Are there any relationships between the fecundity of bilateral ovaries in an individual patient and the incidence of apoptotic granulosa cells?

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Many researchers have discussed fecundity on a per patient or per ovarian follicle basis. In contrast, this study was undertaken on a per ovary basis, and tests the hypothesis that there is a relationship between the fecundity of the bilateral ovaries in an individual patient and the incidence of apoptotic granulosa cells. The two ovaries of 10 women undergoing ovulation induction for in-vitro fertilization (IVF) with gonadotrophin-releasing hormone analogue (GnRHa) and gonadotrophins were compared. There was no difference in apoptotic index in granulosa cells and various hormones in the follicular fluids. Our results indicate that, in the ovulation induction protocol for IVF, GnRHa and gonadotrophins, there is no predisposition of one ovary over the other in an individual patient in terms of apoptosis at the time of aspiration, even though the number of oocytes in each ovary is different, because the number of oocytes retrieved may reveal ovarian fecundity before stimulation with GnRHa + human menopausal gonadotrophin (HMG).

Key words: apoptosis/granulosa cells/human/in-vitro fertilization

Introduction

The asymmetry of ovarian function has long been a subject of controversy. In primates, it was suggested that ovulation occurs with an equal frequency in the right and left ovary (Wallach et al., 1973). De Zergues and Holden (1982) reported that there was no definite preference of one ovary over the other during consecutive cycles in humans. Potashnik et al. (1987) showed that, in normally menstruating women, ovulation rate was significantly higher in the right ovary. Also, Driscoll et al. (1996) showed that in ovulation induction for gamete intra-Fallopian transfer (GIFT), there was a slight skew towards right ovary predominance, but it was not statistically significant. Potashnik et al. (1987) suggested that right-side predominance was either genetically determined or due to the differences in the vasculature of the ovaries. He also demonstrated that the length of the follicular phase is correlated with the side of the ovulation: in a cycle with a short follicular phase, the residual corpus luteum from the previous cycle may prevent the development of a dominant follicle in that ovary by the local activity, and ovulation therefore tends to occur in the contralateral ovary. In cycles with a follicular phase >14 days, the diminished local corpus luteum activity enables dominant follicular development from either ovary at random. If this is the case, there is a strong possibility that, in stimulated cycles as in IVF with long down-regulation before the follicular phase, the side of ovulation is affected.

Programmed cell death or apoptosis is a process whereby physiological stimuli activate a genetic programme to implement a specific series of events that culminate in the death and efficient disposal of a cell. Electron microscopic features of apoptosis include nuclear chromatin condensation, compactness of cytoplasmic organelles, and the appearance of pedunculated protuberances on the cell surface. The changes are recognized under the light microscope as pycnosis of nuclei, decrease in cell volume, some extraordinary surface circumvolutions, and explosion of the cell into cytoplasmic fragments containing condensed chromatin (Benedetti et al., 1990).

The incidence of apoptotic bodies has been applied as a morphological marker for physiological cell renewal in the liver and gastroduodenal mucosa (Tilly et al., 1991), as a biomarker for the regression of liver hyperplasia (Bursch et al., 1985), and for prognosis of patients with neoplasms such as non-Hodgkin lymphoma (Leoncini et al., 1993) and prostatic intraepithelial cancer (Wheeler et al., 1994). Apoptosis is also associated with follicular atresia occurring in natural (Yuan and Giudice, 1997) and stimulated ovarian cycles (Nakahara et al., 1997a,b) and also in fetal (Pol et al., 1997) and aged (Perez and Tilly, 1997) ovaries. There have been few reports concerning apoptosis in human IVF-embryo transfer programmes. However, recently Nakahara et al. (1997a,b) reported that the incidence of apoptotic bodies in membrana granulosa can predict the prognosis of ova from patients undergoing ovulation stimulation for in-vitro fertilization (IVF). They found that a higher incidence of apoptotic bodies is associated with low oocyte retrieval, empty follicles, poor oocyte fertilization and poor embryo quality. In their study, the relationship between the incidence of apoptotic granulosa cells and oocyte competence (fertilization and embryo development) was analysed on a per follicle basis (Nakahara et al., 1997b), and the relationship between the incidence of apoptotic granulosa cells and the pregnancy rate was analysed on a per patient basis (Nakahara et al., 1997a). They showed that the incidence
of apoptotic granulosa cells is a very sensitive indicator to
detect the fecundity on a per follicle and per patient basis.
The relationship between the incidence of apoptotic cells and
ovarian fecundity on a per ovary basis was not evaluated.

