Original Article
Correlation between morphological abnormalities in the human oocyte zona pellucida, fertilization failure and embryonic development

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Abstract: Objective: The general patient condition, oocyte and embryo condition and clinical outcomes were studied in patients with a morphologically abnormal zona pellucida (ZP). Methods: The general embryo condition was compared between patients with a morphologically abnormal ZP or a normal ZP. The general embryo conditions in the first and subsequent cycles were compared in patients with a morphologically abnormal ZP. Results: Oestradiol (E2) levels were higher in patients with an abnormal ZP than in those with a normal ZP ($P<0.05$). In the process of promoting ovulation, luteinizing hormone (LH) levels were higher in patients with an abnormal ZP than in those with a normal ZP ($P<0.05$). The MII rate was significantly lower in patients with a morphologically abnormal ZP than in patients with a normal ZP ($P<0.05$). After performing intracytoplasmic sperm injection (ICSI), the 2PN fertilization rate and pregnancy rate were significantly lower in patients with a morphologically abnormal ZP than in those with a normal ZP ($P<0.05$). The gonadotrophin (Gn) dosage for patients with a morphologically abnormal ZP was significantly higher in the second IVF cycle than in the first cycle. The MII rate, 2PN fertilization rate, and pregnancy rate were also significantly higher in the second cycle than in the first cycle ($P<0.05$). The perivitelline space was smaller within oocytes with a morphologically abnormal ZP than within those with a normal ZP ($P<0.05$), but the ZP was thicker than normal ($P<0.05$). Conclusions: Oocytes with a morphologically abnormal ZP had lower maturity, and ICSI can improve the fertilization rate of these oocytes. The application of a modified scheme improved ovum maturation, the fertilization rate, and the pregnancy outcome.

Keywords: Zona pellucida morphological abnormality, IVF total fertilization failure, rescue ICSI, PVS, zona pellucida thickness

Introduction

The fertility rate from conventional in vitro fertilization (IVF) is 60% to 70%. Some patients experience fertilization failure with all oocytes; this incidence is 5% to 20% in the conventional IVF period [1, 2]. Studies have demonstrated that the common reason for total fertilization failure following conventional IVF is that sperm could not penetrate into the cytoplasm through the oocyte zona pellucida (ZP) [3]. The ZP is composed of glycoproteins that are synthesized and secreted by the Golgi apparatus during oocyte generation, and the ZP protects oocytes and fertilized eggs from the outside environment. The ZP of human eggs contains 4 types of glycoproteins, namely, ZP1, ZP2, ZP3, and ZP4 [4]. The ZP, as a sperm barrier, can identify specific substances in the sperm head

