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Embryological Characteristics and Clinical Outcomes of Oocytes with Heterogeneous Zona Pellucida During Assisted Reproduction Treatment: A Retrospective Study

Authors' Contribution:
Study Design A
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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The condition of the zona pellucida can be used to predict human oocyte quality. This study investigated the embryological characteristics and clinical outcomes of oocytes with heterogeneous zona pellucida (HZP) during *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI).

Material/Methods: This was a retrospective study of IVF and ICSI cycles undertaken at The First Affiliated Hospital of Wenzhou Medical University between June 2006 and March 2016. Cycles involving oocytes with HZP (HZP group) were compared with those involving non-HZP oocytes retrieved on the same day (non-HZP group). Embryological characteristics and clinical outcomes were compared.

Results: There were 29 IVF and 46 ICSI cycles in the HZP group, and 521 IVF and 206 ICSI cycles in the non-HZP group. In ICSI cycles, the rates of MII oocyte and high-quality embryo were lower in the HZP group ($p<0.05$ *vs.* non-HZP). In IVF cycles, the MII oocyte ($p<0.001$), normal fertilization ($p<0.001$), and cleavage ($p<0.001$) rates were lower, while the abandoned transfer rate ($p<0.001$) was higher in the HZP group compared with the non-HZP group. The positive human chorionic gonadotropin (HCG), implantation, pregnancy, and miscarriage rates were similar between groups. Multivariate analysis revealed that the woman's age (OR=0.916 95% CI 0.873–0.962; $p<0.001$) and the number of D3 high-quality embryos (OR=1.120 95% CI 1.004–1.249; $p=0.043$) were associated with pregnancy in IVF cycles, but no significant factors were found in ICSI cycles.

Conclusions: ICSI may help increase the number of viable embryos in cycles with oocytes showing HZP. However, both IVF and ICSI cycles can achieve pregnancy.

MeSH Keywords: Fertilization • Oocytes • Pregnancy • Zona Pellucida

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Background

The use of assisted reproduction technologies accounts for over 1 million births each year worldwide [1]. The morphological assessment of oocytes and subsequent embryos is a crucial part of assisted reproduction techniques such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) [2] because it helps ensure the selection of metaphase II (MII) oocytes with an optimal potential to successfully develop into viable embryos for transfer [3].

The human zona pellucida (ZP) is an extracellular glycoprotein matrix that is synthesized and secreted by oocytes during follicular development. The ZP is made of 4 glycosylated proteins (ZP1, ZP2, ZP3, and ZP4) [4,5]. The human ZP is vital for specific sperm-oocyte binding, acrosome reaction induction, and polyspermy prevention [6]. In addition, *in vivo*, the ZP protects the embryo during its passage from the oviduct to the uterus or, *in vitro,* the ZP protects the embryos from mechanical and chemical stress [7,8].

The ZP shows different morphologies among oocytes of women undergoing assisted reproductive technology treatment. Abnormal ZP morphology can be observed in 2–5% of all oocytes [9]. It is likely that any abnormality in composition, shape, color, and thickness of the ZP may affect the outcomes of IVF. Oocytes with an oval-shaped ZP have a high risk of abnormal embryo cleavage and are associated with lower rates of implantation and pregnancy after IVF [10,11]. The fertilization rate and clinical outcomes may depend upon variations in ZP thickness [12,13]. In contrast, the fertilization or clinical outcomes seems unaffected by a dark appearance of the ZP [14–16], but contradictory results have been reported [17]. Oocytes with heterogeneous zona pellucida (HZP) have a bright vitreous appearance with an irregular outer edge. A study with a limited sample size found that HZP is associated with reduced oocyte maturity and reduced rates of fertilization and high-quality embryos [18]. Further investigation into the outcome of oocytes with HZP is needed.

Therefore, this study investigated the embryological characteristics and clinical outcomes of oocytes with HZP during IVF and ICSI treatments, as compared to oocytes with normal zona pellucida (non-HZP).

