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Case Report

A Case Report of Conjoined Oocytes with Independent Zona Pellucida from Polycystic Ovary Syndrome

Ayaka Tanaka, Hitomi Nakamura*, Keiichi Kumasawa, Tateki Tsutsui, Kiichiro Furuya, Namhyo Kim, Kaori Koizumi, Tadashi Kimura

Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Osaka, Japan

Email address:

ayaka0726lynn@gmail.com (A. Tanaka), hitomi@gyne.med.osaka-u.ac.jp (H. Nakamura), kokoko52@hotmail.com (K. Kumasawa), tatekitsutsui@icloud.com (T. Tsutsui), kiishin529@yahoo.co.jp (K. Furuya), nonnon.net@gmail.com (N. Kim), koichy555@gmail.com (K. Koizumi), tadashi@gyne.med.osaka-u.ac.jp (T. Kimura)

*Corresponding author

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Abstract: The occurrence of more than two ova from the same follicle (polyovular follicle) in humans has been observed. In this case report there was a high frequency of conjoined oocytes retrieved with individual zona pellucida (40% of oocytes retrieved, 4.3% of mature follicles) from women with polycystic ovary syndrome (PCOS). We may have to be more aware of the risk of multiple pregnancies in infertility treatment for PCOS women.

Keyword: Conjoined oocytes, Polyovular Follicle, Binovular Follicle, Polycystic Ovary Syndrome (PCOS), Assisted Reproductive Technology (ART) Treatment, Case Report

1. Introduction

Since 90 years ago, the occurrence of polynuclear ova and more than two ova from the same follicle (polyovular follicle) in mammals has been reported [1]. Since then, polynuclear ova and polyovular follicles in women have been observed sporadically.

One histological analysis using surgically removed ovaries from 165 patients observed polynuclear follicles in 58% of the follicles found in women under the age of 20, 13% in women between 20 to 39 years, and none in women over 40 years [2]. The histological analysis using laparoscopic bilateral ovarian biopsies sample from a patient aged 27 years with primary amenorrhea observed approximately 1 in 20 polyovular primordial or primary follicles [3]. Other histological analysis using 36 pairs of ovaries and 81 biopsies sample showed 98% of women aged 18 to 52 had polyovular follicles and polynuclear oocytes and only 2% of women had neither polyovular follicles nor polynuclear oocytes [4]. It was not age dependent and varied between 0.06% to 2.44% of the total follicular population and 97.1% of these polyovular follicles contained 2 oocytes (binovular follicles) [4]. On the other hand, the autopsy study shows that tumorlike structure in 29%, binovular follicles in 52% and binuclear oocytes in 19% were identified in pediatric autopsies (pre-term to 15 years old, n=222), but not in adult (20-40 years old, n=22) [5]. It seems that the frequency of binovular follicles could be higher in neonates and prepubertals than in adults in much the same way as in mammals [4, 6-9].

Since the 1980's human assisted reproductive technology (ART) treatment has been performed around the world. Since then, the polyovular follicles from ART treatment have been reported [10-18]. Jones *et al.* [10] and Dandekar *et al.* [11] retrieved more than one oocyte from 8% of the aspirated follicles via laparoscopy in ART treatment. However, they did not mention how the oocytes connected. Later, Ron-EI *et al.* presented the frequency of binovular follicles in 0.3% of all retrieved follicles (15 of 4695 follicles, 9 adjacent cumulus complexes, 1 separate corona radiata and common cumulus mass, 5 two oocytes within a single zona pellucida) via

laparoscopy (n=69) and vaginal ultrasonography (n=562) in ART treatment [12]. There is a big discrepancy in the frequency of polyovular follicles in humans by depending on the methods and the authors doing the analysis.

In approximately two thirds of binovular follicles, conjoined oocytes were reported with discordancy in maturity [11]. In animal studies, an oocyte needs to be located at a certain position inside a follicle to reach an appropriate size that allows it to resume meiosis [18]. The position of oocytes inside of a follicle may play an important role in the normal maturation of oocytes. At least one of the conjoined oocytes was at immature germinal vesicle (GV) stage or degenerated in 18 of 19 reported cases from ART treatment [14]. The conjoined oocyte from binovular follicles may have difficulty reaching maturity and most of them may degenerate or not reach secondary follicle stage. For that reason, the frequency of polyovular follicles in histological studies could be higher than in ART treatment.

