Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Obstetrics & Gynecology and Reproductive Biology 110 (2003) 181–185
EUROPEAN JOURNAL OF OBSTETRICS & GYNECOLOGY AND REPRODUCTIVE BIOLOGY
www.elsevier.com/locate/ejogrb

Apoptosis in human granulosa cells after induction of ovulation in women participating in an intracytoplasmic sperm injection program

Ana Clavero[a], Jose A. Castilla[b,*], Ana I. Núñez[b], Mª García-Peña[b], Vicente Maldonado[b], Juan Fontes[b], Nicolás Mendoza[b], Luis Martinez[b]

[a]*Servicio de Análisis Clínicos, Hospital Virgen de las Nieves, Granada, Spain*
[b]*Unidad de Reproducción, Hospital Universitario Virgen de las Nieves Avda, Fuerzas Armadas s/n, 18014 Granada, Spain*

Received 4 June 2002; received in revised form 28 February 2003; accepted 26 March 2003

Abstract

Objectives: To investigate whether analysis of granulosa cell apoptosis can be useful in assessing follicular and oocyte maturation and the regulation of granulosa cell apoptosis by follicular fluid steroids in preovulatory follicles of stimulated women. *Study design*: Apoptosis in aspirated granulosa cells ($n = 64$) was measured using the Annexin V-affinity assay by flow cytometry. Follicular fluid steroids were determined by ELISA and RIA. Statistics were evaluated using the Levenne test, Student *t*-test and simple linear regression analysis. *Results*: No significant differences in the number and percentage of apoptotic granulosa cells per follicle were observed according to the maturity and fertilizability of the oocytes by intracytoplasmic sperm injection within these follicles. No correlations were found between levels of steroid hormones in follicular fluid and the number and proportion of granulosa cells undergoing apoptosis. *Conclusions*: The percentage of apoptosis in granulosa cells is not related to oocyte maturity and fertilizability by ICSI or to follicular quality in stimulated cycles of normal women. However, the possibility cannot be discarded that this parameter may be of importance at other phases of follicular development or in natural cycles when no treatment that influences follicular physiology is being applied.

Keywords: Annexin V; Apoptosis; Flow cytometry; Human granulosa cells; ICSI

1. Introduction

Tissue homeostasis is maintained by a delicate balance between cell proliferation and cell death. The latter may arise not only from externally inflicted mechanical or chemical damage (necrosis), but also from a suicidal process that is controlled from within the cell (apoptosis). Apoptosis differs from necrosis in that the cell plays an active role in its own destruction. It has characteristic ultrastructural features (condensation and fragmentation of chromatin) and biochemical changes (caspase proteolytic cascade) [1,2]. The process is genetically controlled and can be triggered by an internal clock or by extracellular agents such as hormones, cytokines and numerous chemical, physical or viral agents. From a biochemical point of view, one of the most important characteristics of the apoptotic process is the loss of phospholipid asymmetry of the plasma membrane. Apoptotic cell death is accompanied by a change in plasma membrane structure by surface exposure of phosphatildylserine (PS), while the membrane integrity remains unchallenged. Surface exposed PS can be detected by its affinity for Annexin V, a phospholipid binding protein, using fluorescently labelled Annexin V and flow cytometry [3]. PS externalisation is a very early phenomenon during apoptosis, initiated at a time following the caspase proteolityc cascade but preceding nuclear condensation and fragmentation of chromatin [3].

In mammalian species atresia is a prominent feature of ovarian follicular development [4]. Less than 0.1% of all follicles present at birth mature and ovulate, whereas more than 99.9% of the follicles undergo atresia. It has been shown that follicle atresia is caused by apoptosis which occurs at first in granulosa cells (GCs) [5]. GCs are essential in ovarian folliculogenesis, due to the production of factors that are necessary in a normal follicular maturation process, like steroids and growth factors. For an adequate follicular response to gonadotropins, it is necessary a suitable number of GCs, which is determined by both the rate of proliferation and the rate of cell death by apoptosis [6].

* Corresponding author. Tel.: +34-58-020052; fax: +34-58-020226.
E-mail address: jcastilla@hvn.sas.junta-andalucia.es (J.A. Castilla).

