Prognostic Implication of Apoptosis in Human Luteinized Granulosa Cells During IVF–ET

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Purpose: To investigate the prognostic significance of apoptosis in granulosa cells recovered from patients participating in IVF–ET.

Methods: Seventy-three women underwent ovarian hyperstimulation for IVF and embryo transfer. After follicle aspiration, recovered granulosa cells were stained by BCL2 monoclonal antibody with FITC and propidium iodide (PI). Fluorescence was detected by flowcytometry, then the apoptotic index (AI) and BCL2 positivity were assessed.

Results: The pregnant group showed a significantly lower apoptotic index in granulosa cells compared with the nonpregnant group ($p < 0.0001$). Patient's age, basal serum FSH concentration, serum E₂ concentrations at hCG day, number of retrieved oocytes, fertilization rates, number of embryos transferred, and BCL2 positivity were not different between the two groups. Linear regression analysis of AI to serum basal FSH showed a positive correlation. AI in granulosa cells of 6.14% or below could predict a successful pregnancy with a sensitivity of 87.5% and a specificity of 73.7%.

Conclusion: Our results indicate that apoptotic analysis within granulosa cells can be used as a prognostic indicator for IVF success.

KEY WORDS: Apoptosis; flowcytometry; granulosa cells; IVF–ET.

INTRODUCTION

Although there are numerous prognostic indicators suggestive of IVF–ET outcomes (1–3), they are still evolving through understanding basic mechanisms of ovarian follicle biology. During the development of the ovary, granulosa cells (GCs) appear to be critical determinants of oocyte fate. For instance, only those oocytes that become surrounded by a layer of GC

enter dictyotene and survive to become primordial follicles (PFs), whereas the other oocytes without GC development die instead of entering dictyotene (4,5). After PFs are first formed, some are recruited to grow and develop through the preantral (FSH independent) and early antral periods of folliculogenesis (6). The target tissue for FSH is GCs from antral stage and undergoes a well-defined response to FSH stimulation in terms of growth and cytodifferentiation. Moreover, recent studies strongly suggest that morphogens produced by the oocyte, such as GDF-9 and BMP-15 play an important role in determining GC growth in response to FSH stimulation (7–9). This concept implies that the full-grown oocyte could play a critical role in determining the primary FSH responses within the developing dominant follicle (9). Given the central role of FSH in the regulation of selection and dominant follicle (DF) formation, it is

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becoming increasingly clear that the oocyte and granulosa interactions are mandatory for the selection of DF. Thus, considering the tight relationship between the oocyte and granulosa cells, it appears that the status of granulosa cells could reflect oocyte condition.

It has long been appreciated that apoptosis is associated with follicular atresia occurring in natural (10), and stimulated cycles (11–14). Apoptosis is a process of physiologic, active cell death to implement efficient disposal of cells within tissues (15–17). Apoptosis shows distinct morphological features quite different from that in necrosis. Under the electron microscope, nuclear chromatin condensation, compacted cytoplasmic organelles, and the appearance of pedunculated protuberances on the cell surface were commonly observed. Fragmentation of DNA into the nucleosomal level leads to the typical “step-ladder” running patterns seen on gel electrophoresis (16). Fragmentation of the cell or condensed chromatin results in the formation of an apoptotic body, one of the typical, latest findings in the apoptotic cascade.

During IVF–ET, low oocyte retrieval, empty follicles, poor oocyte fertilization, poor embryo quality, and therefore, poor IVF success have been reported in correlation with apoptotic bodies in membrana granulosa (12,13). However, those methods seem to be impractical as they require time-consuming laborious work to count the apoptotic bodies under fluorescent or electron microscope. Instead, flowcytometry has more advantages in its ability to measure quantitatively large numbers of cells in a short time as well as to evaluate the cell cycle stage of granulosa cells.

In this study, we introduce a simple, quantitative method using flowcytometry for the identification of apoptosis within granulosa cells obtained from patients undergoing IVF to correlate apoptosis of granulosa cells with pregnancy outcome.