Hartman described three hypotheses to explain the occurrence of polyovular follicles: (i) a polynuclear oocyte may divide into two or more; (ii) a few independent oocytes may fuse or adhere to cumulus cells; (iii) granulosa cells may fail to separate two germ cells at the early stage of folliculogenesis [1]. Vicdan *et al*. and Rosenbusch have described the most likely explanation is the third assumption [11, 13, 17]. In the beginning of ovarian development, the primordial germ cells generated from yolk sac migrate to the posterior body wall mesenchyme. Germ cells and proliferative cells of mesonephros and celomic epithelium are mixed at genital ridges, but granulosa cells from cortical sex cords begin to isolate germ cells with regression of medulla. Each complex develops into an independent primordial follicle [19]. Polyovular follicles may develop due to failure of isolation and form these units accidentally. However, it is still unexplained.

In mice it has been reported that neonatal exposure to diethylstilbestrol (DES), estrogen, or the phytoestrogen genistein induces formation of multioocytic follicles [20-22]. In wildlife the alligators exposed to environmental estrogenic contaminants displayed multioocytic follicles (often three to four oocytes per follicle) [6]. In humans, one study has suggested that the frequency of binovular follicles in ovarian tissue from mature teratoma tends to be higher compared with normal ovarian tissue [23]. The association of polyovular follicles with polycystic ovary syndrome (PCOS) has been discussed since 1958. Shettles *et al* observed in their histological study that 19 in 30 cases of polycystic ovaries with Stein-Leventhal syndrome had polyovular follicles or polynuclear oocytes, compared to polyovular follicles but no polynuclear oocytes in 2 of 30 normal ovaries [24]. Later, there was one histological study which compared polycystic ovary with no sign of Stein-Leventhal syndrome (n=1) and Stein-Leventhal syndrome ovary (n=1) [25]. It showed that there were not remarkable differences between polycystic ovary and Stein-Leventhal syndrome in the degeneration and maturation rate of oocytes, but a binovular follicle was observed only in the Stein-Leventhal ovary. On the other hand, it is considered that the higher incidence of polyovular follicles in polycystic ovaries than in normal ovaries might be due to the higher number of observed follicles, rather than to a secondary effect due to pituitary hormonal stimulation [26]. The association between polyovular follicles and PCOS is still controversial [4, 13, 26].

The present report describes a case of conjoined oocytes with individual zona pellucida from a woman with PCOS in a program of ART treatment.

2. Methods

The controlled ovarian hyperstimulation (COH) for ART treatment was started with administering 150 IU of recombinant FSH (Follistim®, MSD Co., Ltd., Tokyo, Japan) intramuscular injection on day 4 of the menstruation cycle. When the dominant follicle reached a diameter of 13 mm, 0.25 mg of daily GnRH antagonist (Cetrotide®, Merck Serono Co., Ltd., Tokyo, Japan) subcutaneous injection was started and continued until the day of human chorionic gonadotropin (hCG) administration. Total 750 IU of recombinant FSH was used for COH. The oocyte retrieval was performed 35 hours after a 5000 IU of hCG (Gonatropin®, ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) intramuscular injection.

As a case report, this did not require Institutional Review Board approval, but was reported with the patient's informed consent.

3. Case Report

A 30 year old woman, gravida 0, was admitted to our hospital for infertility treatment and presenting with PCOS and a history of stage IA grade 1 endometrioid adenocarcinoma of the uterus.

In her previous hospital, she was diagnosed with PCOS according to Japan Society of Obstetrics and Gynecology PCOS guideline [27], and stage IA grade 1 endometrioid adenocarcinoma of the uterus. She received tumorectomy under uterine incision laparotomy and ovarian drilling at her insistence on preserving her uterus in previous hospital. The hysterectomy was not performed because the biopsy of uterine myometrium during the surgery revealed no myometrial invasion. After the tumorectomy she received medroxyprogesterone acetate (MPA) therapy (600 mg/day) for 6 months. Periodic endometrial tissue sampling was carried out by dilation and curettage and was assessed by the pathological response to MPA treatment. After 6 months of MPA treatment, the endometrial biopsies did not reveal the recurrence or the remains of the cancer and she was allowed to receive infertility treatment.

After MPA treatment for 6 months, polycystic ovaries on ultrasonography were observed and her basal serum luteinizing hormone (LH) level was high (8.7 mIU/ml), but basal serum follicle stimulating hormone (FSH) level (6.6 mIU/ml), total testosterone level (0.24 ng/ml) and free testosterone level (< 0.4 pg/ml) were normal. She was not presented with hirsutism or any sign of virilization. Her Body

Mass Index (BMI=19.8) and insulin resistance (HOMA-R=0.75) were normal. She and her husband had normal somatic karyotypes. She attempted her first in-vitro fertilization (IVF) treatment after her 5th failed intrauterine insemination (IUI) treatment. The ovarian hyperstimulation was induced by gonadotropin releasing hormone (GnRH) antagonist protocol.