0301-2115/$ – see front matter © 2003 Published by Elsevier Ireland Ltd.
doi:10.1016/S0301-2115(03)00243-4

The apoptotic process is regulated by different mechanisms. One of the most important is the modulation produced by gonadotropins and gonadal steroids. Treatment with estrogens increases follicular growth, ovarian weight and the mitotic index of GCs [7]. Gonadotropins and estrogens have an antiatretogenic effect mediated by the prevention of GC apoptosis and the stimulation of GC mitosis, whereas androgens enhance GC death by apoptosis [8]. A close relationship has been reported between apoptosis and assisted reproduction techniques, based on the incidence of apoptotic GC in the follicle. A lower number of apoptotic GCs is associated with a higher number of oocytes after ovarian stimulation [9] and with oocytes with a better outcome [10–12].

In an attempt to define the regulation of GC apoptosis by follicular fluid (FF) steroids, we studied the relation between the percentage of apoptosis in GCs in a preovulatory follicle and the levels of steroids in FF. To determine whether analysis of GC apoptosis can be useful in assessing follicular and oocyte maturation in preovulatory follicles of stimulated women, we measured these parameters in GCs aspirated at the time of oocyte retrieval for intracytoplasmatic sperm injection (ICSI) using the classic method of the Annexin V-affinity assay. The number and proportion of GCs in apoptosis was related to the maturity and fertilizability of the oocytes within these follicles.

2. Study design

GCs were obtained from a total of 64 follicles from 55 couples with male infertility. Repeated semen analyses for the husband were determined pathological according to World Health Organization recommendations (World Health Organization, 1999). All women had regular menstrual cycles and Day 3 FSH and PRL, and Day 21 progesterone were normal. Tubal and uterus normality was based on histerosalpingography and ultrasonography studies. The patients ages ranged from 24 to 36 years.

The women were down-regulated with gonadotrophin releasing hormone agonist triptorelin (Decapeptyl 0.1, Lasa, Barcelona, Spain) for 3–5 weeks, starting on cycle Day 21 until the administration of FSHr, when the doses where reduced by half. After 10–14 days, they received FSHr (Gonal F, Serono, Madrid, Spain) until the follicles reached maturity. When more than three follicles had a diameter of >18 mm, ovulation was stimulated by the administration of 5000 UI of human chorionic gonadotrophin (hCG) (Profasi HP, Serono, Madrid, Spain). Oocyte retrieval was performed by transvaginal needle-guided ultrasound aspiration 36 h after hCG injection. Each follicle was systematically washed in order to avoid any spill to the next follicle. Oocytes were enzymatically denuded of cumulus cells with a solution containing 80 IU hyalorunidase/ml (HYASE, IVF Science Scandinavia, Gothenburg, Sweden). Corona radiata cells were removed mechanically with the fire-polished tip of a Pasteur pipette. Oocyte maturity was judged by morphological criteria. The ICSI procedure was carried out only in morphologically normal metaphase II oocytes that had extruded the first polar body. Motile spermatozoa were selected by the Swim-up procedure, which consists of washing the semen with the Gamete medium (IVF Science Scandinavia, Gothenburg, Sweden) by centrifugation at 300 × g. The supernatant was discharged and in the same tube 1 ml of the medium was added and left standing for 30 min to allow the sperm to swim up from the pellet. Just before injection 2 μl of the prepared sperm suspension were added to 5 μl of a polyvinylpyrrolidone (PVP) solution (ICSI, IVF Science Scandinavia, Gothenburg, Sweden) to slow down the spermatozoa. Only one spermatozoon was aspirated, tail first, from the PVP solution and was injected into the cytoplasm of the oocyte through the zona pellucida. The injection pipette was withdrawn slowly from the oolema and oocytes were placed in 1 ml of IVF medium (IVF Science Scandinavia, Gothenburg, Sweden) on a four-well dish. Between 16 and 20 h after the ICSI procedure, oocytes were observed under the stereomicroscope (Eclipse TE-200, Nikon, Tokyo, Japan) for the presence of pronuclei and polar bodies.

After removal of the oocyte from the aspirated FF, it was centrifuged for 5 min at 400 g, and the supernatant was collected and stored at −80 °C until its analysis. The pelleted cells were resuspended in PBS, and were carefully overlaid on Ficoll-Hypaque (Sigma Diagnostics, St. Louis, MO, USA) and centrifuged at 400 × g for 30 min. GCs were collected from the interphase and washed twice with phosphate-buffered saline. The total number of cells isolated were counted on a Neubauer camera.

The FF concentrations of estradiol and progesterone were determined by ELISA immunoassay commercial kits (Biomerieux, Marcy-l'Etoile, France). Levels of testosterone in FF were measured by commercial RIA kits (Orion Diagnostica, Espoo, Finland).