through receptors on the glycoprotein shell, such as ZP3, which was the first identified receptor. When sperm is encountered, ZP3 induces the sperm acrosome reaction, resulting in ZP penetration and completion of the fertilization process. The structure and function of the ZP play vital roles in signal transmission and material exchange between eggs, embryos, and the internal environment. Therefore, ZP abnormalities may affect oocyte quality and cause fertilization failure and serious problems with embryo quality and potential development. Mannikko [5] reported several single nucleotide polymorphisms (SNPs) in human ZP gene sequences. The loci of the ZP SNPs are associated with the ZP3 genotype and IVF fertilization failure. In laboratory experiments, one type of oocyte with ZP morphological abnormalities
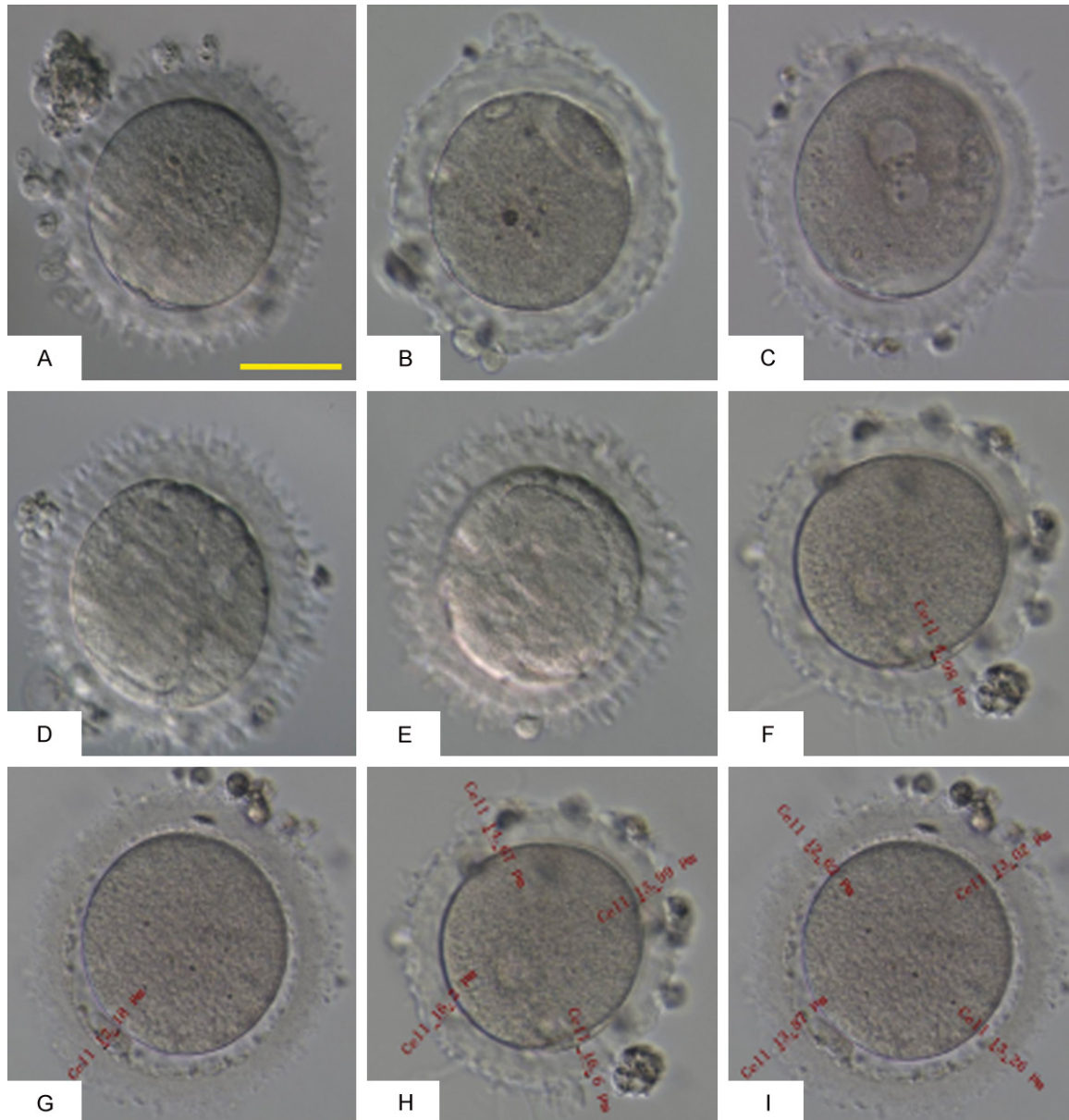
Figure 1. (A and B) Oocytes with morphological abnormalities. (C) A fertilized egg with an abnormal ZP. (D and E) An embryonic abnormal ZP after 48 hours (D) and 72 hours (E). (F and G) Measurements of the perivitelline space in oocytes with a morphologically abnormal (F) or normal (G) ZP. (H and I) Thickness measurements of oocytes with a morphologically abnormal (H) or normal (I) ZP. Scale bar = 50 μm.

was conspicuous because the ZP exhibited hyaline changes, serrated changes, pyknosis, lucency, high refraction, even formation of the cytoplasm, a small perivitelline space, and poor maturity. Fertilization of such oocytes usually failed during IVF. Some scholars have studied eggs with a morphologically abnormal ZP and decreased ZP3 protein expression by immuno-fluorescence staining [6]. Other scholars have examined the ovum ZP using electron micros-copy; the results demonstrated that the ZP structure was abnormal [7]. Methods to improve the maturity and fertilization rate of these oocytes with a morphologically abnormal ZP have not been reported. This study analysed the basic condition of the oocytes and embryos in patients with a morphologically normal or abnormal ZP during IVF and compared the patient, oocyte, and embryo conditions of patients with a morphologically abnormal ZP

between the first and subsequent cycles to explore options for improving oocyte maturation, the fertilization rate, and the pregnancy rate.

