in pregnancies generated in our centre during the period of January 1996 to December 1999. This variable was compared in 129 gestations that resulted from blastocyst transfer (occurring from September 1998 to August 1999) with 814 pregnancies produced by transfers of 4- to 8-cell embryos. Follicular development was induced with human menopausal gonadotrophin and urinary FSH during 1996 and 1997 and with recombinant FSH during 1998 and 1999. Blastocysts were cultured in sequential media using S1 or G1 up to 72 h and S2 or G2 to day 5. Five of the 129 pregnancies generated by blastocyst transfers were complicated by MZT gestation (3.9%). In comparison, only six of 814 pregnancies occurred from 4- to 8-cell transfers (0.7%), a difference that is statistically significant ($P < 0.001$ with Yates correction). The results confirm an increase of MZT in pregnancies from intracytoplasmic sperm injection as compared to the natural incidence. Moreover, the frequency of MZT was significantly higher when transfers were performed at the blastocyst stage, suggesting that extended in-vitro culture of embryos may be associated with alterations of the zona pellucida and the hatching process.

Key words: blastocyst/ICSI/IVF/monozygotic twinning

Introduction

The most prevalent and serious complication of assisted reproductive techniques is multiple pregnancy. The routine use of ovarian stimulation and the transfer of several embryos to achieve acceptable pregnancy rates results in an excessive number of embryos implanting in a significant proportion of the patients. As expected, the large majority of these cases are non-identical twins. Monozygotic twinning (MZT) is a rare phenomenon in humans and occurs in 0.42% of spontaneous pregnancies (Bulmer, 1970). However, both ovarian stimulation in hypogonadotrophic women and IVF have also been associated with a significant increase in the incidence of identical twins. Derom *et al.* (1987) described a higher incidence of monozygotic twins in a series of hypogonadotrophic women in which follicular development was induced with human menopausal gonadotrophins, and suggested that the therapy could produce alterations on the zona pellucida of the oocytes ovulated by these patients (Derom *et al*., 1987). Edwards *et al.* (1986) were the first to suggest that the incidence of MZT is increased with IVF, and to describe a possible relationship with embryo culture conditions (Edwards *et al*., 1986).

More specifically, several authors have directed attention to alterations of the zona pellucida in embryos produced *in vitro*, and their possible association with MZT incidence. Cohen *et al.* (1990) suggested that both abnormal in-vitro zona hardening and assisted hatching could favour MZT (Cohen *et al*., 1990). Hershlag *et al.* (1999) reported a possible relationship of MZT with mechanical assisted hatching. The authors described an increase in pregnancy rates on poor prognosis cases in which assisted hatching was used with a concomitant elevation on the number of identical twins (Hershlag *et al.*, 1999). Alikani *et al.* (1994) presented six cases of MZT in patients treated with IVF (Alikani *et al.*, 1994). The common feature among the embryos of these women was a naturally thin zona, or its artificial breach by assisted fertilization or hatching. However, more recently a large retrospective analysis reported by Scott Sills *et al*. (2000) did not find significant differences in the incidence of MZT between zona intact and zona manipulated groups after IVF (Scott Sills *et al*., 2000).

Prolonging embryo culture *in vitro* to the blastocyst stage has been presented as an effective form of embryo selection that results in increased implantation rates. This approach has gained popularity due to the excellent results recently reported (Gardner and Schoolcraft, 1999), with the introduction of sequential media. On the other hand, the ideal culture conditions for an embryo to reach its biological potential are not com-

pletely defined, and prolonged exposure of the embryo to laboratory conditions may not be free of risk.

Five pregnancies complicated with monozygotic twins, after intracytoplasmic sperm injection (ICSI) and transfer of embryos at the blastocyst stage, are presented here. These pregnancies occurred during a period of 8 months and represent an important increase in the historical incidence of MZT in our centre.

Materials and methods

Prolonged in-vitro embryo culture was introduced in our centre in September 1998. A retrospective review was performed of the incidence of monozygotic gestations in pregnancies generated in our centre by ICSI during the period January 1996 to December 1999. The incidence of monozygotic twins in 129 gestations that resulted from blastocyst transfers was compared with 814 pregnancies produced by transfers of 4- to 8-cell embryos.

