Monozygotic bichorionic twinning after transfer of a single frozen/thawed embryo that has undergone quarter laser-assisted zona thinning: A case report¹

A. Allegra,² F. Sammartano,² P. Scaglione,² F. Coffaro,² A. Marino,² and A. Volpes^{2,3}

Submitted May 6, 2005; accepted June 2, 2005

Purpose: To present the occurrence of a monozygotic bichorionic twinning after the transfer of a single frozen/thawed embryo has undergone assisted zona hatching (AZH), using a non contact laser.

Methods: A 33-year-old woman who underwent single frozen embryo replacement (FER). Before transfer, the patient was treated with GnRH agonist and with substitutive therapy (estrogens and progesterone).

Conclusions: We are absolutely certain that we are reporting a monozygotic bichorionic pregnancy: a single frozen/thawed embryo was transferred and no concomitant spontaneous conception was possible. Moreover, molecular biology assessment for polymorphisms confirmed clinical findings. The possibility of the occurrence of a monozygotic bichorionic twinning after the transfer of a single frozen/thawed embryo should be borne in mind and its incidence will have to be studied further.

KEY WORDS: Cryopreservation; laser-assisted hatching; monozygotic twinning; single-embryo transfer.

Monozygotic twinning (MZT) is estimated to occur in 0.42% of all births in in vivo conception (1) and is associated to an increase in perinatal morbidity and mortality.

However, in vitro fertilization (IVF) techniques have increased the numbers of this type of pregnancy. Many publications, from the 1980s (2) to today (3,4), concerning this topic are available. Alikani *et al.* (5) reported an overall occurrence of MZT at 1.88% (81/4305) of all clinical pregnancies, but only 10% of these were bichorionic.

In this case report, we describe a bichorionic MZT after the transfer of a single frozen/thawed embryo

has undergone assisted zona hatching (AZH) using a non contact laser.

CASE REPORT

A 33-year-old nulliparous woman with 2 years of infertility was referred to our Reproductive Medicine Unit for Intracytoplasmic Sperm Injection (ICSI).

She had a normal hormone profile and normal hysterosalpingogram. Her husband's semen analysis showed severe oligoasthenoteratozoospermia.

Ovarian stimulation was performed in November 1999 and nine mature oocytes were recovered. Seven embryos were obtained by ICSI: one did not cleave, three of them were transferred and three were cryopreserved at the eight-cell stage.

No pregnancy was established, and 4 months later a frozen embryo transfer was scheduled. After thawing, none of the embryos survived, and the embryo transfer was not performed.

¹ The abstract of this case report was presented, as poster presentation, at the 13th World Congress on In Vitro Fertilization, Assisted Reproduction and Genetics, Istanbul, Turkey, May 26–29, 2005.

² ANDROS Day Surgery, Reproductive Medicine Unit, Via Ausonia 43/45, Palermo I-90144, Italy.

³ To whom correspondence should be addressed; e-mail: volpes@centroandros.it.

In July 2000, the patient underwent a second ovarian stimulation. Thirteen oocytes were recovered and nine of these were mature. Six zygotes were obtained by ICSI. Embryo transfer (ET) was not performed due to a high risk of Ovarian Hyperstimulation Syndrome (E2 level on the day of Human Chorionic Gonadotrophin administration was 4124 pg/mL), and all the zygotes were frozen at the pronuclear stage.

Four months later, after adequate endometrial preparation (GnRH analogue, estradiol valerate and natural progesterone), three zygotes were thawed and ET was performed 24 h after thawing. Laser assisted hatching was not performed, as the laser system was not yet available. The patient conceived and delivered a healthy boy at 38 weeks gestation by elective caesarean section.

In October 2003, the patient requested the transfer of the last three cryopreserved zygotes. GnRH analogue administration (Buserelin, Suprefact s.c., Aventis Pharma, Italy) was started, 0.1 mL twice a day subcutaneously, on the 21st day of the cycle. Six days after the onset of the following menstruation, estradiol valerate (Progynova 2 mg, Schering, Italy) was started, twice a day for 1 week, and was continued three times a day for a further week.

