

## GnRH agonist versus GnRH antagonist in IVF/ICSI cycles with recombinant LH supplementation: DNA fragmentation and apoptosis in granulosa cells<sup>☆</sup>

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### ABSTRACT

**Objective:** To compare the level of apoptosis and DNA fragmentation in the human granulosa cell (GC) layer exposed to an agonist or antagonist of GnRH in intracytoplasmic sperm injection (ICSI) cycles supplemented with recombinant LH (rLH).

**Study design:** Patients without ovulatory dysfunction, aged  $\leq 37$  years and in their first ICSI cycle were prospectively randomised to receive either a long GnRH agonist protocol or a multi-dose antagonist protocol. In both groups, recombinant FSH supplemented with rLH was used for ovarian stimulation, and the GCs were collected during oocyte denudation. The GCs were then analysed for DNA fragmentation by TUNEL assay and for apoptosis using the annexin-V assay. The outcomes were given as the percentage of GCs with DNA fragmentation and apoptosis out of the total number of GCs analysed. Comparison of the agonist versus the antagonist group was performed using the Mann–Whitney test.

**Results:** DNA fragmentation: 32 patients were included in either the GnRH agonist group ( $n = 16$ ) or the antagonist group ( $n = 16$ ). The percentage of GCs with positive DNA fragmentation did not differ significantly ( $P = 0.76$ ) between the agonist group ( $15.5 \pm 9.4\%$ ) and the antagonist group ( $18.8 \pm 13.3\%$ ). Apoptosis: 28 patients were included in either the GnRH agonist group ( $n = 14$ ) or the antagonist group ( $n = 14$ ). The percentage of GCs positive for apoptosis did not differ significantly ( $P = 0.78$ ) between the agonist group ( $34.6 \pm 14.7\%$ ) and the antagonist group ( $36.5 \pm 22\%$ ).

**Conclusions:** The results suggest that therapy with either an agonist or antagonist of GnRH is associated with comparable levels of DNA fragmentation and apoptosis in granulosa cells in ICSI cycles supplemented with rLH.

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## 1. Introduction

Apoptosis, or programmed cell death, is a suicidal process crucial for the maintenance of ovarian homeostasis. It is responsible for the selection of the dominant follicle and the collapse of other follicles recruited at the beginning of the cycle and for the degeneration of the corpus luteum [1]. In this way, granulosa cells (GCs) play a crucial role in the oocyte maturation processes, and apoptosis and necrosis may influence oocyte and embryo quality. Several factors can act as triggers of apoptosis in

GCs, including the exogenous hormones used for in vitro fertilisation (IVF).

The discovery of gonadotropin-releasing hormone (GnRH) receptors in GCs at the mRNA and protein levels [2] led researchers to study the effects of GnRH analogues on these cells. It has been established that GnRH agonists (GnRH-a) directly induce the apoptosis of GCs [3]. It was also reported that GnRH antagonists (GnRH-ant) reduce apoptosis in rat GCs and in human GCs in vitro [4,5]; when the effects of GnRH-a and GnRH-ant were compared, however, no differences in the apoptosis rate of GCs were found [6,7]. In contrast, it has been demonstrated that LH supplementation during IVF cycles using a GnRH-a or GnRH-ant improves the clinical and laboratory outcomes of the cycle [8,9]. In addition to a relationship with GC physiopathology, the inclusion of LH in the stimulation protocol of patients with GnRH-a-induced pituitary desensitisation seems to prevent apoptosis of GCs [10].

Therefore, to better comprehend the action of GnRH analogues on ovarian activity, the present randomised study aimed to

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compare the effects of GnRH-a and GnRH-ant on DNA fragmentation and apoptosis of GCs supplemented with recombinant LH during IVF/intracytoplasmic sperm injection (ICSI) cycles.

## 2. Materials and methods

### 2.1. Participants

This study was conducted on women undergoing their first ICSI cycle. The inclusion criteria were as follows: aged  $\leq 37$  years, first IVF/ICSI cycle, BMI  $< 30$  kg/m<sup>2</sup>, regular menses and the presence of two normal ovaries. The indications for the procedure were male infertility (55%), idiopathic infertility (23%), and tuboperitoneal factors (22%). Written consent was given by all patients, and the study was approved by the local Research Ethics Committee.

