Incidence of apoptosis in granulosa cells from immature human follicles

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The aim of this study was to investigate the incidence of apoptosis in granulosa cells from immature human follicles undergoing *in vitro* maturation (IVM) and to compare the incidence of apoptotic granulosa cells (i) between FSH-primed and unprimed normal ovaries and (ii) between polycystic and normal ovaries. Furthermore, the incidence of apoptosis was related to maturation and subsequent fertilization and cleavage of the oocytes from the corresponding ovary. Seventy women undergoing 70 IVM cycles were included. Group 1 consisted of patients with normal ovaries ($n = 52$) and group 2 consisted of patients with polycystic ovaries ($n = 18$). Patients in group 1 were subdivided into two groups according to priming with FSH before aspiration. In group 1a ($n = 27$ cycles) oocytes were obtained in unstimulated cycles. In group 1b ($n = 25$ cycles) oocytes were obtained after priming with recombinant FSH for 3 days initiated on day 3 after spontaneous menstruation. In group 2 all patients were primed with recombinant FSH for 3 days before aspiration. Aspiration was performed transvaginally and cumulus-enclosed

oocytes were matured for 28–30 h before fertilization. Granulosa cells were collected from follicular aspirates. An APOPTAG[®] detection kit was used to stain the granulosa cells and to detect apoptosis. The incidence of apoptosis in granulosa cells was decreased in follicles from FSH-primed normal ovaries compared with follicles from unprimed normal ovaries and FSH-primed polycystic ovaries. No difference was found between granulosa cells from FSH-primed polycystic ovaries and granulosa cells from unstimulated normal ovaries. No differences in maturation rate, fertilization rate, cleavage rate and implantation rate were observed when oocytes from a polycystic ovary were compared with oocytes from an unstimulated normal ovary. In unstimulated cycles, the ovaries were grouped according to the presence of a dominant follicle. The incidence of apoptosis was significantly higher in granulosa cells from an ovary without a dominant follicle compared with granulosa cells from an ovary with a dominant follicle. The rates of maturation, fertilization and cleavage did not differ between the two groups.

Introduction

Apoptosis is involved in regulation and selection of human ova from the primordial follicle pool (De Pol *et al.*, 1997) and is also thought to play a major role in the depletion of oocytes from ovarian tissue during ageing (Wu *et al.*, 2000). Apoptosis can be initiated in at least four different cell compartments in follicular development (theca cells, granulosa cells, cumulus cells and in the oocyte itself). Apoptosis in granulosa cells and cumulus cells is associated with follicular atresia during natural (Yuan and Giudice, 1997) and stimulated (Nakahara *et al.*, 1997a; Oosterhuis *et al.*, 1998; Høst *et al.*, 2000) ovarian cycles. However, the mechanism by which oocytes and granulosa cells interact to escape or promote apoptosis is poorly understood (Driancourt and Thuel, 1998).

A correlation between low incidence of apoptosis in cumulus–granulosa cells and improved embryo development is suggested when oocytes and cumulus–granulosa cells are obtained after controlled ovarian hyperstimulation (Seifer *et*

al., 1996; Nakahara *et al.*, 1997a; Oosterhuis *et al.*, 1998). Lower incidence of apoptosis from a single cumulus–oocyte complex correlates with maturation to metaphase II and better outcome of the corresponding oocyte in terms of fertilization rate (Høst *et al.*, 2000) and *in vitro* development (Nakahara *et al.*, 1997b). These findings indicate that the incidence of apoptosis in granulosa and cumulus cells may be useful to assess the fertility potential of oocytes recruited after controlled ovarian stimulation for *in vitro* fertilization (IVF).

Increased efforts have been devoted to *in vitro* maturation (IVM) of human oocytes (Tounson *et al.*, 1998; Cha *et al.*, 2000; Mikkelsen *et al.*, 2000). However, apoptosis has not been reported in a human IVM embryo transfer programme. The aim of the present study was to investigate the incidence of apoptotic changes in granulosa cells from immature follicles in women undergoing IVM. The incidence of apoptosis was compared between normal and polycystic ovaries and related to rates of maturation, fertilization and cleavage of the oocytes. In unstimulated cycles, the ovaries were grouped according to the presence of a dominant follicle. The incidence of apoptosis was compared between the two groups and the rates of maturation, fertilization and

cleavage were compared between oocytes originating from the ovary with a dominant follicle and oocytes originating from the ovary without a dominant follicle.