After 2 weeks of estradiol administration, the transvaginal ultrasound examination showed satisfactory endometrial thickness (10 mm) and 200 mg of natural Progesterone (Progeffik 100 mg, Effik, Italy) was started three times a day by vaginal route for 14 days. When the progesterone administration was started, GnRH analogue administration was suspended.

After thawing, a single zygote survived and was left in culture for 24 h. The following day, the embryo reached the four-cell stage without any fragmentation. Laser assisted zona hatching was performed by thinning one quarter of the Zona Pellucida (ZP) 30' before the embryo transfer. A Saturn Laser System with Cronus Image Analysis Software (Research Instruments Ltd.) was used.

Two days after the embryo transfer, estradiol valerate was decreased to 4 mg/day.

The β -HCG level was positive 12 days after the embryo transfer (168 mIU/mL). Estradiol and progesterone were continued for 8 weeks.

A transvaginal ultrasound examination was performed at 7-weeks gestation and showed an ongoing bichorionic diamniotic twin pregnancy. In each gestational sac, a foetal heartbeat was detected. Twenty-five days later, the transvaginal ultrasound examination confirmed the previous observation and the

"lambda" sign was seen as evidence of bichorionicity. The patient delivered spontaneously at the 29th week of gestation because of premature rupture of the membranes.

After birth, molecular biology assessment for polymorphisms was performed using buccal cells to confirm clinical findings (see Fig. 1).

Amplification and typing of the 13 core CODIS loci (CSF1-PO, FGA, TH01, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51 and D21S11) using the multiplex STR systems AmpF/STR Profiler Plus and AmpF/STR Cofiler (Applied Biosystems, Foster City, CA) showed an absolute overlapping of the genetic profile of the twins.

Finally, it is worth noting that the three offspring born to our patient had been conceived simultaneously and that both pregnancies were obtained from frozen embryos.

DISCUSSION

Several factors are involved in the aetiology of MZT in IVF.

It has been reported that culture conditions might be responsible for the high rate of MZT (6); the authors stated that the rate of some components of the sequential media utilized for blastocysts culture could increase apoptotic phenomena in the embryonic cells. During the hatching process, apoptosis might determine the splitting of the inner cell mass (ICM), leading to two separate ICM and monozygotic twinning (7).

Indeed, embryo transfer at the blastocyst stage compared to embryo transfer at the cleavage stage seems to increase the MZT rate (8,9). Da Costa *et al.* achieved an overall MZT rate of 3.9% after blastocyst transfers (5/129) compared to an overall MZT rate of 0.7% after 4- to 8-cell transfer (6/814). Milki *et al.* reported 5.6% of MZT (11/197) after blastocyst transfer and 2.0% of MZT (7/357) after day 3 embryo transfer.

Other reports have suggested that gonadotrophin treatment itself can determine a higher incidence of MZT (10).

The authors reported 1.5% of MZT (2/129) after ovulation induction or controlled ovarian hyperstimulation, whereas the incidence of MZT after conventional IVF or IVF-ICSI/assisted hatching was 0.72% (1/139) and 0.86% (4/463) respectively.

Fig. 1. Microsatellite DNA polymorphism analysis.

Therefore, it is suggested that the actual administration of gonadotrophins predisposes to zygotic division or enhanced survival of MZT (11).

Another theoretical mechanism involved in the increase of MZT is ZP manipulation. A higher MZT rate was reported after ICSI cycles, when compared to conventional IVF treatments. Abusheika *et al.* (12) found 0.9% of MZT rate after IVF compared to 8.9%

of MZT after ICSI. On the other hand, Schachter *et al.* (10) as previously reported, did not find any statistically significant difference in the incidence of MZT comparing conventional IVF (0.72%) and IVF-ICSI/assisted hatching (0.86%).

Schieve *et al.* (13) found that MZT was more than twice as likely to occur among IVF-ET cycles in which embryos were treated with assisted hatching

methods; very recently, Elizur *et al.* (14) stated that ZP manipulation procedures are not associated with an increased incidence of MZT in IVF-ET treatment cycles.