Material and Methods

Patients

This study retrospectively examined the IVF, and ICSI treatment cycles carried out at The Reproductive Medicine Center of The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China, between June 2006 and March 2016. Only the cycles completed under controlled ovarian hyperstimulation with the protocol using gonadotropin-releasing hormone (GnRH) agonist were included. Twenty-nine IVF and 46 ICSI cycles with HZP oocytes were included (HZP group). Another 521 IVF and 206 ICSI cycles with oocytes retrieved on the same day were included as controls (non-HZP group). The detailed baseline information from the women is shown in Table 1.

The exclusion criteria were: 1) known previous poor ovarian response to ovarian stimulation; and 2) endometriosis, polycystic ovary syndrome (PCOS), hydrosalpinx, or uterine pathology.

This study was reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Wenzhou Medical University (No. 2019-099), and the requirement for informed consent was waived due to the retrospective nature of the study.

Table 1. Baseline patient information.

Characteristics	ICSI cycles			IVF cycles		
	HZP	Normal	P value	HZP	Normal	P value
Total, n	46	206		29	521	
Female age (year), mean±SD	32.6±3.6	31.8±4.7	0.315	31.1±4.3	31.9±4.6	0.304
BMI (kg/m^2), mean±SD	21.0±2.7	21.2±2.9	0.781	20.6±2.6	21.2±2.8	0.222
Duration of infertility (year), mean±SD	6.6±3.5	4.8±3.6	0.002	4.8±3.0	3.7±2.7	0.003
Diagnosis, n (%)			<0.001			<0.001
Primary infertility	41 (89.1)	110 (53.4)		20 (69.0)	159 (30.5)	
Secondary infertility	5 (10.9)	96 (46.6)		9 (31.0)	362 (69.5)	

ICSI – intracytoplasmic sperm injection; IVF – *in vitro* fertilization; HZP – heterogeneous zona pellucida; SD – standard deviation; BMI – body mass index. P<0.05 indicates a statistically significant difference.

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Study design

The patients were grouped according to the presence or absence of HZP in the oocytes used during IVF/ICSI. When all retrieved oocytes showed HZP and no normal oocytes could be found, the HZP oocytes were used for the procedure and assigned to the HZP group. The control (non-HZP group) group consisted of cycles with oocytes retrieved on the same day and considered to be morphologically normal.

Stimulation regimens

During the study period, the luteal long downregulation protocol (LP) or the short flare-up protocol (SP) were routinely used, as previously described [19]. Specifically, in the LP, a 0.5-0.9 mg depot of a GnRH agonist (diphereline, 3.75 mg; IpsenPharma Biotech) was administered in the mid-luteal phase. Stimulation using gonadotropin (Gonal-F; EMD Serono) was initiated at 14 days, the injection of diphereline (usually on cycle day 3-9) or when pituitary downregulation was achieved, as previously described [19]. Downregulation was confirmed by an endometrial lining of up to 5 mm, serum estradiol <50 pg/L, and serum luteinizing hormone (LH) <5 U/L. For SP, the GnRH agonist (decapetyl, 0.1 mg; Ferring GmbH) was given as a daily dose of 0.1 mg starting on cycle day 2, followed by gonadotropin (Gonal-F; EMD Serono) starting on day 3, as previously described [19]. For both protocols, Gonal-F (150 or 225 U/day) was given for 5-6 days and adjusted based on follicle growth and serum levels of estradiol. Human chorionic gonadotropin (HCG; Livzon, China) was administered at 5000–10 000 IU when at least 3 follicles were observed to have reached 17 mm in diameter as assessed by B-mode ultrasound; oocyte retrieval was carried out 34-36 h later, as previously described [20].

