THE EFFECTS OF ART ON MZ TWINNING: MICROMANIPULATION & THE RISK OF MULTIPLE PREGNANCY

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Introduction

In the past 20 years micromanipulation has become an important aspect of all human assisted reproduction programmes with the goal to improve fertility treatment results and overcome male infertility. The commonest micromanipulation techniques used are:

Intracytoplasmatic Sperm Injection (ICSI) for the treatment of male infertility.

Cytoplasmic transfer to investigate and improve embryo development, Assisted Hatching (AH), procedure used to make a "hole " in the Zona Pellucida to facilitate implantation of the embryo in the endometrium, Blastomere biopsy for the diagnosis of genetic disease and aneuploidy, Preimplantation Genetic Diagnosis (PGD). The use of those techniques has been associated with an increase in Monozygotic Twins.

Micromanipulation Techniques

Of all micromanipulation techniques, Intracytoplasmic Sperm Injection (ICSI), Assisted Hatching and Embryo biopsy for Preimplantation Genetic Diagnosis (PGD) most commonly are responsible for the increased incidence in Monozygotic (MZ) twins and multiple pregnancies in vitro fertilization cycles (IVF). All are used in efforts to increase fertilization rates in partners with low sperm counts, to increase rates of endometrial implantation, and to provide preimplantation genetic information. Studies in animal models suggest that MZ twinning can be induced through ovum or embryo "manipulation", either mechanical or chemical. In particular zona manipulation in placental animals appears to increase monoamniotic twinning.[1]

Although the mechanisms by which MZ twins are formed is not completely known, many investigators postulate that the splitting of the inner cell mass at an early stage of development may cause the duplication of the embryo.[1] Splitting of the zygote may occur at any time during the first 2 weeks after fertilization, resulting in the various forms of MZ twinning. When division of the embryonic cell mass occurs earlier than 72 hours after fertilization, diamniotic dichorionic MZ twins will evolve. Division after the inner cell mass has formed, between day 4 and day 8, gives rise to diamniotic monochorionic twins. Splitting after day 8 will lead tomonochorionic monoamniotic twins. This process probably begins with the protrusion of some tropho-ectoderm cells through a small opening in the zona pellucida.

Factors that appear to predispose to this splitting include alterations in the thickness of the zona pellucida, time of implantation, and the assisted reproductive techniques. In particular micromanipulation of zona pellucida in ART (Subzonal insemination, ICSI and Assisted hatching) increases the frequency of twins and monozygotic twins.[1] The artificial opening introduced in the zona pellucida caused may alter the process of hatching, in that the expansion during the expulsion of the blastocyst through the artificial opening may cause constriction and bisection of the trophoblast and inner cell mass, which results in twinning. Multiple gaps in the zona pellucida may even lead to multiple herniations, possibly contributing to higher-order monozygotic pregnancies.[6]

Intracytoplasmatic Sperm Injection (ICSI)

Spermatozoa sometimes fail to fertilize an egg during conventional IVF. Failure in IVF are particularly common in the presence of grossly abnormal semen parameters or when

the number of spermatozoa is insufficient. In majority of the instances gamete micromanipulation is the only way to overcome this problem. Initially techniques

focused on the obstacle to sperm penetration, the zona pellucida (ZP), by thinning it through exposure to enzymes or creating an opening through localized chemical digestion, mechanical breach, or even photoablation. Although associated with a fertilization rate of 20%, these techniques have been abandoned due to the requirement for numerous functional spermatozoa with good progressive motility and complications such as multiple sperm penetration.

ICSI, on the other hand entails the deposition of a single spermatozoa directly within the cytoplasm of the oocyte, and bypassing the ZP and the oolemma. The ability of ICSI to achieve higher fertilization and pregnancy rates regardless of the sperm characteristics makes it the most powerful micromanipulation procedure as yet

to treat male factor infertility. In fact, the therapeutic possibilities of ICSI range from instances in which, after sperm selection, the spermatozoa show poor progressive motility, to application in azoospermic men where spermatozoa are surgically retrieved from the epididymis and the testis. ICSI is also useful when specific oocytes are considered for PGD. As removal of the polar body requires the stripping of cumulus corona cells, ICSI is the only option to avoid polyspermy.