Data and methods

Study participants

Between January 2011 and November 2014, 92 patients with complete fertilization failure in the first cycle of IVF, including 28 patients with a morphologically abnormal ZP and 64 patients without an abnormal ZP, were identified at the Reproductive Center of the First Affiliated Hospital of Zhengzhou University. The characteristics of an abnormal oocyte ZP were fewer perivitelline granular cells, a loose structure, and no radioactive shape. The granulosa cells were removed 4 h after IVF, and the oocyte ZP was observed under optical microscopy to assess the presence of hyaline changes, serrated changes, pyknosis, lucency, high refraction, thick ZP, even formation of the cytoplasm, and a small perivitelline space. During intracytoplasmic sperm injection (ICSI), the elasticity of the ZP worsened, the ZP became harder, and the injection points on the ZP were not easy to recover. This type of egg was called a morphologically abnormal oocyte (**Figure 1**).

There were 13 patients with total fertilization failure due to a morphologically abnormal ZP in the first cycle of IVF-assisted reproduction from January 2011 to November 2014. ICSI treatment was then performed in the subsequent cycle.

Methods

Endocrine determination

Venous blood was extracted in the morning of the second or third day of the menstrual cycle. Serum hormones, including oestradiol (E2), progesterone (P), luteinizing hormone (LH), and human chorionic gonadotrophin (hCG), were analysed during the controlled ovarian hyperstimulation (COH) period.

Ovulation stimulation control

Patients who exhibited complete fertilization failure in the first cycle underwent additional therapy to improve the outcome. A long down-regulation protocol of short-acting triptorelin was performed in these patients. After attaining the standards of down-regulation, gonadotrophin (Gn) therapy was initiated. When a type-B ultrasonic instrument showed a follicular diameter of 18 mm in two oocytes, 10000 U of hCG was administered instead of Gn. Oocyte retrieval was performed by transvaginal ultrasound-guided puncture 36 h later. A modified super-long protocol of long-acting triptorelin was performed in patients with a morphologically abnormal ZP in the second cycle. After attaining the standards of down-regulation, human menopausal gonadotrophin (HMG) therapy was initiated.

Treatment of oocytes and sperm

Oocyte treatment: Follicular fluid was observed under an anatomic lens. Oocyte-granular cell components were identified and then collected. The oocyte-granular cell components were washed with a series of culture media and then incubated in a 6% CO_2 atmosphere at 37°C for 2-3 h.

Sperm treatment: Sperm collected through masturbation was liquefied, treated using the density gradient centrifugation method, and stored at 37°C and 6% CO_2 for later use.

Observation of embryo condition during insemination and after fertilization

Short-term fertilization: Fertilization was performed 39-40 h after hCG injection. Droplet fertilization was performed, and the perivitelline granular cells were removed 4 h after fertilization. The discharge was observed under a microscope. If a second polar body was present in more than 50% of the oocytes, the oocytes were incubated. If a second polar body was present in less than 50% of the oocytes, the oocytes were observed after an additional 2 h. If the second polar body was still only present in less than 50% of the oocytes, rescue ICSI was adopted.

ICSI method: Perivitelline granulosa cells were removed 2 h after the egg was removed. Single sperm injection was performed 1-2 h after removal of the perivitelline granulosa cells. The pronucleus condition was observed 16-20 h after fertilization. Embryo cleavage was investigated 48 and 72 h after egg removal.

