Recovery and Maturation of Immature Oocytes in Patients at Risk for Ovarian Hyperstimulation Syndrome

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Purpose: Our purpose was to examine the rate of immature oocyte recovery and their potential for in vitro maturation from canceled human menopausal gonadotropin cycles due to the risk of having ovarian hyperstimulation syndrome develop.

Methods: Patients underwent ultrasound-guided immature oocyte pickup. The number of oocytes recovered from these patients was recorded, and then cultured in vitro. Cumulus expansion and the stage of nuclear maturation were observed after 24 and 48 hr, respectively.

Results: Seventeen patients underwent 20 immature oocyte recoveries. A total of 162 oocytes (8.1 oocytes/patient) was obtained. All of the oocytes were enclosed in dense layers of cumulus cells. Among them, 78.4% showed cumulus expansion after 24 hr and 66% completed meiotic maturation to metaphase II after 48 hr in culture. There was only one immature oocyte pickup in which no oocytes were recovered (95% recovery rate). None of the patients had ovarian hyperstimulation syndrome develop.

Conclusions: Immature oocytes can be recovered from canceled human menopausal gonadotropin cycles in patients who are at potential risk for severe hyperstimulation syndrome. These oocytes can be matured in vitro and can be used for clinical and research purposes as well.

KEY WORDS: embryo; human; hyperstimulation; in vitro maturation; oocyte.

INTRODUCTION

The shortage of human immature oocytes for research is the limiting factor in developing methods for using

them in clinical practice. During each menstrual cycle, several preantral follicles enter into the pool of growing follicles. Only one of the antral follicles is destined to ovulate, while others undergo atresia (1). Ovarian stimulation is carried out in assisted reproduction programs to increase the number of oocytes available for fertilization. Patient responses to stimulation vary considerably and a number of those cycles have to be canceled due to the possible risk for ovarian hyperstimulation syndrome (OHSS). Cycles with elevated serum estradiol levels and a large number of growing follicles during ultrasound are at higher risk for OHSS (2). Several preventive steps have been suggested, ranging from cycle cancellation to reduction of the human chorionic gonadotropin (hCG) dosage (2). Unfortunately, several potentially recoverable immature oocytes are lost when these cycles are canceled. These immature oocytes can be used for in vitro maturation if a simple recovery technique is available and culture conditions can be perfected.

The oocyte is arrested at the diplotene stage of prophase I in the growing follicle. It remains in this meiotic arrest until the ovulatory luteinizing hormone (LH) surge, which stimulates the resumption of meiosis in the Graafian follicle (3). Mammalian oocytes also undergo spontaneous maturation when removed from the follicular environment and placed in a supportive medium (4). In farm animals (5,6) as well as in humans (7–11), it has been shown that immature oocytes removed from small antral follicles can be fertilized in vitro and result in pregnancies after transfer into the uterus. However, the success rate of this procedure is very low compared to that in in vivo matured oocytes. Hormonal supplementation and somatic cell cocultures in either simple or complex medium were used to improve the in vitro maturation rate (12–14). The optimal culture conditions for in vitro maturation of oocytes are not known. Moreover, the availability

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of immature human oocytes on which to experiment to improve the maturation rate is very limited.

The goal of this study was to evaluate whether immature oocytes may be recovered through the routine mature oocyte pickup technique when the human menopausal gonadotropin (hMG) cycle is canceled and matured in vitro.

MATERIALS AND METHODS

Patient Stimulation

Either a long (Lupron Depot, 3.75 mg) or a short gonadotropin-releasing hormone (GnRH) (Buserelin) protocol was used. After down-regulation of the pituitary, 75–225 IU of hMG (Humegon) was administered i.m. daily until risks for severe hyperstimulation syndrome were foreseen. The risks were characterized as serum estradiol 17- β levels exceeding 20,000 pM, average ovarian dimensions (height, width, depth) exceeding 6 cm, or the presence of at least 20 follicles in each ovary (except one case) with diameters of 15 mm or less and a serum estradiol 17- β level of more than 5000 pM. Patients with canceled cycles between 10 May 1996 and 15 May 1997 were asked to undergo immature oocyte retrieval with subsequent in vitro maturation. Couples who agreed to undergo immature oocyte pickup were asked to sign a consent form. In all cases, hCG was not administered.

