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APOPTOTIC ANALYSIS OF CUMULUS CELLS FOR THE SELECTION OF COMPETENT OOCYTES TO BE FERTILIZED BY INTRACYTOPLASMIC SPERM INJECTION (ICSI)

Liana Bosco[1]* --- Giovanni Ruvolo[2] --- Roberto Chiarelli[3] --- Maria Agnello[4] --- Maria Carmela Roccheri[5]

[1,3,4,5]Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche. Università degli Studi di Palermo, Viale delle Scienze Ed.16, Palermo, Italy

[2]Centro di Biologia della Riproduzione, Via Villareale, Palermo, Italy

ABSTRACT

Oocyte quality is one of the main factors for the success of in vitro fertilization protocols. Apoptosis is known to affect oocyte quality and may impair subsequent embryonic development and implantation. The aim of this study was to investigate the apoptosis rate of single and pooled cumulus cells of cumulus cell–oocyte complexes (COCs), as markers of oocyte quality, prior to intracytoplasmatic sperm injection (ICSI).We investigated the apoptosis rate by TUNEL assay (DNA fragmentation) and caspase-3 immunoassay of single and pooled cumulus cells of COCs. The results showed that DNA fragmentation in cumulus cells was remarkably lower in patients who achieved a pregnancy than in those who did not. Cumulus cell apoptosis rate could be a marker for the selection of the best oocytes to be fertilized by intracytoplasmatic sperm injection.

Keywords: Human cumulus cells, Apoptosis, DNA fragmentation, Caspase-3, Fertilization rate.

Contribution/ Originality

This study is one of very few studies which have investigated the apoptosis rate by TUNEL assay and caspase-3 immunoassay of single and pooled human cumulus cells of COCs. The apoptosis rate could be a marker for the selection of the best oocytes to be fertilized by intracytoplasmatic sperm injection.

1. INTRODUCTION

Several studies have been devoted to define criteria for predicting oocyte quality [1, 2] and selecting the most suitable oocytes for *in vitro* fertilization (IVF) cycles. Oocyte quality, as an expression of nuclear and cytoplasmic competence, is closely related to successful fertilization and

**Corresponding Author*

implantation. Oocyte quality, nuclear maturation, fertilization and implantation can be affected by different factors, such as ovarian hyperstimulation, patient age, the so-called 'poor responder' condition and endometriosis [3, 4].

Most embryologists select oocytes on the basis of cumulus cell–oocyte complex (COC) morphology and oocyte features. It is currently understood that pregnancy and implantation rates are closely associated to the number of metaphase II (MII) oocytes retrieved [5]; however introduce of new criteria for oocyte selection could guarantee good rates of fertilization, pregnancy and implantation.

Many researchers have investigated the relationship between oocytes and their surrounding somatic cells [6, 7]. Cumulus cells form a multilayer mass around oocytes during the maturation process, consequently protecting them and providing several molecules to the oocytes, through the gap junctions [8]. In an earlier paper, we demonstrated that a reduced rate of cumulus cell apoptosis improves rates of pregnancy and implantation [9], as a result of better oocyte quality [10]. It is possible that through gap junctions or other molecular pathways, the direct activation of the apoptotic process from cumulus cells moves to the oocytes, affecting the quality and developmental and implantation potential of the embryos [11, 12].

The aim of the present study is to verify our hypothesis that the apoptosis rate of the cumulus cells of individual COCs can be used as a marker for selecting the best oocytes to be fertilized by intracytoplasmatic sperm injection (ICSI), through investigating the specific clinical outcomes of pregnancy rate.

Usually, in a spontaneous menstrual cycle, one or two follicles are selected from a pool of follicles. The dominant follicle induces atresia on the other follicles through apoptotic pathway, operating a selection that assures a pregnancy rate that in general population is about 40%. To treat infertility, the patient included in an assisted reproductive technology (ART) program, receives an hormonal stimulation to produce more than 1 or 2 spontaneous follicles. Usually more than 6 follicles are produced by the ovaries including follicles than, in a natural cycle, should be eliminated by atresia. To date, it is unknown if the quality of the oocytes collected after hormonal stimulation, that escape from atretic selection, is comparable with the dominant oocytes selected in a natural cycle.

