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Article

The Impact of Assisted Hatching on Monozygotic Twinning is Related to Female Age and Insemination Method: A New Perspective

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Abstract

Whether assisted hatching (AH) is associated with a higher incidence of monozygotic twinning (MZT) in women undergoing assisted reproductive technology remains controversial; the aim of the study was to demonstrate the relationship between AH and MZT. A total of 8900 clinical pregnancies were selected among embryo transfer cycles from January 2011 to October 2019. Women receiving day (D) 3 embryos were divided into groups A–C: group A ($n = 1651$) and group B ($n = 1045$) included women aged ≤37 or ≥38 years, respectively, with zona pellucida (ZP) thinning; group C ($n = 3865$) included women aged ≤37 years without AH. Women aged ≤37 years who underwent blastocyst transfer and/or blastocyst ZP breaching were included in group D ($n = 2339$). The incidence of MZT was compared among groups A, B and C, and between groups C and D. The incidence of MZT in group B (2.2%) was significantly higher than in group A (1.0%), especially following intracytoplasmic sperm injection (ICSI), while the incidence of MZT in group A (1.0%) was significantly lower than in group C (2.2%). The MZT rate with in vitro fertilization was higher in group D (2.8%) than in group C (2.2%), but the MZT rate following ICSI was not significantly different between the two groups. ZP thinning of D3 embryos may increase the risk of MZT in older women (≥38 years), but decrease it in younger women (≤37 years). ZP breaching may be useful to reduce the incidence of MZT in ICSI-generated blastocysts.

Keywords: Blastocyst; female age; intracytoplasmic sperm injection; laser-assisted hatching; monozygotic twinning

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Human monozygotic twinning (MZT) is rare following natural conception, but the incidence of MZT increases following assisted reproductive technology (ART; Derom et al., 1987, Hattori et al., 2019, Saito et al., 2000). Compared with natural pregnancies, gametes and embryos are exposed in vitro and manipulated artificially in ART, and therefore the culture environment in vitro is not exactly the same as in vivo, which might be a cause for the increase of MZT. However, what leads to the association between ART and MZT remains unclear. Papanikolaou et al. (2010) studied the relationship between MZT and the etiology of infertility, including tubal factors, male factors, endometriosis, polycystic ovarian syndrome, idiopathic and combined causes, none of which were related to MZT. Knopman et al. (2014) found that among in vitro fertilization (IVF) cycles, oocytes recovered from young women and extended culture were associated with a higher incidence of MZT, while frozen-thawed embryo transfer and intracytoplasmic sperm injection (ICSI) did not increase the incidence. A multicenter large sample cohort study showed that the risk of MZT was higher with the transfer of fresh day (D) 5–6 embryos or following assisted hatching (AH) of D2–3 embryos (Luke et al., 2014). In addition, the use of donor oocytes, lower doses of follicle stimulating hormone and gonadotropin-releasing hormone agonist suppression were also associated with a higher incidence of MZT, all of which reflected the ART treatment of younger women (i.e. younger oocytes; Hviid et al., 2018; Luke et al., 2014). A recent systematic review and meta-analysis of MZT in couples undergoing ART also found that blastocyst transfer significantly increased the risk of MZT compared with cleavage-stage embryos, and younger maternal age was associated with an increased risk of MZT, while it failed to document any association between frozen-thawed embryo transfer and the occurrence of MZT (Busnelli et al., 2019). Conventional IVF had a milder but still statistically significant higher risk of MZT pregnancy compared with ICSI (Busnelli et al., 2019), which was converse to the result of Hviid et al. (2018). Busnelli et al. (2019) found that AH might increase the risk of MZT, but this was not confirmed after limiting the analysis to a high-quality cohort of infertile couples (Newcastle-Ottawa scale score >7 for quality of nonrandomized studies in meta-analyses) and to case control studies (Busnelli et al., 2019). This might be because factors such as maternal age and the embryo stage of using AH have not been considered in most studies. The purpose of AH is to improve the pregnancy rate, and it is generally used in poor prognosis ART treatment cycles such as those in older women and when using frozen-thawed embryos (Martins et al., 2011; Zeng et al., 2018). Embryo freezing does not increase the incidence

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of MZT, but AH might do so. The age of the woman and the transferred embryo stage have important effects on the incidence of MZT (Busnelli et al., 2019). Therefore, it is necessary to separate the two factors and compare the impact of AH on MZT.