### 2.2. Randomisation

Double randomisation was performed. First, a computer-generated table indicated the protocol of ovarian stimulation for each case. At the moment the protocol of ovarian stimulation was prescribed, lots were drawn to determine the case number of each patient (e.g., case #1 and/or case #10). In this manner, each patient was randomly allocated into one of the two groups: (1) the GnRH-a group, which received long GnRH agonist (leuprolide acetate, Lupron<sup>®</sup>, Abbott, Brazil), or (2) the GnRH-ant group, which received a multi-dose GnRH antagonist (cetorelix, Cetrotide<sup>®</sup>, Serono, Brazil).

### 2.3. Ovarian stimulation

#### 2.3.1. GnRH agonist protocol

First, pituitary down-regulation was started during the luteal phase of the previous menstrual cycle with the GnRH-a at a dose of 1 mg/day for 14 days. Then, ovarian stimulation was started with a fixed dose of 150–225 IU recombinant FSH (rFSH/Gonal F<sup>®</sup>; Serono, SP, Brazil) with 75 IU/day recombinant LH (rLH/Luveris<sup>®</sup>; Serono, SP, Brazil) for 7 days. On the 8th day of ovarian stimulation, follicular development was monitored by transvaginal ultrasound. The dose of rFSH was adapted according to the ovarian response, and rLH supplementation was increased to 150 IU/day when one or more follicles measuring  $\geq 10$  mm in diameter were found.

#### 2.3.2. GnRH antagonist protocol

On the 3rd day of the menstrual cycle, ovarian stimulation was started with a fixed dose of 150–225 IU rFSH and 75 IU/day rLH for 5 days. On the 8th day of the menstrual cycle (6th day of ovarian stimulation), follicular development was monitored by transvaginal ultrasound. The dose of rFSH was adapted according to the

ovarian response, and supplementation with rLH was increased to 150 IU/day when one or more follicles measuring  $\geq 10$  mm in diameter were found. The GnRH-ant, at a dose of 0.25 mg/day s.c., was started when at least one follicle  $\geq 14$  mm was observed on ultrasound.

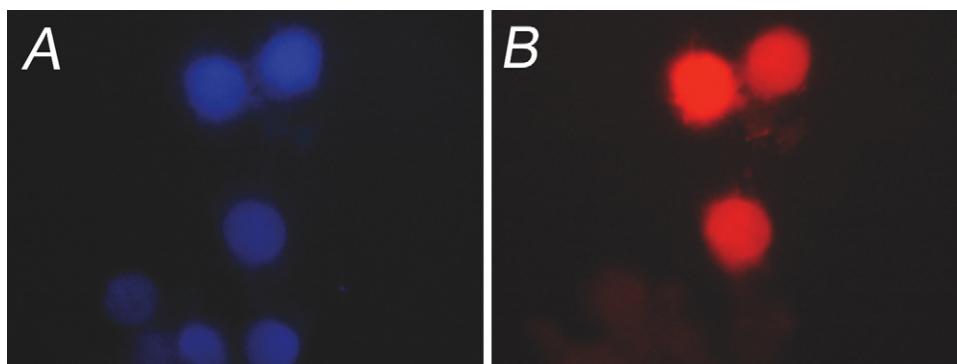
For both groups, 250  $\mu$ g recombinant hCG (rhCG/Ovidrel<sup>®</sup>; Serono, SP, Brazil) was administered s.c. when at least two follicles reached a diameter of  $\geq 17$  mm during final oocyte maturation. Oocyte retrieval was performed by transvaginal aspiration under ultrasound guidance 34–36 h after rhCG injection.

### 2.4. Isolation of granulosa cells

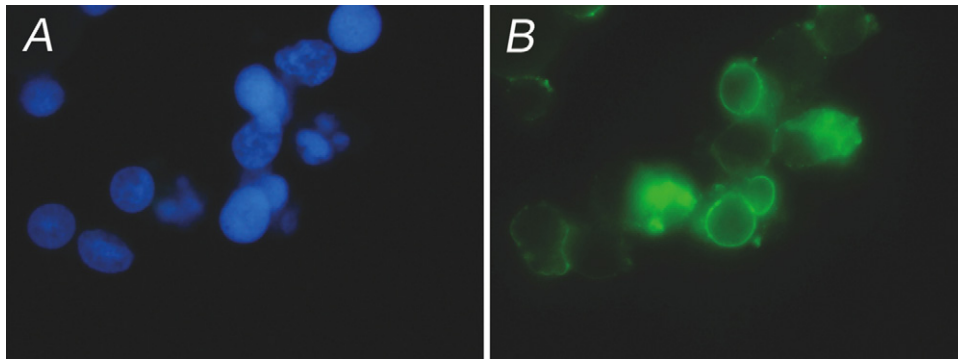
The retrieved oocytes from both groups were incubated in culture medium (P1; Irvine Scientific, Santa Ana, CA, USA) at 37 °C and 5.5% CO<sub>2</sub> for 1 h. The GCs were removed by exposing the oocytes to modified human tubal fluid medium (mHTF, Irvine Scientific, Santa Ana, CA, USA) containing 40 IU/mL hyaluronidase (Irvine Scientific, Santa Ana, CA, USA) for 30 s, after which the coronal cells were manually removed using a stripper (Cook, Australia). The GCs were separated from blood cells by centrifugation on a 60% Isolate<sup>®</sup> (Irvine Scientific, USA) solution. Cells were subsequently subjected to the DNA fragmentation and apoptosis analyses.