Almost all the MZT reported after assisted reproductive techniques are monochorionic, both monoamniotic and diamniotic (6,12). The incidence of bichorionic MZT is very rare. Two different mechanisms, which might occur in two different stages of embryonic development, can be invoked to explain bichorionic MZT; first, early breaking of the ZP and early division (within 72 h of fertilization) of the embryo or, as recently described, an atypical hatching at the blastocyst stage through a small hole in the ZP with a partial escape of both trophectoderm and inner cell mass (15,16).

However, the occurrence of bichorionic MZT is underestimated, because when more than one embryo is transferred, bichorionic pregnancies are referred to an implantation of two distinct embryos.

Theoretically, to account for bichorionic MZT, its occurrence after IVF should be assessed only by considering transfers of a single embryo that lead to a multiple pregnancy. Blickstein *et al.* (17) analyzed data provided by the Human Fertilization and Embryology Authority (HFEA) including 15,644 cycles involving single-embryo transfer in 7832 women. The rate of multiple pregnancies after single-embryo transfer was 2.3%; the rate was similar after the transfer of fresh embryos and after the transfer of embryos that had been frozen and thawed; the rate was similar after both stimulated cycles and unstimulated cycles.

On the contrary, in the case of IVF treatments, multiple pregnancies, which seem to be monozygotic bichorionic, can be dizygotic bichorionic. This event could be due to a concomitant spontaneous conception. Recently, it has been described how a spontaneous conception during an IVF cycle led to a quadruplet pregnancy after the transfer of only two blastocysts and the birth of one boy and three girls (18). The authors stated that in every IVF patient with patent fallopian tube and normal semen parameters, as a consequence of ovarian hyperstimulation, an oocyte could be fertilized spontaneously and could be implanted.

Only if the cycle has been treated with GnRH agonist and substitutive therapy (estrogens and progesterone) and a single embryo has been transferred, will the pregnancy be a monozygotic bichorionic.

Indeed, in our case report, GnRH agonist was started on the 21st day of the cycle and afterwards endometrial maturation was carried out by using estradiol valerate and natural progesterone; for this reason, no follicular development was achieved, so we can totally exclude a concurrent spontaneous conception.

Furthermore, only a single frozen/thawed embryo was transferred. The case we are reporting is certainly an incontrovertible bichorionic MZT and can be considered a paradigmatic *in vivo* model.

Moreover, we performed AZH by laser to overcome hardening due to frozen/thawed procedures, which can also determine biochemical changes in the ZP (19); even though it is difficult to assess and quantify the hardness of the zona, it has been reported that AZH can improve both pregnancy and implantation rates after transfer of cryopreserved embryos (20,21).

We believe that AZH played a role in the activation of one of the mechanisms described above (early division or atypical hatching) and therefore in the production of a bichorionic MZT.

Probably both ZP hardening due to freezing/thawing procedure and laser ZP thinning can determine an early breaking of the ZP and an early division of the embryo, or, at the blastocyst stage, a small hole in the ZP with a partial escape of both trophectoderm and inner cell mass.

In conclusion, we report for the first time, as far as we know, a bichorionic MZT after transfer of a single frozen/thawed embryo that has undergone assisted zona hatching using a laser at four-cell stage.

Further studies should be carried out to define the real incidence of bichorionic MZT after IVF; indeed, it will be necessary to analyze all bichorionic pregnancies after IVF treatments by using microsatellite DNA polymorphism analysis

1. to include possible bichorionic MZT that are usually mistakenly attributed to the implantation of different embryos, when more than one embryo is transferred and also
2. to exclude possible concomitant spontaneous conceptions when a single embryo is transferred.

It will also be very interesting to fully understand the mechanisms that lead to bichorionic MZT; in fact, the possible role played by ZP hardening and AZH procedures in increasing bichorionic MZT is still unclear. However, even though an increase in monochorionic MZT after AZH is documented, one might

hypothesize that, as a consequence of the described mechanisms, bichorionic MZT undergoes a relatively greater increase than the monochorionic.

