



## Pregnancy Outcome Following ICSI of Oocytes with Abnormal Zona Pellucida: Three Case Reports

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### Abstract

Three couple were classified as unexplained infertility, and treated by the staff of Reproductive Medicine Center of Hainan Women and Children's Medical Center. It was found that conventional IVF fertilization failed due to abnormal zona pellucida of the oocyte. The zona pellucida of this type of oocyte has a ring-shaped burr, with obvious protrusions like antennae. After ICSI treatment, fertilization, good embryo and clinical outcome were obtained. Finally, one case showed no pregnancy and another two patients had positive serum hCG pregnancy on day 12 after embryo transfer of which a patient suffered a medical abortion due to the absence of yolk sac and embryonic bud in fetuses, another patient gave birth to a healthy pair of a boy-girl twin. It is hypothesized that the spinous ring-shaped zona pellucida can lead to IVF fertilization failure and may be the great obstacle to natural pregnancy. However, fertilization and pregnancy can be achieved by changing the fertilization method, using ICSI and laser-assisted-hatching.

**Keywords:** Spinous ring-shaped Zona Pellucida; Fertilization Failure; ICSI

### Introduction

Over the past 40 years, Assisted Reproductive Technology (ART) has evolved significantly, thus providing better pregnancy opportunities for many infertile patients. However, we still often face challenging cases in routine clinical practice, for example, gamete abnormalities, particularly oocyte anomalies, are mainly observed in individuals with idiopathic infertility [1]. Based on the published data, it is very clear that severe cytoplasmic deviations of the oocyte (such as organelle clustering, centrally severe granulation, excessive vacuolization) do impair the developmental and implantation potential of the embryo [2]. Among them, Zona Pellucida (ZP) abnormalities have been associated with less than 5% of oocyte anomalies [3,7].

The ZP, a thick membrane surrounding the ovum, is essential for binding sperm and protection from polyspermy [3]. The mature Oocytes of normal morphology should be round, uniform cytoplasmic, small egg space, uniform particles, no granules in the perivitelline space. Normal zona pellucida should be 15  $\mu$ m to 20  $\mu$ m thick, and should be uniform and clear in appearance (Figure 1). Abnormal zona pellucida can affect fertilization, embryo development potential and planting rate. The types of abnormal zona pellucida mainly include: The color deepening, abnormal thickening (20  $\mu$ m) or too thin (13  $\mu$ m) [8], the uneven thickness, double layer or with interlayer, extremely dense zona pellucida and surface roughness, burr or irregular shape, etc. [7,8].

At present, there is still no clear definition of abnormal zona pellucida, so we try to characterize one special type, spinous ring-shaped zona pellucida, based on our own data. This abnormal type

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Received Date: 30 Jan 2024

Accepted Date: 09 Feb 2024

Published Date: 14 Feb 2024

#### Citation:

Hu J, Lu Z, Lu H, Li Y, Wang A, Zhao L, et al. Pregnancy Outcome Following ICSI of Oocytes with Abnormal Zona Pellucida: Three Case Reports. *Ann Clin Case Rep.* 2024; 9: 2576.

ISSN: 2474-1655.

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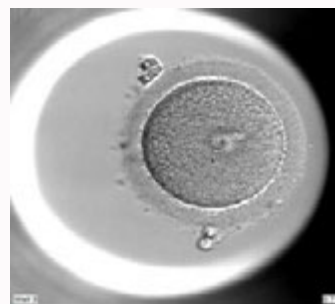
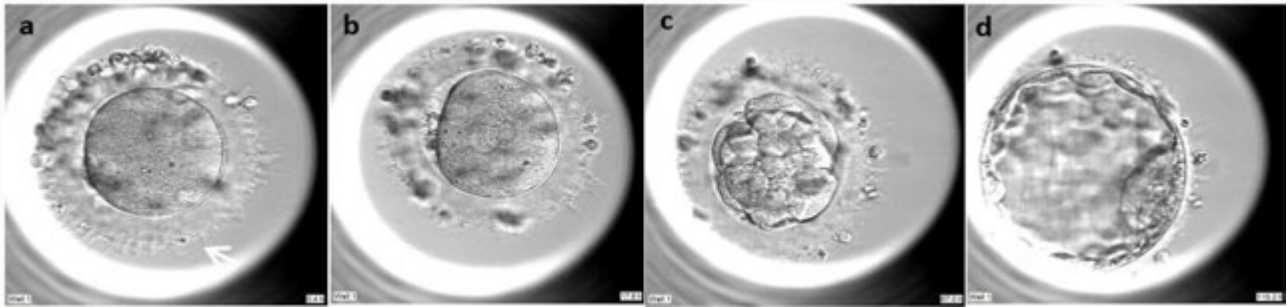