Materials and Methods

Subjects

The patients were recruited in the immature oocyte retrieval programme from patients referred for IVF-ICSI (intracytoplasmic sperm injection) due to male factor infertility, tubal factor infertility or polycystic ovary syndrome (PCOS). The procedure was explained to the patients and they gave informed consent before the procedure. Seventy IVM cycles from 70 consecutive women enrolled in the IVM programme were included in the study.

Group 1 included patients with normal ovaries ($n = 52$) referred for IVM due to male factor infertility ($n = 34$) or tubal disease ($n = 18$). Group 2 included patients with polycystic ovaries ($n = 18$), defined as > 10 follicles in one plane (Adams *et al.*, 1985). All the women were < 38 years of age with a body mass index between 18 kg m^{-2} and 29 kg m^{-2} . The basal FSH concentration was $< 15 \text{ iu l}^{-1}$ (Scott *et al.*, 1989) and the basal inhibin B concentration was $> 45 \text{ pg ml}^{-1}$ (Seifer *et al.*, 1997). Patients with infertility caused by endocrine abnormalities such as hyperprolactinaemia and patients with one ovary or ovarian endometriosis were excluded. Patients with possible poor quality oocytes (patients with a low ($< 20\%$) cleavage rate after controlled ovarian hyperstimulation and IVF or intracytoplasmic hyperstimulation) were also excluded. Moreover, patients who had previously failed more than three times to conceive with IVF-ICSI were not included. The cycle was cancelled (on day 3 of the cycle) if an ovarian cyst $> 10 \text{ mm}$ in diameter was observed.

The IVM-IVF and embryo transfer procedure had been approved by the local ethics committee and written informed consent was obtained from all participants.

Experimental design

Women with normal ovaries were subdivided into two groups. Group 1a included 27 women undergoing unstimulated cycles. Oocyte recovery was performed on the day after a follicle 10 mm in diameter and an endometrium of 5 mm in thickness could be detected by ultrasonography (Mikkelsen *et al.*, 2000). Group 1b included 25 women primed with 150 iu recombinant FSH day^{-1} (Gonal-F; Serono, Genova) for 3 days, initiated on day 3. Oocyte recovery was performed on days 8–9 after deprivation of FSH for 2–3 days (Mikkelsen *et al.*, 1999).

All women with polycystic ovaries were primed with 150 iu recombinant FSH for 3 days initiated on day 3, and oocyte recovery was performed on days 8–9 after deprivation of FSH for 2–3 days (group 2).

For each patient the incidence of apoptosis in granulosa cells was recorded for each ovary. Granulosa cells from the

aspirated follicles were collected for each ovary. In unstimulated cycles with a dominant follicle the granulosa cells from this follicle were separated and not pooled with granulosa cells from the remaining follicles.

In unstimulated cycles the ovaries were grouped according to the presence of a dominant follicle. The following factors were recorded for each ovary: the number of oocytes retrieved, the number of oocytes reaching metaphase II, the number of oocytes cleaved and the number of embryos transferred.

Oocytes from unprimed normal ovaries (group 1a) and FSH-primed polycystic ovaries (group 2) were matured in the same medium and in these groups the rates of maturation, fertilization, cleavage and implantation were compared.

Ultrasonography

An ultrasound examination was performed on day 3, days 6–7 and either once a day or at 2 day intervals until the day of aspiration using a 7.5 MHz transvaginal transducer (B & K Medical, Gentofte). The follicular diameter was calculated as the mean of the longest follicular axis and the axis perpendicular to it. A clinical pregnancy was defined as evidence of intrauterine fetal heart activity after embryo transfer visualized by ultrasonography.

Oocyte recovery, maturation, fertilization and embryo culture

These procedures have been described in detail previously by Smith *et al.* (2000) and Mikkelsen *et al.* (2000). In brief, oocyte recovery was performed transvaginally with a 17G single lumen needle (K-OPSC-1225; Cook, Queensland) connected to a syringe to induce the aspiration vacuum, and the follicular aspirates were transferred into tubes containing Ham's F-10 medium with heparin (Life Technologies, Roedovre) at 37°C . The aspirates were filtered (Falcon 1060; $70 \mu\text{m}$ mesh size) to remove erythrocytes and small cellular debris. The oocytes were isolated under a stereomicroscope and washed twice. The oocytes and their cumulus investments were classified as follows: multilayered cumulus, sparse cumulus, nude or atretic. Only oocytes that were classified as having a multilayered or sparse cumulus were used for the experiments.