The aims of this study are to observe: (i) if there is a
difference in the incidence of apoptotic cells in human granu-
losa cells, follicular fluid hormones and number of oocytes
retrieved between the right and left ovaries in stimulated cycles
of patients undergoing IVF and (ii) if there is a difference in
apoptotic cells index, follicular fluid hormones and oocyte
maturity when the bilateral ovaries of each patient are defined
as high or low fecundity according to the number of oocytes
retrieved from each ovary.

Materials and methods

Patients and follicle aspiration

Ten IVF cycles from 10 patients were analysed between January and
February 1998 at Yamagata University Hospital, Yamagata, Japan.
Patients with endometriosis were excluded because of the effect of
such disease on steroidogenesis and the incidence of apoptosis.

The study was approved by the Yamagata University Hospital
Committee for Research on Human Subjects; written informed consent
was obtained from all patients, and clinical information concerning
them was concealed.

The ovulation induction protocol was performed using a
gonadotrophin-releasing hormone analogue (GnRHa), buserelin
acetate (Suprecur nasal; Hoechst, Tokyo, Japan) in a long suppression
protocol starting from the mid-luteal phase. Human menopausal
gonadotrophin (HMG) (150–300 IU/day; Humegon; Sankyo, Tokyo,
Japan) with or without follicle stimulating hormone (FSH)
(Fertinom P; Serono, Tokyo, Japan) was started on day 3 of the
menstrual cycle. Human chorionic gonadotrophin HCG (10 000 IU;
Mochida, Tokyo, Japan) was administered when one follicle achieved
a mean diameter of 16 mm. At 35 h after the administration of HCG,
follies were aspirated by transvaginal ultrasound retrieval (6.5 MHz;
Mochida). All follicles with a mean diameter of ≥11 mm were
aspirated using a 20 ml syringe. Follicular fluids from each ovary
were gathered for the analysis of each ovarian status.

Cell fixation and quantification of apoptotic cells

The aspirated follicular fluid was transferred into tissue culture dishes
(Falcon 3002; Becton Dickinson and Company, Lincoln Park, NJ,
USA). Oocyte–cumulus cell complexes were isolated under a dis-
secting microscope (SZH-ILLB; Olympus, Tokyo, Japan) using
×10–20 magnifications and were put into an organ tissue culture
dish (Falcon 3037; Becton Dickinson) with human tubal fluid (HTF)
medium. The oocyte–cumulus cells complexes were classified into
two groups according to Saito’s criteria (Saito and Hiroi, 1986). These
criteria, with two categories, are simpler than Marrs’ criteria
(Marrs et al., 1984), which have four morphological categories.
Saito’s have two categories. The complexes with large, loose cumulus
and distinct corona radiata were judged as mature, and the complexes
with a small, dense cumulus and opaque corona radiata were defined
as immature. The mural granulosa cells were put into another dish
with HTF medium for washing. Then the granulosa cells were placed
on a slide glass and hyaluronidase (Sigma, St Louis, MO, USA)
(0.1% wt/vol in HTF medium) was added. The cell masses were then
pipetted thoroughly for 1 min, and fixed with 4% wt/vol of neutral
buffered formalin. This process was completed within 1 h after the
follicle aspiration to avoid post-aspiration cell death. After fixation,
the slides were washed by phosphate-buffered saline for 1 h and the
nuclei of cells were stained with 0.5 μg/ml of Hoechst 33258
(fluorescent dye; Wako, Osaka, Japan) with 5% wt/vol of DABCO
(1,4-diazabicyclo 2,2,2-octane; Sigma) in 90% glyceral; 10% 0.2 M
Na2HPO4. The DABCO blocked the bleaching effect of fluorescence
and allowed the slides to be rechecked within a few weeks.

