Fertilization and Development of Conjoined Human Oocyte from a Binovular Follicle Containing Two Individual Oocytes Surrounded by a Zona Pellucida

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Abstract

The most reliable definition of conjoined oocytes is the inclusion of two oocytes within a common zona pellucida (ZP) or their fusion in the zonal region. Because the available data are extremely limited, the significance of conjoined oocytes in IVF laboratory setting has been questionable. We analyzed the characteristics of 18 patients with conjoined oocytes in our center. The maturation of conjoined oocytes were asynchronous. Most of the cases were that one oocyte in each pair of conjoined oocytes was in the MII stage and the other was in the GV stage. The maturation rate and fertilization rate of conjoined oocytes were significantly lower than that of normal oocytes. There was no significant difference in the rate of cleavage and D3 High-quality embryo, but the rate of blastocyst formation of conjoined oocytes decreased significantly. The area of larger oocytes in conjoined oocytes was not different from that of normal oocytes, and its companion was significantly smaller than that of normal oocytes. The mean ZP thickness of all oocytes, larger or smaller, was significantly thinner than that of normal oocytes. After analyzing the clinical characteristics of the patients, it was suggested that the higher estrogen level or the more number of maturation follicles on HCG day were more likely to promote the emergence of conjoined oocytes.

Introduction

Polyovular follicles occur in the ovaries of many mammals, including women. They are common in possum and bitch¹, and 17% of follicles in bitch appear to be polyovular. Of all the species studied so far, follicles with multiple eggs are mainly binovular follicles: 0.10–2.40% of female follicles are polyovular follicles, 97% of polyovular follicles are binovular, 2.5% of polyovular follicles are triovular and 0.4% have > 3 eggs². Conjoined oocytes usually come from polyovular follicles. Data on human conjoined oocytes produced by binovular follicles are very limited in the literature. Conjoined oocytes are mainly defined as two oocytes contained in the common zona pellucida or fused in the zonal region³. Because of the limited data available, the significance of conjoined oocytes in clinical in vitro fertilization laboratory environment has been questionable. In assisted reproductive techniques (ART), there are few reports of single follicles after ovulation induction producing two attached oocytes surrounded by a single pellucida.

There are three hypotheses to explain the formation of conjoined oocytes: (i) The abnormal meiosis may result in the fusion of two oocytes in a single pellucida⁴, (ii) fusion of two separate follicles⁵, (iii) the failure of connective tissue to separate adjacent oocytes⁶. The most likely mechanism is the latter^{7,8}, as binovular follicles is a natural polymorphism rather than a pathological phenomenon⁹.

Zeilmaker et al. (1983) reported the first conjoined oocytes in humans containing two mature oocytes without pregnancy⁶. Two case studies reported that conjoined oocytes combined with intracytoplasmic sperm injection (ICSI) allowed fertilization and development to form cleavage embryos¹⁰. Transplantation of conjoined oocytes after blastocyst formation can lead to pregnancy and live birth¹¹. However, four

recent cases reported that conjoined oocytes can be fertilized normally, but lack of developmental ability, which should be considered abnormal ¹².

In contrast, our understanding of the clinical significance conjoined oocytes is limited to a few case studies. The occurrence of conjoined oocytes was rare. More information is needed on maturity status, fertilization, and developmental ability of conjoined oocytes. We analyzed the information of 18 patients with conjoined oocytes in our center, and measured the area and pellucida thickness of conjoined oocytes, which provided basic data for analyzing the formation mechanism of conjoined oocytes.

Results

Baseline characteristics of the patients with conjoined oocytes.

The data of conjoined oocytes were collected from 18 patients with 19 oocyte-collecting cycles over a 10-year period (see Table 1). Conjoined follicle was present in each of 19 retrievals of 18,000 retrievals (1.06%). In one patient, conjoined oocytes were found in both consecutive oocyte collection cycles. In one patient, two pairs of conjoined oocytes appeared in the same cycle. The mean age of the 18 patients was 32.0 ± 3.1 years with no statistical difference from the mean age of our IVF population (33.2 ± 4.8). In ovulation stimulation protocols, most of them were long protocol, accounting for 63.2 percent. Fallopian tube factors accounted for about half of the case of infertility. The average Gn days were 9.4 days and the estrogen level was 4488.3 pg/ml on HCG days. The hormone level in patients was relatively high. The number of mature follicles was about 10.1, which was more than the mean number of mature follicles (7.7) found in the total IVF cycles.