Apoptosis is the molecular pathway of atresia. For this reason it was decided to use DNA fragmentation, that is the molecular aspect of final steps of apoptotic process, as the molecular marker of oocyte quality. It was hypothesized that CoCs with a lower number of apoptotic cells could represent a model in which the surviving pathways prevail. This is a condition that allows to the oocyte to complete the maturation process, prior to ovulation, consisting in the synchronization of nuclear and cytoplasmic maturation, that seems to represent the basis of oocyte competence [12]. It is known that cumulus cells supply the oocytes, particularly during oogenesis, with nutrients that maintain meiotic arrest and participate in the induction of meiosis by driving the luteinizing hormone (LH) signal to the oocytes [13].

This study was approved by the Institutional Review Board of the Clinic. The patients involved in the study underwent ICSI for a severe male factor infertility (< 1 million/ml motile sperm) and provided written informed consent.

The study consisted of two different trials. The policy of the clinic was to fertilize a maximum of 3 oocytes for each patient included in the study. In the first trial, three oocytes, out of the total number of oocyte collected after hormonal stimulation, were selected for fertilization. We investigated the apoptosis rate of individual cumulus cells derived from each of the three oocytes selected to be fertilized by ICSI. The three COCs were previously selected, from the total of the oocytes collected, using the morphological criteria by Veeck [1]. In the second trial, for each patient included in the study, the apoptosis rate of the pooled cumulus cells derived from the selected 3 COCs were compared with the apoptosis rate of the pooled cumulus cells of non selected and not fertilized (supernumerary MII oocytes) (when more of five COCs at least were aspirated after pick-up). The aim of this second trial was to verify if the morphological selection by Veeck [1] was effective in selecting the best oocytes, according to the evaluation using molecular markers, comparing oocytes collected in the same patient, in the same cycle and after the same hormonal stimulation.

Twenty-two consecutive couples undergoing cycles of ICSI were included in the first trial, and 20 couples in the second trial, thus providing a total of 42 couples. The mean age of patients and COCs collected are described in Table 1.

Investigations concerning the extent of DNA fragmentation were performed using the TUNEL assay (terminal deoxynucleotidyl transferase [TdT]). The aim of this assay is to recognize the terminal 3'OH ends of DNA by adding modified nucleotides (for this purpose was used the exogenous enzyme Terminal transferase (TdT).

Moreover, to validate whether DNA fragmentation was related to the apoptotic process we performed in the same cells cleaved-caspase-3 immunoassay, one of the most important effector molecules of apoptosis.

2. RESULTS

The study was performed using cumulus cells isolated from single or pooled COCs collected from women undergoing cycles of ICSI.

2.1. First Trial

No differences in the mean of female and male ages, in the total number of COCs collected and selected, was found between the 2 trials as reported in Table 1.

In the first trial, a total of 154 COCs were recovered, from which 58 MII oocytes were selected for ICSI treatment. Out of the 22 patients involved in this trial, 8 were pregnant (36% A Group) and 14 were not (63% B Group). Clinical outcomes are reported in Table 2. Twenty-four oocytes from A Group (n = 8), and 34 oocytes from B Group (n = 14) from the first trial were microinjected with sperm. No statistical difference was observed in fertilization rates (95.8% [A Group] vs 85.2% [B Group]) or morphological quality of the transferred embryos (expressed as grade A and B using Bolton's definition) [14].