MATERIALS AND METHODS

Patients Selection

The protocol was reviewed and approved by IRB of Seoul National University Hospital, Clinical Research Center. Follicular fluids were obtained from 73 patients undergoing ovarian hyperstimulation and IVF–ET. Patients were selected randomly from those who gave their consent to this study. The causes of infertility were as follows: male factor ($n = 11$), female factor ($n = 47$), combined male and female factors ($n = 8$), and unexplained factor ($n = 7$). Female factors other than tubal factor were excluded.

Ovarian Stimulation and ART

Ovarian stimulation was performed with highly purified FSH (Metrodin-HP, Serono, Aubonne, Switzerland) after pituitary suppression using GnRH agonist (Decapeptyl, Ferring, Malmo, Sweden) with either long ($n = 61$) or flare protocol ($n = 12$). Ovarian response was monitored frequently with transvaginal ultrasonography. When a leading follicle reached 18 mm or more in diameter, 10,000 IU of hCG (Profasi; Serono, Aubonne, Switzerland) was administered. Transvaginal oocyte aspiration was performed 36 h after hCG injection, and oocyte–cumulus cell complexes were isolated. Oocytes were incubated 4–6 h before insemination. In case of male factor, ICSI was performed as previously described (18). Fertilization was confirmed 16 h later by determining the presence and number of pronuclei. Embryos were graded and scored for quality using the cumulative embryo score (CES) as previously described (19). Embryos were replaced transcervically into the uterus 48–72 h after insemination. Luteal support was provided by 50 mg of progesterone in oil (Progest, Samil Pharmacy, Seoul, South Korea) daily from the day of ET. Pregnancy was defined by the presence of intrauterine gestational sac(s) with pulsating fetal heart beats 6 weeks after ET.

Preparation of Sample

Each tube of follicular fluid containing granulosa cells was mixed within the same patient. Preparation of granulosa cells was performed immediately after follicular collection and flowcytometry was also performed without cell freezing. Cells were prepared as previously described with modification (20). In brief, after isolation of oocyte–cumulus cell complexes, the remaining follicular fluid from each patient was centrifuged at 300g for 10 min. Resultant cell pellets were washed with phosphate buffered saline (PBS) (pH 7.4) and resuspended in mixture of Ham's F-10 culture medium and 90% percoll solution (1:1, v/v). Red blood cells were removed by treating with Red Blood Cell Lysing buffer (Sigma, Cat. No. R7757, U.S.A.), and then, centrifuged at 300g for 10 min, and washed twice with PBS. The cell suspensions were filtered through a commercial nylon filter (Spectra/Mesh, Spectrum, U.S.A.) with a pore size of 30 μ m, and PBS was added to adjust the final cell concentrations to $0.5\text{--}1.0 \times 10^6/\text{mL}$. Cells were fixed using $10\times$ volume of 70% ethanol for at least 30 min, then centrifuged at 300g for 10 min and washed by PBS. Cell pellets were suspended with 5 mL of lysis

solution, and centrifuged at 300g for 10 min. The lysis solution was prepared by mixing PBS with Triton X-100 (0.5%), EDTA (0.2 mg/mL), and bovine serum albumin (1.0%). Cell pellets were resuspended with 1 mL of lysis solution and reacted with 20 μ L of FITC-labelled BCL2 monoclonal antibody (Pharmingen, Cat. No. 6511KK, U.S.A.) for 30 min. After washing with PBS, cell pellets were resuspended with 800 μ L of PBS, and treated with 100 μ L of RNase (1 mg/mL, Sigma, U.S.A.) and 100 μ L of propidium iodide (PI, 0.4 mg/mL, Sigma, U.S.A.) for at least 15 min.

Flowcytometry

Fluorescence was detected using a flowcytometer (FACSCalibur, Becton Dickinson, U.S.A.), equipped with 488 nm argon laser (15 mW). The wavelength for the detection of FITC was 510–530 nm and for PI, 550–590 nm was used. BD DNA Quality Control Particles (Becton Dickinson, U.S.A.) was used for testing and documenting optimal instrument performance for DNA analysis. Vial A, chicken erythrocyte nuclei (CEN), was used to monitor coefficients of variation (CVs) which was less than 3%. DNA histograms were analyzed using the software CELLQuest program (ver. 3.1) installed within the cytometer. The cell cycle phase was determined as previously described (21). Apoptotic index (%) was defined as the proportion of area left to the diploid peak at the PI fluorescence. Fluorescence less than 15% of the diploid peak was excluded from the data analysis. BCL2 positivity (%) was defined as the relative proportion of cells showing FITC fluorescence. At least 10,000 cells were analyzed per patient.

