Effects of Aging on Ovarian Fecundity in Terms of the Incidence of Apoptotic Granulosa Cells

SEYED HOMAYOON SADRAIE,^{1,2} HIDEKAZU SAITO,^{1,3} TOMOKO KANEKO,¹ TAKAKAZU SAITO,¹ and MASAHICO HIROI¹

Submitted: August 3, 1999

Accepted: November 19, 1999

Purpose: The objective was to determine the effects of women's age on the ovarian fecundity as assessed by the incidence of apoptotic granulosa cells.

Methods: Twenty-eight normo-ovulatory women underwent ovulation induction for standard IVF. The husbands of these women showed severe male infertility factors. The women were divided into four groups according to their ages. Women underwent follicle aspiration after the administration of human menopausal gonadotropin plus human chorionic gonadotropin. The nuclei of granulosa cells were examined by using fluorescence microscopy, and the incidence of apoptotic granulosa cells was tabulated.

Results: Granulosa cells in the older women revealed a significant increase in the number of apoptotic cells. The number of total oocytes and the number of mature oocytes obtained significantly decreased with age. However, endometrial thickness and follicular estradiol, progesterone, and free testosterone levels were not significantly different among four different age groups.

Conclusions: Age increases apoptotic changes in granulosa cells and consequently decreases the ovarian fecundity.

KEY WORDS: Aging; apoptosis; granulosa cells; in vitro fertilization; oocyte quality.

INTRODUCTION

Cell death by apoptosis mediates several important physiological and pathological processes and appears to be intrinsically programmed. Apoptosis is a selective process of physiological cell deletion (1,2), and it plays a major role in the control of the shape and

size of cells in normal and abnormal processes (3,4). Apoptosis also exerts a homeostatic function in relation to tissue dynamics, since the steady state of continuously renewing tissues is achieved by a balance between cell replication and cell death (5). Apoptotic cells do not induce an inflammatory reaction even when present in large numbers, but they are targets of immediate phagocytosis either by macrophages already present nearby or by the adjacent viable cells (6).

Apoptosis always occurs in a single cell surrounded by viable cells in tissues such as liver, gastroduodenal mucosa, and ovaries (7,8). Electron microscopic features of apoptosis include nuclear chromatin condensation, compactness of cytoplasmic organelles, and the appearance of pedunculated protuberances on the cell surface (9). The apoptotic changes are recognized under the light microscope as pyknosis of nuclei, decrease in cell volume, extraordinary surface convolutions, and the explosion of the cell into cytoplasmic fragments containing condensed chromatin (9).

In mammals, the growth and development of ovarian follicles continue uninterruptedly throughout female reproductive life. However, more than 99.9% of follicles undergo the degenerative changes known as atresia at varying stages of follicle development (10). Recent studies have been suggested that apoptosis is the underlying mechanism of ovarian follicle degeneration during atresia (11). Apoptosis in the ovary displays a morphological spectrum similar to that in somatic organs including but not confined to the liver, uterine endometrium, prostate, and adrenal cortex (9). Palumbo and Yeh (8) showed that apoptosis in the rat ovary is associated with atretic follicles and that cells involved were for the most part granulosa cells; however, occasionally theca cells also undergo apoptosis.

The incidence of apoptotic bodies has been applied as a morphological marker for physiological cell renewal in the liver and gastroduodenal mucosa (7),

¹ Department of Obstetrics and Gynecology, Yamagata University, School of Medicine, Yamagata, Japan.

² Department of Anatomy, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Evin, Tehran, Iran.

³ To whom correspondence should be addressed at Department of Obstetrics and Gynecology, Yamagata University, School of Medicine, 2-2-2 Iida-nishi, Yamagata 990-9585, Japan.

as a biomarker for regression of liver hyperplasia (1), prostatic intraepithelial neoplasms and prostatic cancer (12), and for prognosis of patients with neoplasms such as non-Hodgkin's lymphoma (13). There have been few reports concerning apoptosis in human in vitro fertilization-embryo transfer (IVF-ET) programs. However, Sugino *et al.* (14) reported the relationship between the incidence of apoptotic granulosa cells and the size of follicles in IVF program. Seifer *et al.* (15), Nakahara *et al.* (16,17), and Oosterhuis *et al.* (18) recently reported that the incidence of apoptosis in granulosa cells derived from follicular aspirations during conventional IVF procedures may be used as a predictor of IVF-ET outcome. They found that the incidence of apoptotic granulosa cells is a very sensitive indicator to detect oocyte quality and consequently the fecundity on a per follicle and per patient bases. On the basis of these investigations, the incidence of apoptotic cells in membrana granulosa was used to examine ovarian function in an IVF program.