Table 1
Baseline characteristics of the patients with conjoined oocytes.

Parameters	Patients
Number of cycles(n)	19
Female age (years)	32.0 ± 3.1
Years of infertility (years)	4.1 ± 2.4
Primary infertility (%)	7(36.8%)
Ovarian stimulation protocol	
long protocol (%)	12(63.2%)
Super-long protocol (%)	3(15.8%)
Antagonist protocol (%)	2(10.5%)
mini-stimulation protocol (%)	2(10.5%)
Cause of female infertility	
Ovarian hypofunction (%)	1(5.3%)
Ovulation dysfunction (%)	4(21.0%)
Tubal factor(%)	10(52.6%)
Endometriosis (%)	1(5.3%)
Other reasons (%)	1(5.3%)
Unexplained infertility (%)	2(10.5%)
Days of Gn	9.4
Dosages of Gn	2102.6
Estradiol level on day of HCG(pg/ml)	4488.3
No. of mature follicles on day of HCG	10.1
Mature follicles:The follicles diameter are greater than 16mm.	
Data are expressed as mean ± SD or percentage.	

The area and zona pellucida thickness of conjoined oocytes.

Figure 1.A showed photograph of two oocytes in a cumulus cell mass at the day of oocyte pick-up. We observed different zonal pellucida fusion patterns, such as two oocytes sharing a common complete

ZP(Fig. 1.B), or each oocyte in two oocytes associated with a separate ZP, but fused within a certain region(Fig. 1.C and D). The conjoined oocytes appeared larger than usual, were pear-shaped and each contained two individual oocytes of unequal size. We measured the area and zona pellucida thickness of conjoined oocytes (see Table 2). The average area of larger oocytes was about twice that of smaller oocytes. The area of larger oocytes in conjoined oocytes was the average size of an oocyte derived from a uniovular follicle, but the other was significantly smaller than that of normal oocytes. The mean zona pellucida thickness of smaller oocytes was $11.77 \pm 1.22\mu\text{m}$, significantly thinner than that of larger oocytes. Both larger and smaller oocytes have significantly thinner zona pellucida thickness than normal oocytes. At the junction, the thickness of the zona pellucida was $9.35 \pm 1.22\mu\text{m}$, which was equivalent to that of smaller oocytes.

Table 2
The area and zona pellucida thickness of conjoined oocytes.

Parameters	Conjoined oocytes		Normal oocytes
	Oocyte1	Oocyte2	
Area(μm^2)	7249.25 ± 859.70^a	14092.87 ± 1158.35^b	16086.14 ± 1015.31^b
Zona pellucida thickness (μm)	11.77 ± 1.22^a	17.87 ± 1.40^b	21.21 ± 0.91^c
Data are expressed as mean $\pm$ SD.			
Different letters within an assessment significantly different at $P < 0.05$.			

Fertilization and clinical outcomes among the patients with conjoined oocytes.

Table 3 shows the fertilization and clinical outcomes among the patients with conjoined oocytes. The maturation rate of conjoined oocytes was significantly lower than that of normal oocytes. Most of the cases were that one oocyte in each pair of conjoined oocytes was in the MII stage and the other was in the GV stage, accounting for about 80% of the conjoined oocytes. Two pairs of conjoined oocytes were MI oocytes attached GV oocytes, the remaining conjoined oocytes were MI oocyte attached MI oocyte and GV oocyte attached GV oocyte. Four hours after insemination, sixteen of the forty oocytes were found to have a second polar body(Fig. 2. A); 17-18h after insemination, six of the forty oocytes had normal fertilization with two anterior nuclei, and its companion was not unfertilized (Fig. 2. B). On the third day after insemination, the fertilized oocytes cleaved to the eight-cell stage (Fig. 2. C), and two reached the blastocyst stage on day 5(Fig. 2.D). The fertilization rate of conjoined oocytes was 15%, which was significantly lower than that of normal oocytes. Cleavage rate and D3 High-quality embryo rate were not different from normal oocytes. The rate of blastocyst formation was 40%, which was also

significantly lower than that of normal oocytes. The conjoined oocytes did not form high quality blastocysts, possibly because of the small number of cultured embryos. All patients transplanted embryos formed by normal oocytes. The implantation rate, pregnancy rate, live birth rate and abortion rate after transplantation were consistent with the data of patients without conjoined oocytes in our center.