Oocytes from groups 1a and 2 were matured in TCM-199 (Sigma, Roedovre) supplemented with 0.3 mmol sodium pyruvate l^{-1} (Sigma), 1500 iu penicillin G ml^{-1} (Sigma), 50 mg streptomycin sulphate ml^{-1} (Sigma), 1 mg oestradiol ml^{-1} (Sigma), 0.075 iu recombinant FSH ml^{-1} (Gonal-F; Serono), 0.5 iu hCG ml^{-1} (Profasi; Serono) and heat-inactivated serum from the patient (10%). Oocytes were cultured separately in $25 \mu\text{l}$ droplets of culture medium under paraffin oil at 37°C in $5\% \text{ CO}_2$ and humidified air for 28–30 h.

After maturation the oocytes were denuded with hyaluronidase (IVF Science, Gothenburg) and mechanical

pipetting. Motile spermatozoa were prepared by Puresperm™ (Cryos, Copenhagen) gradient separation. For ICSI, denuded oocytes were placed individually into droplets of sperm prep medium (Medi-Cult, Copenhagen) and 2 µl sperm suspension was placed into a 10 µl droplet of polyvinyl peroxidase (IVF Science). ICSI was performed on all metaphase II oocytes. The oocytes were then cultured in Falcon Petri dishes in droplets of IVF medium (Medi-Cult) until day 2 or 3 after fertilization at which time suitable embryos (maximum two embryos) were transferred into the uterus.

Images were recorded at $\times 300$ magnification at the start of IVM, after 22–23 h of IVM and at the end of IVM (28–30 h from the start of aspiration) after the oocytes were prepared for ICSI. All of the oocytes were inseminated by ICSI to confirm the presence of the first polar body microscopically, which was the criterion used to classify oocytes as matured to metaphase II. Fertilization was defined as the presence of two pronuclei.

Endometrial priming consisted of administration of oestradiol (2 mg orally three times per day) started on the day of oocyte retrieval. On day 2 after aspiration, treatment with intravaginal progesterone suppositories was initiated (100 mg three times a day) and continued until the pregnancy test. Oestrogen and progesterone treatment were continued if the pregnancy test was positive until day 50 of gestation.

Granulosa cell preparation, analysis and assessment of apoptosis

The granulosa cells were collected from the follicular aspirates during aspiration and washed twice in Earle's balanced salt solution (EBSS; Medi-Cult). The pellet with granulosa cells was pipetted onto a Silan-coated microscope slide and a thin smear was prepared. Slides were air-dried and fixed in 96% (v/v) ethanol for 5 min. An APOPTAG® *in situ* apoptosis detection kit–peroxidase (INTERGEN Oxford, catalogue no S7100-KIT) with some modifications for staining of the granulosa cells for the detection of apoptosis (Høst *et al.*, 1999) was used. The APOPTAG® kit is designed to label the free 3'-OH DNA termini *in situ* with chemically labelled and unlabelled nucleotides. The nucleotides contained in the reaction buffer are added enzymatically to the DNA by terminal deoxynucleotidyl transferase (TdT). TdT catalyses a template-independent addition of nucleotide triphosphates to the 3'-OH ends of double- or single-stranded DNA. The incorporated nucleotides from an oligomer composed of digoxigenin are allowed to bind an anti-digoxigenin antibody that is conjugated to peroxidase. The localized peroxidase enzyme then catalytically generates an intense signal from chromogenic substrates. A control without enzyme was used to determine background staining and mixed water was used in reaction buffer instead of TdT enzyme as a negative control. Background staining was < 3%.

Fig. 1. Micrograph showing two granulosa cells with apoptosis (brown coloured) and two granulosa cells without apoptosis (green coloured). Scale bar represents 10 nm.

Apoptosis and morphology were scored according to peroxidase–3,3'-diaminobenzidine staining. About 300 granulosa cells on each slide were analysed to evaluate the degree of apoptosis. The cells were viewed under a microscope by oil immersion at $\times 1000$ magnification and bright light field illumination (Fig. 1).

The slides were assessed by the same observer twice, as the slides were reassessed randomly. The intra-observation variation was < 2%.

The incidence of apoptosis was examined on a per ovary basis. The apoptotic index is the degree (%) of apoptotic activated granulosa cells per ovary.

Statistical analysis

Statistical analyses were performed using chi-squared test or Fisher's exact test. Mann–Whitney rank sum test or Kruskal–Wallis test was used for comparison between apoptosis in the cumulus cells. Values were considered significant at $P < 0.05$.

Results

The incidence of apoptosis in granulosa cells from FSH-primed normal ovaries was lower than the incidence of apoptosis in granulosa cells from unprimed normal ovaries and FSH-primed polycystic ovaries. No difference in the incidence of apoptosis was found between unstimulated normal ovaries and FSH-primed polycystic ovaries (Table 1). More oocytes were obtained from polycystic ovaries (median 8, range 4–17 oocytes per aspiration) compared with unstimulated normal ovaries (median 5, range 2–9 oocytes per aspiration). A total of 153 oocytes was recovered from polycystic ovaries and, of these, 88 (58%) cumulus-enclosed oocytes were used in the experiment.