Apoptotic cells were revealed as cytoplasmic fragments containing
condensed chromatin (i.e. apoptotic bodies) or as the cells with
fragments of condensed chromatin when examined by fluorescence
microscopy (Figure 1). The apoptotic cells were identified and counted
among 1000 granulosa cells at random at ×1000 magnification.

Follicular fluid collection and assay for intrafollicular steroids

After the oocytes and granulosa cells masses were retrieved, each
sample of follicular fluid was centrifuged at 400 g for 10 min and
the supernatants were stored at −20°C until the assay for oestradiol,
progesterone, free testosterone, FSH, luteinizing hormone (LH),
prolactin and HCG. Quantification of hormonal concentration was
performed using commercial available immunoassay kits: oestradiol,
progesterone, LH, FSH, prolactin and HCG by chemiluminescent
enzyme immunoassay system (Immulyse; Diagnostic Products
Corporation, Los Angeles, USA) and free testosterone by radio-
immunoassay (Diagnostic Products Corporation).

Statistical analysis

Comparison between groups of ovaries was performed using the Sign
test or Signed rank test. Comparison between groups of patients was
done using the Mann–Whitney U-test.

Results

Comparison of right ovary versus left ovary

In order to clarify differences in fecundity between right and
left ovaries, we examined the number of retrieved oocytes, the
number of mature oocytes, the rate of mature oocytes (calcu-
lated as the mean of % oocyte maturity for each patient),
apoptotic cells in mural granulosa cells, follicular fluid volume
and hormones concentrations in follicular fluid were compared.
Individual aspirates from each ovary showed no difference
between right and left ovaries (Table I).

Comparison of the ovary groups with high and low fecundity

We separated the two ovaries of each patient into two groups
according to the number of oocytes retrieved from each ovary.

Group A: ovaries which have more oocytes retrieved than
the opposite ovary. These ovaries were thought to have higher
fecundity.

Group B: ovaries which show fewer oocytes retrieved than
the opposite ovary. Their fecundity was evaluated as lower.

We then compared the number of mature oocytes, the rate of
mature oocytes, apoptotic index, follicular fluid volume and
hormone concentrations between the two groups (Table II).
The number and the rate of mature oocytes were significantly
higher in group A than in group B (P < 0.05). Apoptotic
index, follicular fluid volume and hormone concentrations
showed no significant difference between the two groups.

Comparison of the patient groups with high and low fecundity
to total oocyte number

We separated the patients, instead of the ovaries, into two
groups according to the total number of oocytes retrieved from
Figure 1. Granulosa cells stained with Hoechst 33258. Right, normal granulosa cell with normal nucleus. Middle and left, apoptotic granulosa cells. Left shows a condensed and shrinking nucleus and middle shows condensed and fragmented nuclei.

### Table I. Comparison between right and left ovaries in 10 patients

<table>
<thead>
<tr>
<th></th>
<th>Right ovary*</th>
<th>Left ovary*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF volume (ml)</td>
<td>17.8 ± 3.5</td>
<td>13.8 ± 2.5</td>
</tr>
<tr>
<td>No. of oocytes</td>
<td>5 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>No. of mature oocytes</td>
<td>4 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Rate of mature oocyte (%)</td>
<td>80.0 ± 11.5</td>
<td>69.4 ± 11.3</td>
</tr>
<tr>
<td>Apoptotic index (/1000)</td>
<td>1.3 ± 0.3</td>
<td>3.0 ± 0.6</td>
</tr>
</tbody>
</table>