Table 3
Comparison of fertilization and clinical outcomes among the patients with conjoined oocytes

Parameters	Patients with conjoined oocytes	
	Normal oocytes	Conjoined oocytes
MII rate,%(n)	84.01 ^a (247/294)	40.00 ^b (16/40)
2PN Fertilization rate,%(n)	64.63 ^a (190/294)	15.00 ^b (6/40)
2PN Cleavage rate,%(n)	98.42 ^a (187/190)	100 ^a (6/6)
High-quality embryo rate,%(n)	67.38 ^a (126/187)	66.67 ^a (4/6)
Blastocyst formation rate,%(n)	57.24 ^a (83/145)	40.00 ^b (2/5)
High-quality blastocyst rate, %(n)	42.17 ^a (35/83)	0 ^b (0/2)
Implantation rate, %(n)	43.75(7/16)	-
Pregnancy rate, %(n)	66.67(6/9)	-
Live birth rate, %(n)	55.56(5/9)	-
Miscarriages rate, %(n)	16.67(1/6)	-
Data are expressed as percentage.		
Different letters within an assessment significantly different at P < 0 .05.		

Discussion

Data on human conjoined oocytes produced by binovular follicles are very limited in the literature. Therefore, a variety of terms and definitions have been used to describe follicles containing multiple oocytes⁵. The failure of two individual germ cells to separate during early follicle formation is considered to be the most likely and recognized reason of binovular follicle formation³. In the same sense, two oocytes surrounded by a zona pellucida are so opposed that they can not be surrounded by granulosa cells alone. Thus, during the folliculogenesis, the zona pellucida becomes a continuous intact layer surrounding two oocytes. We observed different zonal pellucida fusion patterns, such as two oocytes

sharing a common complete ZP, or each oocyte in two oocytes associated with a separate ZP, but fused within a certain region. It is believed that this different zonal fusion may be due to the previous distance between the two germ cells^{3,12}.

Conjoined oocytes are usually asynchronously maturation, or oocytes usually come from polyovular follicles. We analyzed the maturity status of conjoined oocytes, which was significantly lower than that of normal oocytes. Most of the cases were that one oocyte in each pair of conjoined oocytes were in the MII stage and the other was in the GV stage, accounting for about 80% of the conjoined oocytes. Two pairs of conjoined oocytes were MI oocytes attached to GVs, the remaining conjoined oocytes were MI oocyte attached MI oocyte and GV oocyte attached GV oocyte. We analyzed 22 cases of conjoined oocytes reported to date. Of these reported cases, 14 contained MII oocytes attached to GVs, 3 contained two GVs, 1 contained MI oocyte and GV, 2 contained two MII oocytes^{3,11-15}. A pair of conjoined oocytes were analyzed by fluorescence in situ hybridization to conclude that each oocyte in the conjoined oocytes represents an individual gamete whose chromosome composition corresponds to its maturation stage¹⁰. A study of rabbit¹⁶ showed that oocytes must occupy a certain position within the follicle and reach the size that allows meiosis to be resumed. The time interference of preovulation development has adverse effects on post-fertilization development. This limitation of growth ability can be explained by the material gradient within the follicle, and the central egg is more favorable. There is only a limited amount of this substance in each follicle. We believe that there is a relationship between the topographic status of oocytes in follicles and their growth potential. Therefore, it is difficult for oocytes not in the right position to mature, which may lead to the unequal area and zonal pellucida thickness of the two oocytes in the conjoined oocytes.