The mean incidence of DNA fragmentation of cumulus cells from individual oocytes selected for ICSI treatment, evaluated by TUNEL assay, was lower in A Group than B Group (mean 6,7% [range 2,.2-13.3%] vs 13.2% [range 6.2-34.9%]; $P< 0.05$) (Figure 1, a,). Similarly, the results

obtained with immunoassay anti-cleaved caspase-3 showed that there was lower caspase-3 activity in cumulus cells from women who were pregnant than in those who did not were pregnant (mean 5.2% [range: 1.2–8.6%] vs 11.8% [range: 5.6–14.8%]; $P < 0.05$) (Figure 1, b,). The percentage of cumulus cells undergoing apoptosis (as analysed by TUNEL assay and caspase-3 immunoassay) is shown in Table 3. It is noteworthy that most women who became pregnant had a DNA fragmentation rate (TUNEL) of less than 10% and a caspase-3 activity rate less than 7% in cumulus cells from at least one COC.

2.2. Second Trial

In the second trial, as shown in Table 4, we compared the mean apoptosis rate of cumulus cells from the three oocytes selected for ICSI with the apoptosis rate of cumulus cells from remaining COCs, in order to verify whether COCs morphological selection criteria were related to lower rates of apoptosis.

In the second trial, 4 of the 20 patients were pregnant (20%; A Group) and 16 were not (B Group). A total of 12 oocytes from A Group (n = 4) and 48 oocytes from B Group (n = 16) were microinjected.

Particularly, 10 patients out of the 20 involved in this trial had at least 5 MII oocytes and then were included in the study. 2 patients of them were pregnant after ICSI cycle (A Group). In most of the patients from both groups, the rates of cumulus cells apoptosis were lower among the COCs selected for ICSI than in the unselected pooled COCs.

Specifically, we found a difference in DNA fragmentation rate (18.2 vs 27.1, $p<0.05$) and in Caspase-3 immunoassay (12.9 vs 19.9, $p< 0.05$) between A Group and B Group, when it was evaluated the differences between the COCs selected by morphological criteria. No differences, both in TUNEL assay and in Caspase 3 immunoassay was found in the pooled cells of the supernumerary COCs (A group 25.75 vs B Group 29.2 for TUNEL assay; A Group 22.3 vs B Group 23.1 for Caspase 3 immunoassay) as shown in Table 4.

However, pooled cumulus cells from two patients in B Group had a lower rate of apoptosis than the pooled cumulus cells from the three selected COCs. In this case, molecular criteria, based on the evaluation of apoptosis rate of cumulus cells, would be useful to establish oocyte quality, suggesting that COCs morphological criteria, per se, are not entirely representative of good oocyte competence.

3. DISCUSSION

The aim of this study was to identify new markers of quality for human oocyte selection prior to ICSI. We hypothesized that low rates of apoptosis in cumulus cells associated with morphological COCs selection could be an innovative method for oocyte selection.

Investigators have previously demonstrated that high rates of cumulus cell apoptosis are associated with a poor outcome of assisted reproductive technology (ART) procedures [15, 16]. Differently from Lee, et al. [16], our investigation concerns molecular evaluation of apoptosis in cumulus cells of individual COCs of the oocytes undergoing ICSI fertilization, previously selected by traditional morphological criteria. In our opinion, this is a potential approach that directly

correlates the oocyte quality, in terms of reduced rate of cumulus cell apoptosis, to ART outcomes expressed as fertilization and pregnancy rates.

As cumulus cells are fundamental to oocyte maturation [8, 17], it is necessary to use a non-invasive method to evaluate indirectly the oocyte quality through cumulus cell analysis.

An increased rate of apoptosis of cumulus cells may affect oocyte maturation and could be a marker of poor oocyte quality. Conversely, oocytes play an important role in determining functional differences between different granulosa cell lineages and in maintaining anti-apoptotic pathways in cumulus cells [18-20].