Table 1. Incidence of apoptosis in granulosa cells from unstimulated follicles of normal human ovaries (group 1a), stimulated follicles from normal ovaries (group 1b) and stimulated follicles from polycystic ovaries (group 2)

	Group 1a	Group 1b	Group 2
Number of IVM cycles	27	25	18
Apoptosis median (%)	45.9 ^a	26.2 ^b	40.5 ^a
Interquartile range (%)	29.3–63.3	21.0–39.0	25.7–49.5

IVM: *in vitro* maturation.
^{ab}Values with different superscripts are significantly different ($P < 0.05$).

Table 2. Comparison between normal unstimulated human ovaries (group 1a) and FSH-stimulated polycystic ovaries (group 2)

	Group 1a (unstimulated)	Group 2 (stimulated)
Number of cycles	27	18
Number of oocytes	132	153
Number of oocytes for IVM	88	88
Metaphase II (%)	51 (58.0)	59 (67.1)
Two pronuclei (% metaphase II)	33 (64.7)	38 (64.4)
Cleavage (% metaphase II)	19 (37.3)	30 (50.8)
Embryos transferred	17	25
Implantation (%)	2 (11.8)	3 (12.0)
Apoptotic index of granulosa cells (%)	45.9	40.5

IVM: *in vitro* maturation.
There were no significant differences between groups.

A total of 132 oocytes was recovered from normal unstimulated ovaries and, of these, 88 (67%) cumulus-enclosed oocytes were used in the experiment. The maturation rate, fertilization rate, cleavage rate and implantation rate of oocytes from FSH-primed polycystic ovaries were not significantly different from those of oocytes obtained from normal unstimulated ovaries (Table 2).

No correlation was found between maturation rate and apoptotic index for each ovary (data not shown).

The two ovaries of each patient undergoing regular menstruation in unstimulated cycles were grouped according to appearance of a dominant follicle on the day of aspiration. The incidence of apoptosis was significantly higher in granulosa cells from the ovary without a dominant follicle compared with granulosa cells from the ovary with a dominant follicle. The rates of maturation, fertilization and cleavage were not significantly different between the two groups (Table 3).

Discussion

Previous studies of human IVF showed that a low incidence of apoptosis in granulosa cells and cumulus cells resulted in a better outcome for the oocytes from the corresponding

Table 3. Comparison between human ovary with a dominant follicle and ovary without a dominant follicle on the day of aspiration (unstimulated normal ovaries)

	Ovary with a dominant follicle	Ovary without a dominant follicle
Number of ovaries	27	27
Number of oocytes	62	70
Oocytes for IVM (%)	36 (51.1)	52 (74.3)
Metaphase II (%)	24 (66.7)	27 (51.9)
2PN fertilization	14 (58.3)	19 (70.3)
Cleaved	9 (37.5)	10 (37.0)
Apoptotic index of granulosa cells (%)	41.9 ^a	59.3 ^b

2PN fertilization: presence of two pronuclei.
^{ab}Values with different superscripts are significantly different ($P < 0.05$; analysed by Mann–Whitney U test).

follicle (Nakahara *et al.*, 1997b; Høst *et al.*, 2000). However, there is little information about the importance and necessity of apoptosis in granulosa cells of immature human follicles. Høst *et al.* (2000) reported a significantly higher degree of apoptosis in cumulus cells from immature oocytes compared with cumulus cells from metaphase II oocytes when oocytes were obtained after controlled ovarian hyperstimulation. Maturation of these immature oocytes *in vitro* has demonstrated that their developmental competence is markedly lower than that of their counterparts matured *in vivo* (Kim *et al.*, 2000). These oocytes may represent an inferior population as they failed to mature although the follicles were exposed to supraphysiological concentrations of gonadotrophins.

The aim of the present study was to obtain oocytes that have been able to develop into viable embryos after IVM in previous studies. There is evidence that competent oocytes in cows could originate from non-growing or early atretic follicles (Blondin *et al.*, 1997). A reduced follicular growth has been shown to be advantageous before oocyte collection for IVM. In unstimulated cycles in humans the recovery of oocytes has to coincide with selection of the dominant follicle (Mikkelsen *et al.*, 2000). In stimulated cycles in humans a time interval between FSH administration and aspiration improves the developmental capacity of oocytes (Mikkelsen *et al.*, 1998). In the present study, the recovery of oocytes was performed according to these criteria.

