Effects of Controlled Ovarian Hyperstimulation on Oocyte Quality in Terms of the Incidence of Apoptotic Granulosa Cells

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Submitted: March 7, 2000

Accepted: June 5, 2000

Purpose: The aim was to investigate which ovarian hyperstimulation protocol performed in the same patients causes development of oocytes of good quality.

Methods: Twenty normo-ovulatory women underwent three different controlled ovarian hyperstimulation protocols for in vitro fertilization–embryo transfer. Patients underwent follicle aspiration after administration of human chorionic gonadotropin (hCG). The total number of retrieved oocytes, the number of mature oocytes, and the rate of mature oocytes were examined. Recovered granulosa cells were stained with Hoechst 33258 and examined by fluorescence microscopy to estimate the incidence of apoptotic cells.

Results: The total number of oocytes and the number of mature oocytes in gonadotropin-releasing hormone agonist (GnRHa) + human menopausal gonadotropin (hMG) + hCG and hMG + hCG cycles were higher than those in the natural cycle ($P < 0.0001$). The rate of mature oocytes in hMG + hCG cycle was the highest among the three protocols ($P < 0.04$). In the mural granulosa cells, the incidence of apoptotic cells in the GnRHa + hMG + hCG cycle was significantly higher than those of the natural ($P < 0.002$) and hMG + hCG cycles ($P = 0.0002$). The incidence of apoptotic cumulus granulosa cells in the GnRHa + hMG + hCG cycle was significantly higher than those of natural and hMG + hCG cycles ($P < 0.002$). Moreover, the incidence of apoptotic cumulus granulosa cells in the hMG + hCG cycle was significantly lower than that in the natural cycle ($P < 0.01$).

Conclusions: These results indicated that hMG + hCG is the most appropriate controlled ovarian hyperstimulation

protocol among the three examined with regard to oocyte quality.

KEY WORDS: Apoptosis; controlled ovarian hyperstimulation; granulosa cells; in vitro fertilization; oocyte quality.

INTRODUCTION

In the last decade, the development of assisted reproductive technologies including in vitro fertilization (IVF) has expanded the use of gonadotropins and increased our knowledge regarding the use of these agents in women with normal ovulatory cycles (1–3). Gonadotropins may be useful in the medical treatment of forms of infertility other than anovulation such as unexplained infertility, male factor, cervical factor infertility, endometriosis, tubal factor infertility, and luteal phase defect.

Gonadotropin-releasing hormone agonists (GnRHa) have been shown to prevent the endogenous luteinizing hormone (LH) surge (4,5), an event that is associated in an IVF program with a decrease in mature oocytes and generally lower pregnancy rate (6,7). GnRHa are widely used for the stimulation protocol in assisted reproductive technology. However, GnRHa increased apoptotic DNA fragmentation in the ovary in a time- and dose-dependent manner in estrogen-treated hypophysectomized rats (8). Testert *et al.* (9,10) also reported that the total number of embryos was increased after GnRHa treatment but the proportion of embryos of good quality was reduced.

Apoptosis has been investigated in terms of folliculogenesis and has been found to be responsible for the perinatal attrition of oogonia and oocytes,

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the postnatal loss of follicles through atresia, and regression of the corpus luteum in diverse species. Follicular atresia was found to be associated with apoptosis in chickens, swine (11), and humans (12). Formation of apoptotic bodies is one of the morphological hallmarks of apoptosis. The incidence of apoptotic bodies has been used as a morphological marker for physiological cell renewal (11) and for prognosis of patients with neoplasms (13,14). Recently, we reported a relationship between the incidence of apoptotic granulosa cells and the prognosis of ova from patients undergoing ovulation induction stimulation for IVF (15–17). A higher incidence of apoptotic granulosa cells is associated with low oocyte retrieval, empty follicles, poor fertilization, and poor embryo development. The incidence of apoptotic granulosa cells is a very sensitive indicator with which to estimate the quality of follicles and oocytes in an IVF program.