The relationship between apoptosis rates in cumulus cells and the maturity or quality of the corresponding oocytes has been studied by other authors. Høst, et al. [21] demonstrated a clear correlation between the rates of cumulus cell apoptosis and immature oocytes. In a recent paper [9], it was demonstrated that LH administration to specific patients undergoing IVF protocol significantly reduced rates of cumulus cell apoptosis, thereby improving clinical outcomes. Therefore a significant reduction in the number of immature oocytes collected and a consequent increase in the number of embryos to be transferred, as well as an improvement in implantation and pregnancy rates. Data from trial 1, where 3 COCs were investigated separately, demonstrated that morphological evaluation of COCs is not useful, per se, in selecting the best oocytes with higher implantation potentiality. It was concluded that cumulus cells, which maintain their physiological function for longer, could provide greater support to the nuclear and cytoplasmic maturation of the oocyte until ovulation, thus allowing the collection of oocytes with better 'intrinsic' qualities, as is necessary for sustaining fertilization and early phases of embryogenesis. However, Moffatt, et al. [22], demonstrated that the incidence of cumulus cell apoptosis and the quality oocytes depend on patient age and exposure to sperm, but are unrelated to oocyte nuclear maturation. Nonetheless, it is agreed that there is an intimate relationship between cumulus cells and their corresponding oocytes. Høst, et al. [23] demonstrated that the developmental potential of an oocyte is compromised if the related cumulus cells show a high rate of apoptosis; this may also impair the fertilization rate of MII oocytes after ICSI and the implantation potential of the derived embryos. This study seems to demonstrate that lower apoptosis rate of the cumulus cells, analyzed in individual COC, is a stronger and useful marker than traditional morphological criteria. The molecular marker could be used in selecting oocytes in older aged patients, in the case of repeated failure cycles and in patients with severe endometriosis [24].

4. CONCLUSION

Data from the first trial, where the three cumulus oocyte complexes were analyzed separately, show that there is a direct relationship between higher apoptosis rate in cumulus cells and pregnancy failure.

The low percentage of apoptosis in cumulus cells in patients who achieved pregnancy also shows the importance of the chromatin integrity.

The evidence from two patients in B Group, who showed a lower rate of apoptosis in pooled cumulus cells from non selected oocytes than in the selected ones, reinforces the notion that

molecular selection of COCs, based on evaluation of cumulus cells apoptosis, could be more sensitive than classical morphological criteria.

We have shown that all women in this study who became pregnant had a lower rate of cumulus cell apoptosis than those who did not achieve a pregnancy. Our data showed that in most pregnant patients, at least 1 of the three oocytes fertilized by ICSI had a lower apoptotic rate of the cumulus cells (usually less than 8%), compared with the oocytes of non pregnant ones. Thus, 8% cumulus cells apoptotic rate could be considered a cut-off threshold for the oocyte selection.

In conclusion, we found that evaluation of cumulus cell apoptosis in individual COCs may be a selective marker for the best quality oocytes for ICSI.

5. MATERIALS AND METHODS

5.1. ICSI Procedure and Cumulus Cell Analysis

Ovarian hyperstimulation was performed using the standard long gonadotrophin-releasing hormone agonist (buserelin, 0.2 ml/day) protocol, starting from day 21 of the previous cycle, followed by a daily dose of 225 IU of recombinant human follicle-stimulating hormone (r-hFSH; GONAL-f®; Merck Serono S.p.A., Rome, Italy, an affiliate of Merck KGaA, Darmstadt, Germany). Follicular growth and serum oestrogen levels were monitored from the sixth day of stimulation, and the dose of r-hFSH was modified accordingly. When at least three or four dominant follicles were ≥18 mm in diameter, 10,000 IU of human chorionic gonadotrophin (Gonasi®, AMSA SRL, Rome, Italy) was administered.

A total of 58 COCs from the first trial and 60 COCs from the second trial were selected and denudated separately in hyaluronidase (40 IU/ml, SAGE® IVF, Trumbull, CT, USA), 30' or at least 2 hours after oocyte collection [25].