Table 1. Comparisons of the general condition and ovulation stimulation outcomes between patients with and without a morphologically abnormal zona pellucida

	Patients with a morphologically abnormal zona pellucida	Patients with a normal zona pellucida	*P*
Cases (n)	28	64	
Age (years)	31.05±4.46	32.07±3.90	0.072
Infertility duration (years)	7.57±4.70	6.58±3.70	0.331
Primary infertility, n (%)	100 (28/28)	71.9 (46/64)	0.002
Secondary infertility, n (%)	0	28.1 (18/64)	
Basic FSH (IU/L)	7.81±1.77	7.27±1.71	0.184
Basic LH (IU/L)	5.38±3.58	5.12±1.78	0.283
Basic P (nmol/L)	0.49±0.21	0.51±0.26	0.958
Basic E2 (pmol/L)	46.49±16.04	39.96±15.69	0.024
Gn days	11.14±1.80	11.40±1.70	0.989
Gn dose (IU)	2163.10±857.77	2308.99±785.46	0.505
Quantity of >14-mm follicles on hCG injection day (pcs)	8.67±4.45	9.26±3.72	0.518
Peak E2 (pmol/L)	4196.37±2160.89	5029.98±2478.56	0.595
P on hCG injection day (nmol/L)	0.73±0.44	0.97±0.55	0.064
LH on hCG injection day (IU/L)	2.12±0.97	1.72±0.94	0.033
Basic follicular quantity (pcs)	11.10±6.07	11.32±5.37	0.650

Notes: Basic FSH, LH, P and E2 were determined on the second or third day of the menstrual period; Peak E2, P and LH on hCG injection day were determined on the hCG injection day.

Perivitelline space and ZP thickness measurements: OCTX software was used to measure the perivitelline space and the ZP thickness under a microscope at 200× magnification (**Figure 1**).

Embryo transfer: Embryo transfer was performed on the second or third day after oocyte retrieval. A maximum of 3 of the best quality embryos were transplanted based on the patient's age and treatment period.

Pregnancy diagnosis: A blood hCG level >5 at 14 days after embryo transfer was the biochemical definition of pregnancy. Clinical pregnancy was detected by pregnant bursa and heart tubes on ultrasound 5 weeks after transplantation.

Statistical analysis

Statistical treatment was performed with SPSS17.0 software. Measurement data were expressed as mean ± standard deviation, and were compared between the two groups with t test. Numeration data were expressed as rate (%), and were compared between the two groups with Chi square test or Fisher-exact probability test. Statistical significance was established at P<0.05.

Results

Comparisons of the general condition and ovulation stimulation outcomes between patients with and without a morphologically abnormal ZP

Among the patients with total fertilization failure, 28 had a morphologically abnormal ZP, and 64 had a normal ZP. The patients with an abnormal ZP had primary infertility. The incidence of primary infertility was higher among patients with an abnormal ZP than among those with a normal ZP. E2 levels were higher in patients with an abnormal ZP than in patients with a normal ZP (P<0.05). During ovulation stimulation of patients with and without an abnormal ZP, there were no obvious differences in the number of follicles, the days or dose of Gn treatment, or the quantity of >14-mm follicles on the hCG injection day. There were no significant differences in the E2 and P levels between the two groups. The LH level was significantly higher in patients with an abnormal ZP than in patients with a normal ZP (P<0.05) (**Table 1**).

Table 2. Comparison of semen from the partners of patients with and without a morphologically abnormal zona pellucida

	Partners of patients with a morphologically abnormal zona pellucida	Partners of patients with a normal zona pellucida	P
Sperm density (×10⁶/mL)	54.39±15.52	52.09±32.83	0.36
Living rate (%)	58.21±16.67	52.33±19.19	0.139
Progressive Motility (%)	37.87±16.13	31.13±14.55	0.140
Sperm deformity rate (%)	41.33±17.05	45.63±18.25	0.146

Notes: Progressive Motility = Grade a sperm + Grade b sperm. (Progressive Motility: Sperm that are swimming from one place to another and not just twitching or going in small circles).

Table 3. Comparison of the ICSI results between patients with and without a morphologically abnormal zona pellucida

	Patients with a morphologically abnormal zona pellucida	Patients with a normal zona pellucida	*P*
Required egg quantity (pcs)	7.38±4.99	11.42±5.64	0.001
PVS (μm)	4.94±2.0	13.86±2.13	0.000
Zona pellucida thickness (μm)	14.38±1.13	11.64±1.28	0.000
MII egg quantity (pcs)	4.13±3.59	11.15±5.43	0.000
MII rate (%)	55.9 (99/177)	97.5 (613/629)	0.000
2PN fertilization rate (%)	70.7 (70/99)	82.5 (506/613)	0.005
2PN cleavage rate (%)	88.6 (62/70)	97.0 (491/506)	0.001
3PN fertilization rate (%)	6.1 (6/99)	4.9 (30/613)	0.642
3PN cleavage rate (%)	100 (6/6)	100 (30/30)	
High-quality embryo rate (%)	58.1 (36/62)	63.5 (312/491)	0.007
Embryo utilization rate (%)	75.8 (47/62)	67.0 (329/491)	0.16
Pregnancy rate (%)	3.6 (1/28)	26.6 (17/64)	0.011