It has been speculated that MZT might be caused by hardening of the zona pellucida (ZP) in vitro, thereby trapping the inner cell mass and splitting it when the blastocyst hatches (Alteri et al., 2018). The density of the embryo's ZP can increase following extended culture in vitro (Kilani et al., 2006). It has been demonstrated that ZP breaching (creating a hole) results in more completely hatched blastocysts than ZP thinning (Chailert et al., 2013; Yano et al., 2006). Thus, would it reduce the incidence of MZT if the blastocyst ZP were to be opened with a big hole? Here, we compared the effects of ZP thinning in cleavage-stage embryos at different maternal ages on the incidence of MZT and examined whether the higher MZT rate of blastocysts subjected to AH was caused by ZP changes such as increased density caused by prolonged embryo culture in vitro.

Materials and Methods

Study Design

It has been demonstrated that there is no association between frozen cycles and MZT pregnancies (Busnelli et al., 2019); therefore, it is not necessary to separate frozen-thawed embryo transfer and fresh embryo transfer cycles. In total, 8900 clinical pregnancies derived from ART treatment cycles were selected from 2011 to October 2019 at the Reproductive Centre of Zhanjiang Jiuhe Hospital, excluding preimplantation genetic diagnosis screening cycles. According to the procedures of the embryo laboratory, for women aged ≥38 years, AH using frozen-thawed embryos or embryos was carried out. These were divided into four groups according to the woman's age, stage of embryo transferred and AH method (ZP breaching or thinning) as follows:

Group A ($n = 1651$): women aged ≤37 years receiving D3 embryos subjected to ZP thinning; Group B ($n = 1045$): women aged ≥38 years receiving D3 embryos subjected to ZP thinning; Group C ($n = 3865$), women aged ≤37 years receiving D3 embryos without AH; Group D ($n = 2339$), women aged ≤37 years undergoing D5/D6 blastocyst transfer and ZP breaching.

To analyze whether the uncertain effect of AH on MZT was associated with the woman's age, the incidences of MZT were compared between Groups A and B, and between Groups A and C. To test whether the higher MZT of blastocysts than in D3 embryos was associated with changes to the ZP such as increasing density caused by prolonged embryo culture in vitro, the blastocyst's ZP was breached with a 60–80 µm hole, and the incidences of MZT were compared between Groups C and D.

Transvaginal ultrasonography was performed at gestational weeks 6–7 for patients with a positive venous blood pregnancy test, and clinical pregnancy was defined as the detection of one or more gestational sac and fetal heartbeat. MZT gestations were documented based on one of the following criteria: (1) the number of fetal heartbeats exceeded the number of embryos transferred; or (2) more than one fetal heartbeat was identified in a single gestational sac.

AH Procedures

AH was carried out using laser equipment (Hamilton Thorne ZILOS-tk®, Beverly, MA, USA) according to previous studies (Hiraoka et al., 2008; Yano et al., 2006) with the laser in clinical mode at a power of 100% and a pulse of 400 µs. Briefly, the ZPs of D3 embryos were thinned to a final length of 40–60 µm, and those of D5/D6 blastocysts were breached with a hole of 60–80 µm.