**FF steroid concentrations**

<table>
<thead>
<tr>
<th></th>
<th>Right ovary*</th>
<th>Left ovary*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol concentration (ng/ml)</td>
<td>551.8 ± 38.4</td>
<td>556.1 ± 53.4</td>
</tr>
<tr>
<td>Progesterone concentration (ng/ml)</td>
<td>6110 ± 1537.3</td>
<td>5200 ± 613.6</td>
</tr>
<tr>
<td>Free testosterone (pg/ml)</td>
<td>70.2 ± 7.6</td>
<td>61.7 ± 8.5</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>2 ± 0.66</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>3.9 ± 0.4</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>31.2 ± 4.2</td>
<td>32.6 ± 4.4</td>
</tr>
<tr>
<td>HCG (mIU/ml)</td>
<td>96.4 ± 10.5</td>
<td>97.1 ± 9.4</td>
</tr>
</tbody>
</table>

*All values are means ± SEM.
There were no statistical differences between the two groups.

P value: analysed by the Sign test.

FF = follicular fluid.

both ovaries: group C: patients with a total of eight or more oocytes retrieved from the two ovaries; group D: patients with a total of seven or fewer oocytes retrieved from the two ovaries.

We analysed the same parameters as described above between the two groups (Table III). The follicular fluid volume and the number of mature oocytes were significantly higher in group C than in group D (respectively *P* = 0.02, *P* = 0.01).

The patients in group C showed a lower incidence of apoptotic granulosa cells than those in group D (*P* = 0.02).

### Discussion

In most previous studies on the prognosis of IVF, prognostic parameters were analysed on a per follicle basis or on a per patient basis. Those studies have used follicular fluid volume and hormones concentration, the number of oocytes retrieved, and granulosa cell apoptotic index as factors in IVF programme outcome. Kemeter and Feichtinger (1991) and Arnott et al. (1995) found a correlation between follicular fluid volume and fertility potential of the oocyte. Follicular fluid gonadotrophins, prostaglandins, cyclic AMP, prolactin, progestosterone, oestriadiol and androgens have all been suggested as potential predictors of oocyte quality (Carson et al., 1982; Botero-Ruiz et al., 1984; Uehara et al., 1985; Basuray et al., 1988; Firmin et al., 1991; Bodis et al., 1993). Arnott et al. (1995) also showed that the more follicles recruited, the more oocytes are retrieved and the larger the number of embryos available from which to select for transfer. Recently, the incidence of apoptotic bodies in membrana granulosa has been shown to be of prognostic significance for oocytes from patients participating in IVF programmes (Siefer et al., 1996; Nakahara et al., 1997a,b). Siefer et al. examined the percentage of apoptotic granulosa cells harvested from nine women with day 3 serum FSH <= 6 mIU/ml and from eight women with FSH >= 10 mIU/ml and concluded that women undergoing IVF with high FSH showed an increase in the percentage of cells undergoing apoptosis as compared with women with low FSH. They also concluded consequently that the women with high FSH produced a lower number of oocytes than the women with low FSH. In Nakahara’s studies, the pool of follicles showed difference in terms of fertility and apoptosis: follicle atresia could occur in some follicles, thus resulting in empty follicles; and the oocytes with a higher incidence of apoptotic bodies corresponded to a poor rate of oocyte fertilization and poor embryo quality. Although the asynchronous follicular development during ovarian stimulation is less prominent if prior suppression with a GnRH agonist is employed, the variation in follicles still remains. Each oocyte in a growing follicle is exposed to its own unique environment, and its maturity and fecundity capacity is therefore also unique. Since individual follicles showed various differences, the bilateral ovaries were consequently thought to have different capacities for follicular development.