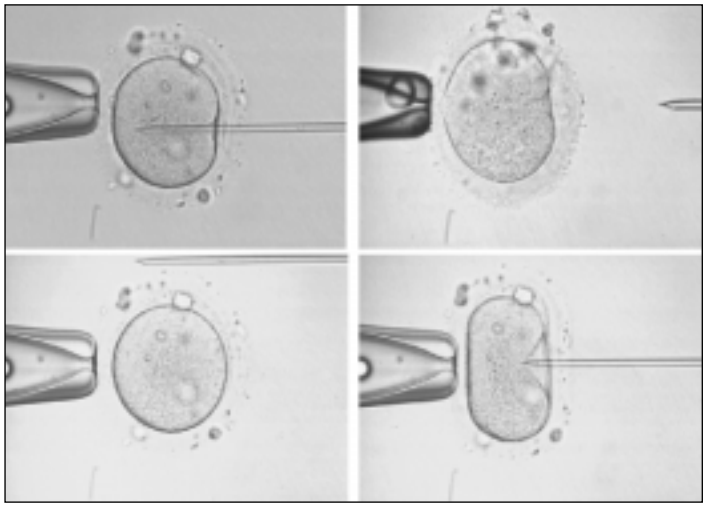

Figure 1. *ICSI procedure*

Three types of micromanipulation procedures were developed to improve fertilization rates, in those cases with associated male factor sterility in ART programmes. The first procedure involved the creation of an artificial gap in the zona pellucida, either by manually dissecting a hole using needles or by using a chemical agent to "burn" a hole in the zona. This procedure was described as Partial Zona drilling (PZD). The second was a more invasive procedure that totally bypassed the zona by depositing spermatozoa directly into the perivitelline space, the so-called Subzonal insemination (SUZI). The third step was the direct injection of a single spermatozoon into the ooplasm of the oocyte (ICSI).[1] The first ICSI pregnancy was created in 1992 (by Palermo) after a human spermatozoon was accidentally injected into the ooplasm of a human oocyte during a SUZI procedure.[16] Initially, this technique only led to a limited improvement of the fertilization rates compared to SUZI.[2] As of the present writing time the incidence of congenital malformation in babies born after ICSI is not higher than in the general population, but patients should be counselled about the higher risk of transmitted

chromosomal aberrations, of sex chromosomal aberrations, and the risk of transmitting fertility problems to the offspring.[3]

Much has been written about ICSI and the increased incidence of twins, specially MZ twins. Tarlatzis et al,[4] recently published a study comparing conventional IVF- blastocyst transferred cycles with ICSI-blastocyst transferred cycles. The overall incidence of MZ was 3.3% per cycle (in both groups), however statistically significant increase in the rates of MP(10,8% vs 2.6%) and MZ (5.9% vs 0%) was observed in the ICSI group compared with the conventional IVF group respectively.

Abusheika et al,[5] reported an incidence of 8.9% of MZ twins subsequent to ICSI compared with 0.9% after conventional IVF. The effect of zona pellucida manipulation on MZ twins was also confirmed in another study that showed significant increase in the MZ twins after mechanical assisted hatching (1.2%) as compared with non hatching (0%).[6] These findings are probably due to the artificial opening created by mechanical trauma, assisted hatching or ICSI, rather than natural autolysis of the zona which is responsible for splitting of the inner cell mass, as there were no cases of MZT in the nonmanipulated zona pellucida.

A.L.E da Costa, et al[7] reported that during 1996 to 1999, 6 out of 814 (0.7%) MZ pregnancies occured after ICSI and transfer of 2 to 8 cell stage embryos. From that time these investigators introduced day 5 transfer in September 1998 until September 1999, 5 out 129 (3.9%) pregnancies resulting from ICSI and blastocyst stage embryo transfers were complicated by MZ twinning. The increased incidence was statistically significant: x2 = 7.33785 with Yates correction ; odds ratio = 5.606; P= 0.00675.