Figure 1: Morphological appearance of normal oocyte zona pellucida.



Case 1: The whole process of embryo development (a-d).

of the zona pellucida reported in this paper was different in detail from the wax like zona pellucida reported in the past. The wax like zona pellucida, such as gel, has bright refraction and no obvious protrusion around it [11]. In this study, the zona pellucida of this type has a ring-shaped burr, with obvious protrusions like antennae, such as antennae, so we named this type by spinous ring-shaped zona pellucida, which was novel one of the morphological abnormalities of zona pellucida. There were few reports on the clinical outcome of Oocytes of this type (spinous ring-shaped) zona pellucida. This study analyzes the final clinical outcome of this case and provides a reference for the selection of insemination methods of Oocytes with spinous ring-shaped zona pellucida.

## Ovarian Stimulation and Embryo Culture

### Case 1

A married couple was referred for Half-ICSI treatment in the reproductive medicine center at Hainan Women and Children's Medical Center. They were presented with a 3 years' history of primary infertility. The husband's age was 29 years and his semen analysis showed normozoospermia. His wife was a 31 years-old lady with regular ovulatory cycles and FSH, LH, AMH levels was 8.05 IU/L, 7.7 IU/L, 4.19 ng/mL, respectively, and BMI was 21.6. From 2020 to 2021, the couple underwent multiple cycles of treatment at other hospitals and did not get pregnant. On March 28<sup>th</sup>, 2022, ultrasound-guided oocyte retrieval was performed 36 h after HCG administration in our center and yielded 10 cumuli, including Oocytes of normal appearance. At the egg collection day, the sperm volume was 3.0 mL, concentration  $70 \times 10^6/\text{mL}$ , and motility 60%. We selected half-ICSI as fertilization treatment considering the long duration of infertility.

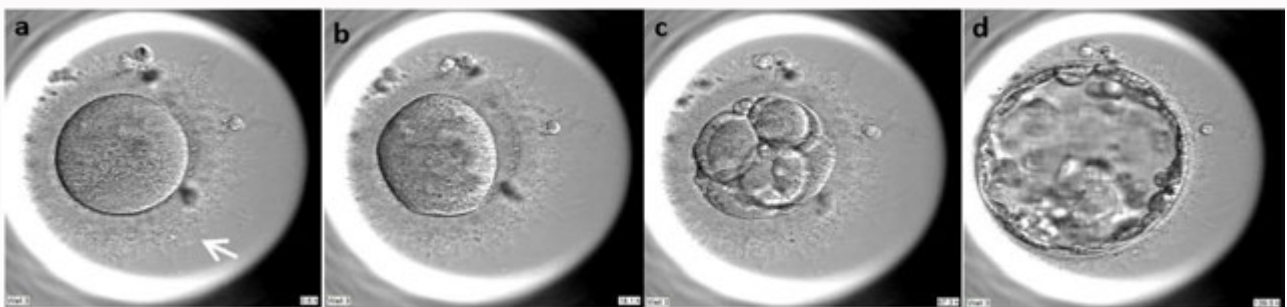
None of the IVF eggs were fertilized. The zona pellucida of this oocyte has a ring-shaped burr, with obvious protrusions like antennae and a small amount of sperm attached. However, after removing granulosa cells in ICSI eggs, the Oocytes were at metaphase II with