REFERENCES

1. Bulmer MG: The Biology of Twinning in Man, Oxford, UK, Clarendon Press, 1970
2. Edwards RG, Mettler L, Walters DE: Identical twins and *in vitro* fertilization. *J IVF-ET* 1986;3:114–117
3. Unger S, Hoopmann M, Bald R, Foth D, Nawroth F: Monozygotic triplets and monozygotic twins after ICSI and transfer of two blastocysts: Case report. *Hum Reprod* 2004;19:110–113
4. Zikopoulos K, Platteau P, Kolibianakis E, Albano C, Van Steirteghem A, Devroy P, Quintuplet pregnancy following transfer of two blastocysts: Case report. *Hum Reprod* 2004;19:325–327
5. Alikani M, Cekleniak NA, Walters E, Cohen J: Monozygotic twinning following assisted conception: an analysis of 81 consecutive cases. *Hum Reprod* 2003;18:1937–1943
6. Cassuto G, Chavrier M, Menezo Y: Culture conditions and not prolonged culture time are responsible for monozygotic twinning in human *in vitro* fertilization. *Fertil Steril* 2003;80:462–463
7. Menezo YJR, Sakkas D: Monozygotic twinning: is it related to apoptosis in the embryo? *Hum Reprod* 2002;17:247–248
8. Da Costa ALE, Abdelmassih S, De Oliveira FG, Abdelmassih V, Abdelmassih R, Nagy ZP, Balmaceda JP: Monozygotic twins and transfer at the blastocyst stage after ICSI. *Hum Reprod* 2001;16:333–336
9. Milki AA, Jun S, Hinckley MD, Behr B, Giudice LC, Westphal LM: Incidence of monozygotic twinning with blastocyst transfer compared to cleavage-stage transfer. *Fertil Steril* 2003;79:503–506
10. Schachter M, Raziel A, Friedler S, Strassburger D, Bern O, Ron-El R: Monozygotic twinning after assisted reproductive techniques: a phenomenon independent of micromanipulation. *Hum Reprod* 2001;16:1264–1269
11. Derom C, Vlietinck R, Derom R, Van den Berghe H, Thiery M: Increased monozygotic twinning rate after ovulation induction. *Lancet* 1987;1:1236–1238
12. Abusheika N, Salha O, Sharma V, Brinsden P: Monozygotic twinning and IVF/ICSI treatment: a report of 11 cases and review of literature. *Hum Reprod Update* 2000;6:396–403
13. Schieve LA, Meikle SF, Peterson HB, Jeng G, Burnett NM, Wilcox LS: Does assisted hatching pose a risk for monozygotic twinning in pregnancies conceived through *in vitro* fertilization? *Fertil Steril* 2000;74:288–294
14. Elizur SE, Levron J, Shrim A, Sivan E, Dor J, Shulman A: Monozygotic twinning is not associated with zona manipulation micromanipulation procedures but increases with high-order multiple pregnancies. *Fertil Steril* 2004;80:462–463
15. Van Langendonck A, Wyns C, Godin PA, Toussaint-Demyle D, Donne J: Atypical hatching of a human blastocyst leading to monozygotic twinning: a case report. *Fertil Steril* 2000;74:1047–1050
16. Behr B, Milki AA: Visualization of atypical hatching of a human blastocyst *in vitro* forming two identical embryos. *Fertil Steril* 2003;80:1502–1503
17. Blickstein I, Jones C, Keith LG: Zygotic splitting rates after single-embryo transfer in *in vitro* fertilization. *N Engl J Med* 2003;348:2366–2367
18. Milki AA, Hinckley MD, Grumet FC, Chitkara U: Concurrent IVF and spontaneous conception resulting in a quadruplet pregnancy. *Hum Reprod* 2001;16:2324–2326
19. Carroll J, Depypere H, Matthews CD: Freeze-thaw induced changes of the zona pellucida explains decreased rates of fertilization in frozen-thawed mouse oocytes. *J Reprod Fertil* 1990;90:547–553
20. Tao J, Tamis R: Application of assisted hatching for 2-day-old, frozen-thawed embryo transfer in a poor-prognosis population. *J Assist Reprod Genet* 1997;14:128–130
21. Gabrielsen A, Agerholm I, Toft B, Hald F, Petersen K, Feldinger B, *et al.*: Assisted hatching improves implantation rates on cryopreserved-thawed embryos. A randomised prospective study. *Hum Reprod* 2004;19:2258–2262