Oocyte Pickup and In Vitro Maturation

Immature oocyte pickup was performed 1 day after the last hMG injection under routine i.v. sedation (5 mg midazolam and 100 mg fentanyl) and vaginal ultrasound guidance with reduced aspiration pressure (100 mm Hg). All of the visible antral follicles were aspirated using a normal beveled Svemed needle. The follicular aspirates were poured into 60-mm dishes (Falcon, No. 3004) as a thin layer to search for immature oocytes; these were mostly invisible on the first look under a dissecting stereomicroscope. A second search was also performed on the same dish after red blood cells were sedimented; this gave better visibility of the immature oocytes. The dishes were examined by at least two biologists. All of the oocytes and some of the granulosa cell pieces were transferred into HEPES-buffered medium with 10% synthetic serum substitute (SSS; Irvine Scientific, Santa Ana, CA) and washed twice. Then they were cocultured in 50 μ l of either human tubal fluid (HTF) or Ham's F-10 (Irvine

Scientific) supplemented with 10% SSS, 75 mIU/ml hMG, and 500 mIU/ml hCG under preequilibrated mineral oil (R. E. Squibb & Sons Inc., Princeton, NJ) in center-well dishes (Falcon, No.3037) for 48 hr. Each drop contained as many as 5 immature oocytes with 10–20 granulosa cell pieces. Figure 1 shows the coculture of the immature oocyte with pieces of granulosa cells. Cumulus cell expansion was assessed after 24 hr of culture (Fig. 2) and graded as follows: grade 0, no expansion; grade 1, outer two layers of cumulus cells expanded; and grade 2, more than two layers expanded. Cumulus–oocyte complexes were decoronated after 44 hr in culture using a 160- μ m capillary pipette following exposure to 80 U/ml hyaluronidase (Sigma, St. Louis, MO) for 30 sec. The stage of nuclear maturation of the oocytes was checked under an inverted microscope and classified as germinal vesicle, metaphase I, metaphase II, or degenerated.

RESULTS

Oocyte Recovery

A total of 17 patients underwent 20 immature oocyte pickup cycles. Patient diagnoses in terms of infertility were as follows: 6 unexplained, 7 male factor, 10 polycystic ovary, 1 tubal, and 1 cervical (some patients had more than one diagnosis). These patients were given an average of 23 ampoules of hMG (range, 7.5–30.5 ampoules) for an average of 9 days (range, 5–20 days). The mean estradiol level on the day of cancellation was 10,689 pM (range, 3447–25,000 pM). The total number of follicles was 889, with an average of 44 follicle per cycle. There were 678 follicles (76%) with a diameter of less than 10 mm and 211 follicles (24%) with diameters of between 11 and 15 mm. The size of leading follicles, with the exception of two cycles, was less than 14 mm in diameter. The leading follicle in eight cycles (40%) was less than 10 mm. A total of 162 oocytes (range, 0–14 oocytes; 8.1 oocytes/patient) was obtained (Table I). The oocyte recovery rate per follicle present as identified by ultrasound in both ovaries was 18.2%. The recovery rate per follicle was similar when patients were grouped according to leading follicle size (17.4% when the leading follicle was greater than 10 mm and 19.4% when it was 10 mm or less). There was only one immature oocyte pickup in which no oocytes were obtained (95% successful recovery). The average time for oocyte pickup was 11 min, while that for oocyte identification was 135 min. None of the patients had OHSS develop.

Fig. 1. Immature human oocytes obtained from patients at risk for OHSS. Oocytes were cocultured with pieces of granulosa cells from the same patient.

Cumulus Expansion and Oocyte Maturation

All of the oocytes were enclosed in dense layers of cumulus cells. There was no sign of cumulus expansion at the time of oocyte recovery. After 24 hr of incubation, 22% of the oocytes were grade 0, 21% were grade 1, and 57% were grade 2. The nuclear maturation of 162 oocytes was checked after 44 hr by removing the cumulus cells. There were 107 metaphase II (66%), 20 metaphase I (12%), 25 germinal vesicle (16%), and 10 degenerated oocytes (6%).

DISCUSSION

Multiple follicular growth can be stimulated by exogenous gonadotropins, and this method therefore is applied to obtain a high number of oocytes for assisted reproduction. However, a certain percentage of these stimulated cycles has to be canceled due to the potential risk of hyperstimulation. Subsequently,

several potentially recoverable oocytes are lost. These patients at risk of hyperstimulation must start a new cycle and undergo the same costly and lengthy stimulation protocol. The availability of a technique to obtain oocytes from these patients to be used for clinical purposes will alleviate the financial burden and emotional stress. The results of the present study demonstrate that immature oocytes can be recovered from these patients and matured in vitro. An average of 8.1 oocytes was obtained from each patient. This number is comparable to the number of immature oocytes obtained by other groups. Russell *et al.* (10) retrieved approximately 11 oocytes from unstimulated patients whose endometrial priming started at either early or midfollicular phase. Trounson *et al.* (8) obtained an average of 13.8 and 13.1 immature oocytes from anovulatory and ovulatory patients with polycystic ovary, respectively. Although the number of oocytes obtained in this study is comparable to those in the aforementioned reports, the overall recovery rate in

Fig. 2. Immature human oocytes after 24 hr in culture. Two oocytes show grade 2 cumulus expansion, while the other two did not expand (grade 0). A piece of granulosa cell is also shown (dark).

terms of the number of oocytes obtained per antral follicle punctured was relatively low. The patients in the present study were given hMG, which caused more

follicles to be produced compared with patients in previous studies (8,10). One reason for the low recovery rate may be the result of our using a regular aspiration needle in this study, whereas Russell *et al.* (10) and Trounson *et al.* (8) used a short, beveled aspiration needle. Another more likely explanation came from Lindenberg (15), who showed that when the follicles are in their growth phase and actively secreting estrogen under the influence of follicle-stimulating hormone, the oocytes are tightly attached to the follicle and are more difficult to recover. However, follicle-stimulating hormone withdrawal followed by dropping estradiol levels increased the oocyte recovery rate. In our study, immature oocyte pickup was performed on patients whose estradiol levels were higher than normal and who had had their last hMG injection fewer than 24 hr before oocyte pickup; that may also have contributed to the overall low oocyte/follicle recovery rate.