Aged women have been shown to have lower fecundity in many studies that pointed out that the poor quality of oocytes is one of the major determinants (19–22). However, there is little information about effects of age on ovarian fecundity assessed by checking the incidence of apoptotic granulosa cells during IVF treatment.

The aim of the present study was to investigate the effects of aging on the incidence of apoptosis in granulosa cells of normo-ovulatory women in different age groups undergoing follicular aspiration after ovarian hyperstimulation in an IVF program due to male infertility in their husbands' history. We also examined the number of oocytes retrieved, the number of mature oocytes, endometrial thickness, and the follicular fluid hormones among different age groups.

MATERIALS AND METHODS

Patient Selection and Follicle Aspiration

Between March and July 1998, a total of 28 IVF cycles were analyzed at Yamagata University Hospital, Yamagata, Japan. The women with a normal menstrual cycle assigned for IVF program ranged in age from 26 to 43 years. The hormone levels of gonadotropin, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) of the women in the present study were in normal range. The women were divided into four groups by their age (A: ≤ 31 , $n = 7$; B: 32–35, $n = 7$; C: 36–39, $n = 7$; D: ≥ 40 years, $n = 7$). Patients with endometriosis, uncollected tubal disease, and/or

other ovarian disorders were excluded because such diseases might have effects on steroidogenesis and the incidence of apoptosis. Therefore, couples with male factor infertility (the existence of one or more factors in semen analysis, that is, severe oligozoospermia, asthenospermia, teratospermia) were selected for this study. The research was approved by the Yamagata University Hospital Committee for Research on Human Subjects and written informed consent was obtained from all patients; identified clinical information on patients was concealed.

Patients underwent a standard protocol of controlled ovarian hyperstimulation for IVF by using a gonadotropin-releasing hormone (GnRH) analogue, buserelin acetate (Suprecur nasal; Hoechst, Tokyo, Japan), in a long suppression protocol starting from midluteal phase (16). Human menopausal gonadotropin (hMG) (150 to 300 IU/d, Humegon; Sankyo, Tokyo, Japan), with or without FSH (Fertinum P; Serono, Tokyo, Japan), was started on day 3 of the menstrual cycle. Human chorionic gonadotropin (hCG) (10,000 IU; Mochida, Tokyo Japan) was administered when multiple follicular maturation was achieved. Thirty-four to thirty-six hours after the administration of hCG, follicles were aspirated by transvaginal ultrasound retrieval (6.5 MHz; Mochida). All follicles with a mean diameter of >11 mm were aspirated using a 20-ml syringe for suction.

Cell Fixation and Quantification of Apoptotic Cells

Aspirated follicular fluid (FF) was transferred into culture dishes (Falcon 3002; Becton Dickinson and Company, Lincoln Park, NJ). Oocyte-cumulus cell complexes were isolated under a dissecting microscope (SZH-ILLB; Olympus, Tokyo, Japan) at the magnifications of $\times 10$ to $\times 20$ and were put into an organ tissue culture dish (Falcon 3037; Becton Dickinson and Company) with human tubal fluid (HTF) medium (23).

The oocyte-cumulus cell complexes were classified into two groups: complexes with a large, loose cumulus and distinct corona radiata, which were judged as mature, and complexes with a small, dense cumulus and opaque corona radiata, which were defined as immature (24). The mural granulosa cell masses were picked out from the FF, and the mural granulosa cell masses were transferred into a different culture dish to be washed. The mural granulosa cells were then placed on a glass slide, and hyaluronidase (Sigma, St. Louis, MO) (0.1% wt/vol) in HTF medium was added to the cell masses (0.05% wt/vol of final concentration). The cell masses were dispersed thoroughly in

5 min by a fine Pasteur pipette and fixed with 4% wt/vol of neutral buffered formalin for 15 min. This process was completed within 1 hr after follicle aspiration to avoid postaspiration cell death. After fixation, the slides were washed by phosphate-buffered saline three times and the nuclei of cells were stained with 0.5 g/ml of Hoechst 33258 (fluorescent dye; Wako, Osaka, Japan) with 5% wt/vol of DABCO (1,4-diazabicyclo-2,2,2-octane, Sigma) in 90% glycerol: 10% 0.2 M Na₂HPO₃. The DABCO blocked the bleaching effect of fluorescence and allowed the slides to be rechecked within a few weeks. We used a fluorescence microscope to observe apoptotic cells, which were revealed as the granulosa cells containing fragments of condensed chromatin or the cytoplasmic fragments containing condensed chromatin. The apoptotic cells were identified and counted among 1000 granulosa cells at random at $\times 400$ magnification.