From these findings, one ovary can be expected to be different from the other in an individual patient in terms of fecundity. As Potashnik et al. (1987) mentioned, the two ovaries were not equal in term of frequency of ovulation in
Table II. Comparison between ovary with higher number of oocytes retrieved and ovary with lower number of oocytes retrieved from each patient

<table>
<thead>
<tr>
<th></th>
<th>Group A* (more oocytes retrieved)</th>
<th>Group B* (less oocytes retrieved)</th>
<th>P valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of ovaries</td>
<td>10</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>FF volume (ml)</td>
<td>17.6 ± 3.6</td>
<td>14.1 ± 2.4</td>
<td>NS</td>
</tr>
<tr>
<td>No. of mature oocytes</td>
<td>4 ± 1</td>
<td>1 ± 0</td>
<td>0.002</td>
</tr>
<tr>
<td>Rate of mature oocyte (%)</td>
<td>90.0 ± 4.3</td>
<td>59.4 ± 13.2</td>
<td>0.04</td>
</tr>
<tr>
<td>Apoptotic index (/1000)</td>
<td>1.6 ± 0.3</td>
<td>2.7 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>FF steroid concentrations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestradiol concentration (ng/ml)</td>
<td>547.3 ± 39.1</td>
<td>560.6 ± 52.8</td>
<td>NS</td>
</tr>
<tr>
<td>Progesterone</td>
<td>5310 ± 691</td>
<td>6000 ± 1510.6</td>
<td>NS</td>
</tr>
<tr>
<td>Free testosterone (pg/ml)</td>
<td>69.7 ± 8.4</td>
<td>62.1 ± 7.8</td>
<td>NS</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>2.13 ± 0.67</td>
<td>1.7 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>3.7 ± 0.4</td>
<td>4 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>31.5 ± 4.1</td>
<td>32.3 ± 4.5</td>
<td>NS</td>
</tr>
<tr>
<td>HCG (mIU/ml)</td>
<td>95.8 ± 10.2</td>
<td>97.6 ± 9.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

All values are means ± SEM.
NS = not significant (P > 0.05).

aP value: analysed by the Sign test.

FF = follicular fluid.

Table III. Comparison between patients with a total of eight or more oocytes retrieved and patients with a total of seven or fewer oocytes retrieved

<table>
<thead>
<tr>
<th></th>
<th>Group C* (≥8 oocytes retrieved)</th>
<th>Group D* (≤7 oocytes retrieved)</th>
<th>P valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>5</td>
<td>5</td>
<td>0.02</td>
</tr>
<tr>
<td>FF volume (ml)</td>
<td>42.6 ± 1</td>
<td>20.8 ± 3.4</td>
<td>0.01</td>
</tr>
<tr>
<td>No. of mature oocytes</td>
<td>8 ± 0.4</td>
<td>4 ± 1</td>
<td>0.02</td>
</tr>
<tr>
<td>Apoptotic index (/1000)</td>
<td>3.4 ± 0.2</td>
<td>5.2 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>FF steroid concentrations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestradiol concentration (ng/ml)</td>
<td>628.4 ± 40</td>
<td>479.5 ± 65.9</td>
<td>NS</td>
</tr>
<tr>
<td>Progesterone</td>
<td>5550 ± 527</td>
<td>3871.9 ± 507.3</td>
<td>NS</td>
</tr>
<tr>
<td>Free testosterone (pg/ml)</td>
<td>70.7 ± 10.6</td>
<td>61.2 ± 8.4</td>
<td>NS</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>2.73 ± 0.80</td>
<td>1.3 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>3.9 ± 0.6</td>
<td>4.3 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>37.9 ± 5.6</td>
<td>25.9 ± 5.6</td>
<td>NS</td>
</tr>
<tr>
<td>HCG (mIU/ml)</td>
<td>95.5 ± 18.8</td>
<td>82 ± 13.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are given as means ± SEM.
NS = not significant (P > 0.05).

aP value: analysed by Mann–Whitney U-test.

FF = follicular fluid.

the natural cycle. Indeed, ovulation in primates is usually a consistently alternating process. Marinho et al. (1982), using pelvic ultrasonography, provided good evidence that ovulation occurs from the alternate ovaries in 80% of healthy subjects with regular menstrual cycles. Predominance of the right side was indicated by Potashnik et al. (1987).