Notes: PVS: perivitelline space; ZP: Zona Pellucida; MII: Metaphase II; 2PN: Two-pronuclear; 3PN: Three-pronuclear.

Comparison of semen from the partners of patients with and without a morphologically abnormal ZP

A comparison of the semen from the partners of patients with and without a morphologically abnormal ZP revealed that the sperm density, living rate, forward movement, and sperm deformity rate were not significantly different (**Table 2**).

Comparison of the ICSI results between patients with and without a morphologically abnormal ZP

The number of eggs required, MII egg quantity, and MII rate were significantly lower in patients with a morphologically abnormal ZP than in patients with a normal ZP (P<0.05). After res-

cue ICSI, the 2PN fertilization rate, cleavage rate, embryo rate, and pregnancy rate were significantly lower in patients with an abnormal ZP than in patients with a normal ZP (P<0.05). Embryo utilization was not significantly different between the two groups. The oocyte perivitelline space was smaller in patients with an abnormal ZP than in patients with a normal ZP (P<0.05). The ZP was thicker in patients with an abnormal ZP than in patients with a normal ZP (P<0.05) (**Table 3**).

Comparison of the patient and embryo conditions in those with a morphologically abnormal ZP in the first and second cycles

The Gn dosage in patients with an abnormal ZP was higher in the second cycle than in the first cycle (P<0.05). The MII rate, 2PN fertilization rate, and pregnancy rate were also significantly higher in the second cycle (P<0.05). E2 levels on the hCG injection day in patients with an abnormal ZP were significantly lower in the second cycle than in the first cycle (P<0.05) (**Table 4**).

Discussion

In this study, the oocyte ZP with hyaline changes exhibited fertilization failure during short-term fertilization. After rescue ICSI, the 2PN fertilization rate, cleavage rate, embryo rate, high-quality embryo rate, and pregnancy rate were significantly lower among patients with an abnormal ZP than among those with a normal ZP.

The ZP is produced during oocyte growth and reflects the integrity of the follicle and the qual-

Table 4. Comparison of the patient and embryo conditions between the first and second cycles in patients with a morphologically abnormal zona pellucida

	First cycle	Second cycle	P
Gn days	11.15±1.82	11.75±2.18	0.377
Gn dose (IU)	1756.73±597.23	2363.54±763.69	0.031
Follicles >14 mm on hCG injection day (pcs)	9.5±4.7	8.1±4.5	0.357
E2 on hCG injection day (pmol/L)	5598.13±2791.00	3745.26±2269.39	0.038
P on hCG injection day (nmol/L)	0.71±0.29	0.54±0.30	0.08
LH on hCG injection day (IU/L)	2.4±1.39	1.71±1.25	0.098
Required egg quantity (pcs)	9.6±6.2	8.0±4.4	0.850
MII quantity (pcs)	3.15±3.08	4.77±3.6	0.115
MII rate (%)	38.7 (41/106)	61.4 (62/101)	0.001
2PN fertilization rate (%)	53.7 (22/41)	82.3 (51/62)	0.002
High-quality embryo quantity (pcs)	0.89±0.93	2.1±2.8	0.116
Pregnancy rate (%)	0	38.5 (5/13)	0.000

Notes: MII: Metaphase II; 2PN: Two-pronuclear.

ity of the oocyte. The ZP plays an important role in the sperm-egg union. This study revealed that any ZP glycoprotein abnormality affects the union of sperm and oocyte, the rate of high-quality embryo formation, and the embryo cultivation rate [9, 10], causing fertilization failure [8]. ZP protein abnormalities may directly reflect oocyte maturation defects [9]. Therefore, the decreases in oocyte quality and embryo developmental potential are related to the ZP abnormality. Bertrand et al. found that oocytes with a thinner ZP had a higher fertilization rate. Other scholars have proposed that unexplained patient infertility is due to the thickness of the oocyte ZP. In IVF treatment, ZP thickening was the main factor associated with reduced embryo implantation and pregnancy rates, and it affected the hatched blastocyst [10]. In patients with a morphologically abnormal ZP, the ZP was thicker and acted like armour for the embryo, limiting proper embryo development. Even with normal embryos, a good pregnancy rate cannot be obtained. For these patients with an abnormal ZP, hatched blastocysts may increase the embryo implantation rate.