In women undergoing regular menstruation, an increased percentage of granulosa cells from unstimulated follicles was apoptotic compared with granulosa cells from follicles stimulated with FSH before aspiration. It was not possible to examine the clinical significance with regard to apoptosis of granulosa cells and subsequent developmental competence in the present study, as the oocytes from FSH-primed normal ovaries and oocytes from unprimed normal ovaries were cultured in different culture media.

There are conflicting data regarding the maturation rate

of *in vitro*-matured oocytes from normal ovaries retrieved after FSH-priming *in vivo* (Wynn *et al.*, 1998; Mikkelsen *et al.*, 1999). Wynn *et al.* (1998) reported an increased number of oocytes matured *in vitro* after FSH priming compared with women without priming. Mikkelsen *et al.* (1999) could not demonstrate any differences in the rates of maturation, fertilization or cleavage between the two groups.

Several studies report the use of *in vitro*-matured and fertilized oocytes in women with irregular cycles and PCOS (Trounson *et al.*, 1998; Cha *et al.*, 2000; Chian *et al.*, 2000). The maturation rate of immature oocytes retrieved from patients with PCOS has been reported to be impaired compared with that of women undergoing regular cycles (Barnes *et al.*, 1996). Suikkari *et al.* (2000) proposed priming with FSH before oocyte pick-up to compensate for this impaired maturation rate.

In the present study, the incidence of apoptosis of granulosa cells originating from FSH-primed polycystic ovaries did not differ from the incidence of apoptosis of granulosa cells originating from unstimulated normal ovaries. Furthermore, the rates of maturation, fertilization and cleavage were not significantly different between the two groups. These findings support the concept that at least some antral follicles in the ovaries of women with PCOS are not committed irreversibly to atresia and, therefore, can be rescued by exogenous FSH.

There is conflicting evidence regarding the importance of a dominant follicle on the day of aspiration before IVM. Cobo *et al.* (1999) reported that once selection of the leading follicle has occurred, the developmental potential of the oocytes is impaired. The explanation for these findings should be a negative effect of the selection process. However, Whitacre *et al.* (1998) and Thorton *et al.* (1999) have raised some doubts about the significance of the dominant follicle. Selection of a dominant follicle from a growing cohort is controlled by feedback mechanisms between the ovary and the hypothalamic–pituitary axis and by intra-ovarian communication. Intra-ovarian communication can include an endocrine regulation as well as follicle–follicle interactions. Hence, a follicle can maintain dominance by inhibiting challengers (Macklon and Fauser, 2000). According to these findings, the oocytes from the ovary with a dominant follicle might be expected to be different from the oocytes originating from the ovary without a dominant follicle. It has been considered that the dominant follicle can be recognized by ultrasonography when it has reached 10 mm in diameter (Fauser and van Heusden, 1997). In the present study, we aimed to collect oocytes to coincide with selection of the dominant follicle. No differences in the rates of maturation, fertilization and cleavage were detected between oocytes originating from the ovary with a dominant follicle and oocytes originating from ovaries without a dominant follicle. No conclusion can be drawn with regard to implanted embryos because the embryos transferred originated from both ovaries in most of the patients.

A lower incidence of apoptosis was observed in the granulosa cells from the ovaries with a dominant follicle compared with ovaries without a dominant follicle. The explanation for this difference is unknown. For an individual patient undergoing a GnRH and human menopausal gonadotrophin (hMG) ovulation protocol, no difference in the incidence of apoptosis of one ovary over the other has been demonstrated (Moreira *et al.*, 1999).

The data in the present study were measured on a per ovary basis and a higher incidence of granulosa cell apoptosis on a per ovary basis cannot be associated directly with each oocyte of compromised quality. Our results show an increased incidence of apoptosis in granulosa cells of immature follicles from unstimulated normal ovaries compared with granulosa cells from FSH-primed normal ovaries. In unstimulated cycles, it was found that follicles from the ovary with a dominant follicle had a decreased incidence of apoptosis compared with follicles from the ovary without a dominant follicle, whereas the rates of maturation, fertilization and cleavage were unaffected by a dominant follicle. Oocytes obtained from unstimulated normal ovaries did not differ from oocytes obtained from polycystic FSH-primed ovaries with respect to incidence of apoptosis of granulosa cells and maturation and fertility potential of the oocytes.

This study was supported in part by Medi-Cult.

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Received 4 December 2000.

First decision 22 January 2001.

Final revision received 19 March 2001.

Accepted 29 March 2001.