The aim of the present study was to determine which protocol of controlled ovarian stimulation allowed development of oocytes of good quality, by analyzing the incidence of apoptotic granulosa cells in the same patients treated with three different controlled ovulation stimulation protocols.

MATERIALS AND METHODS

Patients

A total of 24 patients undergoing IVF treatment were entered into three different stimulation protocols between July 1997 and March 1999 at Yamagata University Hospital, Yamagata, Japan. All patients did not receive any hormone stimulation (natural cycle). In case pregnancy was not achieved they were entered into GnRHa + human menopausal gonadotropin (hMG) + human chorionic gonadotropin (hCG) stimulation protocol followed by hMG + hCG protocol if necessary. During this period, four patients left the study because they achieved pregnancy. Twenty patients (mean $\pm$ SD age, 34 ± 6 years) were entered into the three stimulation protocols. The diagnosis included patients with unexplained ($n = 12$) and tubal disease ($n = 8$). This study was approved by Yamagata University Hospital Committee for Research on Human Subjects. Written informed consent was obtained from all patients and their clinical information was concealed.

Stimulation Protocol

Three different controlled ovarian hyperstimulation protocols were performed in each patient. Between the first and second trial (natural and GnRHa + hMG + hCG), there was an interval of one menstrual cycle, three menstrual cycles between the GnRHa + hMG + hCG and hMG + hCG. Protocol I: Natural cycle; patients did not undergo controlled ovarian hyperstimulation and follicle monitoring was performed. Only hCG (10,000 IU; Mochida, Tokyo, Japan) was administered when one follicle achieved a mean diameter of 16 mm or greater. Protocol II: GnRHa + hMG + hCG cycle; the ovulation induction protocol used the GnRHa, buserelin acetate (600 μ g/day; Suprecur nasal®; Hoechst, Tokyo, Japan), in a long suppression protocol starting from midluteal phase in the previous cycle. Administration of hMG (150 to 300 IU/day, Humegon; Sankyo, Tokyo, Japan) with or without follicle-stimulating hormone (FSH) (Fertinom P®; Serono, Tokyo, Japan) was started on day 3 of the menstrual cycle. Protocol III: HMG + hCG cycle; hMG with or without FSH was started on day 3 of the menstrual cycle. In the latter two stimulation protocols, hCG also was administered when one follicle achieved a mean diameter of 16 mm or more. In all protocols, the development of follicles was monitored by transvaginal ultrasound every day starting on day 8 of the menstrual cycle.

Oocyte Collection

Thirty-five hours after administration of hCG, follicles were aspirated by transvaginal ultrasound retrieval (6.5 MHz; Mochida, Tokyo, Japan). All follicles with a mean diameter of ≥ 11 mm were aspirated using 20-ml syringes. Aspirated follicular fluid (FF) was transferred into tissue culture dishes (Falcon 3002; Becton Dickinson and Company, Lincoln Park, NJ). Cumulus–oocyte complexes were isolated under a dissecting microscope (SZH-ILLB; Olympus, Tokyo, Japan) and were put into organ tissue culture dishes (Falcon 3037; Becton Dickinson and Co.) containing human tubal fluid (HTF) medium.

Oocyte Maturity

Oocyte maturity was evaluated morphologically by analyzing the conditions of corona radiata and cumulus granulosa cells were classified as mature or immature (18). Oocyte–corona–cumulus complexes with

large and loose cumulus and distinct corona radiata were defined as mature and complexes with a small and dense cumulus and opaque corona radiata were defined as immature.