The aetiological factor of polyovular follicles occurrence in hamsters is heredity¹⁷ and the proportion of polyovular follicles in some inbred mice is much higher. Polyovular follicles are found in immature ovaries, but most of them disappear in late fetus and early newborn. They can also happen in adult women, but it has been suggested that the stimulation of gonadotropins may increase their incidence during the treatment cycle¹⁰. This conclusion is supported by our cases: most patients were highly responsive to ovarian stimulation; obviously, estrogen levels in patients and the total number of oocytes collected were higher than other patients in our center. Polyovular follicles may be associated with polycystic ovary¹⁸ and may play a role in the transformation or development of ovarian teratoma¹⁹. Furthermore, studies in different mammals have concluded that experimental postnatal exposure to diethylestradiol or bisphenol A can cause the occurrence of polyovular follicles²⁰⁻²². In alligators, exogenous substances with estrogen-like effects are thought to promote the formation of multinuclear oocytes and multicellular follicles²³. It would be meaningful to understand how these exogenous materials affect the occurrence of human polyovular follicles.

The fertilization rate and blastocyst formation rate of conjoined oocytes were significantly lower than these of normal oocytes. It has been reported that mature oocytes contained in the conjoined oocyte complex have limited developmental potential after fertilization and should be regarded as abnormal¹².

There were evidences that these conjoined oocytes can obtain healthy live births. Cummins L et al. first reported a healthy live births from conjoined oocytes, confirmed by array CGH as aneuploidy¹¹. In 2017, yano et al. reported another case of successful pregnancy and live birth of a transplanted blastocyst formed by fertilization of a mature oocyte from two mature conjoined oocytes¹³. A pair of conjoined blastocysts formed by a pair of conjoined oocytes after ICSI have recently been reported and dizygotic twins was born after transplantation¹⁴. Therefore, it can be speculate that the production of a pair of mature conjoined oocytes from the polyovular follicles may be one of the reasons for the formation of dizygotic twins.

In conclusion, it is reasonable to assume that the use of exogenous gonadotropins during controlled ovulation increases the incidence of abnormal follicles, including conjoined oocytes, especially in highly reactive ovarian populations. To date, only three successful live births after conjoined oocyte transplantation have been reported in the literature, indicating that the developmental ability of conjoined oocytes after fertilization was limited. We believe that other normally developed embryos should be transplanted first, not from conjoined oocytes, even if conjoined oocytes can be fertilized and subsequently developed. Transfer of these embryos should be avoided unless other embryos were not available and that they must be genetically screened before transfer if possible.

Methods

Patients.

This study was a retrospective research of patients who came to our center for IVF treatment. The present study was approved by the institutional ethics committee review board of the Affiliated Yuhuangding Hospital of Qingdao University, Shandong, China and all methods were carried out in accordance with relevant guidelines and regulations. From January 2012 to December 2019, a total of 18 patients with conjoined oocytes were included in this research. All patients gave written informed consent prior to the start of the study. The patient's age ranges from 24 to 43.

Ovarian stimulation and oocyte retrieval.

Most patients used the long protocol were down regulated with GnRH agonist triptorelin (Decapeptyl; Ipsen-biotech, Paris, France), then administered with follicle-stimulating hormone (Lizhu Group Co., Ltd, Zhuhai, China; Merck Serono, Geneva, Switzerland) daily on the basis of patient's age and ovarian reserve. Other protocols were also adopted in ovarian stimulation, such as antagonist protocol or mini-stimulation protocol according to the patients' response to stimulation. To induce the oocyte maturation, recombinant human chorionic gonadotrophin (Merck Serono, Geneva, Switzerland) or HCG (Lizhu Group Co., Ltd, Zhuhai, China) was injected when ≥ 3 follicles reached the diameter of 17–18 mm. After HCG injection, oocytes were collected at 36–38h and cultured at incubators until insemination.

Sperm manipulation and insemination.