Hormone Assay

Blood was taken from each patient on the day of hCG injection for the measurement of serum estradiol concentration and on menstrual day 3 for FSH measurement. The sera were stored at -20°C until assay. Quantification of hormonal concentration was performed using commercially available radioassay kits for estradiol (Serono Diagnostics, Switzerland) and FSH (American International Plc, U.K.). Intraassay and interassay coefficients of variation were 1.3 and 0.4% for estradiol, 1.6 and 4.7% for FSH respectively. The radioimmunoassay was based on second international standard (2nd IS). The conversion equations from the 2nd IS to the 3rd IS are as follows respectively: for LH, 3rd IS = (2nd IS $\times$ 0.52) + 0.5, for FSH, 3rd IS = (2nd IS $\times$ 0.525) + 0.4, for β -hCG, 3rd IS = 2nd IS $\times$ 2.07.

Data Analysis

Data are presented as mean $\pm$ SD. The statistical significance of differences was determined by student's *t* test and linear regression analysis where appropriate. Sensitivity and specificity were calculated, and the receiver operating characteristic (ROC) curve was plotted by changing criteria of sensitivity and specificity. A *p* value below 0.05 was considered statistically significant.

RESULTS

The mean age of the females was 33.4 ± 4.0 years, the mean number of trials was 2.5 ± 1.9 , and the mean duration of infertility was 5.5 ± 3.6 years respectively. The mean basal serum FSH concentrations in the female was 12.2 ± 5.7 mIU/mL.

Outcomes were compared between pregnant ($n = 16$) and nonpregnant ($n = 57$) groups. Among the various parameters shown in Table I, only apoptotic index was significantly lower in the pregnant group compared to the nonpregnant group.

A positive correlation was observed between the apoptotic index and basal serum FSH concentrations (Pearson's correlation test, $n = 22$, $r = 0.667$, $p = 0.038$) (Fig. 1). However, the apoptotic index had no correlation with female age, serum E_2 concentrations

Table I. Comparison of Variable Parameters Between Pregnant and Nonpregnant Group

	Pregnant ($n = 16$)	Nonpregnant ($n = 57$)
Age of female (years)	33.3 ± 2.7	33.5 ± 4.4
Female basal serum FSH (mIU/mL)	12.8 ± 5.9	12.2 ± 5.8
Duration of infertility (years)	4.2 ± 2.9	5.9 ± 3.7
Number of trial	2.8 ± 1.8	2.5 ± 1.9
Ovarian hyperstimulation		
Long protocol + FSH	14 (87.5%)	47 (82.5%)
Flare-up protocol + FSH	2 (12.5%)	10 (17.5%)
Peak E_2 at hCG day (pg/mL)	2158 ± 1153	1983 ± 872
Number of oocytes aspirated	8.5 ± 3.5	7.6 ± 4.2
Number of ICSI cases	4 (25.0%)	19 (33.3%)
Number of metaphase II oocytes	7.8 ± 4.8	6.9 ± 3.8
Number of oocytes fertilized	5.8 ± 3.4	4.9 ± 3.1
Fertilization rate (%)	69.1 ± 22.3	70.9 ± 22.4
Number of embryos transferred	4.1 ± 1.5	4.0 ± 1.8
CES	73.4 ± 49.1	66.4 ± 48.8
Number of AH cases	2 (12.5%)	6 (10.5%)
Apoptotic index (%) ^a	4.68 ± 2.72	13.61 ± 9.26
Positive BCL2 (%)	42.88 ± 17.31	36.28 ± 17.89

Note. CES: cumulative embryo score; AH: assisted hatching using acid Tyrode's solution.

^a $p < 0.0001$ (Student's *t* test, two-tailed).