Oocyte insemination and embryo culture

IVF and ICSI were carried out according to standard protocols, as previously described [17,19]. Specifically, ICSI was used when sperm concentration was below 5×10^6/mL or when sperm motility was <10%. Insemination was performed by IVF or ICSI 3–6 h after oocyte retrieval. Sixteen to eighteen h later, fertilization was confirmed based on the observation of 2 pronuclei and 2 polar bodies. Fertilized oocytes were cultured in fresh 20 µl of G1 plus medium (Vitrolife AB, Sweden) in individual microdroplets covered with mineral oil at 37°C in an atmosphere of 6% CO_2. In the morning of day 3, embryos were graded according to morphology, blastomere number, blastomere size, and fragmentation, as described by the Istanbul Consensus [21]. The embryos with 7, 8, or 9 blastomeres, and those with less than 20 fragments were considered to be of high quality.

Embryo transfer and pregnancy detection

On day 3, embryo transfer was performed under abdominal ultrasound guidance, as previously described [22]. Specifically, 2 or 3 transferred embryos were selected according to embryo quality. If there were only poor-quality embryos or no embryos from the IVF/ICSI cycle, embryo transfer was canceled.

Pregnancy was confirmed as previously described [19]. Specifically, 2 weeks later, an HCG test was performed. In the case of positive signals, clinical pregnancy was defined as the presence of fetal heartbeat 4 to 5 weeks after embryo transfer, confirmed by ultrasound. Early abortion was defined as spontaneous abortion before 20 weeks of gestation.

Clinical data collection and examination methods

Observation of the ZP

The ZP was evaluated under an OLYMPUS IX73 microscope (OLYMPUS, Japan) equipped with a digital camera at 200× magnification. We defined HZP as a ZP with the bright and vitreous appearance and an irregular outer edge, as previously described [18]; an oocyte with HZP had overtly absent or extremely narrow perivitelline space (Figure 1).

Definitions and outcomes

The numbers of oocytes retrieved, MII oocytes, fertilized oocytes, cleaved embryos, high-quality embryos, and available embryos for transfer were recorded. The corresponding rates were calculated as previously described [19], including MII oocyte rate (MII oocyte number by that of all retrieved oocytes×100), normal fertilization rate (number of fertilized oocytes by that of all retrieved oocytes [for IVF] or MII oocytes [for ICSI]×100), cleavage rate (number of cleaved embryos by that of all fertilized oocytes×100), high-quality embryo rate (the number of high-quality embryos [7, 8, or 9 blastomeres with less than 20 fragments] divided by that of cleaved embryos×100) and abandoned transfer rate (number of cycles of abandoned transfer divided by that of all cycles×100). For successfully transferred embryos, the number of transferred embryos, positive or negative HCG, implantation, pregnancy, gestational period, and miscarriage status were recorded. The positive HCG rate was defined as the total cycles of clinical, biochemical, intrauterine, and ectopic pregnancies divided by all transfer cycles×100. The implantation rate was defined as the number of fetal heartbeats divided by that of transferred embryos×100. The pregnancy rate was defined as the number of patients with 1 or more gestational sac(s) visualized by ultrasound at 6–7 weeks' gestational age by that of transferred cycles×100. The miscarriage rate was defined as the number of miscarriage cycles divided by that of cycles with