The cumulus cells were collected in a test tube containing 2 ml of basic culture medium (Quinn's Advantage® Medium with Hepes, SAGE® IVF) and centrifuged twice at 300*g* for 10 minutes. The oocytes were transferred to a culture medium (fertilization medium, SAGE® IVF) and incubated at 37°C in 5% CO_2 until the ICSI procedure. For the ICSI procedure, the semen samples were taken 2 hours after oocyte collection, washed in Hepes medium (SAGE® IVF) and centrifuged for 7 minutes at 300*g*. Fertilization medium (50 µl) (SAGE® IVF) was layered on the pellet and the sperms were incubated at 37°C in 5% CO_2 until ICSI. After 13–16 hours, the oocytes cultured in cleavage medium (SAGE® IVF) were checked for the pronuclear presence. After 48 hours, embryos were transferred using a catheter.

5.2. Fluorescent in Situ TUNEL Assay (TdT)

Apoptosis was tested in at least 80 cumulus cells for each of the COCs by TUNEL assay. Cumulus cells were fixed in 3.7% paraformaldehyde for 60 minutes. After centrifugation at 300*g* for 5 minutes, the supernatant was removed and phosphate buffer saline (PBS)–glycine buffer was added (0.1 M glycine in PBS and 0.3 mg/ml bovine serum albumin [BSA]). The cells were then mounted on polylysine-coated glass slides by cytocentrifugation.

Cumulus cells, mounted on polylysine-coated glass slides, were washed with PBS (137 mM NaCl, 2.68 mM KC1, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4) and permeated for 10 minutes with

0.1% Triton X-100 and 0.1% sodium citrate in PBS at 4°C, and then washed three times in PBS at room temperature for 5 minutes. Subsequently, the cell preparations were incubated for 60 minutes at 37°C in a humidified chamber in 50 µl of a mixture containing 5 µl nucleotide mix, 1 µl TdT enzyme and 45 µl equilibration buffer (Dead-End™ Fluorometric TUNEL System, Promega Italia, SRL).

Cumulus cells were incubated with the same mixture without the TdT enzyme (negative control) while another sample was treated for 10 minutes with a DNAse buffer solution containing 10 unit/ml of DNAse I (positive control).

The reaction was blocked with saline sodium citrate buffer 2× for 15 minutes, then washed for 5 minutes with PBS. The cumulus cells were stained with propidium iodide (1 µg/ml) for 10 minutes at room temperature and observed under an Olympus BX 50 microscope (Olympus, Copenhagen, Denmark) equipped with a reflected light fluorescent attachment (Olympus), and a 20 × 0.40 objective.

Apoptosis rate: total apoptotic nuclei labelled in green (TUNEL assay)/total nuclei labelled in red (Propidium iodide) x 100.

5.3. Immunoassay of Cleaved-Caspase-3

Apoptosis was tested in at least 80 cumulus cells for each of the COCs by caspase-3 immunoassay.

After three washes in PBS, cumulus cells were permeated for 10 minutes at 4°C with 0.1% Triton X-100 and 0.1% sodium citrate in PBS. After three rinses with PBS, the cells were incubated overnight at 4°C with anti-caspase-3 cleaved polyclonal antibody (Cell Signaling Technology®) (1:50 dilution) in PBS containing 3% BSA. In negative controls, the primary antibody was omitted. After three rinses with PBS, anti-rabbit immunoglobulin fluorescein-linked secondary antibody (Amersham) (1:60 dilution in PBS containing 1% BSA) was added to the samples for 60 minutes. Then, secondary antibody excess was removed by three washings with PBS. Cumulus cells were stained for 10 minutes with propidium iodide (1 µg/ml), mounted in 10 µl DABCO solution (de-ionized H_2O, 1 M Tris-HCl pH 8, 2 mM DABCO, glycerol) and observed under an Olympus BX 50 microscope equipped with a reflected light fluorescent attachment (Olympus), and a 40 × 0.55 objective. Apoptosis rate detected by anti-caspase-3 cleaved immunoassay was calculated as described for TUNEL assay.