It has been proposed that hCG is needed for the release of oocytes and granulosa cells from the follicular wall to resume meiosis. Failure to take hCG or faulty hCG preparations were used to explain the

Table I. Recovery of Immature Oocytes from the Patients at Risk of Hyperstimulation

Number of patients	17
Number of pickups	20
Number of patients with no oocytes recovered	1
Cycle day on the day of pickup	11.7 (9–21) ^a
Number of days on hMG	9 (5–19)
Number of ampoules of hMG injected when risks were seen	23 (7.5–30.5)
Estradiol on the day of cancellation (pM)	10,689 (3329–25,000)
Number of follicles <10 mm	678 (11–60)
Number of follicles >10 mm	211 (0–38)
Number of oocytes/patient	8.1 (0–14)
Oocyte recovery/follicle present (%)	18
Number of patients who had OHSS develop	0

^a Numbers in parentheses are ranges.

empty follicle syndrome (16–18). In the current study, we were able to obtain oocytes from 18% of the identified follicles as determined by ultrasound, although none of the patients received hCG and they were under GnRH-agonist suppression to prevent LH surge. This seems contradictory to the proposed etiology of the empty follicle syndrome in which no eggs were found in the follicular fluid despite acceptable estradiol levels and follicular size. One of the major differences between the empty follicle syndrome and this report is in the leading follicle size. In this study, the leading follicle was 15 mm in diameter, whereas the leading follicle size in the described cases of empty follicle syndrome is always greater than 15 mm. It could be that the oocyte–cumulus complexes within follicles 16 mm or greater became more attached to the follicle wall and more difficult to aspirate. However, we must point out that, in all our patients, no oocytes were seen at first evaluation and the dishes had to be searched for oocytes at least two more times by two biologists after red blood cells were sedimented. The average recovery time for immature oocytes in our laboratory is 135 min, compared to 10 min in regular in vitro fertilization cases. This might be another reason for the absence of oocytes in empty follicle syndrome. It may be worth trying to search thoroughly for immature oocytes rather than mature ones when empty follicle syndrome is present.

It was demonstrated long ago in rabbits that oocytes will resume meiosis when they are removed from the follicular environment and placed in culture medium (19). Human oocytes were also successfully matured (4) and fertilized in vitro (20). Recent studies have also shown that embryos obtained from in vitro matured oocytes could be transferred and produce pregnancies (7–11). Information regarding optimal culture conditions for maturation of immature human oocytes is limited, and various culture media were used by different groups. Among those, culture in Ham's F-10 (7), tissue culture medium 199 (9,10), Eagle's minimal essential medium (8,10), and HTF (11) resulted in pregnancies.

Patients in this study received GnRH agonist and hMG. All the oocytes were immature and enclosed in dense cumulus cells without any sign of expansion. Bomsel-Helmreich *et al.* (21) also reported that all of the oocytes obtained from follicles less than 16 mm in diameter were immature in hMG-stimulated patients. Beneficial effects of gonadotropins and coculture with granulosa cells during oocyte maturation have been reported (12–14). Oocytes were cultured with gonadotropins and autologous granulosa cells in the present

study. The maturation rate obtained in this report (66%) is comparable to published data, although the rates in the literature vary according to the culture conditions and the source of oocytes. Cha *et al.* (7) reported 36 or 56% maturation in Ham's F-10 supplemented with fetal calf serum or mature follicular fluid, respectively. Russell *et al.* (10) reported that oocytes obtained from patients who had early follicular-phase endometrial priming had a lower maturation rate compared with those with midfollicular-phase endometrial priming. Similarly, oocytes from regularly cycling women had a higher maturation rate compared to those from anovulatory patients (22). Another example that the source of oocytes can affect the nuclear maturation rate is obtained by the finding that oocytes from hMG- or follicle-stimulating hormone-stimulated cycles showed a significantly higher maturation rate than those from unstimulated cycles (23,24).

This report shows that immature oocytes can be recovered from patients who are at potential risk for hyperstimulation syndrome and matured in vitro. These oocytes could be used to research the optimal culture conditions. Once the oocyte maturation protocol is perfected, these oocytes can be fertilized and transferred to establish pregnancies. This will give an alternative to cancellation of cycles.

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