Cell Preparation of Cumulus and Mural Granulosa Cells

Cumulus cell masses were separated mechanically from oocytes using 26-gauge needles and transferred into tubes (Falcon 2001; Becton Dickinson and Co.). After collecting oocyte-cumulus cell complexes, aspirated FF was gathered into tubes and left for 10 min to allow sedimentation of mural granulosa cells. Precipitated mural granulosa cells were transferred to another tube containing 5 ml of medium and then gently resuspended. One ml of 80% Percoll was carefully layered at the bottom of the tube followed by centrifugation at 600 *g* for 10 min. The mural granulosa cells formed a layer on the Percoll solution, while the blood cells sedimented to the bottom of the tube. The mural granulosa cells were transferred to another tube and washed again with 5 ml of medium. The tube was centrifuged at 300 *g* for 5 min and the supernatant was decanted. Hyaluronidase solution (0.1% wt/vol in HTF) (Sigma, St. Louis, MO) was added to the tubes containing cumulus cell masses or mural granulosa cell masses (final concentration of 0.05% wt/vol). Both kinds of cells were mechanically dispersed by pipetting for 2 min. The cells were washed and centrifuged twice with phosphate-buffered saline (PBS), then the precipitated cells were fixed with 4% wt/vol of neutral buffered formalin and placed on slides.

Analysis of Apoptosis by Fluorescence Microscopy

After drying, the slides were washed with PBS three times and the nuclei of cells were stained with 0.5 μ g/ml of Hoechst 33258 (fluorescent dye; Wako, Osaka, Japan) with 5% wt/vol of DABCO (1,4-diazabicyclo-2,2,2-octane, Sigma, St. Louis, MO) in 90% glycerol: 10% 0.2 M Na₂HPO₃ to block the bleaching effect of fluorescence.

Apoptosis was defined as the incidence of apoptotic cells containing condensed or fragmented condensed chromatin, or with fragmented cytoplasm containing condensed chromatin. We counted the apoptotic cells with fragmented condensed chromatin or fragmented cytoplasm containing condensed chromatin in 1000 granulosa cells chosen at random.

Oocyte Quality

Oocyte quality was scored by the incidence of apoptotic granulosa cells with condensed and fragmented nuclei.

Statistical Analysis

The data are presented as means $\pm$ SEM. For statistical comparisons between groups, Friedman's test and ANOVA were applied. *P* values < 0.05 were considered significant.

RESULTS

To clarify differences in fecundity with the three different stimulation protocols, the total number of retrieved oocytes, the number of mature oocytes, and the rate of mature oocytes (mature oocytes/total oocytes for each patients) were tabulated (Table I). The total number of oocytes and the number of mature oocytes in the GnRHa + hMG + hCG cycle and hMG + hCG cycle were higher than those in the natural cycle (*P* < 0.0001). The rate of mature oocytes was highest in the hMG + hCG cycle among the three stimulation protocols examined (*P* < 0.04).

The incidences of apoptotic cells among mural and cumulus granulosa cell populations are shown in Fig. 1. The incidence of apoptotic cumulus granulosa cells in the GnRHa + hMG + hCG cycle was significantly higher than those of natural and hMG + hCG cycles (*P* < 0.002). Moreover, the incidence of apoptotic cumulus granulosa cells in the hMG + hCG cycle was significantly lower than that in the natural cycle (*P* = 0.01). The incidence of apoptotic mural granulosa cells in the GnRHa + hMG + hCG cycle was significantly higher than those of the natural and hMG + hCG cycles (*P* < 0.002).

DISCUSSION

Assisted reproductive technologies including IVF have been developed for treatment of infertility patients. There has been a great deal of discussion regarding which stimulation protocol is most appropriate for IVF, but each protocol has its own benefits and no consensus of opinion has yet been reached.

Recently, we reported a relationship between the incidence of apoptotic granulosa cells and the prognosis of ova from patients undergoing IVF treatment

Table I. Comparison Among the Three Different Ovulation Induction Protocols

Parameters	Natural ^a	GnRHa + hMG + hCG	hMG + hCG	<i>P</i> value
No. of total oocytes retrieved	0.8 ± 0.1	5.8 ± 0.9	4.2 ± 0.8	<0.0001
No. of mature oocytes	0.6 ± 0.1	3.6 ± 0.5	3.4 ± 0.6	<0.0001
Rate of mature oocytes (%)	60 ± 11.3	65 ± 2.3	85 ± 4.6	<0.04

^a Values are means ± SEM.