Transvaginal oocyte retrieval was performed with an 18-gauge needle, aspirating 8 cumulus-oocyte complexes (COCs) from a total of 46 punctures of mature follicles. The two COCs contained two oocytes each (Fig. 1a, 1e) sharing corona radiata, cumulus oophorus and granulosa cells (Fig. 1b, 1f). These two conjoined COCs were not separated mechanically, they were separated by oocyte denudation using 80 mU/ml hyaluronidase (NAKA medical Inc., Tokyo, Japan). The two conjoined COCs contained two oocytes each and the each oocyte had independent zona pellucida (Fig. 1c-d, 1g-h).

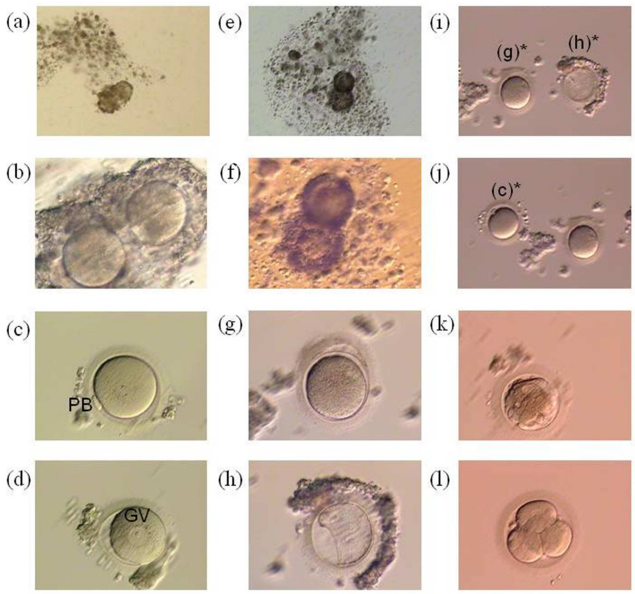

Figure 1. *Two pairs of conjoined oocytes and other oocytes.*

Binovular cumulus-oocyte complexes (COCs) after 4 hours of oocyte retrieval (a, e). (b) shows an enlarged view of (a). (f) shows an enlarged view of (e). The conjoined oocytes shared corona radiata, cumulus oophorus and granulosa cells (b, f). These were not be mechanically separated. Two associated oocytes in mature metaphase II (MII) (c) and immature germinal vesicle (GV) stage (d) from binovular COC (a-b). The second binovular COC (e-f) contained an immature metaphase I (MI) stage oocyte (g) and a degenerated oocyte (h). All oocytes from binovular COCs had independent zona pellucida (c-d and g-h). The size of oocytes between 2 oocytes (g* and h*) from the same binovular COC (e-f) was not apparently different (i). The size of a MII stage oocyte (c*) from binovular COC (a-b) was apparently similar to the size of other a MII stage oocyte from uniovular COC (j). After ICSI, the mature MII stage oocyte (c) developed cleavage stage (k). A healthy baby was born after the transfer of the cleavage-stage of embryo from uniovular COC (l).
PB: first polar body

The first conjoined COC contained oocytes at metaphase II (MII) (Fig. 1c) and germinal vesicle (GV) stage (Fig. 1d). The second conjoined COC contained a metaphase I (MI) stage oocyte (Fig. 1g) and a degenerated oocyte (Fig. 1h). The GV (Fig. 1d) and MI (Fig. 1g) stage of oocytes did not reach MII stage. The size of oocytes from conjoined COCs were similar to each other (Fig. 1i) or other ordinary MII oocyte (Fig. 1k).

The intracytoplasmic sperm injection (ICSI) was performed for MII stage oocytes including the one from the first conjoined COC (Fig. 1c, 1j). The one from the first conjoined COC was fertilized and then developed into the cleavage stage of embryo (Fig. 1k) after ICSI procedure.

All embryos were cryopreserved due to the risk of ovarian hyperstimulation syndrome (OHSS). She was conceived after the first frozen-thawed single embryo transfer using a single embryo (Fig. 1l) from a single oocyte from a single COC in an artificial hormonally-controlled cycle using sequentially administrated exogenous estrogen and progesterone. A healthy female baby weighting 2,122 g was delivered with Apgar score 8/9 by elective cesarean section at 36th weeks of gestation.