Table I.

	Case number					
	1	**2**	**2**	**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 HG administration (pg/ml)	3795	2431	3984	2050	4238	2270
Number of blastocysts transferred	3(2Ex/1E)	3(3E)	4(2Ex/1H/1E)	2(1E/Ex)	4(2H/2Ex)	

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

Table II. *by the same group,[7] shows the early echographic characteristics and pregnancy outcome of the MZ twins.*

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; yolk

Age and cycle outcome are shown in Table I by A.L.E. da Costa et al.[7] 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 transfer.

Assisted Hatching (AH)

The main function of the ZP after fertilization is the protection of the embryo and the maintenance of its integrity. It is postulated that the blastomeric cells are only weakly connected and that the ZP is needed during the migration of the embryo through the reproductive tract to maintain its physical integrity. Implantation has

been observed after replacement of zona free morulas or blastocysts, whereas the transfer of zona free precompacted embryos results in the inadherence to the oviductal walls or to one another. A possible protective role against hostile uterine factors has also been described. The first report on the use of AH in human embryos was published by Cohen et al in 1990.[8] These authors reported an important increase of implantation rates with mechanical AH in embryos from unselected IVF patients.

The mechanism by which AH promotes embryo implantation remains unclear.As the implantation window represents the critical period when the endometrium reaches its ideal receptive state for implantation, a precise synchronization between the embryo and the endometrium is essential. In a randomised study, Liu et al demonstrated that implantation occurred significantly earlier in patients whose embryos were submitted to AH compared to the control group, possibly by allowing earlier embryo endometrium contact.[9] Furthermore, although most molecules are able to cross the ZP, the rate of transport may be related to zona thickness. The presence of an artificial gap may alter the two-way transport of metabolites and growth factors across the ZP, permiting earlier exposure of the embryo to vital growth factors. In addition to facilitating embryonic hatching, it has been postulated that AH allows earlier hatching, therefor aiding earlier embryo-endometrium contact. Using hCG production as the marker, Liu et al, showed that implantation occurred a day earlier in hatched embryos compared with unhatched controls.[9] AH may therefore promote successful implantation in embryos with defective mechanisms which would otherwise cause late hatching, or in embryos with retarded development by enabling contact with the endometrium earlier and within the implantation window. This is doubly important when one considers that delayed implantation is been associated with a higher incidence of miscarriage.

Whatever the underlying cause, the simple logic of AH is to artificially create a slit or hole through which the blastocyst can escape. This can be achieved mechanically by partial zona dissection, chemically using acid Tyrode,s or using Lasers. Hershlag A. et al, [6]published a study of the incidence of MZ twinning after mechanical AH. In A retrospective comparative analysis of hatched versus nonhatched consecutive ART cycles. The results shown in Table III, that are the pregnancy rate (PR) per embryo transfer (ET) in the hatching group increased from 25.2% to 37.1% and the multiple pregnancy rate from 6.8% to 13.1%. In the non hatched series there were no MZ twins compared with eight cases in the hatched series (1.2% per ET).

Table III. *Comparison of nonhatched and hatched IVF cycles.*[6]

	Study Group		
Outcome	**Nonhatched**	**Hatched**	***p value***
Clinical pregnancy rate per transfer*	25.2(141/559)	37.1(250/674)	<.0001†
Viable pregnancies per transfer‡	21.8(122/559)	33.5(226/674)	<.0001†
Multiple pregnancies per transfer§	6.8(38/559)	13.1(88/674)	<.0003†
High-order multiple pregnancies per transfer∫	3.0(17/559)	4.3(29/674)	<.245†
Monozygotic twins per transfer	0(0/559)	1.2(8/674)	<.01#
Ectopic prengancies per transfer	0.5(3/559)	1.2(8/674)	<.363#

Note: Valuse are perecentages wih numbers in parentheses unless otherwise indicated.

**Fetal sac observed by ultrasound at 5 weeks after transfer.*

† Determined by X2 test.

‡ Fetal heartbeat(s) observed by ultrasound at 5 weeks after transfer.