### 2.5. Determination of DNA fragmentation (Fig. 1)

DNA fragmentation was measured using the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) assay, which was performed using an in situ cell death detection kit with tetramethylrhodamine-labelled dUTP (Roche, Monza, Italy). The slides with GCs from the GnRH-a and GnRH-ant groups were air-dried, fixed in formaldehyde solution for 30 min, and permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate at 4 °C for 10 min. After washing with phosphate-buffered saline (PBS), the slides were processed for the TUNEL assay. The TdT-labelled nucleotide mix was added to each slide and incubated in the dark in a humidified atmosphere for 2 h at 37 °C. After the enzymatic reaction was stopped, the slides were rinsed twice in PBS and then counterstained with Vectashield Mounting Medium with 4,6-diamidino-2-phenylindole (DAPI; 1.5  $\mu$ g/mL; Vector Laboratories, Burlingame, CA, USA). The final evaluation was performed using a fluorescent microscope, and the percentage of TUNEL-positive GCs was determined. The number of cells per field stained with DAPI (blue) was counted; in the same field, the number of TUNEL-positive cells (red) was expressed as a percentage of the cells with DNA fragmentation. The same technician, blinded to subject identity, performed all examinations.



**Fig. 1.** Determination of DNA fragmentation. (A) Cell identification: cells stained with DAPI are blue and (B) DNA fragmentation: cells that fluorescence red are TUNEL-positive. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)



**Fig. 2.** Determination of apoptosis. (A) Cell identification: viable cells are Hoechst 33342-positive (blue) and (B) identification of apoptotic cells: apoptotic GCs are positive for annexin V (green) and negative for red propidium iodide dye. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

### 2.6. Determination of apoptosis (Fig. 2)

The presence of green annexin V fluorescence can serve as a marker of early apoptosis because annexin V specifically and reversibly binds to externalised phosphatidylserine. The GCs were incubated with 3  $\mu$ L annexin-V, 1  $\mu$ L propidium iodide (Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit; Molecular Probes, Eugene, OR) and 1  $\mu$ L Hoechst 33342 at room temperature for 15 min in the dark. Propidium iodide (PI) is a red fluorescent dye that allows the identification of necrotic cells because it is impermeable to live cells. After incubation, the suspension was centrifuged at  $800 \times g$  for 10 min, and the pellet was mounted on poly-L-lysine-coated slides for examination by fluorescence microscopy. Normal cells were Annexin V(-)/PI(-), early apoptotic cells were Annexin V(+)/PI(-) and necrotic cells were Annexin V(+/-)/PI(+). The percentages of apoptotic (those GCs stained green by annexin-V but unstained red by the propidium iodide dye) were determined. The same technician, blinded to subject identity, performed all examinations.

### 2.7. Statistical analysis

The data were analysed using InStat version 3.0 (GraphPad Software, San Diego, CA, USA) on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA). The comparison of the agonist and the antagonist groups was performed using Student's *t*-test, the Mann-Whitney *U* test or a chi-squared test when appropriate. The significance level was set at  $P < 0.05$ .