4. Discussion

In this case 2 sets of conjoined oocytes in the same oocyte retrieval cycle were found, but all of them had individual zona pellucida. This frequency of conjoined oocytes (40% of oocytes retrieved, 4.3% of mature follicles) was higher than in current reports from ART treatment [12, 14]. This case report is the first report which observed high frequency of binovular follicles in PCOS without clinical signs of hyperandrogenism. PCOS is heterogeneous condition and its main features are hyperandrogenism, chronic anovulation and infertility. PCOS patients in Japan show less frequency of hyperandrogenism features and in 2007 the Japan Society of Obstetrics and Gynecology established a new criteria of diagnosis and treatment of PCOS in Japan [27].

In recent years, conjoined oocytes sharing a common and intact zona pellucida or two oocytes with an individual zona pellucida that are connected in a defined region from ART treatment have been reported [14]. Previous studies have shown the 19 cases of conjoined oocytes in human ART treatment [14, 16]. The majority of them shared common zona pellucida. There were only 2 case reports of conjoined oocytes with independent zona pellucida, which are the same as our case [14-15]. In our case the 2 sets of conjoined COCs containing two oocyte each with individual zona pellucida were found, but it was not clear whether the zona pellucida was connected at the surface. These oocytes were easily separated after oocyte denudation using hyaluronidase. This fact can rule out the possibility that the zona pellucida connected tightly, but can not rule out if it connected loosely on the surface. Because these oocytes had individual zona pellucida, there is a small possibility that they were each originally coming from uniovular follicles and they were attached to each other in the aspiration needle during the oocyte retrieval using vaginal ultrasonography. However, if so, it could easily be separated mechanically, which it did not in this case. In this case, all conjoined oocytes shared corona radiata, cumulus oophorus and granulosa cells, but had independent zona pellucida, even though the majority of

reported conjoined oocytes shared common zona pellucida. It might be related to PCOS.

In mice, neonatal exposure of DES increases the frequency of polyovular follicles and ihnibin alpha mRNA expression in ovary [28]. The transgenic mouse study has shown that the overexpression of rat inhibin alpha-subunit gene from a metallothionein-I promoter increases the incident of polyovular follicle and the hormone profile of these mice resemble human PCOS [29]. However, women with PCOS have high serum concentration of total inhibin but not of inhibin A or inhibin B [30]. It suggests that PCOS women have an impaired processing of alpha-inhibin precursor proteins [30]. The water channel aquaporin-8 (AQP8) deficient mice show increasing incidence of multi-oocyte follicles [31]. In humans, recently one single-nucleotide polymorphism (SNP) within AQP8 associated with PCOS has been reported [32]. AQP8 is expressed in ovarian granulosa cells, and inhibin alpha subunits and mature inhibin are produced by ovarian granulosa cell. However, the relation between AQP8 and inhibin is unknown.

There are some researchers and clinicians who have been questioning whether polyovular follicles may have actually been causing dizygotic twinning for a long time [15]. Dizygotic twinning occurs when two different eggs are fertilized by two different sperms in the same menstrual cycle. Spontaneous dizygotic twinning occurs in 1- 4% of women [33]. The hypothesis of double ovulation as an origin of dizygotic twinning is regarded as an established fact, but double ovulation from different follicles has never been observed or proven to be a cause of dizygotic twinning [34]. Not much evidence has surfaced so far that multiple ovulation is a direct origin of human multiple pregnancy. Oocytes of a binovular follicle are heterogeneous, and it is suggested that some binovular follicles could occasionally lead to ovulation [4]. Furthermore, if oocytes derived from binovular follicles reach ordinary maturity, their ability to fertilize does not appear to be different from oocytes of normal follicles [13]. In fact, one of the conjoined oocytes was able to be fertilized in 9 of 19 reported cases and 8 of 9 cases developed into the cleavage stage of embryo, resulting in one live-birth case [4, 16]. Therefore, if both of the conjoined oocytes derived from a binovular follicle are favorably mature and ovulated, and then are fertilized with two individual sperms, they may lead to dizygotic twinning. There is still not enough data yet to conclude that the frequency of binovular follicles is higher in ovaries with PCOS than without PCOS. However, results from mice and some human studies assume that PCOS is related to binovular follicles.

5. Conclusion

This report shows a high frequency of binovular follicles from women with PCOS (40% of oocytes retrieved, 4.3% of mature follicles) in ART treatment. This case report is the first report which observed a high frequency of binovular follicles in PCOS without clinical signs of hyperandrogenism.

So far, there is no evidence to rule out the possibility that dizygotic twining occurs from polyovular follicles. If the incidence of polyovular follicles are related to the pathophysiology of PCOS, we may have to be more aware of the risk of multiple pregnancies in infertility treatment for PCOS women.

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