§ > 1 fetal hearts.

∫ > 2 fetal hearts.

 # Determined by Fisher's exact test.

Table IV. *Relationship between assisted hatching and pregnancy, multiple gestation, and MZ, USA 1996. From L.A. Schieve.*[6]

Assisted hatehing	No.of cycles	pregnancy (%)[a] (pregnanciev/transfer procedure	Multiple gestation (%) (multiple gestation/ pregnancy[b])	At least one MZ multiple gestation (%) (MZ multiple gestations/pregnancey[b])
No assisted hatchiag	21.490	33.3	41.5	0.13[c]
Assisted hatching with some transferred embryos	3.310	40.1	46.2	0.16
Assisted hatching with all transferred embryos	10.703	33.9	39.1	0.33
Total	35.503	34.1	41.3	0.20

[a] *Based on 12.095 pregnancies.*

[b] *Percent multiple gestation and pereent at least one MZ multiple gestation based on 11.247 pregnancies (848 subject were missing necessary data).*

[c] *P<05 for X2 test for trent comparing rates across assisted hatching categories.*
Schieve. Assisted hatching and monozygotic twinning. Fertil Steril 2000.

Laura A. Schieve et al,[17] reviewed all the IVF-ET cycles initiated in U.S. clinics in 1996, a total of 35.503 cycles and 11.247 pregnancies, and concluded that the risk of MZ twinning was increased considerably when AH was performed. The percentage of pregnancies with multiple fetuses was 41%, and AH did not materially affect the multiple gestation rate. The percentage of pregnancies that contained at least one set of MZ multiples was 0.20%. However, this rate varied considerably depending on AH status.

Preimplantation Genetic Diagnosis (PGD

In the mid eighties, the development of PCR strategies for amplification of specific fragments of DNA from single cells paved the way for PGD of inherited disease from one or more cells biopsied from embryos at preimplantation stages after IVF. As the human oocyte and embryo are enclosed within the ZP up to the expanded blastocyst stage any sampling procedure requires micromanipulation to penetrate this glycoprotein layer. This is followed by removal of target cells with minimal damage to the embryo, a process again requiring micromanipulation.The first PGD cycles were carried out in late 1989 at the Hammersmith Hospital, London, UK, in a series of couples at risk of X-linked disease. The sex of each embryo was identified by biopsying single cells from cleavage stage embryos by PCR amplification of a Y-linked sequence and transferring female embryos which could carry the defect but should not be affected.

Micromanipulation techniques and Multiple Pregnancies

Some authors have linked the use of ovulation induction drugs to MZ twinning (Derom et al.,1987).[11] Others have calculated an increased incidence of MZ twins in the setting of IVF, with or without zona manipulatio.[12,13,6,14,15] One well accepted hypothesis to explain the higher incidence of monozygosity suggests that manipulation of the zona pellucida encourages inner cell mass herniation during hatching, or that the increase in twinning is simply due to the presence of more embryos in the uterine cavity after embryo transfer. Regrdless, various rates of MZ twinning after ART with or without micromanipulation are described below.

The 1.2% of al MZ twinning after OI observed by Derom et al was significantly higher than the expected frequency of 0.45% after spontaneous ovulation.[12] Edwards et al calculated an incidence of MZ twinning after IVF of 1.33 suggesting that the artificial conditions of in-vitro media are the likely causes of increased incidence of MZ twins.[12] Blickstein et al reported on conventional IVF single embryo transfer, finding MZ twinsin 5% (4/82) with no cases after ICSI (Blickstein et al., 1999). Alikani et al., reported their results after IVF-ET, describing 6 sets of MZ twins or triplets on incidence of 0.84%, twice the expected frequency. The authors proposed that zona manipulation procedures were possible causes of MZ twins.