Fig. 1. A positive correlation between basal serum FSH and apoptotic index (%) in granulosa cells obtained from follicular fluids in IVF patients (Pearson's correlation test, $n = 22$).

on the day of hCG injection, number of oocytes aspirated, fertilization rates, CES, or number of embryos transferred (data not shown). BCL2 positivity had a negative correlation with the apoptotic index (Pearson's correlation test, $p = 0.037$).

An apoptotic index of less than 6.14% could predict a successful pregnancy significantly with a sensitivity of 87.5% and a specificity of 73.7% (Table II). A BCL2 positivity of greater than 37.61% could also predict successful pregnancies with a sensitivity of 68.7% and a specificity of 61.4% (Table II).

Table II. Result of Receiver Operating Characteristic Curve for Predicting a IVF Success in Various Parameters

	Cut-off (AUC, 95% CI)	Sen	Spe
Age of female (years)	≤ 38 (0.501, 0.381–0.620)	100%	17.5%
Basal serum FSH (mIU/mL)	≤ 8.6 (0.512, 0.293–0.728)	50.0%	80.0%
Peak E ₂ at hCG day (pg/mL)	> 2733 (0.522, 0.400–0.663)	38.5%	83.7%
Oocytes aspirated	≤ 7 (0.588, 0.466–0.703)	60.0%	61.4%
Embryos transferred	> 4 (0.528, 0.408–0.646)	50.0%	59.6%
CES	> 18 (0.551, 0.429–0.668)	100%	19.3%
Positive BCL2 (%) ^a	> 37.61 (0.630, 0.509–0.741)	68.7%	61.4%
Apoptotic index (%) ^a	≤ 6.14 (0.832, 0.727–0.909)	87.5%	73.7%

Note. AUC: area under the curve; CI: confidence interval; Sen: sensitivity; Spe: specificity.

^aCompared by AUC between positive BCL2 and apoptotic index; $p < 0.05$.

Fig. 2. Receiver operating characteristic curve showing that apoptotic index (%) have a better predictability of a successful pregnancy compared with positive BCL2 (%) in granulosa cells obtained from follicular fluids in IVF patients (difference between areas = 0.202, $p = 0.025$).

However, the apoptotic index showed higher predictability power than BCL2 positivity in predicting a successful pregnancy after IVF (Fig. 2). There was no statistical difference in apoptotic index and BCL2 positivity between the two regimens for ovarian hyperstimulation.

DISCUSSION

In the human, when an appropriately high FSH threshold is reached in one Graafian follicle (GF), it is selected to become a dominant follicle (DF). By contrast, the small GFs in the cohort with sub-threshold levels of FSH become nondominant and will eventually undergo apoptosis. If exogenous gonadotropins are added, some of the follicles destined to become atretic can be rescued. However, after gonadotropic ovarian stimulation with currently used protocols, only 80% of these follicles contain healthy metaphase II oocytes, while 10% are already in the process of apoptosis and 10% have not achieved meiosis (22). This suggests that rescue of some follicles by gonadotropin stimulation may cause multiple selection lacking in normal changes, which should occur during the stages of physiological GF development. These major changes occurring in normal ovaries involve cytodifferentiation of granulosa cells as well as

cytoplasmic and nuclear maturation of the oocyte. Recent studies strongly suggest that morphogens produced by the oocyte play an important role in determining which genes are expressed in the GC in response to FSH stimulation (7–9). This concept implies that the full-grown oocyte could play a critical role in determining the primary FSH responses within the developing dominant follicle. Thus, it may be hypothesized that GCs status can be the mirror image of oocyte quality. It is noteworthy that this could be a very useful concept in the clinical field, because there is a lot of asynchronism between nuclear and cytoplasmic maturation of the oocyte that hinders accurate diagnosis of oocyte quality directly at the time of retrieval. If proper evaluation on the status of granulosa cells can be obtained, it can raise diagnostic accuracy on oocyte condition. It has long been appreciated that apoptosis is associated with follicular atresia occurring in natural (10), and stimulated cycles (11–14). Therefore, proper evaluation of the apoptosis in granulosa cells, in turn, can predict whether an oocyte will be successfully fertilized and become pregnant during assisted reproduction.