(15–17). The incidence of apoptotic granulosa cells with condensed and fragmented nuclei can be used as a sensitive indicator to estimate the quality of oocytes in an IVF program. This method has two advantages for estimating the quantification of apoptosis; i.e., the granulosa cells with fragmented nuclei are so clear that they can be easily detected, and this method requires only small numbers of cells for quantification of apoptosis.

Our results clearly showed that the hMG + hCG protocol significantly decreased the incidence of apoptotic granulosa cells. These observations sug-

gested that the oocyte quality in the hMG + hCG protocol was best among the three protocols examined in terms of the incidence of apoptotic granulosa cells. Therefore, this protocol is of value for infertility patients failing to retrieve good oocytes in previous cycles in which the other controlled ovarian stimulation protocols were employed.

The use of GnRHa in IVF has been reported to have many benefits; that is, larger numbers of oocytes were collected, higher fertilization rates, longer luteal phases, and higher pregnancy rates (19,20). GnRHa also prevents the premature endogenous LH surge

Fig. 1. Differences in the incidence of apoptotic granulosa cells between the three ovarian hyperstimulation protocols used. Values are means ± SEM of 20 patients. The GnRHa + hMG + hCG cycle showed a significantly higher incidence of cumulus and mural granulosa cells than the other cycles (**P* < 0.002 and #*P* = 0.0002). The hMG + hCG cycle showed a significantly lower incidence of cumulus granulosa cells than the natural cycle (*P* < 0.01).

(4,5), an event that is associated with IVF programs with a decrease in mature oocytes.

On the other hand, detrimental effects of GnRHa on granulosa cells were also demonstrated in rats (8), pigs, and humans (21). GnRHa increases apoptosis in granulosa cells cultured in vitro in a dose-dependent manner (21), and at a concentration of 5 pg/ml buserelin significantly increased the incidence of apoptosis in granulosa cells. Saito *et al.* (22) investigated the plasma levels of buserelin in humans after intranasal administration of 300 μ g of this agent. The maximum concentration of buserelin in plasma was 117 ± 42.5 pg/ml and was still more than 5 pg/ml 4 hr after administration. Considering the dosage used in our study (600 μ g/day), the plasma levels of buserelin in our patients definitely have the possibility of inducing apoptosis in granulosa cells. Our study showed that the GnRHa + hMG + hCG protocol increased the incidence of apoptotic granulosa cells as compared with the hMG + hCG protocol. This may result from the administration of GnRHa and may in turn cause oocytes to be of poor quality.

The incidence of apoptotic granulosa cells in natural cycle IVF is lower than that in GnRHa + hMG + hCG. Taking into account the problems associated with controlled hyperstimulation protocols (23), although the natural cycle protocols yields one oocyte or less, this protocol is an alternative method for follicle development in IVF treatment.

The mechanism by which superovulation improves cycle fecundity in couples with unexplained infertility remains speculative (24). It is possible that hMG may improve a subtle form of ovulation dysfunction that has been suggested to exist in many of these women. These are abnormalities in follicular growth, low estradiol production, and the luteinized unruptured follicle syndrome. Superovulation may lead to an increased probability of fertilization and pregnancy, by increasing the number of fertilizable oocytes ovulated per cycle, the chances of ovum pickup, and the density of gametes in the female reproductive tract. In our study, the hMG + hCG cycles showed a lower incidence of apoptosis than the natural cycle. Therefore, hMG + hCG also may increase fecundity by improving oocyte quality.

In conclusion, considering quality of oocytes, hMG + hCG is the most appropriate controlled ovarian hyperstimulation protocol for IVF in terms of the incidence of apoptotic granulosa cells in patients with no premature endogenous LH surge.

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