On the day of the administration of hCG, the endometrial thickness and the maximal follicle diameter of each patient had been measured by transvaginal ultrasound and registered. In addition, after follicle aspiration, the number of oocytes retrieved and the number of mature oocytes were counted under a dissecting microscope.

FF Collection and Assay for Intrafollicular Steroids

After the oocytes and granulosa cell masses were retrieved, each sample of FF was centrifuged at 3000 rpm for 10 min and stored at -20°C until the assay for estradiol (E₂), progesterone (P), and free testosterone (free T). The quantification of hormonal concentration was performed using commercially available

immunoassays: E₂ by time-resolved fluoroimmunoassay (DELFA Estradiol; Pharmacia, Tokyo, Japan); P and free T by radioimmunoassay (RIA) (DPC Progesterone Kit and DPC Free Testosterone Kit; DPC Corporation, Tokyo, Japan). Reliability criteria for all assays were established. The interassay coefficient of variation was 3.3% for E₂, 7.9% for P, and 10.8% for free T. The interassay coefficient of variation was 5.2% for E₂, 7.2% for P, and $<13\%$ for free T. Hormones were assayed in duplicate within the same assay. The sensitivities of the assays were as follows: E₂, 13.6 pg/ml (conversion factor to SI unit, 3.671); P, 0.05 ng/ml (conversion factor to SI unit, 3.180); and free T, 0.24 pg/ml (conversion factor to SI unit, 3.467).

Statistical Analysis

The data are expressed as mean $\pm$ SEM. For statistical comparison between groups, the Kruskal–Wallis test, analysis of variance (ANOVA), and the Fisher test were applied. $P < 0.05$ was considered as statistically significant.

RESULTS

The overall results of the four age groups are given in Table I. The number of oocytes retrieved from the youngest groups (group A) was significantly higher than those of groups B, C, and D [9.8 ± 1.4 vs. 5.3 ± 1.6 ($P < 0.02$); 5.0 ± 0.8 ($P < 0.01$); and 4.1 ± 0.9 ($P < 0.01$), respectively (mean $\pm$ SEM)]. Similarly, the number of mature oocytes in group A was significantly higher than those of groups B, C, and D [7.8 ± 1.1 vs. 4.1 ± 1.4 ($P < 0.05$); 4.1 ± 0.9

Table I. Comparison Between Different Age Groups^a

Parameters	A: ≤ 31 ($n = 7$)	B: 32–35 ($n = 7$)	C: 36–39 ($n = 7$)	D: ≥ 40 ($n = 7$)
No. of oocyte retrieved	9.8 ± 1.4^b	5.3 ± 1.6	5.0 ± 0.8	4.1 ± 0.9
No. of mature oocytes	7.8 ± 1.1^c	4.1 ± 1.4	4.1 ± 0.9	3.1 ± 0.7
Maximal follicle diameter (mm)	17.2 ± 0.4	18.9 ± 2.7	19.2 ± 0.8	18.1 ± 0.8
Endometrial thickness (mm)	10.9 ± 0.7	9.9 ± 0.8	9.5 ± 0.9	8.8 ± 0.5
Day of follicle aspiration	13.2 ± 0.4	12.4 ± 0.7	13.1 ± 0.6	13.0 ± 0.6
Incidence of apoptotic cells (/1,000)	3.5 ± 0.6^d	5.0 ± 0.4	6.1 ± 0.4	6.4 ± 0.5
FF steroid levels E ₂ level (ng/ml)	446.0 ± 75.9	337.0 ± 41.5	446.0 ± 84.6	313.7 ± 58.9
P level ($\mu\text{g/ml}$)	4.7 ± 0.4	6.1 ± 1.0	5.2 ± 0.7	5.4 ± 1.2
Free T level (pg/ml)	58.7 ± 12.3	43.4 ± 5.8	61.0 ± 24.1	42.4 ± 16.7

^a All values are mean $\pm$ SEM.

^b Significantly different from groups B, C, and D in the number of oocytes ($P < 0.02$; $P < 0.01$, and $P < 0.01$, respectively).

^c Significantly different from groups B, C, and D in the number of mature oocytes ($P < 0.02$; $P < 0.02$, and $P < 0.01$, respectively).

^d Significantly different from groups C and D in the apoptotic index ($P < 0.01$).