Apoptosis was measured using the commercial kit Annexin V-FITC (R&D Systems, Minneapolis, USA; Fig. 1). Cells were stained with Annexin V, a phospholipid binding protein that detects early apoptosis, and with propidium iodide (PI), a membrane impermeable stain, to discriminate between dead and apoptotic cells. Briefly, the Annexin V incubation reagent was prepared by mixing 10 μl of 10× binding buffer, 10 μl of PI, 1 μl of Annexin V-FITC and 79 μl of distilled water. The cells were gently resuspended in the Annexin V incubation reagent in the dark for 15 min at 18–24 °C. Finally, 400 μl of 1× binding buffer were added to each sample. After staining, cells were analyzed by flow cytometry (Cytoron Absolute, Ortho, Madrid, Spain) within 1 h for maximal signal. Vital cells are negative for both PI and Annexin V, apoptotic cells are Annexin V positive and PI negative, while necrotic cells are positive for both PI and Annexin V. Positive control samples consisted of lymphocytes treated with 10^{-4} M dexamethasone for 8 h [9], while negative controls consisted of GCs without staining with the Annexin V-FITC kit.

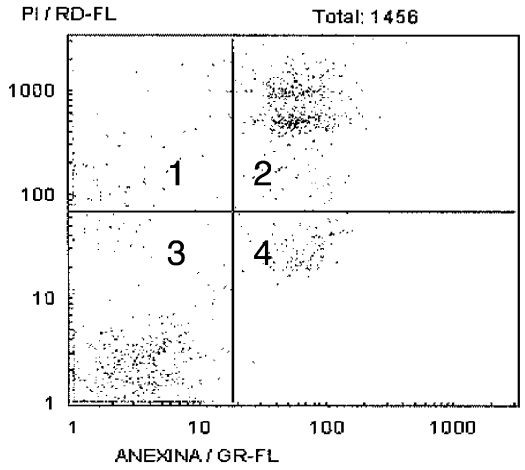

Fig. 1. Granulosa cell apoptosis measured with Annexin V-FITC kit. Cells in quadrant 4 are apoptotic. Quadrants 1 and 2 represent damaged and dead cells, respectively, and vital cells are situated in quadrant 3.

Shapiro-Wilk's test was used to test whether data were normally distributed. The data not conforming to a normal distribution (number of apoptotic GCs) were mathematically transformed (log) prior to analysis. Statistics were evaluated using the Levenne test to check the variance homogeneity. If this test was significant we used the Welch test to compare the means, and if it was not, we used the Student *t*-test. Simple linear regression analysis was used to show the degree of linear association between the percentage of apoptosis in GCs and the hormones studied.

3. Results

Apoptotic GCs were observed in all FFs analyzed, and ranged between 250 and 123,000 cells per follicle, with a mean and standard deviation of $8899 \pm 17{,}057$ apoptotic GCs. The proportion of apoptotic GCs in preovulatory follicles averaged $3.3 \pm 1.9\%$ of the studied cells, with a range of 0.4–8.0%.

Table 1 shows that the number of apoptotic GCs per follicle was not significantly different between follicles with metaphase I oocytes and those with metaphase II oocytes (8381 ± 7536 versus $9064 \pm 19,193$ apoptotic GCs). No significant differences in the percentage of apoptosis in GCs were found in follicles with metaphase I oocytes and those with metaphase II oocytes. There was no difference in the number of apoptotic GCs from FF from which fertilized oocytes were obtained and those with oocytes that remained unfertilized after ICSI (Table 2). The percentage of apoptosis in GCs from FF from which fertilized oocytes were obtained and those with oocytes that remained unfertilized after ICSI was similar ($3.1 \pm 0.3\%$ versus $3.5 \pm 0.5\%$ apoptotic GCs). No correlations were found between levels of steroid hormone in FF and the number and proportion of GCs undergoing apoptosis (Table 3).

4. Comment

It has been claimed that the incidence of apoptosis in GCs could be indicative of the ovarian function and a prognosis factor in a program of assisted reproduction techniques. Oocytes that come from follicles with a low percentage of GCs in apoptosis have good prognoses, that is, such oocytes are fertilized and grow into good-quality embryos after IVF [10–12].

