Aneuploidy in human granulosa lutein cells obtained from gonadotrophin-stimulated follicles and its relation to intrafollicular hormone concentrations

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Proliferation of granulosa cells is inversely related to differentiation and hormone production. The purpose of this study was to evaluate the intrafollicular and serum steroid concentrations and to compare these results to granulosa cell proliferation as measured by DNA flow cytometry. Human granulosa lutein cells in follicular fluid of in-vitro fertilization (IVF) patients were investigated with regard to ploidy, percentage of S-phase cells and proliferation index (PI: percentage of cells in the S- and G1/M-phase). The study was originally designed to indicate an additional marker for the outcome of IVF treatment by DNA flow cytometric measurements of granulosa lutein cells. Follicular fluids of 160 follicles (45 patients) were evaluated: 45.6% \( (n = 73) \) of the follicles showed aneuploid granulosa lutein cells and 5.6% \( (n = 9) \) of the follicles contained multiploid granulosa cells, defined as at least two aneuploid populations of cells with different DNA indices. A total of 48.8% \( (n = 78) \) of the follicles had only diploid cells. Thus >50% of the investigated follicles showed aneuploidy. In all, 73% (33 of 45) of patients had at least one follicle containing aneuploid granulosa lutein cells. The PI of the aneuploid cell populations significantly exceeded that of the diploid cell populations (median: aneuploid: 15.5; diploid: 7.4; \( P < 0.0001 \)). The intrafollicular concentrations of testosterone, progesterone and dehydroepiandrosterone sulphate (DHEA-S) were significantly lower in follicles with aneuploid granulosa cell populations. Luteinizing hormone concentration was significantly higher in follicles with aneuploid granulosa cells. Intrafollicular concentrations of oestradiol, follicle stimulating hormone and the serum concentrations of all steroid hormones did not show any significant correlation to ploidy. Although aneuploidy has been reported for oocytes (in ~17% of the oocytes), no study, to our knowledge, has observed such a high incidence of aneuploidy in granulosa lutein cells after gonadotrophin stimulation. Except for aneuploidy found in tissues with some characteristics of neoplastic growth (colon adenoma, borderline tumours, endometriosis with atypical cells, etc.), it is unique for non-malignant human cells. The correlation with intrafollicular steroid concentrations points to a possible pathophysiological or physiological relevance of these findings. However, it was impossible to correlate the outcome of IVF with DNA flow cytometry results.

Key words: aneuploidy/flow cytometry/granulosa lutein cells/IVF/proliferation

Introduction

Granulosa cells are a substantial cellular component of follicular fluid. At 36 h after human chorionic gonadotrophin (HCG) injection, follicular fluid contains luteinized and non-luteinized granulosa cells and leukocytes, mainly macrophages and monocytes, which make up 5–15% of all cells in the follicular fluid (Loukides et al., 1990). During the follicular phase of the human menstrual cycle, granulosa cells proliferate and differentiate under follicle stimulating hormone (FSH) control to become eventual sites of luteinizing hormone (LH)-responsive hormone synthesis in the pre-ovulatory follicle and corpus luteum. FSH initiates this programmed sequence of events through binding to its receptor on the granulosa cell surface. FSH activates intracellular adenyl cyclase and cyclic AMP-dependent protein kinases, leading to increased expression of genes encoding for steroidogenic enzymes (Doody et al., 1990). Functional LH receptors are also coupled to steroid synthesis via adenylyl cyclase. Post-ovulation, however, LH stimulates increased secretion of progesterone. The follicle luteinizes and apparently ceases to grow. Thus, FSH appears to support both cytoproliferation and differentiation of granulosa cells, while LH stimulates cell function (steroidogenesis) and differentiation but not proliferation. This reveals a development-related inverse relationship between proliferation and expression of differentiated function in human granulosa cells. When maximal progesterone production is reached, there is an almost complete cessation of DNA synthesis and cell proliferation (Yong et al., 1992).

Proliferation dynamics of granulosa cells can be assessed by means of DNA flow cytometry (FCM). The technique of DNA FCM is an accepted and simple way of measuring the DNA content of cells.

The purpose of this study was to examine the relationship between clinical parameters (fertilization and pregnancy rate), biochemical parameters (intrafollicular and serum steroid concentrations) and the proliferation index (PI) and other flow...
cytometric parameters of granulosa lutein cells in the follicular fluid of patients undergoing a gonadotrophin stimulation in an in-vitro fertilization (IVF)/embryo transfer programme. Since granulosa cells are responsible for the intrafollicular environment, both their proliferative and steroidogenic properties are important for the fate of the oocyte.