($P < 0.02$); and 3.1 ± 0.7 ($P < 0.01$), respectively]. The number of apoptotic cells per 1000 granulosa cells in group A was significantly lower than those of groups C and D (3.5 ± 0.6 vs. 6.1 ± 0.4 and 6.4 ± 0.5 , respectively, $P < 0.01$).

There was a correlation between the incidence of apoptotic cells in granulosa cells and women's age. There also was a progressive increase in the incidence of apoptotic cells with age (Fig. 1). In addition, the endometrium in the youngest group was thicker than that of the oldest group (group A vs. group D; $P = 0.06$), but this difference was not significant. The maximal follicle diameter, the day of follicle aspiration, and the steroid levels in follicular fluid did not show any significant difference or trend among the four groups.

DISCUSSION

Age-dependent decrease in human female fertility begins by the late 30s and by the late 40s fertility is virtually lost. Fertility of women decreases with age not only in a natural cycle but also in assisted reproductive procedure cycles. Attempts to elucidate the factors responsible for the detrimental effect of aging on female fertility historically have pointed toward either poor oocyte quality (ovarian aging) (19–22) or impaired uterine receptivity (uterine aging) (25–28).

Navot *et al.* (19) suggested that poor oocyte quality rather than implantation failure was the leading cause of the age-related decline in female fertility. They showed a high pregnancy rate in older women who received oocytes of younger ones. This would seem

to demonstrate that reproductive aging in the human female is due exclusively to a reduction in oocyte quality, rather than uterine aging. Another study indicated that the oocytes of older mice might already be of poor quality for fertilization at the time of ovulation, whereas the oocyte quality of young mice was good at ovulation (22). Nakahara *et al.* (17) recently concluded that the incidence of apoptotic granulosa cells of women who underwent an IVF program can estimate the quality of oocytes and also can predict the prognosis of oocytes of individual follicles. They also suggested that a poor prognosis for IVF might be associated with a higher incidence of apoptotic cells on a per patient basis and that ovarian function on a per patient basis in an IVF program could be evaluated using this incidence. We found a significantly higher incidence of apoptotic cells in mural granulosa cells in women >40 years than in younger women. This result may mean a poor prognosis in the IVF treatment of aged patients. However, Nakahara *et al.* (16) could not find the difference in the incidence of apoptotic cells among the different age groups. This may be because the patients in his study included endometriosis patients. The endometriosis patients reveal the higher incidence of apoptotic granulosa cells than normal women (16,29). This factor may affect the incidence of apoptosis in younger group; but the patients in this study were in an IVF program due to male infertility factor in their husbands' histories and their ovaries were intact. Thus, the age effects on the incidence of apoptotic granulosa cells was directly revealed without any effect of ovarian lesions.

In our study, the number of oocytes retrieved from older women was significantly lower than that in younger women. This result generally agrees with Nakahara and co-workers' (16) finding that the patients who had higher numbers of oocytes retrieved had a lower incidence of apoptosis in granulosa cells compared to the patients with lower numbers of oocytes retrieved.

Steinkampf (30) mentioned that ovulation induction was most likely to fail in older women due to the diminishing pool of ovarian follicles available for development. In addition, Fujino *et al.* (22) also have shown that age has a profound influence on the number of oocytes harvested in mice stimulated by gonadotropin.

We also measured FF steroids (E_2 , P, and free T) but no significant differences were found among all age groups. Nakahara *et al.* (17) did not find a difference in steroid concentrations in the follicles that contained an oocyte either. The reason for this result is due to a

Fig. 1. The incidence of apoptotic granulosa cells of each case plotted according to their age. The incidence of apoptotic granulosa cells is correlated with age. Each point represents one case. The incidence of apoptotic granulosa cells = $-1.867 + 0.201 \times \text{Age}$ ($r = 0.58$, $P < 0.001$).

unique mechanism of compartmentalization of steroidogenic organelles in the perinuclear region and migration of the multicatalytic proteinase, the proteasome, to the apoptotic blebs (31). As Amsterdam (32) has suggested, steroidogenesis can be maintained in the apoptotic cells as long as the steroidogenic organelles bearing the steroidogenic apparatus remain intact.

CONCLUSIONS

The results of the present study demonstrated that with age, the incidence of apoptotic cells significantly increased in granulosa cells in healthy women with regular menstrual cycles who underwent an IVF program. These findings may have a responsibility for the decline of fertility in women ≥ 40 years old. Further investigations are required to determine the precise mechanisms that produce follicular atresia through apoptosis of granulosa cells in older women.

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