We found no relationship between the percentage of apoptosis and oocyte maturity and fertilizability by ICSI. These results are in accordance with Saito et al. [13], who did not find any relationship between GC apoptosis and oocyte fertilizability by ICSI. However, these authors and others, such as Nakahara et al. [11], found a correlation between the two parameters when IVF was used as the assisted reproduction technique. These findings can be explained by the requirement of the IVF procedure of physiologic sperm-oocyte interactions that succeed only with a good-quality oocyte (i.e. one with less apoptosis of GCs), whereas ICSI bypasses these natural barriers by its invasive nature and thus does not

Table 1
Relationship between number and percentage of apoptosis in GCs and oocyte maturity

	Metaphase I ($n = 16$)	Metaphase II ($n = 48$)	t_{exp}	P
Number of apoptotic cells	8381 ± 7536 (1650–28500)	9064 ± 19193 (250–123000)	0.13	N.S.
Percentage of apoptosis	3.8 ± 0.5 (0.6–7.0)	3.2 ± 0.3 (0.4–8.0)	1.05	N.S.

Values are mean ± S.D.. Numbers in parentheses are ranges.

Table 2
Relationship between number and percentage of apoptosis in GCs and oocyte fecundability

	Unfertilized ($n = 14$)	Fertilized ($n = 34$)	t_{exp}	P
Number of apoptotic cells	5657 ± 3642 (2000–15800)	10886 ± 23011 (250–123000)	0.77	N.S.
Percentage of apoptosis	3.5 ± 0.5 (1.0–7.9)	3.1 ± 0.3 (0.4–8.0)	0.73	N.S.

Values are mean ± S.D.. Numbers in parentheses are ranges.

Table 3
Pearson's simple linear correlation coefficients between number of apoptotic cells and percentage of apoptosis in GCs and levels of steroids in FF ($n = 64$)

	E_2	Progesterone	Testosterone
Number of apoptotic cells	0.19	0.07	0.10
Percentage of apoptosis	0.12	−0.01	−0.03

require a good-quality oocyte. These results indicate that if the quality of the oocyte, measured by the apoptosis in GCs, is low, it has a greater chance of being fertilized with ICSI than with the IVF procedure.

Oosterhuis et al. [12] also found an increase in the number of embryos obtained by IVF from patients with a low percentage of GC apoptosis. The differences described can also be attributed to the use of a pool of FF of each patient, whereas we studied this percentage in FF from individual follicles. The separation of FF allowed us to study the relationship between GC apoptosis and the maturity and fertilizability of the corresponding oocyte. In follicles stimulated with FSH, a survival factor, GC apoptosis tends to be low [14], and so it is understandable that we did not find any relation between GC apoptosis and parameters of oocyte quality. However, in other stages of follicular development GC apoptosis could be of importance in the regulation of follicular physiology.

A further factor that should be taken into account is that we studied women in treatment due to male factor, whereas the above authors studied women with gynaecologic diseases. It has been shown that women with endometriosis [10] and with deficit of ovarian reserve [9,15] have a high percentage of apoptosis. We consider it necessary to exclude these patients from the study group for a correct analysis of this parameter.

In our study, the incidence of apoptosis was 3.3% of the GCs present in FF. These results contrast with the 0.5% of GC apoptosis obtained by Seifer et al. [9], a discrepancy that may be attributed to the use of different techniques of measuring apoptosis. The loss of plasma membrane asymmetry is an early event in apoptosis, irrespective of the cell type, resulting in the exposure of PS residues at the outer plasma membrane leaflet. Annexin V specifically binds the PS residues that are exposed on the outer leaflet of the plasma membrane of apoptotic cells, but is not able to bind to normal vital cells since the molecule is not able to penetrate the phospholipid bilayer. In dead cells, the inner leaflet of the membrane is available for binding of extrinsically applied Annexin V, since the integrity of the plasma membrane is lost. The availability of biotin or FITC labelled Annexin V provides a useful tool to measure apoptosis in nucleated cells. In this way, vital apoptotic and dead cells can be discriminated on the basis of a double-labelling for Annexin V and a membrane impermeable DNA stain, such as PI, and analysed afterwards by flow cytometry [3]. Note that the PS exposure analysed in the Annexin V assay is a very early phenomenon during apoptosis, preceding nuclear condensation and breakdown of intracellular cytoskeletal and nuclear matrix constituent [17]. O'Brien et al. [16] showed that PS externalizations can be measured prior to the detection of DNA strand breaks; therefore it is reasonable that Seifer et al. [9] should have found a lower rate of apoptosis in GCs due to the use of a technique that detects the presence of DNA fragmentation, an event that occurs later in the process of apoptosis.