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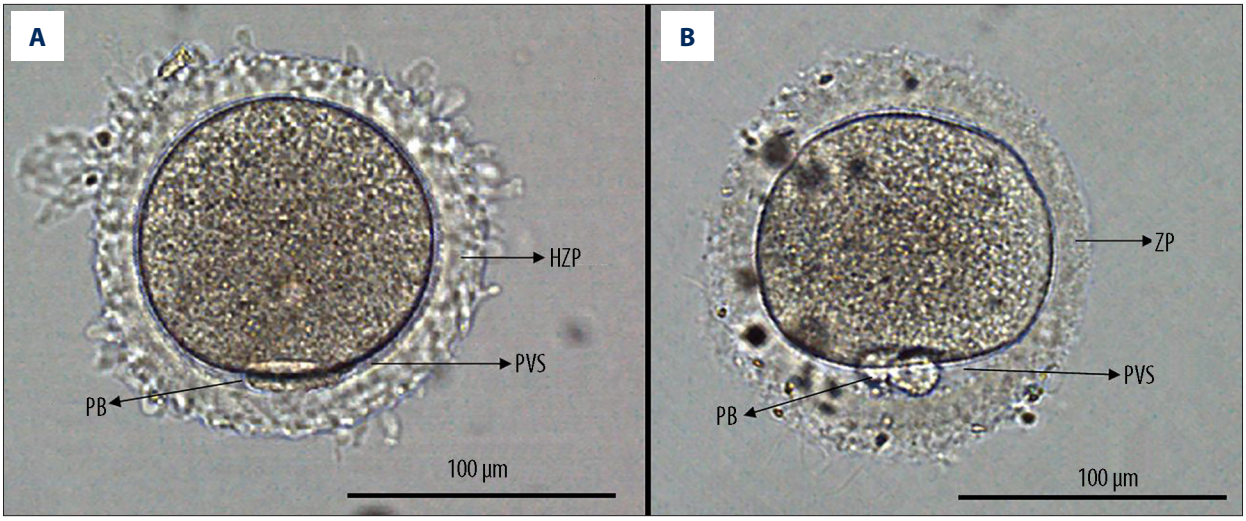

Figure 1. Human mature oocytes. (**A**) Oocyte with heterogeneous zona pellucida. The perivitelline space is extremely reduced or absent. (**B**) Oocyte with normal zona pellucida. Scale bar – 100 µm. ZP – zona pellucida; PB – polar bodies; PVS – perivitelline space.

clinical pregnancy×100. The live birth rate was defined as the number of cycles with premature or mature delivery divided by that of transferred cycles×100.

Statistical analysis

Statistical analyses were carried out with SPSS version 16.0 (SPSS, Inc., USA). Continuous variables are presented as means±standard deviations (SD); comparisons between groups were performed using the *t* test. Categorical variables were reported as frequencies (percentages) and compared by the chi-square test. Multivariable logistic regression models were used to assess the associations of risk factors with the main outcome (pregnancy or not) in both ICSI and IVF cycles. P<0.05 was considered statistically significant.

Results

Baseline features of the included patients

When the ICSI cycles of both groups were compared, age and BMI were similar, but the duration of infertility was longer in the HZP group (p=0.002), and the diagnosis of infertility was more likely to be primary infertility (p<0.001) *vs.* the non-HZP group. When IVF cycles were compared between the 2 groups, the same factors were significant: the duration of infertility was longer in the HZP group (p=0.003), and the diagnosis of infertility was more likely to be primary infertility (p<0.001) compared with the non-HZP group, but age and BMI were similar. Other baseline features are shown in Table 1.

Embryo characteristics

Embryological outcomes are shown in Table 2. For ICSI cycles, no differences were observed between the HZP and non-HZP groups in the number of oocytes retrieved, normal fertilization rate, and cleavage rate. The rates of MII oocytes and high-quality embryos were lower in the HZP group *vs.* the non-HZP group (p<0.05). In IVF cycles, no differences were detected between the HZP and non-HZP groups in the number of oocytes retrieved or high-quality embryo rate. MII oocyte rate, normal fertilization rate, and cleavage rate were lower in the HZP group *vs.* the non-HZP group (all p<0.001).

Clinical outcomes

Clinical outcomes are shown in Table 2. In ICSI cycles, 36 embryo transfers were performed in the HZP group and 161 in the non-HZP group. There were no significant differences in the abandoned transfer rate per retrieval cycle, positive HCG, implantation, pregnancy, and miscarriage rates between the 2 groups.

