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Assisted reproductive technologies and monozygous twins: implications for future study and clinical practice

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That the zona pellucida (ZP) plays a prominent role in the physiology of some human twinning is an attractive, albeit incompletely proven, medical hypothesis. Indeed, an association has been proposed between manipulation of the ZP and/or native ZP microarchitecture and monozygotic (MZ) twins. Ovulation induction also has been theoretically linked to in vivo ZP alterations facilitating MZ twin development. In vitro fertilization (IVF) relies on necessary (and, in some cases extended) embryo culture techniques potentially creating subtle ZP changes and subsequent MZ twinning. With growing experience in the assisted reproductive technologies and particularly IVF, some preliminary reports have noted an increased frequency of MZ twins after procedures that artificially breach the ZP (i.e., intracytoplasmic sperm injection [ICSI], or 'assisted hatching'). Such ZP manipulations ostensibly enhance oocyte fertilization or facilitate blastocyst hatching, thus improving pregnancy rates for couples undergoing fertility treatment. Evidence exists both to challenge and support the connection between these phenomena and MZ twins. This report outlines the fundamental embryological processes believed responsible for these conflicting observations; the current literature on the subject of human ZP micro-manipulation and MZ twins is also discussed. Twin Research (2000) 3, 217–223.

Keywords: twinning, monozygotic, zona pellucida, in vitro fertilization

Introduction

In mammals, when a single fertilized ovum splits into two genetically identical embryos, MZ twins are thought to result. While the actual frequency of such twinning at the early pre-embryo or blastocyst stage escapes clinical detection, MZ twins are believed to represent about 0.4% of all deliveries.[1] Of the events or conditions precipitating the division of the early conceptus, little is known. Even the 'identical' genome long thought to be shared between MZ twins[2] has recently been questioned.[3]

The subjects of MZ twins and infertility treatment began to mix with the awareness that IVF might be associated with an increased frequency of such twinning.[4] This observation was echoed by a questionnaire study of 42 IVF centers in the United States, where increased numbers of mono-amniotic multiple gestations following ZP manipulated cycles were described.[5]

In contrast to dizygotic (DZ) or 'fraternal' twinning, the rate of MZ twinning initially appeared to be relatively unaffected by race, age, family history of parity.[1] However, later studies of families with very high frequencies of MZ twinning[6,7] have suggested a heritable component to this process. A specific gene or promoter sequence associated with induction of MZ twinning has yet to be localized in any species. While such an intrinsic regulator of embryonic twinning may be isolated and confirmed in the future, currently non-genomic elements are regarded (perhaps by default) as causative. Specifically, observations of clinical outcomes following assisted reproduction have implicated only a few phenomena possibly influencing MZ twinning in humans: ovulation induction[8] and zona pellucida architecture or micro-manipulation.[9] In this report, we discuss how these two concepts are related and how they might modulate MZ twin development. Insights regarding future directions for human twin research are also offered.

Developmental and functional importance of the human zona pellucida

According to an early description of the human ZP in an 1858 obstetrics lecture, 'the ovule itself is found to consist of an external membrane, the zona pellucida'. These observations were foreshadowed by even earlier but less specific work by de Graaf

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(1673) and von Baer (1827).[10] Modern appreciation of multiple yet critical ZP functions has gradually coalesced with increased knowledge of reproductive physiology, and meticulous observation of animal gamete interactions over time has facilitated a better understanding of this structure.

Direct oocyte secretion of extracellular protein matrix is thought to be responsible for ZP growth and development, with conservation of its three constituent zona-binding proteins. Indeed, the species-specific spermatozoa barrier mediated by the ZP is among its most important functions. Since the ZP is important in the prevention of polyspermy at fertilization, maintenance of spatial integrity of the preimplantation embryo, and transportation of the early conceptus to the intrauterine cavity, ZP competency is mandatory to insure the sequence of normal reproductive events from ovulation to nidation. Although iatrogenic ZP damage or removal during laboratory processing in IVF is common and such oocyte are generally discarded,[11] the fact that only one naturally occurring case of congenital absence of the ZP has ever been encountered in the human attests to the significance of this structure (Palermo, personal communication, 1997). It is likely that absence of the ZP is not heritable and signals a lost capacity for human reproduction.