In this study, patients with a morphologically abnormal ZP had primary infertility and high baseline levels of E2, which promoted high LH levels. The oocytes in these patients exhibited low maturity, a small perivitelline space, and a thicker ZP, all leading to a low pregnancy rate. The patients with a morphologically abnormal ZP in the first cycle had the same abnormality in subsequent cycles. Changes in the hormonal

environment during the egg-laying period and the increase in oestrogen may impact the thickness and hardness of the ZP. These ZP abnormalities may be due to a congenital malformation caused by incorrect fibre connections within the ZP, which have been correlated with oocyte morphology and the degree of maturity. The degree of oocyte maturity was significantly lower in patients with an abnormal ZP than in patients with a normal ZP. An investigation of the ZP maturity process in mice using electron microscopy revealed constant changes in the location, density, and distribution of the glycan connections. These changes also occur in the human oocyte ZP and are hypothesized to be associated with the mature state of the ultrastructure. The relationship between ultrastructural changes in the ZP and oocyte maturity indicates the significance of the regulation of the sperm and ZP union. Oocyte maturity refers to the completion of the first meiotic division. This process is necessary for egg fertilization and embryo development [11]. Scholars previously analysed 30,000 genes from oocytes associated with total fertilization failure using gene chip technology. Meiosis-, cell growth-, and apoptosis-related genes were associated with fertilization failure. Due to the poor maturity of a morphologically abnormal ZP, the question of whether oocyte fertilization failure is associated with meiosis genes needs to be further researched.

The modified super-long protocol was applied to study patients with a morphologically abnor-

mal ZP in subsequent cycles. The LH level decreased, and the oocyte maturation level, fertilization rate, and pregnancy rate were significantly higher than in the first cycle. The improved solutions are generally utilized for patients with endometriosis and adenomyosis, in whom they have improved the quantity and quality of mature oocytes, increased the number of oocytes required, improved the embryo quality, yielded better LH levels on the hCG injection day, stabilized the pregnancy rate, and decreased the spontaneous abortion rate. There have been few reports of these effects in patients with endometriosis and adenomyosis.

In patients with an abnormal ZP, the modified super-long protocol was applied in the second cycle, which significantly improved the oocyte maturity, fertilization rate, and pregnancy rate. Influencing oocyte quality with the long down-regulation protocol was an effective solution for improving patient prognosis [12], and the application of the modified super-long protocol in the subsequent cycle was an effective solution for increasing oocyte maturity and quality. In previous applications of IVF-assisted reproduction, some patients exhibited general fertilization failure, with a 30%-50% risk of recurrent fertilization failure [13] if the fertilization failure was due to abnormal sperm penetration and oocyte penetration failure. ICSI, which was applied in the subsequent cycle, effectively increased the fertilization and clinical pregnancy rates. Fertilization failure associated with oocytes with a morphologically abnormal ZP was remedied by ICSI, and patients with an abnormal ZP in the first cycle underwent ICSI. Both strategies resulted in normal fertilized eggs and cleavage embryos, which indicated that the obstacles associated with the union of sperm and egg are the main reasons for the association between a morphologically abnormal ZP and total fertilization failure. ICSI was an effective approach for producing normal fertilized eggs.

In conclusion, oocytes with ZP hyaline changes were less mature and resulted in poor potential embryo development and low pregnancy rates. The improved ovulation schedule increased oocyte maturity and the fertilization and pregnancy rates, and ICSI was effective in patients with an abnormal ZP. However, due to ethical limitations and the limited sample size, this study did not thoroughly investigate ZP ultra-structure and gene expression, which should be investigated in the future.

Disclosure of conflict of interest

None.

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