The semen was collected by masturbation after 2–7 days of abstinence and liquefied at room temperature for 30min. After liquefaction, the sperm suspension was mixed and a 5µL aliquot was loaded into a Makler chamber. Semen parameters (concentration and motility) were assessed under a Makler-chamber. Sperm was collected by conventional discontinuous density gradient centrifugation and swim-up method according to the World Health Organization (WHO) guidelines and then stored in an incubator for subsequent insemination. Oocytes were incubated in culture medium for 2-3h after being picked up and then incubated with motile sperm. The oocytes were incubated at a concentration ratio of 50 000 sperm/ml.

Fertilization assessment and embryo culture.

Fertilization was evaluated by the formation of pronucleus approximately 17-18h after insemination. Oocytes with two pronuclei were considered normal fertilization, and oocytes with one or more than two pronuclei were considered abnormal fertilization. Embryo culture was carried out according to the operating instructions of Vitrolife(Vitrolife, Gothenburg, Sweden). Briefly, the activated oocytes would be cultured in G1 medium for subsequent development. On day 3, the embryo quality score was carried out, and the embryos with 7–9 symmetrical blastomeres and the fragmentation rate of less than 10% were rated as high-quality embryos. Embryos were transferred into GII medium on the afternoon of day 3 for further development. Blastocyst formation score was performed on days 5 and 6, only blastocysts with dilated blastocyst cavity, clearly visible inner cell masses and compact trophoblast cells were considered as available blastocysts.

Clinical pregnancy.

On the third day after oocyte retrieval, 1–2 good quality embryos were transplanted under ultrasound guidance. The embryo transfer tube (Cook, Ireland Ltd, Ireland) was used to embryo transfer (ET). After ET, the tube was examined to make sure that there were no retained embryos. All ET patients were administered duphaston or injected progesterone for luteal support. About seven weeks after embryo transfer, patients were checked for clinical pregnancy by the presence of a gestational sac. The clinical pregnancy rate was defined as the number of clinical pregnancy divided by the number of patients.

Measurement of area and zona pellucida thickness of conjoined oocytes.

The area and ZP thicknesses of conjoined oocytes were measured on Day 0. All area and ZP thickness assessments were performed directly under an inverted microscope equipped with Hoffman modulation contrast optics. Oocytes images were recorded using a colour digital camera mounted on an inverted

microscope by Hoffman modulation contrast. The setting for microscopic observations (200× magnification) and bright fields were kept constant throughout the study. Clear images of oocytes stored on the computer were displayed. The area and ZP thickness were measured using a public domain Image J program and ZP thickness was measured by selecting 4 sites on zona pellucida as 0:00, 3:00, 9:00 and 12:00. The calculated average ZP thickness is $ZP\ average = (ZP1 + ZP2 + ZP3 + ZP4)/4$. To minimize experimental distortion, the same technician performed all image recording and measurements.

Statistical analysis.

The software used was Statistics Package for Social Science (SPSS22; SPSS Inc., Chicago, IL, USA). Percentage data were arc sine transformed and analyzed with ANOVA when each measure contained more than two groups or with an independent t-test when each measure had only two groups; a Duncan multiple comparison test was used to locate differences. Categorical variables were expressed as the percentage. $P < 0.05$ was considered significant.

Declarations

Author contributions

Each author has met the requirements for authorship. X.Y. L & G.Z.J designed and wrote the main manuscript text. Acquisition of data was made by L.L.C & S.Y.H. Analysis and interpretation of data were made by J.H.W & H.X. All authors have provided final approval for the submission of this manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Different zonal pellucida fusion patterns of conjoined oocytes. (A) A conjoined oocyte with cumulus cell. (B) A conjoined oocyte: One oocyte was mature while the other was GV; two oocytes contained within a common zona pellucida. (C) The zona pellucida of the two oocytes fused at the junction. (D) The two oocytes had their individual zona pellucida or very small fused parts. $\times 200$.

Figure 2

Different developmental stages of a conjoined oocyte. (A) Four hours after insemination, the larger oocyte had two polar body (2PB) and the other was at the GV stage. (B) Day 1: The larger oocyte was fertilized with two pronuclei (2PN). (C) Day 3: One oocyte fertilized of the conjoined pair, cleaved to eight-cell stage. (D) Day 5: The conjoined oocyte developed to the blastocyst stage. $\times 200$.