The rationale for zona tampering

Application and refinement of medical technology have made the once impossible a reality for many aspects of human reproduction, and many cases of subfertility have been remedied as a result. Yet, until recently, two troublesome areas still posed formidable obstacles for the reproductive scientist and infertility practitioner: oocyte fertilization and embryo implantation. As the structure specifically limiting sperm access to the oocyte, and later constraining blastocyst contact with the endometrium, the ZP became a prime topic of investigation.

Historically, extreme male factor infertility was problematic in conventional IVF since successful droplet insemination generally requires a minimum motile spermatozoa concentration of 50 000 to 100 000/ml to achieve fertilization. Accordingly, several approaches have been developed to assist fertilization by ZP micromanipulation: partial zona dissection (PZD),[12] sub-zonal sperm insertion (SUZI),[13] and intracytoplasmic sperm injection (ICSI).[14] Of these ZP breaching procedures (which are reviewed elsewhere[15]), ICSI has been shown to be the superior choice in the management of male factor infertility[16] and consequently is the technique most often used in modern clinical practice.

An important problem in IVF continues to be that a significant number of morphologically normal embryos fail to implant. The therapeutic rationale for assisted hatching (AH) derives from the assumption that weakening the zona either by drilling a hole in it, by thinning it, or by altering its stability, will help promote hatching of embryos that otherwise would not escape from their zonae during blastocyst expansion. Zona openings formed during AH are generally 20–30 μm in diameter. Such dimensions are small even by embryo standards, but are still much larger than the defect associated with ICSI. ZP openings created by AH probably show some variation from center to center,[17,18] whereas the ZP puncture after ICSI is more standardized. The overall prevalence of AH with IVF is not known definitively, although it is performed more widely in the United States than in Europe. In contrast, ICSI is now part of an established assisted fertilization repertoire offered essentially worldwide.

Evidence supporting zona manipulation and subsequent MZ twinning

Animal experiments targeted the ZP as an important factor in the twinning equation. One microsurgical approach subjected sheep embryos to zona ripping, blastomere aspiration, and repair of the ZP breach by an agar 'microbandage' and was successful in producing some MZ twin sheep.[19] Other veterinary research established that viable MZ twins could be produced by in vitro 'splitting' of day-8 heifer embryos[20] (Figure 1). Serious developmental malformations in bovine offspring made this microsurgical technique unacceptable for human application, although some investigators have expressed interest in revisiting this technique as an adjunctive therapy for some cases of human infertility.[21]

Talansky and Gordon[22] were among the first to propose that ZP tampering facilitated twinning by inducing structural changes in some mouse blastocysts. Specifically, some blastocysts with zona puncture assumed an analemma or 'figure of eight' shape as the cells attempted to pass through the artificial ZP hole. Some investigators found complete murine blastocele expansion unimpeded by the zona constriction after hatching in most cases,[23] but 'trapping;' was noted in some (59/132, or 45%) embryos that received zona drilling when observed 5 days post-manipulation. One human blastocyst subjected to PZD 'hatched partially' on day 8, and was noted to 'fold double and split' resulting in two distinctly separate but grossly unequal blastoceles.[23] An additional 'biamniotic twin pregnancy' was