Table V. *Shows the most recent and relevant publications looking at multiple pregnancies and monozygosity after assisted reproduction cycles with or without micromanipulation techniques. From M.Schachter et al.*[10]

Author	Type of ART described	Type of MZT	No.of cases	Perecent of all pregnancies
Control population	None	Monozygotic twins	-	0.42-045%
Derom et al.(1987)	OI, CC DZ triplets and HMG	18/1485 MZ twins	1.2%	
Edwards et al, (1986)	conventional IVF	MZ twins	9/600	1.33%
Blickstein et al, (1999	conventional IVF	MZ twins	4/82	4.99%
Avrech et al., (1993)	conventional	DZ triplets	3	-
Steiner and Ojakangas (1994)	IVF			
Inion et al (1998)				
Biljan et al (1995)	convential IVF	trizygotic quadruplet	1	-
Salat -Baroux et al (1994)	conventional IVF	trizygotic quintuplet	1	-
Alikani et al, (1994)	micromanipulation (AH, PZD)	MZ twins DZ triplets	6/737	0.84%
Slotnick and Ortega (1996)	micromanipulation (AH, PZD)	MZ twins	5/143	3.49%
Wenstom et al (1993)	micromanipulation (AH, SUZI, PZD)	MZ twins	7/218	3.2%
Herschlat et al., (1999)	micromanipulation (AH only)	MZ twins	8/674	1.2%
Behr et al., (1999)	IVF-ICSI blastocysts	MZ twins	10/199	5%
Abusheikha et al., (2000)	IVF ICSI	MZ twins DZ triptles	11/718	1.53%
Saito et al, (2000)	IVF. ICSI	MZ twins	12/475	2.52%
Sills et al, (2000)	IVF-ICSI	MZ twins	23/1911	1.2%
Schachter, (2000)	OI, IVF	MZ twins	7/731	0.95%
(Current report)	ICSI, AH	DZ triplets		
Total (excluding case reports)	All assisted reproduction	All monozygosity	120/7973	1.51%

Rate calculated in all studies as the number of monozygotic pregnancies per total number of pregnancies.
ART= assisted reproductive techniques; MZ, MZT = monozygotic twins; OI= ovulation induction: CC= clomiphene citrate; HMG= human menopausal gonadotrophin; DZ= dizygotic; AH = assisted hacthing; SUZI = subzonal insemination; PZD= partinal zonal dissection; ICSı = intracytroplasmic sperm injection.

Slotnick and Ortega, who described an incidence of 3.49%(5/143) MZ twinning seven times over the rate of spontaneous twinning.[18]

On the other hand, other studies find to contrary for example, Sills et al,[15] reported on 23 sets of MZ twins from 1911 assisted reproduction pregnancies (1.2%), with no correlation between zonal manipulative techniques and MZ twinning., concluding that the most likely cause was the increased number of embryos transferred to the uterus.

Summary

The connection between assisted embryo hatching and MZ twins remains highly speculative,as blastocoeles have never been observed to divide evenly and completely after passing through an artificial zona opening, either in humans or in animal models

An analysis of factors affecting zona characteristics is appropriate,since the zona pellucida is central to several theories regarding MZ twinning. A synthesis of findings from earlier studies suggests at least three factors are influential in MZ twinning among patients receiving infertility treatments: ovulation induction per se, certain IVF culture conditions, or zona architecture/manipulation. With these three variables occurring together so often in the construct of modern infertility practice it is not easy to analyze the respective contribution of each,

even if multiple regression analysis is used. If the "natural" rate of MZ twins, 0.42%, is considered valid in settings of spontaneous conception and single embryo implantation, then the 3-fold increase in the observed MZ twinning rate reported in IVF patients may be partially explained by the increase number of implantations. The clinical rarity of MZ twinning challenges the study of this phenomenon in the context of IVF, as relevant investigations require large samples (>10.000 cases) to detect meaningful differences with suitable statistical power.

The physiology of both MZ twin evolution and natural blastocyst hatching in the human remain incompletely characterized, and what is known has largely been extrapolated from animal models. It may therefore be premature to attempt to MZ twinning and assisted embryo hatching, given the current limited knowledge regarding both phenomena. Although some studies find the part played by AH and ICSI in MZ twinning negligible, the exact roles of these zona treatments remain incompletely defined in the MZ twinning process. Continued large-scale, clinical studies of sufficient statistical power will therefore be needed.

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