In IVF cycles, 12 embryo transfers were performed in the HZP group and 442 in the non-HZP group. The rate of abandoned transfer per retrieval cycle was higher in the HZP group *vs.* the non-HZP group (p<0.001). Although the number of transferred embryos per cycle in the HZP group was lower than that of the non-HZP group, there were no differences in positive HCG, implantation, and pregnancy and miscarriage rates between the 2 groups.

Univariate and multivariate analyses

Univariate analyses of factors related to pregnancy after ICSI are shown in Table 3. The results revealed that the woman's

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Table 2. Embryological and clinical outcomes for patients in both ICSI and IVF cycles.

Parameter	ICSI cycles			IVF cycles		
	HZP	Normal	P value	HZP	Normal	P value
Total cycles (n)	46	206		29	521	
Retrieved oocytes (mean±SD)	9.4±6.2	10.9±7.5	0.152	8.8±5.1	11.1±6.6	0.071
MII oocyte rate (%)	62.40%	83.50%	<0.001	62.90%	90.40%	<0.001
Normal fertilization rate (%)	77.70%	74.20%	0.221	20.30%	66.10%	<0.001
Cleavage rate (%)	95.70%	94.10%	0.355	76.90%	95.50%	<0.001
High quality embryo rate (%)	23.00%	31.20%	0.019	35.00%	30.10%	0.505
Available embryos for transfer (mean±SD)	4.4±4.4	6.3±5.2	0.016	1.4±2.5	7.0±4.6	<0.001
Abandoned transfer rate (%)	21.70%	21.80%	0.988	58.60%	15.20%	<0.001
Transferred cycles (n)	36	161		12	442	
Transferred embryos (mean±SD)	2.1±0.6	2.2±0.6	0.299	1.8±0.7	2.2±0.5	0.016
Positive HCG rate (%)	47.20%	49.70%	0.789	50.00%	53.20%	0.828
Implantation rate (%)	24.00%	24.90%	0.876	22.70%	27.90%	0.590
Pregnancy rate (%)	38.90%	44.10%	0.568	41.70%	46.40%	0.747
Miscarriage rate (%)	21.40%	12.70%	0.390	20.0%	11.70%	0.572

ICSI – intracytoplasmic sperm injection; IVF – *in vitro* fertilization; HZP – heterogeneous zona pellucida; SD – standard deviation; HCG – human chorionic gonadotropin.

Table 3. Multivariate analysis of the main outcome (pregnancy or not) in ICSI cycles.

	Univariate analysis			Multivariate analysis		
	OR	95% CI	P value	OR	95% CI	P value
Female age	0.894	0.835–0.959	0.002	0.935	0.864–1.012	0.094
Duration of infertility	0.921	0.851–0.998	0.044	0.968	0.885–1.058	0.472
BMI	0.984	0.895–1.081	0.734			
Diagnosis						
Primary infertility	Reference					
Secondary infertility	0.783	0.437–1.402	0.410			
No. of retrieved oocytes	1.077	1.025–1.131	0.003	0.979	0.843–1.137	0.782
No. of MII oocytes	1.097	1.037–1.160	0.001	1.062	0.892–1.263	0.500
HZP						
Normal	1.24	0.592–2.595	0.569			
HZP	Reference					
No. of ET embryos	1.496	0.926–2.417	0.100			
No. of D3 high-quality embryos	1.286	1.109–1.491	0.001	1.164	0.974–1.391	0.095

ICSI – intracytoplasmic sperm injection; HZP – heterogeneous zona pellucida; BMI – body mass index; ET – embryo transfer; No. – number; OR – odds ratio; CI – confidence interval. P<0.05 indicates a significant association.

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Table 4. Multivariate analysis of the main outcome (pregnancy or not) in IVF cycles.