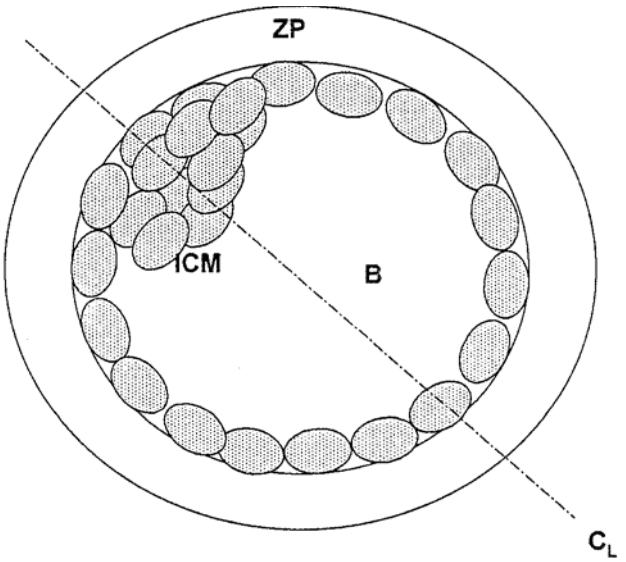

Figure 1 Schematic of post-conception day 6 blastocyst (about 200 cells) depicting the relationships among zona pellucida (ZP), blastocele (B), and inner cell mass (ICM). Mechanical 'splitting' of an embryo has successfully produced monozygotic twins in laboratory animals, and deformation subsequent to blastocyst passage through an artificial zona opening has been proposed to yield similar results. A cleavage plant (C_L) bisecting this early conceptus favors complete duplication of the embryo and development of monozygotic-dichorionic twins

delivered after 'zona rubbing' was performed on a frozen-thawed human blastocyst, although no data on chorionicity were provided.[24]

The link between AH and MZ twins remains speculative, as blastocysts have never been observed to divide evenly and completely after passing through an artificial zona opening, either in humans or in any animal model. Dichorionic MZ twin calves were reported following day 7 transfer of a single blastocyst,[25] yet the in vivo events precipitating this outcome cannot be known. However, other investigators described an unusual spontaneous in vitro cow blastocyst hatching sequence, wherein 'a herniation through a small hole in the zona pellucida' resulted in division of the blastocyst into two equal halves, each containing an inner-cell mass (ICM).[26] One study[9] reviewed six MZ twin pairs (chorionicity not specified) born after IVF, and described some unifying ZP features. Among MZ twins in this small series, the zonae were either thin or AH had been performed. It was concluded from this investigation that ZP tampering or inherent ZP thinness were factors in human MZ twin development.

An important distinction exists between blastocyst splitting and ICM cleavage; both are likely required to produce MZ twins. Human MZ twinning at an early developmental stage could result from a symmetrical ICM 'split'.[23] This splitting hypothesis is supported by animal studies showing that in vitro mechanical cleavage of mammalian embryos along a plane bisecting the ICM could produce MZ twins.[6,7]

Until recently, it was not known if human blastocyst ICM fission could occur spontaneously. Sophisticated laboratory culture techniques have permitted prolonged in vitro surveillance of human blastocysts, and the development of a single blastocyst with two separate but apparently identical ICMs (Figure 2) and dual ICMs joined by a narrow cytoplasmic bridge (Figure 3) have been observed. No deliveries have resulted from such blastocysts, however, so data regarding zygosity, ultimate membrane configuration and pregnancy outcome are lacking.