Ovarian stimulation was achieved using luprolide acetate (0.5–1.0 mg/day s.c.) and recombinant FSH (Gonal F®; Serono, São Paulo, Brasil). Follicular development was monitored with periodic measurements of serum oestradiol and vaginal ultrasound. Human chorionic gonadotrophin (HCG) 10 000 IU (Profasi®; Serono) was administered when at least two follicles reached a mean diameter of 18 mm and oocyte retrieval was performed by ultrasound guided vaginal aspiration 34 h later.

Oocytes collected in pure follicular fluid were immediately sent to the adjacent embryology laboratory, where they were identified using a dissecting microscope at ×50 magnification. After being identified, oocytes were placed in a single well culture dish (3260; Costar, Cambridge, MA, USA) with 1.0 ml of 80 IU/ml of hyaluronidase (Hyase-1®; Scandinavian IVF Science, Gothenburg, Sweden) for 30 s and subsequently washed several times in IVF 50 medium (Scandinavian IVF Science), and one oocyte was put inside one droplet containing 30 µl of IVF 50 medium and covered with light mineral oil (Ovoil 150®; Scandinavian IVF Science) and incubated for 2–4 h at 37°C in an atmosphere with 5% CO_2. At the end of the incubation period, cumulus and radiate cells were removed with an 125 µm gauge pipette, connected to a stripper (Mid Atlantic Diagnostics Inc., Medford, NJ, USA).

Assessment of the stage of oocyte development and quality was performed evaluating its polar body, nuclear status and morphological characteristics using inverted microscope (Nikon Diaphot Microscope®; Nikon Corporation, Tokyo, Japan) at ×200–400 magnification. Metaphase II (MII) oocytes were micro-injected within a period of 2–4 h after collection. Metaphase I (MI) oocytes were analysed as to extrusion of first polar body at 4 h intervals up to 8 h after retrieval and the injection was performed accordingly.

The methods for sperm preparation and injection have been previously reported (Abdelmassih *et al*., 1996). After sperm injection, oocytes were incubated in 1 ml of IVF 50 medium (Scandinavian IVF Science) and covered with mineral oil for 16–18 h. Oocytes were then observed for the presence or absence of pronuclei and polar bodies. Fertilization was considered normal when two clearly distinct pronuclei containing nucleoli were present. If a single pronucleus was observed, a second evaluation was carried out ~4 h later.

Oocytes containing two pronuclei were put separately into droplets of 30 µl of IVF 50 medium (Scandinavian IVF Science) into a Petri dish (3260 Costar) and covered with mineral oil if the transfer was to be performed in day 3 of culture, or into S1 medium (Scandinavian IVF Science) for day 5 transfers and incubated until the next morning.

Table I. Distribution of patients according to aetiology of infertility

	Group I *n* (%)	Group II *n* (%)
Oligoasthenospermia	956 (37)	131 (39)
Tubo-peritoneal	672 (26)	80 (24)
Ovulatory dysfunction	465 (18)	54 (16)
Unexplained	233 (9)	27 (8)
Endometriosis	155 (6)	23 (7)
Other[a]	103 (4)	20 (6)
Total	2584	335

[a]Refers to more than one factor.
Group I = 4- to 8-cell embryos transferred, group II = blastocysts transferred.

Table II. Age and cycle outcome

	Group I Embryos with 4- to 8-cells transferred	Group II Blastocysts transferred
Number of cycles	2584	335
Age (years)	34.11 ± 3.53	35.72 ± 4.67
Number of oocytes per cycle	8.0	8.3
Number of pregnancies (pregnancy rate %)	814 (31.5)	186 (38.5)
Number of MZT (MZT rate %)	6 (0.7)	5 (3.9)
Mean number of embryos transferred	3.90	2.54
Implantation rate	11.50%	18.5%

MZT = monozygotic twinning.

The embryos were observed at 42 h after injection and classified according to the criteria proposed by Palermo *et al.* (1992). Embryos with >50% fragmentation were not transferred. If the transfer was to be held on day 5, the embryos were placed in S2 medium (Scandinavian IVF Science) on day 3 (72 h) and remained in this medium until day 5 (120 h).