Some authors [10,11,13] have determined lower rates of apoptosis than we found in this study. There is, however, an important difference in the techniques used by these authors and the method used in the present study: they measured the percentage of apoptosis in GCs using fluorescence microscopy, while in the present study the cells were analysed by flow cytometry. In the former studies, only the apoptotic bodies of up to 1000 granulosa-lutein cells were counted with the use of fluorescence microscopy. Measuring apoptosis by flow cytometry has the advantage of allowing the analysis of far more GCs (>50,000) in a short time, thereby increasing outcome precision enormously compared with fluorescence microscopy. In addition, our results coincide with those obtained by Oosterhuis et al. [12] who also used flow cytometry to measure the percentage of apoptotic GCs.

We also studied the relationship between GC apoptosis and steroid levels in FF. No correlation was found between these parameters. These results can be explained by the maintenance of steroidogenesis observed in apoptotic GCs [15], due to the fact that steroidogenic organelles (mitochondria, smooth endoplasmic reticulum) remain intact during bleb formation and nuclear fragmentation.

However, our data neither correlate with the inhibition of apoptosis observed in cultivated GCs after addition of estradiol, nor with the enhancement of this process by testosterone [8]. The differences observed can be attributed to the different techniques used to measure apoptosis. We use a technique that detects early apoptosis, and so the results will be different. Moreover, the cited authors used rat GCs, which have a different steroidogenic production from human GCs [18].

In conclusion, the number and percentage of apoptosis in GCs is not related to oocyte maturity and fertilizability by ICSI or to follicular quality in stimulated cycles. Nevertheless, this parameter may be of importance in other moments of follicular development, or in natural cycles not subjected to any treatment influencing follicular physiology.

References

[1] Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251–300.
[2] Williams GT, Smith CA. Molecular regulation of apoptosis-genetic controls on cell death. Cell 1993;74:777–9.
[3] Van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CPM. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 1998;31:1–9.

[4] Hirshfield AN. Development of follicles in the mammalian ovary. Int Rev Cytol 1991;124:43–101.
[5] Tilly JL, Kowalski KI, Johnson AL, et al. Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. Endocrinology 1991;129:2799–801.
[6] Attaran M, Frasor J, Mascha E, Radwanska E, Rawlings RG. The relationship of human granulosa-lutein cell proliferative index to follicular diameter and serum estradiol. Obstet Gynecol 1998;91: 449–53.
[7] Richards JS. Maturation of ovarian follicle: actions and interactions of pituitary and ovarian hormones on follicular differentiation. Physiol Rev 1980;60:51–89.
[8] Billig H, Furuta I, Hsueh AJW. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinology 1993;133: 2204–12.
[9] Seifer DB, Gardiner AC, Ferreira KA, Peluso JJ. Apoptosis as a function of ovarian reserve in women undergoing in vitro fertilization. Fertil Steril 1996;66:593–8.
[10] Nakahara K, Saito H, Saito T, et al. Incidence of apoptotic bodies in membrana granulosa of the patients participating in an in vitro fertilization program. Fertil Steril 1997a;67:302–8.
[11] Nakahara K, Saito H, Saito T, et al. The incidence of apoptotic bodies in membrana granulosa can predict prognosis of ova from patients participating in IVF programs. Fertil Steril 1997;68: 312–7.
[12] Oosterhuis GJ, Michgelsen HW, Lambalk CB, Schoemaker J, Vermes I. Apoptotic cell death in human granulosa-lutein cells: a possible indicator of in vitro fertilization outcome. Fertil Steril 1998;70:747–9.
[13] Saito H, Saito T, Kaneko T, et al. Relatively poor oocyte quality is an indication for intracytoplasmic sperm injection. Fertil Steril 2000;73: 465–9.
[14] Breckwoldt M, Selvaraj N, Aharoni D, et al. Expression of Ad4-BP/ cytochrome P450 side chain cleavage enzyme and induction of cell death in long-term cultures of human granulosa cells. Mol Hum Reprod 1997;2:391–400.
[15] Castilla JA, Clavero A, Expósito A, et al. Apoptosis y estrés oxidativo en células de la granulosa de mujeres sometidas a desarrollo folicular múltiple con niveles elevados de FSH sérica basal. Rev Iber Fertil 2000;17:29–36.
[16] O'Brien IE, Reutelingsperger CP, Holdaway KM. The use of Annexin-V and TUNEL to monitor the progression of apoptosis in plants. Cytometry 1997;29:28–33.
[17] Oberhammer FA, Hochegger K, Froschl G, et al. Chromatin condensation during apoptosis is accompanied by degradation of lamin A + B, without enhanced activation of cdc2 kinase. J Cell Biol 1994;126:827–37.
[18] Maxson WS, Haney AF, Schomberg DW. Steroidogenesis in porcine atretic follicles: loss of aromatase activity in isolated granulosa and theca. Biol Reprod 1985;33:495–501.