Apoptosis as a function of ovarian reserve in women undergoing IVF was initially provided by Seifer *et al.* (11). They demonstrated that women undergoing IVF with day 3 serum FSH concentration greater than 10 mIU/mL have an increased percentage of cells undergoing apoptosis compared to women with a FSH value of less than 6 mIU/mL. This was further supported by other researchers suggesting that apoptotic cell death in granulosa lutein cells could be a possible indicator of IVF outcome (12,14). Our results show similarity with previous reports (11,14). Relatively high level of day 3 FSH in our results is totally due to the differences in the methods that we used. We used the 2nd IS as a reference, which is higher than the 3rd IS. If the day 3 FSH is converted from the 2nd IS to the 3rd IS, the mean serum concentration will be from 12.2 ± 5.7 (2nd IS) to 6.8 ± 3.4 (3rd IS).

The apoptotic index was positively correlated with the serum concentration of day 3 FSH (Fig. 1) and the pregnant group showed a smaller apoptotic index (Tables I and II). Moreover, if the apoptotic index is less than 6.14%, a successful pregnancy could be predicted with a sensitivity of 87.5% and specificity of 73.7% respectively. Thus, this test could be tried limitedly in those patients showing repeated implantation failure with normal oocyte morphology, fertilization rate, and without apparent endometrial pathology.

One thing different from the methodology discussed in previous reports is that for the identification

of apoptotic granulosa cells within follicular fluids, we used PI, which is one of the intercalative dyes, for DNA staining. This method originally was developed to analyze the cell cycle distribution, therefore, it can readily quantify the proportion of cells in G0/G1, S, and G2/M phase when fluorescent dyes are used. Since apoptotic cells typically decrease in size, in part due to nuclear condensation, the flowcytometer is able to detect apoptotic cells as smaller, less fluorescent entities. Although a peak of DNA fluorescence is observed in the hypodiploid region of the cell cycle, this peak is often variable in width and occasionally overlaps with the G0/G1 peak. Nevertheless, we could obtain a sharp, well-separated peak containing apoptotic cells with a careful fixation and staining protocol. Another question that can be raised is the contamination of WBCs after nylon filter, because the presence of WBCs could be confounding variable. To test this, we stained the sample with Giemsa in five different patients after filtration through nylon. All of the samples didn't show any granulocytes (neutrophil, eosinophil, basophil) as well as some agranulocytes (monocyte, erythrocyte). However, lymphocytes could be observed under high power fields in certain areas of slide examined, but not more than 4% (range: 2.1–3.9%) of total cellular counts ($0.5 \times 1.0 \times 10^6$). Thus, we accept that this may be one of confounding variables to the results of our experiment. But this may be a minor effect to our experiment because of significantly higher differences in apoptotic index between pregnant and nonpregnant group than contamination rate of WBCs.

Bcl2 is a well-known antiapoptotic gene expressed in the human granulosa cells (23). In the granulosa cells, BCL2 positivity was significantly higher in the pregnancy group, as expected (Table I). However, because the sensitivity of the test was lower as 68.7%, BCL2 positivity does not appear to be useful as a screening method to predict pregnancy in women undergoing IVF (Table II). This result may be explained by considering the underlying mechanism for granulosa apoptosis: On one side is a positive system involving the suppression of apoptosis (antiapoptotic), on the other hand is a negative mechanism involving a specific component directed toward blocking a trophic response (such as FSH action) which then results in the activation of the apoptotic pathway (proapoptotic). Thus, further studies regarding the relative expression of pro- and antiapoptotic genes are required before use as a new screening method.

Our study was not designed to assess the number of apoptotic granulosa cells per follicle, but per patient. It is speculated that the intensity of apoptosis is different from follicle to follicle, even in the same superovulatory period. Thus, although investigation on the follicular basis seems to be the proper way, this is practically difficult to perform in the clinical setting. Moreover, even after an observation on the follicular basis, it is still not certain which embryo is implanted after transferring two or three embryos. Therefore, more refined future studies are required through investigation on the follicular basis and with single embryo transfer at the blastocyst stage.

In conclusion, our results indicate that detection of the apoptotic index in the luteinized granulosa cells might be used as a prognostic indicator of successful pregnancy in women undergoing IVF.

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