Statistical Analysis

The data were analyzed using IBM SPSS Statistics (v. 22.0, IBM Corp., Armonk, NY, USA). Categorical variables are shown as percentages and were compared using Fisher's exact test; multivariate logistic regression model was used to adjust the p value, excluding the influence of confounding factors on the results. According to the previous literature, eight factors were evaluated for their association with MZT: male infertility factors (obstructive azoospermia, other male factors, no male factors); female infertility factors (unexplained infertility, ovulation disorders, ovarian dysfunction, endometriosis, cavitas pelvis and fallopian tube factors, other female factors and no female factors); suppression protocol (GnRHa and non-GnRHa); methods of insemination (IVF, ICSI); gonadotropin dose (<2000 IU, 2000–2999 IU and ≥3000 IU); infertile duration(≤2 years, 2.01–4 years, 4.01–6 years, 6.01–8 years, >8 years); pregnancy history (primary and secondary infertility); number of embryos transferred(1, 2, 3). Univariate analysis was carried out first, and all variables with $p < .15$ were included in the logistic regression model. Results were reported as odds ratios (*OR*) and 95% confidence intervals (CI), and $p < .05$ was considered statistically significant.

Results

The incidence of MZT in Group B (2.2%) was significantly higher than in Group A (1.0%, $p < .01$), especially following ICSI (Table 1), while the incidence of MZT in group A (1.0%) was significantly lower than in Group C (2.2%, $p < .001$) (Table 2).

The incidence of MZT with IVF was higher in Group D (2.8%) than in Group C (2.2%, $p < .05$). However, the MZT rate following ICSI was not significantly different between Groups C and D (Table 3).

Discussion

Twin pregnancies are usually associated with higher risks than singleton pregnancies for both the mother and fetus, such as fetal or neonatal death, or cerebral palsy in the offspring (Anja et al., 2005; Aston et al., 2008; Scher et al., 2002). In addition, MZT carries a risk of twin-to-twin transfusion syndrome, with an incidence of about 10% (Samawal et al., 2004). Moreover, the mortality rate is higher in MZT compared with dizygotic twins (Christophersen et al., 2013). One study found that it was not clear whether AH would lead to a higher incidence of MZT (Busnelli et al., 2019), while most studies found that a higher risk of MZT was associated with lower female age (Busnelli et al., 2019; Hviid et al., 2018; Luke et al., 2014). However, in our study, the incidence of MZT in older patients (≥38) with ZP thinning in D3 embryos was higher than that in younger patients (≤37), and the incidence of MZT in younger patients with AH was lower than that in those without AH.

AH is generally used in patients with poor prognosis, such as older women and those with failed IVF cycles or poor embryo quality. As we all know, the ovarian function, oocyte quality and embryo quality of older women are decreased. Therefore, in most older women who undergo cleavage embryo transfer, the probability of pregnancy failure is high. According to the statistics of the USA from 2000 to 2010, almost two-thirds of the patients ≥38 had AH, which is far higher than the proportion of patients <38. From 2000 to 2010, the proportion of patients ≥38 who had no history of pregnancy

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Table 1. Effect of AH (ZP thinning) on MZT in D3 embryos from differently aged women

		Fisher's exact test		Multivariable logistic regression model		
	Groups	MZT (%)	*p* value	*OR*	95% CI	*p* value
IVF	Group A	16/1136 (1.4)	.154	1.00 reference		.079
	Group B	17/736 (2.3)		1.93	0.93, 4.01	
ICSI	Group A	0/515 (0.0)	.003#			
	Group B	6/309 (1.9)				
Total	Group A	16/1651 (1.0)	.012	1.00 reference		.007
	Group B	23/1045 (2.2)		2.6	1.30, 5.71	

Note. MZT of Group A was 0.0%, so a logistic regression analysis has not been done. Female infertility factors and methods of insemination (total MZT) were in included in the logistic regression model. Group A, women aged ≤37 years; Group B, women aged ≥38 years; MZT, monozygotic twinning; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.