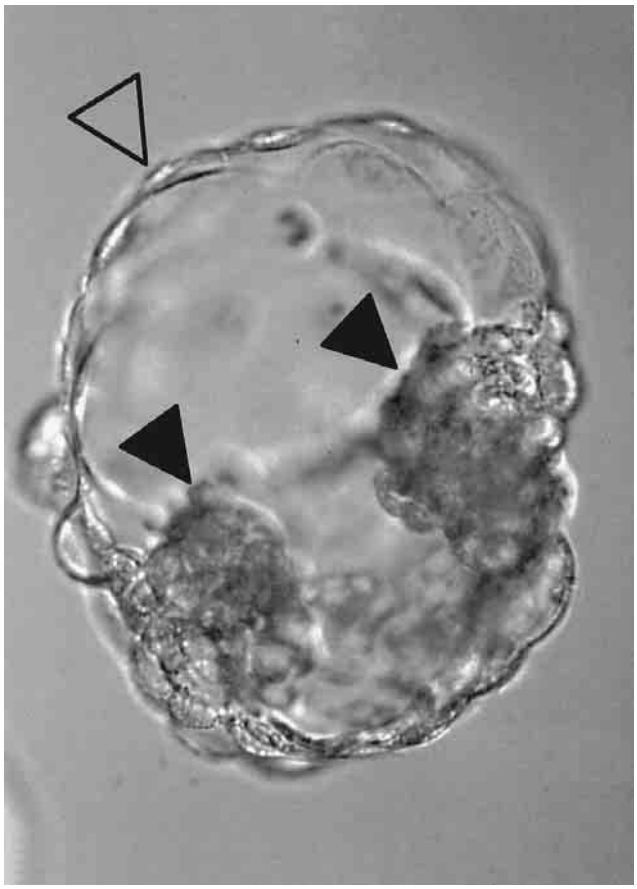

Figure 2 Human blastocyst on post-fertilization day 7 following spontaneous hatching (no zona pellucida micromanipulation). MZ twins may develop from the de novo dual inner cell masses depicted here (black arrows). Expanding mural trophectoderm is also seen (open arrow). This embryo was transferred in utero but the patient miscarried in the first trimester

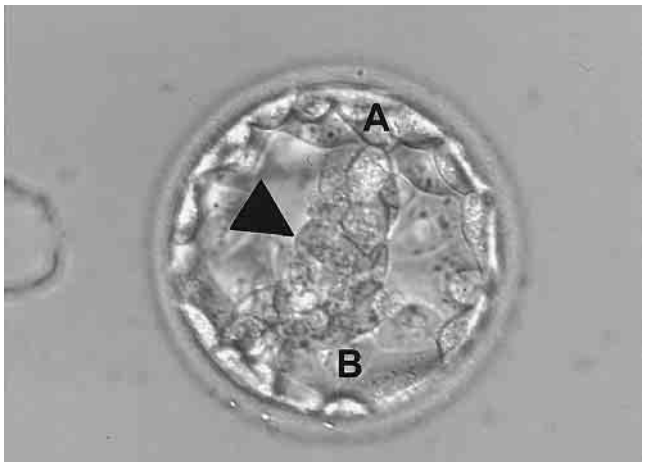

Figure 3 A single human blastocyst at post-conception day 5 developing an 'intracellular bridge' (arrow) between two inner cell masses (A and B). This embryo was not selected for in utero transfer

Ovulation induction or in vitro embryo culture: impact on MZ twinning via ZP change

Since treatments resulting in supraphysiologic oocyte recruitment and release would be expected to increase the number of oocytes available for fertilization, the connection between DZ or 'fraternal' twins and ovulation induction was intuitive. Interestingly, an 8-year review of birth records for a large non-IVF population undergoing ovulation induction revealed increases in both DZ and MZ twinning.[8] Specifically, the rate of MZ twins was significantly increased (1.2% actual vs 0.45% expected, $P < 0.001$) following human menopausal gonadotropin and human chorionic gonadotropin therapy for infertility. The finding that artificial ovulation induction was associated with an increase in MZ twin frequency without IVF was unexpected, and it was concluded that the first biologic mechanism promoting MZ twinning in humans had been discovered.[8] But how would a non-IVF fertility treatment influence the MZ twinning rate?

A hypothetical relationship between ovulation induction and human MZ twinning evolved from related murine embryo work and an improved understanding of ZP and blastocyst physiology. Specifically, early investigations in animal models suggested that important ZP alterations (especially zona 'hardening') could accompany ovarian stimulation.[23] If this effect were generalized to humans undergoing ovulation induction, then related changes in ZP morphology might also occur. Should ZP hardening secondary to ovulation induction be irregular, uneven or non-uniform, this glycoprotein envelope would necessarily contain patches of relative structural instability and weakness. Being thinner, these weak spots would be preferred sites for blastocyst hatching. Extrusion through a narrow ZP lacuna was envisioned to facilitate blastocyst 'herniation' or entrapment. If the conceptus were subject even to slight physiologic or mechanical stress at this moment, the blastocyst might be cleaved into two equal, viable organisms.