clear extrusion of the first polar body, and the membrane of these Oocytes was not elastic and had no resistance during ICSI injection (Figure 2 Case 1a). Four Oocytes were fertilized by ICSI, all of them were 2 PN, the embryo was four-cell grade two on day 2, reaching eight cells on day 3 resulted in 1 embryo frozen, the rest embryos were still cultured until day 5 when 3 blastocysts were obtained. On Day 5 the blastocyst cavity repeatedly expands during the formation of blastocyst, and its trophoblast cannot hatch through the zona pellucida. It is greatly different from normal, all of which may lead to implantation failure because the blastocyst cannot hatch normally. Finally, one blastocyst (4AA on day 5) was selected for transplantation (Figure 2 case 1d) with laser-assisted hatching (local perforation, 1/4 of the zona pellucida), which was confirmed with no pregnant.

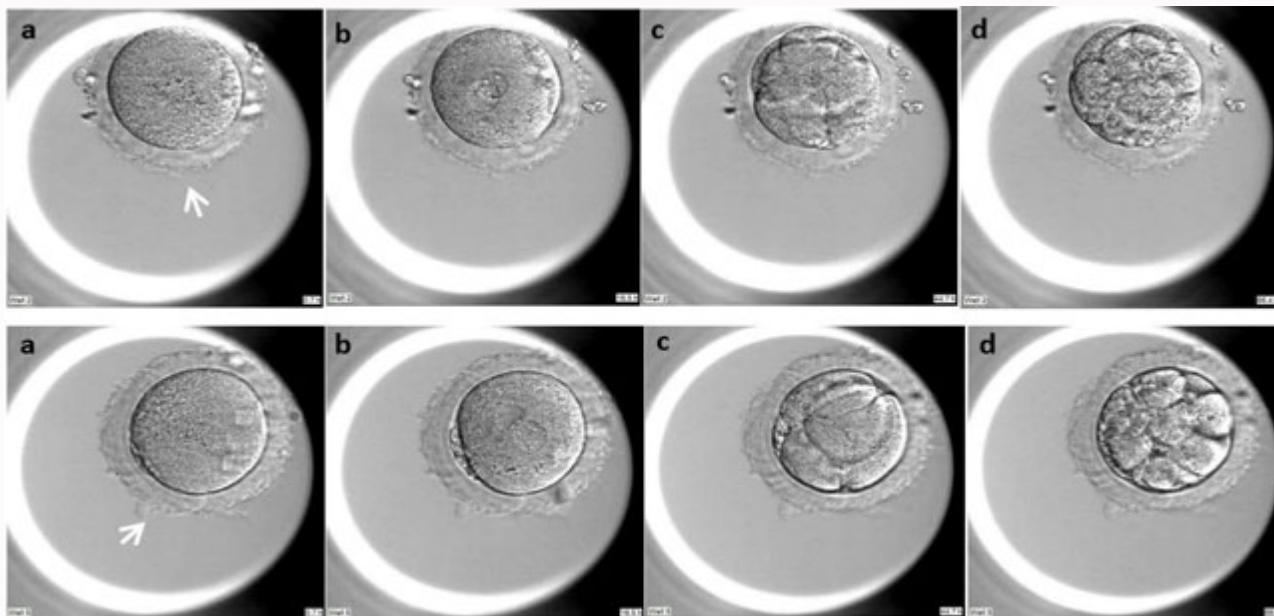
### Case 2

A 34-year-old lady with 3 years of primary infertility due to tubal factor underwent conventional ICSI treatment at Reproductive Medicine Center of Hainan Women and Children's Medical Center. The husband's age was 39 years and his semen analysis showed normozoospermia. FSH, LH, AMH levels of the woman was 4.31 IU/L, 5.15 IU/L, 3.91 ng/mL, respectively, and her BMI was 23.2. They were performed 2 IVF cycles with no pregnancy in other hospital. Then the couple visited the Reproductive Medicine Center of Hainan Women and Children's Medical Center in March 2022. In this treatment cycle, we selected ICSI as fertilization treatment considering the low fertilization rate by conventional IVF in previous cycles.