## 3. Results

### 3.1. DNA fragmentation

Thirty-two patients were evenly allocated between the GnRH-a group ( $n = 16$ ) and the GnRH-ant group ( $n = 16$ ). Basic demographic characteristics were not significantly different between the GnRH-a and GnRH-ant patient groups: age: GnRH-a  $33.7 \pm 2.7$  years (range 29–37) and GnRH-ant  $32.1 \pm 3.1$  years (range 27–37),  $P = 0.12$ ; BMI: GnRH-a  $23.7 \pm 3.1$  and GnRH-ant  $23.4 \pm 2.7$ ,  $P = 0.75$ ; duration of infertility: GnRH-a  $2.8 \pm 1.1$  years and GnRH-ant  $3.9 \pm 3.0$  years,  $P = 0.59$ ; smoking: GnRH-a 0/16 and GnRH-ant 0/16; regular alcohol use: GnRH-a 6.2% (1/16) and GnRH-ant 6.2% (1/16),  $P = 1$ ; and infertility aetiology: GnRH-a male 56.3% (9/16), idiopathic 31.3% (5/16), or tuboperitoneal 12.5% (2/16) and GnRH-ant male 56.3% (9/16), idiopathic 12.5% (2/16), or tuboperitoneal 31.3% (5/16),  $P = 0.27$ . The distribution ( $P > 0.05$ ) of the main characteristics of the ovarian stimulation cycle observed for the GnRH-a and GnRH-ant groups was equal. These data are summarised in Table 1.

The average DNA fragmentation was  $17.1 \pm 11.5\%$  for the general population,  $15.5 \pm 9.4\%$  for the GnRH-a group, and  $18.8 \pm 13.3\%$  for the GnRH-ant group. The percentage of positive DNA fragmentation in the GCs did not differ significantly ( $P = 0.76$ ) between the GnRH-a group and the GnRH-ant group. These data are summarised in Fig. 3.

### 3.2. Apoptosis

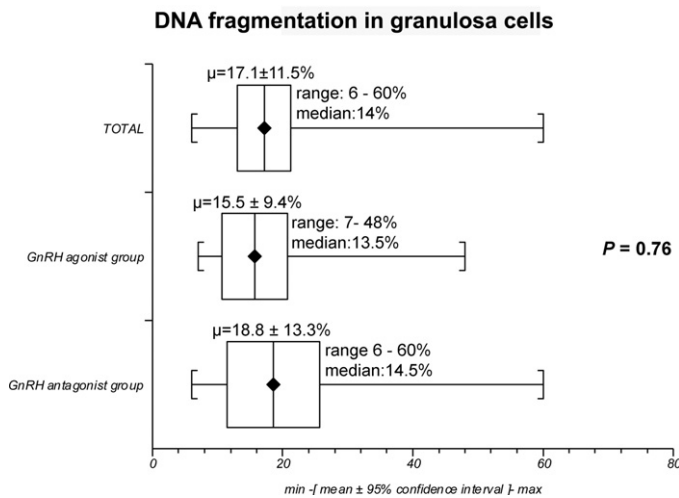
Twenty-eight patients were included in either the GnRH-a group ( $n = 14$ ) or the GnRH-ant group ( $n = 14$ ). Basic demographic characteristics were not significantly different between the GnRH-a and GnRH-ant patient groups: age: GnRH-a  $33.3 \pm 2.5$  years (range 29–37) and GnRH-ant  $32.4 \pm 4.3$  years (range 27–37),  $P = 0.42$ ; BMI: GnRH-a  $22.7 \pm 3.6$  and GnRH-ant  $23.2 \pm 3.02$ ,  $P = 0.71$ ; duration of infertility: GnRH-a  $4.09 \pm 2.9$  years and GnRH-ant  $4.91 \pm 3.8$  years,  $P = 0.78$ ; smoking: GnRH-a 0/16 and GnRH-ant 0/16; regular alcohol use: GnRH-a 6.2% (1/16) and GnRH-ant 6.2% (1/16),  $P = 1$ ; and infertility aetiology: GnRH-a male 50% (7/14), idiopathic 35.7% (5/14), and tuboperitoneal 14.3% (2/14) and GnRH-ant male 57.1% (8/14), idiopathic 14.3% (2/14), and tuboperitoneal 28.6% (4/14),  $P = 0.36$ . As in the DNA fragmentation analysis, there was no significant difference ( $P > 0.05$ ) in the parameters of the cycle of ovarian stimulation between the group of patients who received the long GnRH-a protocol and the group of patients receiving the multi-dose GnRH-ant protocol. These data are summarised in Table 2.

The average level of apoptosis was  $35.6 \pm 18.5\%$  for the general population,  $34.6 \pm 14.6\%$  for the GnRH-a group, and  $36.5 \pm 22\%$  for the GnRH-ant group. The percentage of positive DNA fragmentation in the GCs did not differ significantly ( $P = 0.78$ ) between the GnRH-a group and the GnRH-ant group. These data are summarised in Fig. 4.