In addition to the in vivo effects of ovulation induction described above, certain in vitro culture conditions are conjectured to cause ZP changes. Preliminary data from day 5 human blastocyst transfer suggests that an increased rate of MZ twins is associated with prolonged laboratory culture,[27,28] and not necessarily AH. In vitro conditions for embryonic growth have long been suspected to foster MZ twin development,[29] although it must be recognized that for AH the near-ubiquitous use of controlled ovarian hyperstimulation in IVF confounds analysis for the two variables independently. That the ZP can be modified by peri-ovulatory hormonal factors has been shown by others,[30] and tends to support the notion that in vivo ovulation induction and ZP changes are related. Less secure is the link between any ZP change and MZ twinning, but the central role played by the zona in these hypotheses is apparent and confirms the need for additional research in this area.

Conflicting evidence for human MZ twin induction

In a series of 23 MZ twin sets delivered after ovulation induction and IVF at a single institution, the frequency of twinning was not significantly different between zona-manipulated and zona-intact embryos.[31] Although this cluster of diamnionic-monochorionic MZ twins represents the largest published study to date specifically in an IVF population, statistical power was suboptimal due to data partitioning. The investigation found no relationship between ZP treatments and MZ twins among donor-oocyte recipients, frozen-thawed embryo transfer cycles, or ICSI treatments. This appeared consistent with earlier findings reported when ICSI alone was examined as a potential associative factor in MZ twin development, where no difference in placentation or zygosity was observed when compared with matched, non-ICSI cases.[32]

Unfortunately, most studies of MZ twins and ZP treatments have been based on small (ie $n < 10$) samples with little or no attention to chorionicity. A report describing five cases of MZ twins after AH from 142 pregnancies did find the frequency of monoamniotic multiple gestations higher in zona-

manipulated cycles.[5] This finding was congruent with a somewhat larger study of nine twin pairs where an association between AH and MZ twinning was suggested, but no data on chorion type were reported.[33] While these limited investigations attempted to show a connection between ZP micromanipulation and the development of MZ twinning, none had sufficient statistical power to prove or disprove the association.

When pregnancies resulting from IVF (irrespective of ZP micromanipulation) were recently reviewed, a matched control multi-center study[34] found the incidence of monochorionic placentation to be substantially lower after IVF compared with spontaneously conceived twin pairs (2.3% IVF vs 23.2% control). The number of transferred embryos in IVF would be expected to influence dizygotic twinning frequency in this population, so these data must be interpreted cautiously. Despite this reduced monochorionic MZ twinning observed after IVF, low and discordant birth weight among IVF twins were still more common than in the non-IVF control group.

Although uncommon in current IVF practice, the single-embryo transfer offers an unusual opportunity to understand MZ twinning in an assisted reproductive setting. One center[35] described outcomes following 645 such transfers without any ZP micromanipulation, and reported 82 pregnancies and 4 MZ twin sets (4.9%, i.e. 12 times the expected MZ twinning rate). In contrast, the same institution found no MZ twins among the 94 pregnancies resulting from 920 single-embryo transfer after ICSI only. These data seem to link MZ twinning more closely to gamete treatment and/or embryo culture conditions than to ZP breaching procedures.

The impact of potential disruption of embryo polarity[36] after 'figure-of-eight' blastocyst pinching also invites close scrutiny. Indeed, the cellular processes and geometry associated with passage of the blastocyst halfway through an artificial zona opening at the precise point needed to cause a balanced division of cellular components remain unknown, but is fundamental to the AH-MZ twins hypothesis.