In April 2022, during her first ART cycle in our center, she used the standard antagonist protocol. Following hCG trigger, 9 mature Oocytes were collected, of which 5 were fertilized and were 2PN. The zona pellucida of the oocyte has a ring-shaped burr and obvious protrusions, such as antennae (Figure 2 Case 2a). Subsequently, the fertilized embryo cleavage normally and on day 3, one embryo of good quality was frozen, the rest embryos continued in culture until



Case 2: The whole process of embryo development (a-d).



**Case 3:** The whole process of embryo development (a-d).

**Figure 2:** The whole development process of embryos (a-d) of 3 cases. Compared with the normal embryo (Figure 1), the shape of the embryo's zona pellucida is abnormal, and the edge of the zona pellucida is spinous ring-shaped, with divergent outward projections (white arrow).

day 5, and 3 blastocysts were obtained and frozen. The blastocyst cavity repeatedly expands during the formation of blastocyst. The phenomenon of expansion and contraction has been repeated and its trophoblast cannot hatch through the zona pellucida. In July 2022, a blastocyst of 4BB (4BB on day 6) was thawed and transplanted (Figure 2 Case 2d). Because of the abnormal zona pellucida, the blastocyst underwent laser-assisted hatching (local perforation, 1/4 of the zona pellucida). A pregnancy of two embryos was observed 6 weeks after transfer, but suffered a medical abortion due to the absence of yolk sac and embryonic bud in fetuses.

### Case 3

A married couple with 2 years of primary infertility due to tubal factor underwent conventional Half-ICSI treatment at Reproductive Medicine Center of Hainan Women and Children's Medical Center. The wife's age was 32 years and her husbands had undergone a full male infertility examination and was diagnosed normozoospermic. FSH, LH, AMH levels of the woman was 6.13 IU/L, 5.45 IU/L, 4.54 ng/mL, respectively and BMI was 23.4. In June 2022, during her first ART cycle in our center, she used the Luteal phase full length protocol. In July, ultrasound-guided oocyte retrieval was performed 36 h after HCG administration and yielded 10 cumuli, at the initial time COCs were observed to appear normal morphology, but just after removal of cumulus Oocytes were found with the above type of abnormal zona pellucida, so we selected ICSI for insemination (Figure 2 case 3a). On the egg collection day, the sperm was prepared with volume of 2.5 mL, concentration  $140 \times 10^6/\text{mL}$ , and motility 43%.

Finally, four 2PN embryos and one 3PN embryo were formed by ICSI fertilization, two embryos were selected transferred into the uterus (9C/II, 10C/II on day 3) (Figure 2 case 3d) with laser-assisted hatching. (The zona pellucida was thinned by 70% to 80% and partially perforated). The remaining embryos were incubated for 5 days, but only one blastocyst developed. On day 14 after embryo transfer, a positive serum hCG was reported. A pregnancy of two

embryos was observed 6 weeks after transfer (based on gestational sacs). The pregnancy progressed uneventfully, and the patient gave birth to a healthy pair of a boy-girl twin.

In a word, these three cases were very similar, different from the previously reported types of abnormal zona pellucida. We found that the zona pellucida of the oocyte has a ring-shaped burr and obvious protrusions, such as antennae (Figure 2), Therefore, we named it the spinous ring-shaped zona pellucida. The Oocytes with abnormal zona pellucida lead to complete IVF fertilization failure. The Oocytes were at metaphase II with clear extrusion of the first polar body, and no the perivitelline space. The egg membrane and zona pellucida are not elastic and resistance during ICSI injection. The blastocyst formed but cannot break through the zona pellucida and hatch normally. It is different from the normal development of the blastocyst which can hatch normally. If the blastocyst cannot hatch normally, it may lead to implantation failure. Therefore, laser-assisted hatching is recommended performed before transplantation.