**Table 1**  
DNA fragmentation analysis.

	GnRH agonist group	GnRH antagonist group	<i>P</i>
Patients ( <i>n</i> )	16	16	
Total dose			
FSH (IU)	2140 $\pm$ 707	1798 $\pm$ 636	0.20
LH (IU)	1017 $\pm$ 300	1012 $\pm$ 267	0.90
Time of stimulation (d)	10.1 $\pm$ 1.8	10.1 $\pm$ 2.4	0.95
Follicles $\geq$ 18 mm ( <i>n</i> )	4.2 $\pm$ 1.5	4.2 $\pm$ 1.9	0.71
Oocytes ( <i>n</i> )			
Mature	7.5 $\pm$ 4.6	11.6 $\pm$ 6.5	0.09
Total	11.4 $\pm$ 6.8	15.4 $\pm$ 7.8	0.08

The baseline demographic characteristics of the patients and the characteristics of IVF/ICSI cycle stimulation.



**Fig. 3.** DNA fragmentation in granulosa cells. The average level of DNA fragmentation was  $17.1 \pm 11.5\%$  (range: 6–60%, median: 14%) for the general population,  $15.5 \pm 9.4\%$  (range: 7–48%, median: 13.5%) for the GnRH-a group, and  $18.8 \pm 13.3\%$  (range 6–60%, median: 14.5%) for the GnRH-ant group. The percentage of positive DNA fragmentation in granulosa cells did not differ significantly ( $P = 0.76$ ) between the GnRH-a group and the GnRH-ant group.

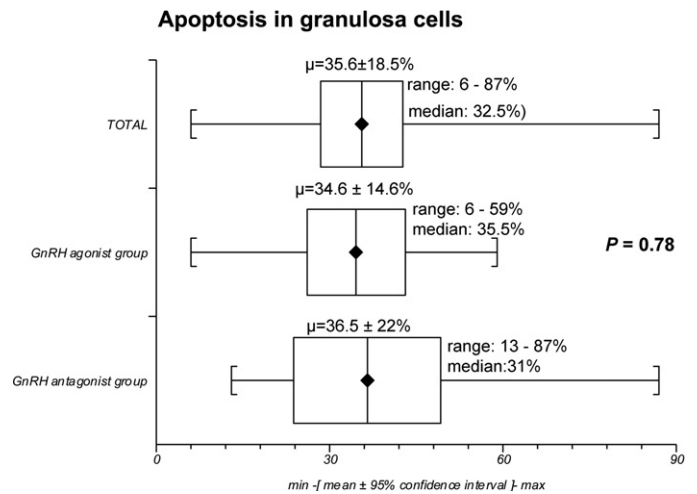
#### 4. Comment

Using the Annexin V and TUNEL assay techniques, this study showed that treatment with GnRH-a or GnRH-ant yielded similar results with respect to the frequency of apoptosis and DNA fragmentation in the population of oocyte-associated granulosa cells. In other studies, no significant differences were evident between the GnRH-a and GnRH-ant treatments in vivo. Giampietro et al. [6] also used the Annexin V and TUNEL assays to report similar levels of apoptosis and DNA fragmentation in GnRH-a- and GnRH-ant-treated granulosa cells suspended in follicular fluid. The differences in these apoptosis levels (agonist:  $7.87 \pm 1.27\%$ ; antagonist:  $9.92 \pm 0.98\%$ ) from those reported in our study may be due to technical variations. The granulosa cells examined in the Giampietro study were incubated in follicular fluid, whereas our study examined cells detached directly from oocytes. Giampietro et al. [6] used flow cytometry instead of immunofluorescence for the analysis of the Annexin V assay. TUNEL assays, however, were analysed by immunofluorescence in both studies, and the results were quite similar (GnRH-a group:  $18.00 \pm 3.87\%$ ; GnRH-ant group:  $20.50 \pm 6.56\%$ ). In contrast, Hassun Filho et al. [7] analysed individual oocyte cumulus complexes with Cytosoft in ViaCount mode and found that the GnRH-a and GnRH-ant protocols induced similar levels of apoptosis in granulosa cells. Considering the low number of studies that are randomised, however, further randomised controlled trials with larger sample sizes will be helpful to corroborate the action of GnRH on GC apoptosis in vivo.