5.4. Statistical Analysis

Paired Chi and *t*-tests, were used for the evaluation of differences between groups. Apoptosis rates (TUNEL assay and caspase-3 immunoassay) are presented as percentage of cells exhibiting fragmented DNA. Differences were considered significant at levels of $P < 0.05$.

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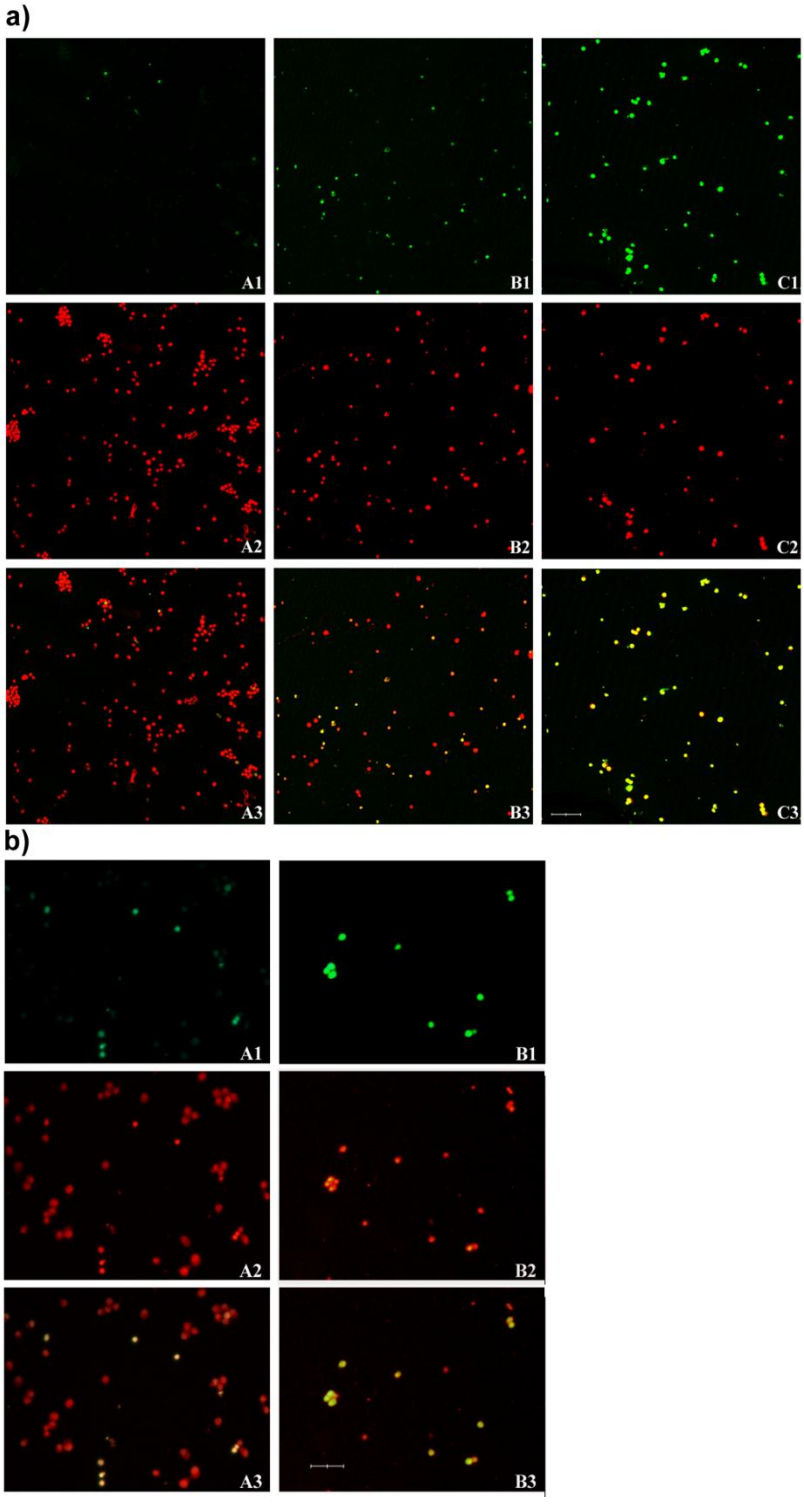

Figure-1. Evaluation of apoptosis of a single cumulus cell–oocyte complex

a) ***TUNEL Assay***.