	Univariate analysis			Multivariate analysis		
	OR	95% CI	P value	OR	95% CI	P value
Female age	0.895	0.855–0.936	<0.001	0.916	0.873–0.962	<0.001
Duration of infertility	0.918	0.855–0.985	0.017	0.951	0.880–1.027	0.201
BMI	1.044	0.979–1.114	0.190			
Diagnosis						
Primary infertility	Reference					
Secondary infertility	0.791	0.528–1.186	0.257			
No. of retrieved oocytes	1.061	1.026–1.097	0.001	0.913	0.788–1.059	0.229
No. of MII oocytes	1.074	1.035–1.114	<0.001	1.132	0.961–1.334	0.139
HZP						
Normal	1.211	0.379–3.874	0.747			
HZP	Reference					
No. of ET embryos	1.306	0.931–1.831	0.122			
No. of D3 high-quality embryos	1.205	1.098–1.323	<0.001	1.120	1.004–1.249	0.043

ICSI – intracytoplasmic sperm injection; HZP – heterogeneous zona pellucida; BMI – body mass index; ET – embryo transfer; No. – number; OR – odds ratio; CI - confidence interval. P<0.05 indicates a significant association.

age (OR=0.894, 95% CI 0.835–0.959; p=0.002), duration of infertility (OR=0.921, 95% CI 0.851-0.998; p=0.044), number of retrieved oocytes (OR=1.077, 95% CI 1.025–1.131; p=0.003), number of MII oocytes (OR=1.097, 95% CI 1.037–1.160; p=0.001), and number of D3 high-quality embryos (OR=1.286, 95% CI 1.109–1.491; p=0.001) were significant factors. The multivariate analysis is shown in Table 3, and no factors were independently related to pregnancy after ICSI.

Table 4 shows the univariate analysis of factors related to pregnancy after IVF. The results demonstrated that the woman's age (OR=0.895, 95% CI 0.855–0.936; p<0.001), duration of infertility (OR=0.918, 95% CI 0.855–0.985; p=0.017), number of retrieved oocytes (OR=1.061, 95% CI 1.026–1.097; p=0.001), number of MII oocytes (OR=1.074, 95% CI 1.035–1.114; p<0.001), and number of D3 high-quality embryos (OR=1.205, 95% CI 1.098–1.323; p<0.001) were significant factors. As also shown in Table 4, the multivariate analysis found that the woman's age (OR=0.916, 95% CI 0.873–0.962; p<0.001) and the number of D3 high-quality embryos (OR=1.120, 95% CI 1.004–1.249; p=0.043) were significantly associated with pregnancy. Whether the oocyte had HZP or not was not found to be related to pregnancy in case embryos were available for transfer in either ICSI or IVF cycle.

Discussion

This study retrospectively analyzed the embryological characteristics and clinical outcomes of IVF/ICSI treatment cycles involving oocytes with HZP and revealed that women with oocytes presenting with HZP could achieve pregnancy during both IVF and ICSI treatments.

A previous study assessing HZP in IVF and ICSI cycles showed that it is associated with lower oocyte maturity and fertilization rate, and high-quality embryo rate [18]. This study also suggested that cycles involving HZP oocytes had a lower MII oocyte rate in ICSI and reduced normal fertilization rate and cleavage rate, and higher abandoned transfer rate per retrieval cycle in IVF. Therefore, the 2 studies are similar in many respects. However, the above study found no successful pregnancies from HZP oocytes in IVF cycles [18]. In the present study, the pregnancy rate after IVF using HZP oocytes was 41.7%, similar to the rate obtained with non-HZP oocytes. Multivariate analysis suggested that there was no association of pregnancy with HZP oocytes in either IVF or ICSI. It is not clear why discrepant results were obtained in both studies, but it may be related to the populations studied, although they were both performed in China, so racial differences are probably slight. This difference may also be due to the small sample size of the above study [18]. Including just 11 IVF cycles with HZP oocytes might be too small a number to draw a reasonable conclusion. Moreover, both their study and ours found that some women with HZP oocytes demonstrated secondary infertility, indicating those women could get pregnant through spontaneous conceiving intercourse. Therefore, it seems likely that those women could achieve pregnancy during IVF treatment.