**Table 2**  
Analysis of apoptosis.

	GnRH agonist group	GnRH antagonist group	P
Patients (n)	14	14	
Total dose			
FSH (IU)	$2012 \pm 746$	$1805 \pm 658$	0.51
LH (IU)	$1034 \pm 288$	$986 \pm 268$	0.89
Time of stimulation (d)	$10.2 \pm 1.7$	$10 \pm 2.5$	0.96
Follicles $\geq 18$ mm (n)	$4.1 \pm 1.6$	$4.6 \pm 1.9$	0.59
Oocytes (n)			
Mature	$8.4 \pm 4.3$	$12.1 \pm 5.9$	0.14
Total	$11.8 \pm 7.0$	$15.6 \pm 6.8$	0.08

The baseline demographic characteristics of the patients and the characteristics of IVF/ICSI stimulation.



**Fig. 4.** Apoptosis in granulosa cells. The average level of apoptosis was  $35.6 \pm 18.5\%$  (range: 6–87%, median: 32.5%) for the general population,  $34.6 \pm 14.6\%$  (range: 6–59%, median: 35.5%) for the GnRH-a group, and  $36.5 \pm 22\%$  (range 13–87%, median: 31%) for the GnRH-ant group. The percentage of positive DNA fragmentation in the GCs did not differ significantly ( $P = 0.78$ ) between the GnRH-a group and the GnRH-ant group.

In our study, regardless of the GnRH analogue used (agonist or antagonist), all IVF/ICSI cycles were stimulated by r-LH in addition to r-FSH. Some studies have reported the beneficial effects of r-LH on ovarian physiology and its clinical outcomes [8,11]. Ruvolo et al. [10] suggested that supplementation with r-LH improves the quality of the chromatin in cumulus cells and protects these cells from apoptosis, possibly by acting directly on granulosa cells or via a paracrine effect. These authors suggested that maintaining the physiological function of the cumulus cells over time would also maintain the nuclear and cytoplasmic maturation of the oocyte, thereby improving the intrinsic quality of the oocytes at the time of retrieval. It should be noted, however, that in our study, in addition to a lack of differences between the agonist and antagonist groups, the overall incidence of apoptosis was consistent with those reported in the literature for IVF/ICSI cycles without r-LH supplementation, even when considering differences in the study designs, populations, and methods of analysis. Ruvolo et al. [10] reported levels of DNA fragmentation in GCs of 12.1% and 18.2% in IVF/ICSI cycles with GnRH-a with or without r-LH supplementation, respectively, according to TUNEL staining. We observed a general level (GnRH-a + GnRH-ant groups) of DNA fragmentation of  $17.1 \pm 11.5\%$ , which is similar to the value reported for IVF/ICSI cycles without r-LH supplementation. Using similar techniques, Giampietro et al. [6] reported similar levels of DNA fragmentation in IVF/ICSI cycles without LH supplementation, but this study reported much lower levels of apoptosis.

An interesting point to consider is the exact origin of the granulosa cells. There are two types of granulosa cells: the cumulus cells, which surround the oocyte, and the mural granulosa cells, which surround the antrum. Several studies have investigated the incidence of apoptosis in GCs and its relationship to IVF/ICSI results, but most of those studies have assessed the apoptosis of cells suspended in follicular aspirates. Thus, those values represent of the level of apoptosis within the mural granulosa cells that had detached from the follicular wall during follicle aspiration [7,12–15]. Trials have demonstrated, however, that cumulus cells in human and animals have a low level of apoptosis compared to mural cells [16–19]. It has been demonstrated that paracrine factors secreted by the oocyte, such as bone morphogenetic proteins 15 and 6, protect cumulus cells from apoptosis [20]. Thus, the clinical significance of the levels of apoptosis in the GCs directly detached from oocytes still requires further investigation. We were

not able to detect a reduction in the levels of apoptosis in the GCs linked to the oocytes. In our study and in that of Hassun Filho et al. [7], both of which only used GCs directly detached from oocytes, higher rates of apoptosis were observed compared with the Giampietro et al. [6] study, which used exfoliated mural granulosa cells.

In conclusion, the results suggest that, despite the different mechanisms of action of the GnRH analogues, both GnRH agonists and antagonists are associated with comparable levels of DNA fragmentation and apoptosis in granulosa cells supplemented with r-LH during IVF/ICSI cycles. However, considering the low number of published trials regarding this issue, particularly randomised in vivo experiments, further clinical studies will be helpful to clarify the in vivo action of GnRH analogues on GCs.

### Conflict of interest

None declared.

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