At the time of transfer, embryos were loaded into 15 µl of IVF 50 or S2 medium using an Edwards Wallace Catheter of 23 cm (Simcare Manufacturing Ltd, Hythe, UK). The patients rested in bed for 20 min after the transfer and later were sent home with instructions to rest for 24 h.

The luteal phase was supplemented with daily administration of 800 mg of oral/vaginal progesterone (Utrogestan®; Laboratoires Besins-Isvovesco, Paris, France) and transdermic 100 µg oestradiol patches changed daily (Estraderm TTS100®; Laboratório Biogalênica, São Paulo, Brasil). Serum βHCG concentrations were measured 12 days after follicular aspiration.

Transvaginal ultrasound was performed in all patients with ascending βHCG titres; presence and number of intrauterine gestational sacs were assessed, as well as the presence of a fetal pole and cardiac activity. Pregnancies were monitored by the patient's obstetrician and the final outcome was reported to the clinic both by the obstetrician and the patient.

Results

During the period from January 1996 to September 1999, six out of 814 (0.7%) pregnancies that occurred after ICSI and transfer of 2- to 8-cell stage embryos resulted in monozygotic twins. Day 5 transfers were introduced in our centre in September 1998. From that date until September 1999, five

Table III. Characteristics of monozygotic twinning cases and mean values for total clinic population

	Case number					
	1	2	3	4	5	Total clinic population
Age (years)	30	37	39	31	38	34
Diagnosis	anovulation	tubal factor	oligozoospermia	tubal factor	oligozoospermia	
rFSH total dosage (IU)	2500	3750	3225	3300	2850	2612
Oestradiol day of HCG administration (pg/ml)	3795	2431	3984	2050	4238	2270
Number of blastocysts transferred	3 (2Ex/1E)	3 (3E)	4 (2Ex/1H/1E)	2 (1E/1Ex)	4 (2H/2Ex)	

E = early; Ex = expanded; H = hatching; rFSH = recombinant FSH.

Table IV. Ultrasound results

Day post-transfer	Case 1	Case 2	Case 3	Case 4	Case 5
23	2GS	1GS	1GS	2GS	3GS
30–40	1GS (2e2am) 1GS (1e1am)	1GS (2e2am) 1GS (1e1am)	1GS (1e1y2am) 3GS (1e1am)	1GS (2e2am)	1GS (2e2am)
Pregnancy outcome	Miscarriage (16 weeks)	Miscarriage (16 weeks)	Miscarriage (7 weeks)	Delivered (29 weeks)	GS reduction delivered (37 weeks)

GS = gestational sac; e = embryo; am = amniotic sac; y = yolk.

out of 129 (3.9%) pregnancies resulting from ICSI and blastocyst stage embryo transfers were complicated by MZT. The increased incidence was statistically significant: $\chi^2 = 7.33785$ with Yates correction; odds ratio = 5.606; $P = 0.00675$.

Table I shows the distribution of patients according to the aetiology of infertility. No significant differences were observed between the two groups.

Age and cycle outcome are shown in Table II. No differences in age or number of oocytes recovered were observed, however the mean number of embryos transferred was lower and the implantation rate higher in day 5 transfers.

Table III shows the characteristics of the MZT cases and includes the means for the total clinic population. In three out of five cases, the ages of the patients were over 37 years. The total dosage of FSH used in four of the cases was higher than the mean for the total patient population. In four of the cases at least one expanded blastocyst, and in three at least one hatching blastocyst were transferred.

The early echographic characteristics and pregnancy outcome of the monozygotic twins are shown in Table IV. Case 1 developed two gestational sacs with three embryos (two of them in one sac) with cardiac activity, but aborted at approximately 16 weeks. Case 2 also evolved normally until week 14 when the diagnosis of poly- and oligohydramnios was made, most probably due to feto-fetal transfusion, and the pregnancy aborted. Case 3 had one gestational sac with two amniotic sacs. One of the sacs had an embryo that reached 5 mm and cardiac activity. The second amnion had only a yolk sac. Case number 4 which had two gestational sacs, one of them with two embryos, evolved without major complications to week 29 of pregnancy. A Caesarean section was then performed due to premature labour and breech presentation. The children went home in good condition after an 8 week period in the neonatal unit. Case 5 had an embryo reduction from five to two sacs (the MZT were reduced), and delivered at 37 weeks.