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The ZP dysmorphology observed in IVF/ICSI cycles may result from extrinsic factors such as the controlled ovarian hyperstimulation (COH) protocol, or intrinsic factors, including heritable molecular defects and patient age. In this study, several women had repeated consistent oocyte dysmorphology despite having different COH during different cycles. Therefore, we speculated that oocytes with HZP might be due to intrinsic factors. One possible explanation is that ZP morphology alterations are caused by patterning problems of the glycoprotein matrix encoded by the ZP 1, 2, 3, and 4 genes. Studies with knockout mice showed all 3 murine ZP proteins have a role in maintaining the structure of the ZP, and deleting 1 of the 3 ZP genes results in abnormal ZP [23–25]. In human oocytes, it was confirmed that sequence variations in human ZP genes are related to abnormal ZP, such as zona splitting, oval zona, and thin and thick zonas [26]. Based on these findings, oocytes with HZP may be attributed to an abnormal encoding of ZP genes.

According to our data, oocytes with HZP had a lower MII oocyte rate in both conventional IVF and ICSI cycles. This finding corroborated other studies. Mei Li et al. found that the immaturity rate is significantly higher in the abnormal ZP group compared with controls [18]. Recently, Sousa et al. also showed oocytes with indented ZP appear to have only a 42% maturity rate, versus 81.2% for normal oocytes [27]. It appears that oocytes fail to reach the MII stage because of 4 main reasons: (1) genetic defects, (2) abnormal meiotic recombination, (3) abnormal microfilament action, and (4) failure to produce key cell cycle regulating factors [28]. Therefore, we speculated that oocytes with HZP have a high risk of having such factors, although further work is required to confirm the exact mechanism.

In this study, oocytes with HZP had a lower fertilization rate than normal counterparts during IVF treatment, while such a difference was not observed during ICSI treatment. This might be explained by the abnormal ZP composition or ultrastructure exerting an effect on sperm-oocyte fusion, leading to lower fertilization [29]. In addition, we found a higher primary infertility rate and longer duration of infertility in the HZP group. We inferred that HZP might also exert a negative effect on *in vivo* fertilization and lead to difficulty in conceiving.

Due to the lower normal fertilization rate and cleavage rates in the HZP group during conventional IVF treatment, we often found there were no suitable or not enough embryos for transfer on day 3. Consequently, the abandoned transfer rate per retrieval cycle was significantly higher, and the number of transferred embryos per cycle was reduced in the HZP group compared with the non-HZP group. In ICSI cycles, we obtained 4.4±4.4 available embryos for transfer per cycle, which generally was enough to choose 2 or 3 embryos for transfer on day 3. Therefore, there were similar abandoned transfer rate per retrieval cycle as well as numbers of transferred embryos per cycle in both groups. After embryo transfer, no differences were detected between the HZP and non-HZP groups in positive HCG, implantation, pregnancy, and miscarriage rates in IVF and ICSI treatments. Based on the above findings, we recommend ICSI treatment when oocytes show HZP. However, as it can be difficult to identify oocytes with HZP during the first ART treatment, some IVF cycles could have oocytes with HZP. In such cases, women could become pregnant if viable embryos are obtained.

Limitations

Firstly, the definition of HZP has no established guidelines and could vary by study. Secondly, patients had different degrees of HZP, which were not graded or distinguished in this study. Thirdly, the sample size was relatively small. A larger study, including patients from multiple centers, might provide more evidence for these findings. Finally, this was a retrospective study, with possible selection bias.

Conclusions

Overall, women with oocytes showing HZP can become pregnant after either IVF or ICSI treatment. However, these women should avoid low fertilization and high abandoned transfer rates by choosing ICSI.

Conflict of interests

None.

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