**Materials and methods**

**Patient group**
Granulosa lutein cells from 45 consecutive unselected infertile patients (aged 23–46 years) who were treated in an IVF/embryo transfer programme were examined by flow cytometric analysis. A total of 28 out of 45 (62.2%) patients had primary infertility and 17 (37.8%) had a secondary infertility. Reasons for infertility were tubal occlusion in 33 out of 45 patients (73.3%), in five patients (11.1%) lasting sterility with endometriosis and no other explanation for infertility, in 33 out of 45 patients (73.3%), in five patients (11.1%) an andrological infertility and in two patients (4%) no explanation could be determined. All patients had normal gonadotrophin serum concentrations. In all, 160 follicular fluid samples from 46 ultrasound guided transvaginal punctures of follicles in 45 patients were analysed (one patient was treated twice during the observation period).

**Stimulation protocol**
Patients were stimulated with pure FSH or human menopausal gonadotrophin (HMG) (step-down regimen) after pituitary down-regulation (starting on day 20 of the previous cycle; leuproleline 0.1 ml/day s.c. until administration of HCG). The step-down regimen is the standard procedure for women <35 years of age in our IVF unit. In the first stimulation cycle, women <35 years of age received a daily dosage of 225 IU of FSH or HMG for the first 3 days, followed by 150 IU per day until ovulation induction. Women ≥35 years old were injected with a daily dosage of 225 IU until ovulation induction.

The gonadotrophin dosage in subsequent cycles was adapted according to the observed ovarian response. In all, 38 (83%) cycles were treated with highly purified FSH (Fertinorm HP®; Serono) and eight (17%) with HMG (Menogon®; Ferring). After adequate follicular growth (at least three follicles ≥18 mm diameter in transvaginal ultrasound), ovulation was induced with 10 000 IU s.c. HCG (Pregnesin 50000®; Serono) and oocyte retrieval was performed 36 h later. From each patient, follicular fluids of the first three to five follicles were separated for hormone analysis and DNA flow cytometry. Each follicle contained an oocyte. On the day of oocyte retrieval, 20 ml blood was obtained for the assessment of hormones.

**Granulosa cell preparation**
Transvaginal oocyte retrieval was performed 36 h after HCG injection. Every follicle was punctured once, the follicular fluid was aspirated and separated after removal of the oocyte. The follicle was then flushed with phosphate buffered saline (PBS) and the fluid was collected in a different vial. The follicular fluid was centrifuged for 10 min at 300 g. Supernatants were removed and stored at -20°C for later assessment of LH, FSH, oestradiol, progesterone, testosterone and dehydroepiandrosterone sulphate (DHEA-S).

Flushing fluid was added to the remaining granulosa cell pellet and a cytological smear from each follicle was prepared by cytopsin to identify the type of cells (Papanicolaou stain). Flow cytometry was carried out immediately after preparation of granulosa cells.

**Flow cytometric analysis of granulosa cell DNA**
Nuclear DNA content of granulosa cells was determined by DNA flow cytometry. From fresh and unfixed fluids cell cycle analyses of granulosa lutein cells were carried out by DNA flow cytometry according to a standardized protocol (Otto et al., 1981) after DAPI staining on a flow cytometer PAS II (Partec GmbH, Münster, Germany). Prior to flow cytometric evaluation, cellular membranes were removed by citric acid. Only nuclei were measured after staining with DAPI. Since there were only nuclei, there was no clustering of granulosa lutein cells. The number of blood cells was kept low by using only fluids from the first follicles, thus obtaining clear follicular fluid. The recognition of aneuploid and diploid cell cycles was done by a standardized method involving trit erythrocytes (Melsheimer et al., 1997). By means of suitable software (Multicycle Ver. 3.5), the percentage of cells in G0/G1, S and G2/M phases of the cycle was calculated. The PI was defined as percentage of cells in S+G2/M phase (Figure 1).

**Hormonal assays**
Concentrations of LH, FSH, oestradiol, progesterone, testosterone and DHEA-S were assessed in follicular fluid and in serum samples obtained at the time of oocyte retrieval. The follicular fluid of seven follicles could not be assessed due to technical reasons. A commercially available ELISA (Boehringer Mannheim, Mannheim, Germany) was used to assess LH, FSH, oestradiol and progesterone. Testosterone was measured by radioimmunoassay (Diagnostic Product Corporation, Los Angeles, CA, USA) and DHEA-S was assessed by enzyme immunoassay (Biochem Immunosystems, Quebec, Canada). The coefficient of variation of the intra-assay variation was <.5% and the interassay variation was 5–12%.

**Statistical analysis**
Results were evaluated by means of a Macintosh Power PC 6100/80 with commercially available software [Word 6.0 (Microsoft), Statview 4.02 (Abacus Concepts), MacDrawPro (Claris), Excel 5.0 (Microsoft)].

Non-parametric procedures were used for tests of significance (Wilcoxon signed rank for dependent variables and Mann–Whitney U for independent variables). The graphical display was done by boxplots, as produced by Statview 4.02.

**Results**

**Aspirated follicles**
A total of 450 follicles was punctured and 332 oocytes were obtained (mean ± SD: 9.8 ± 4.5 per oocyte retrieval). Follicular fluids of 165 follicles were prepared for DNA flow cytometry. In all, 160 follicles (35% of all follicles) could be analysed by DNA flow cytometry, and intrafollicular hormone concentrations were determined from 153 follicular fluid samples; thus 3.6 ± 0.8 follicles (range 2–5) were analysed per oocyte retrieval (Table I).