Table 2. Effect of AH (ZP thinning) on MZT in D3 embryos from young women

		Fisher's exact test		Multivariable logistic regression model		
	Groups	MZT (%)	*p* value	*OR*	95% CI	*p* value
IVF	Group C	62/2833 (2.2)	.129	1.00 reference		.016
	Group A	16/1136 (1.4)		0.5	0.28, 0.88	
ICSI	Group C	22/1032 (2.1)	.001#			
	Group A	0/515 (0.0)				
Total	Group C	84/3865 (2.2)	.002	1.00 reference		<.001
	Group A	16/1651 (1.0)		0.36	0.21, 0.62	

Note. MZT of Group A was 0.0%, so a logistic regression analysis has not been done. Number of embryos transferred, gonadotropin dose and male infertility factors were included in the logistic regression model. Group A, women aged ≤37 years with AH; Group C, women aged ≤37 year without the use of AH; ZP, zona pellucida; MZT, monozygotic twinning; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; AH, assisted hatching.

Table 3. The effect of AH in blastocysts on MZT

		Fisher's exact test		Multivariable logistic regression model		
	Groups	MZT (%)	*p* value	*OR*	95% CI	*p* value
IVF	Group C	62/2833 (2.2)	.171	1.00 reference		.019
	Group D	51/1792 (2.8)		2.05	1.13, 3.75	
ICSI	Group C	22/1032 (2.1)	.440	1.00 reference		.543
	Group D	8/547 (1.5)		0.66	0.17, 2.55	
Total	Group C	84/3865 (2.2)	.383	1.00 reference		.066
	Group D	59/2339 (2.5)		1.69	0.97, 2.95	

Note. Male infertility factors, gonadotropin dose, pregnancy history and number of embryos transferred were included in the logistic regression model. Group C, women aged ≤37 years with D3 embryo transfer and no AH; Group D, women aged ≤37 years with blastocyst (D5/D6) transfer and ZP breaching; MZT, monozygotic twinning; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; AH, assisted hatching.

failure increased (Kissin et al., 2014). The effect of AH on monozygotic multiple births is still controversial, which may be due to the different proportion of older patients with cleavage embryos undergoing AH in different studies. Studies with a high proportion of older patients may conclude that AH increases the incidence of MZT. On the contrary, if there are more younger patients, the result may be that AH reduces the incidence of MZT.

In one study, AH had a slightly lower risk of MZT in women <37 years who received cleavage-stage embryos (Wu et al., 2014), consistent with our results. However, most studies did not limit AH to couples with a single infertility factor or consider the woman's age or stage of embryo transferred (Busnelli et al., 2019; Hviid et al., 2018). According to the most accepted hypothesis, AH involving premature interruption of the ZP might cause embryo splitting by interfering with signaling mechanisms in the embryo (Knopman et al., 2014). It has also been speculated that MZT might be caused by hardening of the ZP (Alteri et al., 2018). In our study, it appeared that D3 embryos of older women were more sensitive to AH. AH may have a greater impact on the ZP of older female embryos, and ZP may become harder, while AH may have a smaller impact

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on the ZP of young female embryos, and the thinning of the ZP may make embryo hatching easier, thus reducing MZT.

It has been confirmed in most studies that blastocyst transfer has a higher risk of MZT than cleavage-stage embryo transfer (Busnelli et al., 2019; Hviid et al., 2018). The density of the embryo's ZP can increase following extended culture in vitro (Kilani et al., 2006), while ZP breaching results in more completely hatched blastocysts than ZP thinning (Chailert et al., 2013; Yano et al., 2006). In our study, the MZT rate in IVF cycles for the blastocyst ZP breaching group was still significantly higher than that of D3 embryos without AH, but the MZT rate was not significantly different following ICSI. Thus, ZP breaching may be useful to reduce the incidence of MZT in ICSI-generated blastocysts.

In conclusion, ZP thinning in D3 embryos increased the risk of MZT in older women (≥38 years), but decreased it in younger women (≤37 years), especially for embryos derived from ICSI. Moreover, ZP breaching may be useful to reduce the incidence of MZT in ICSI-generated blastocysts.

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Conflict of Interest. All authors declare that they have no conflict of interest and approved the publication of the manuscript.

Ethics Approval. This study was approved by the Ethics Committee of Zhanjiang Jiuhe Hospital. All patients in our study signed informed consent for assisted reproduction technology treatment including the use of assisted hatching.

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