### Discussion

Zona pellucida is the layer of translucent membrane wrapped around the oocyte and preimplantation embryo. It plays an important role in the complete development of oocytes, egg recognition, binding and penetration, and promoting the acrosome reaction, preventing polyspermy and protecting embryos from mechanical damage before hatching and implantation [4-6]. It has been proved by many studies that the thickness of the zona pellucida can affect the implantation rate [7,8]. The abnormal zona pellucida can be gradually densified or hardened during the embryo culture process [9]. Nevertheless, the thickened and hardened zona pellucida can lead to the repeated expansion and contraction of the blastocyst trophoctoderm which can cause invalid hatching, cease the development of the blastocyst [9,10]. In these cases, the blastocyst formed but cannot break through the zona pellucida and hatch normally. Laser-assisted-hatching should be considered before embryo transfer.



As to the first case in March 2022 in our report, we selected half-ICSI as fertilization treatment considering the long duration of infertility. We did not find abnormal zona pellucida of oocytes at first, because the appearance of the oocyte-cumulus complex obtained at the beginning was normal, and only after the removal of granular cells did, we find abnormal zona pellucida. The zona pellucida of this type has a ring-shaped burr, with obvious protrusions like antennae. On day 1, the Oocytes from IVF part showed fertilization failure, but from ICSI part are fertilized. Unfortunately, there was no pregnancy followed by transfer of ICSI part embryos.

Second case in April 2022, this couple has received assisted reproductive therapy at other hospitals, but has never been pregnant. Previous medical records indicate a low fertilization rate, but no records of abnormal zona pellucida of oocytes was told. We selected ICSI as fertilization treatment considering the low fertilization rate by conventional IVF in previous cycles. After removing granulosa cells, it was found that the zona pellucida of oocyte also had a ring-shaped burr, with obvious protrusions like antennae, similar to the first case. It was reported that antennae, abnormal zona pellucida may be the main reason for the low fertilization rate of previous IVF [11,12]. After ICSI treatment, fertilization and good embryo were obtained. This patient underwent pregnancy after transferring a blastocyst, but suffered a medical abortion due to the absence of yolk sac and embryonic bud in fetuses. As to the third case in July 2022, at the initial time COCs were observed to appear normal morphology, but just after removal of cumulus Oocytes were found with the above type of abnormal zona pellucida, so we selected ICSI for insemination. The results showed IVF egg fertilization failed, only ICSI egg fertilization. ICSI eggs obtained usable embryos, and a pregnancy occurred after two embryos were transferred, the pregnancy progressed uneventfully, and the patient gave birth to a healthy pair of a boy-girl twin.

To summarize the above three cases, we found a new subgroup of abnormal zona pellucida and its solution. The zona pellucida defect is easy to cause IVF fertilization failure, and it is particularly important to prevent and remedy fertilization failure in clinical practice [11,12]. It was reported that for patients with IVF complete fertilization failure or low fertilization rate caused by abnormal zona pellucida, ICSI treatment can be selected to improve the fertilization rate and clinical outcome [13]. For high-risk groups with fertilization failure, such as patients with long period of primary infertility and unexplained infertility, it is suggested that changing the fertilization method may significantly improve the fertilization outcome. We can choose half-ICSI or short-term fertilization combined with early rescued ICSI, or directly select ICSI in the next cycle and subsequent laser-assisted-hatching can be used to help the embryo hatch and improve the clinical pregnancy rate [11-14]. Finally, it is suggested that proper training of oocyte morphological evaluation should be provided to staff members involved in clinical practice, which is helpful to find oocyte abnormalities in time and use ICSI insemination, in order to achieve an ideal clinical outcome.

## Funding

This work was supported by the Hainan Health Industry Research Project (No. 21A200232; 22A200097), Natural Science Foundation of Hainan Province (822RC857) and the Key R&D Program of Hainan Province (ZDYF2023SHFZ093) and project supported by Hainan Province Clinical Medical Center (OWYH202175).

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