If the 0.4% incidence of human monozygotic twins[1] is accepted as valid, then several statistical computations may be made regarding the sample size necessary to detect meaningful differences among embryos receiving AH, ICSI or both as a function of MZ twinning frequency and chorionicity type. Since the natural incidence of identical twins is quite low, a large study (about 14 000 cases) would be required to discern significant differences with sufficient statistical power. Even with data combined from many IVF centers, the inter-institutional variables of patient selection, non-uniform ovulation induction stimulation protocols, differences in microsurgical technique, and operator experience would yield data too heterogeneous for useful clinical interpretation.

Conclusion

Modern medical science has provided greatly increased understanding of the physiology, classification, and management of twin gestation, yet the search for cause(s) of MZ twinning remains unfulfilled. Could common and well-intentioned assisted reproductive techniques involving the ZP inadvertently alter the MZ twinning rate in humans? For now, the answer seems as elusive as the identical twinning process itself.

The importance of MZ twinning in the context of the assisted reproductive technologies stems from the markedly increased perinatal hazards attendant to pregnancies of this type. Twin–twin transfusion syndrome,[37] fetal entanglement and umbilical cord accidents,[38] caudal regression syndrome,[39] and other developmental anomalies[40] are much more common among MZ twin gestations than in singleton pregnancies.[41]

In any case, the matter of MZ twinning as a sequela of AH has rarely overshadowed the larger debate regarding the efficacy of and selection criteria for AH itself. While several authors have reported improved reproductive outcomes after AH,[42–45] others have found the treatment to be of questionable benefit.[46–49] Still others have introduced a compromise posture of 'selective' hatching,[50] restricting the procedure according to established criteria believed to identify patients for whom AH would be worthwhile. Currently, advanced maternal age, ZP thickness > 17 μm, elevated day three FSH, and/or a history of prior failed IVF have been suggested as criteria for AH.

An analysis of factors affecting the ZP is timely as this structure is central to current theory regarding MZ twinning. In particular, the finding of increased numbers of monoamniotic MZ twins after AH[5] warrants further investigation, because a single amnion is usually associated with embryonic 'splitting' between days 8 and 13. AH is typically performed much earlier (post-conception day 2–5), well before embryo transfer in IVF. Research focusing on why AH has not altered the frequency of dichorionic MZ twins (the membrane configuration expected after partial blastocyst hatching and subsequent bisection) will be highly instructive. To advance knowledge of this embryological phenomenon, the chorionicity of twins should be authenticated and reported as this subject receives further study. We agree with the need to take zygosity – and thus

chorionicity – into account when medically assisted twins are compared with 'natural twins'.[34]

While the role of ICSI in the treatment of male factor infertility appears secure, the advanced reproductive technologies are set to follow a course that may diminish the relevance of AH. Historically, the efficacy of assisted reproductive techniques was low and any optimization of reproductive outcome promised by ancillary procedures like AH was especially welcome. Indeed, with < 20% of embryos placed in the uterus actually implanting after a traditional 'day 3' embryo transfer,[51] a ready clinical niche existed (and may continue to exist) for AH. However, this appears to be changing as improvements in in vitro blastocyst culture have enabled natural hatching to be underway (or complete) by the time of intrauterine transfer.[52] Alternatively, the ZP could be completely removed once a blastocyst has formed,[53] as the embryo would then be ready to implant with little need for the presence of the ZP. The rationale for intervention by AH may therefore be trumped by blastocyst culture modalities permitting (or obviating) natural hatching, thus making AH largely unnecessary.

The physiology of both MZ twins and natural blastocyst hatching in the human remain incompletely characterized, and what is known has chiefly been extrapolated from animal models. Limited observations of human embryos have implicated some ZP features or treatments as associative in MZ twin development, but larger sampling has failed to validate this correlation. It may therefore be premature to attempt a linkage between MZ twinning and ZP status. The role of the assisted reproductive technologies may be marginal in the MZ twinning process, but large-scale clinical studies of appropriate consistency and statistical power will be needed for confirmation. Attention to chorionicity type will likely prove essential as the complex developmental story of MZ twins is elucidated.

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