Discussion

The five cases of monozygotic twins presented here confirm the poor obstetrical prognosis of these pregnancies and that MZT should be considered as a serious complication. Three pregnancies aborted, one ended prematurely with an elevated health cost for the children and economic cost for the family, and one required an embryo reduction.

The detailed mechanism of embryo hatching in humans has not been established. Two processes are known to be important. One is digestion of the zona by embryonal or maternal enzymes. A second one is mechanical pressure exerted by the expanding blastocyst causing the thinning of the zona. Most of the literature that suggests there is a relationship between IVF-related techniques and increased incidence of MZT focuses on zona pellucida alterations and manipulation. Embryos with a naturally thinner zona and those in which some form of zona manipulation occurred appear to be at a higher risk. ICSI and assisted hatching (Alikani *et al*., 1994; Hershlag *et al*., 1999) have all been associated with MZT. One probable mechanism is that artificial piercing of the zona may create a non-natural gap through which the blastocyst herniates prematurely, favouring the embryo splitting.

All cases reported here involved ICSI as the form of fertilization. However, the historical control group also contained only pregnancies achieved with embryos formed after ICSI but transferred at the 2- to 8-cell stage, so the zona manipulation should not be the only explanation for the increased incidence of MZT. Moreover, the report of Scott

Sills *et al*. (2000), in which pregnancies achieved with embryos transferred at 72 h of culture were reviewed, confirms the higher incidence of MZT after an in-vitro embryo culture, but minimizes the possible relationship with zona pellucida manipulation (Scott Sills *et al*., 2000). The authors did not find a difference in the frequency of MZT in pregnancies achieved after ICSI or assisted hatching versus those achieved with conventional IVF.

Unfortunately, routine measurement of the zona thickness is not part of our embryology laboratory routine, so zona characteristics of the embryos transferred were not documented. However, thinning of the zona is more common in older patients (Cohen *et al*., 1992), and in three of the cases the ages of the patients were 37 or more. Furthermore, four out of five cases reported had at least one expanding blastocyst and three a hatching blastocyst transferred which implies that an important proportion of zona thinning occurred *in vitro*.

The physical changes occurring in the zona pellucida after fertilization are commonly known as 'zona hardening' (Wassermann, 1994). Spontaneous hardening has also been described in in-vitro aged unfertilized mouse ova (Long, 1981). Moreover, abnormal zona hardening has been proposed as a result of extended exposure of embryos to laboratory conditions, resulting in an impaired capacity to hatch and implant (Cohen *et al.*, 1990). Enhancement of embryo implantation rates in specific groups of patients, by assisted zona hatching or drilling, are indirect proof that zona hardening affects in-vitro produced embryos in the human (Malter and Cohen, 1989; Cohen *et al.*, 1990; Tucker *et al.*, 1991). This same phenomenon could play a role in favouring MZT. The herniation of the blastocyst through a less flexible zona may favour its division.

As described previously, sequential media were used as the form of embryo culture in cases in which the transfer was performed on day 5. The protocol followed was similar to the one published by Gardner *et al*. (1998), in which excellent pregnancy and implantation rates were achieved. This same group of authors has expanded their original experience, and confirmed the good results obtained with this method of culture (Scholtes and Zeilmaker, 1998; Schoolcraft *et al*., 1999; Marek *et al*., 1999). However, they do not report an increased number of monozygotic twins. Furthermore, none of the large series of blastocyst transfers refers to MZT as a significant complication (Schoolcraft *et al*., 1999). Only recently Peramo *et al.* (1999) described what appear to be the first cases reported, in which two monozygotic twin pregnancies resulted after embryos were transferred at the blastocyst stage.

Our own experience includes only cases in which ICSI was used as the technique of oocyte fertilization. Since this technique is used in most cases performed in our centre, it is not possible to evaluate the relative importance of the prolonged culture and ICSI, other than the comparison with the historical control of transfers performed at the 2- to 8-cell stage shown here. Furthermore, the retrospective nature of the analysis cannot account for all potential differences between the two groups of patients compared. A larger number of cases reported, where pregnancies were achieved after prolonged embryo culture *in vitro*, will be necessary to determine the significance of our experience.

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