In stimulated cycles, GnRHa increased the chance of pregnancy by abolishing the spontaneous release of gonadotrophin by the pituitary gland and protecting oocytes from inappropriate exposure to LH, which is thought to be detrimental to normal maturation of the egg. Rutherford et al. (1988) demonstrated that more oocytes and more embryos were recovered in women given buserelin than in women receiving clomiphene citrate. This implies that GnRHa improved the condition of follicles and every follicle became almost equal by its action. Lass et al. (1997) found that there is no predilection of one ovary over the other in GnRH/HMG ovulation induction protocol for IVF. However, their study was based on a comparison between patients with right oophorectomy versus patients with left oophorectomy. Both ovaries of the same patient were not evaluated.

In our study, both ovaries were present on both sides and we found no predisposition between the right and left ovary groups (Table I). In our comparison of the ovary groups with high and low fecundity (Table II), it was demonstrated that even though the growth of individual follicles and the number of oocytes retrieved are different in individual ovaries; the two ovaries of each patient showed no difference in the potential of fecundity in terms of the incidence of apoptosis and various hormones in the follicular fluids. So, even if the number of recruited follicles in the new follicular cohort of each ovary has already been decided and was different at the time of starting the GnRH agonist, the two ovaries showed the same conditions for developing follicles, at least after
starting follicle stimulation using GnRHa, HMG and HCG. This suggested that down-regulation by GnRHa not only protects the ovaries from inappropriate LH but also maintains the same capacity for follicle development in the two ovaries. Thus the two ovaries can grow follicles in the same conditions and reach the end of the stimulated cycle with an equivalent quality of production (follicular fluid hormone and apoptosis).

In this study, individual patients showed no predominance of either ovary in terms of the incidence of apoptosis. Although the number of patients was small, the patients still indicated the same phenomenon as described by Nakahara et al. (1997a,b) in his analysis on a per patient basis. The patients who had a larger number of oocytes retrieved showed a lower incidence of apoptosis in granulosa cells than the patients who had a smaller number of oocytes retrieved. This means that, on a per patient basis, each patient has their own unique state for developing follicles before starting the stimulation. The state after stimulation started was the same for both ovaries in an individual patient in terms of apoptosis.

We also measured follicular fluid steroids (oestradiol, progesterone and free testosterone), but did not find any significant differences in the three types of comparison. This phenomenon may be due to the maintenance of steroidogenesis in the apoptotic granulosa cells (Breckwoldt et al., 1996; Amsterdam et al., 1997). Aharoni et al. (1995) also proposed a unique mechanism of compartmentation of steroidogenic organelles in the perinuclear region, and migration of the multicatalytic proteins to the proteasome to the apoptotic blebs. The relatively small changes in the rate of apoptosis between the groups in the comparisons may also be responsible for the lack of difference observed in the concentrations of follicular fluid steroids.

Lastly, our study clearly indicates that, for an individual patient undergoing a GnRHa/HMG ovulation protocol, there is no predisposition of one ovary over the other in terms of the incidence of apoptotic granulosa cells and various follicular hormones even though the number of retrieved oocytes in each ovary is different. Although both the number of oocytes retrieved and the incidence of apoptotic granulosa cells are good indicators of the fecundity of ovaries, the number of oocytes reveals ovarian fecundity before starting GnRHa + HMG stimulation, and the incidence of apoptotic cells reveals the ovarian fecundity at present, i.e. the time of oocyte aspiration. If GnRHa + HMG stimulation were not employed, apoptosis would occur more vigorously in many follicles and consequently only one follicle would develop. Thus using GnRHa + HMG stimulation, apoptosis was restrained, and the fecundity of developing follicles increased equally in both ovaries of an individual patient. However, natural fecundity is profoundly different among individual patients, and it may still have affected the number of oocytes retrieved and the incidence of apoptotic granulosa cells even though GnRHa + HMG stimulation was employed. Consequently, patients in whom more oocytes were retrieved had a lower incidence of apoptotic granulosa cells.

References


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