A = A Group; B = B Group; C = Positive control.

Green: DNA fragmentation (A1, B1, C1). Red: nucleic acids stained with propidium iodide (A2, B2, C2). Merge of green and red (A3, B3, C3). Fields observed under fluorescent microscope (20 × 0.40 objective). Bar = 30µm.

b) ***Caspase-3 Immunoassay***.

A = A Group; B = B Group.

Green: immunolocalization of cleaved-caspase-3 (A1, B1). Red: nucleic acids stained with propidium iodide (A2, B2). Merge of green and red (A3, B3). Fields observed under fluorescent microscope (40 × 0.55 objective).Bar = 30µm.

Table-1. Patient characteristics and COCs collected.

	Trial 1	**Trial 2**
Patients, n	22	20
Mean (SD) female age, years	34.5 (4.8)	34.8 (3.8)
Range, years	28–45	31–43
Mean (SD) male age, years	38.3 (8.3)	38.4 (7.2)
Range, years	30–56	31–52
Total number of COCs collected	154	101
Mean (SD) number	7.0 (3.2)	4.9 (2.2)
Total number of COCs selected	58	60
Mean (SD) number	2.9 (0.3)	2.9 (0.3)

COC, cumulus cell–oocyte complex; SD, standard deviation.

Table-2. Oocytes injected, fertilization rates, transferred embryos and clinical outcomes in the two groups studied.

	Trial 1 (n = 22)		**Trial 2 (n = 20)**	
	A Group	**B Group**	**A Group**	**B Group**
Injected oocytes, n	24	34	12	48
Two pronuclear (2PN)-stage oocytes, n (%)	23 (95.8)	29 (85.2)	12 (100)	46 (96)
Grade A or B embryos transferred, n	23	29	12	46
Pregnancies, n (%) No pregnancies, n (%)	8 (36.3) –	– 14 (63.7)	4 (20.0) –	– 16 (80.0)

A Group: women that became pregnant; B Group: women that did not become pregnant. Grade A and B according to Bolton's definition [14].

Table-3. Apoptosis in cumulus cells analysed by TUNEL assay and caspase-3 immunoassay from Trial 1.

		A Group n = 8	**B Group n = 14**
TUNEL assay	Apoptosis in cumulus cells, %	6.7	13.2
	Range	2. 2–13.3	6.2–34.9
Caspase-3 immunoassay	Apoptosis in cumulus cells, %	5.2	11.8
	Range	1.2–8.6	5.6–14.8

Differences were considered significant at levels of $P < 0.05$.

Table-4. Apoptosis in cumulus cells analysed by TUNEL assay and caspase-3 immunoassay in selected COCs compared with pooled COCs in patients with more than three COCs from Trial 2.

	Cumulus cell apoptosis in pooled selected COCs[(a)] (mean), %		**Cumulus cell apoptosis in pooled remaining COCs [(a)] (mean), %**	
	TUNEL assay	Caspase-3 immunoassay	TUNEL assay	Caspase-3 immunoassay
A Group n = 2	(18.2%)	(12.9%)	(25.75%)	(22.3%)
Range	17.2–19.2	12.3–13.5	23.1–28.4	20.5–24.1
B Group n = 8	(27.1%)	(19.9%)	(29.2%)	(23.1%)
Range	10.0–60.3	7.6–45.3	8.8–67.3	7.2–51.4

Differences were considered significant at levels of $P < 0.05$.
[(a)] COC: cumulus cell–oocyte complex.