**Papanicolaou smears**
In none of the cytological smears, performed for each follicle separately, were dysplastic or atypical cells were found. The smears were stained according to Papanicolaou. The vast majority of cells seen in those smears were granulosa lutein cells. A small number of leukocytes and histiocytes was observed.

**Ploidy**
The FCM measurements of follicular fluids of 160 follicles (45 patients, 46 follicle punctures) revealed a high percentage
Aneuploidy in human granulosa lutein cells

Figure 1. Example of a DNA flow cytometric histogram, showing one diploid and two aneuploid cell populations.

<table>
<thead>
<tr>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>Min.</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Follicles</td>
<td>450</td>
<td>9.8</td>
<td>4.5</td>
<td>8.5</td>
<td>2</td>
</tr>
<tr>
<td>Oocytes</td>
<td>332</td>
<td>7.2</td>
<td>3.6</td>
<td>6.5</td>
<td>1</td>
</tr>
<tr>
<td>Pronuclei</td>
<td>220</td>
<td>4.8</td>
<td>3.1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Embryos</td>
<td>113</td>
<td>2.5</td>
<td>0.9</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Flow cytometry and hormone assessment

- **Follicles**: 160, 3.6, 0.8, 4, 2, 5
- **% flow cytometrically analysed follicles**: 35% of all follicles punctured
- **Granulosa lutein cells assessed per follicle**: 22 806 ± 10 176

(n = 46) (n = cases, SD = standard deviation, Max = maximum, Min = minimum).

- **Diploid cycle**
  - Mean G1 = 63.5
  - CV G1 = 83.4
  - Mean G2 = 95.0
  - CV G2 = 85.0
  - % S = 11.1
  - G2/CL = 0.800
  - % TEL = 40.3

- **Aneuploid cycle**
  - Mean G1 = 73.6
  - CV G1 = 90.7
  - Mean G2 = 197.6
  - CV G2 = 84.8
  - % S = 0.0
  - G2/CL = 1.976
  - % TEL = 71.3

Of aneuploidy: 45.6% (n = 73) of the follicles contained aneuploid granulosa lutein cells; 5.6% (n = 9) contained multiploid granulosa lutein cells, defined as at least two aneuploid cell populations with different DNA indices.

The number of aneuploid granulosa cells amounted to 9985 ± 6873 (range: 344–34 062) per follicle containing aneuploid granulosa lutein cell populations (n = 82). On average this represents 43 ± 23% (range: 4–92%) of all cells in follicles containing aneuploid granulosa lutein cells (n = 82). Of 3 649 021 total cells counted, 22% of all granulosa lutein cells analysed were aneuploid.

In all, 74% of the patients (n = 33) had at least one follicle with aneuploid or multiploid granulosa cells, while only 27% (n = 12) of the patients had exclusively follicles containing diploid granulosa lutein cells (Tables II and III).

**S-phase and proliferative index**

The flow cytometric parameters of diploid and aneuploid cell populations were compared. A total of 160 follicles contained 251 cell populations (aneuploid and diploid cell cycles); 13 cell populations could not be evaluated for missing data. Of the remaining 238 granulosa cell populations, 78 were aneuploid and 160 were diploid. Of the aneuploid cell populations, 44 cell cycles and of the diploid cell populations 61 cell cycles could not be included in the evaluation of the parameters of DNA flow cytometry. In those cases, the percentage of cells in the G1-phase, S-phase or G2 phase could not be calculated by the Multicycle program due to technical reasons (higher amount of debris). Thus, 34 aneuploid cell populations were compared to 99 aneuploid cell populations.

In aneuploid cell populations, there was a significantly higher percentage of cells in the S-phase and G2/M-phase in comparison to the diploid cell populations. The PI (sum of the percentage of cells in S-phase and G2/M-phase) was significantly higher in aneuploid granulosa lutein cells. In contrast, the percentage of cells in the G1-phase was significantly lower in aneuploid granulosa lutein cells (Table IV, Figure 2).

There was a significant difference in the percentage of cells in S-phase when aneuploid cell cycles were compared to diploid cell cycles. Comparing the percentage of cells in G2/M, no significant difference could be found.
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Figure 2. Percentage cells in G1-phase, S-phase, G2/M-phase and proliferation index of aneuploid and diploid cell populations.

DNA index (DNA content of aneuploid cells/DNA content of diploid cells) showed in 83.7% hyperdiploidy with a mean of 1.14 ± 0.2. Only 16.5% of the aneuploid cell populations showed a hypodiploid DNA content (Figure 3).

Aneuploid cell cycles showed a significantly higher PI (sum of cells in S- and G2/M-phase as a percentage of the total cell number) than diploid cell cycles (median: aneuploid: 15.5; diploid: 7.4; \( P < 0.0001 \)).

Age and ploidy

The mean age of the patients was 34 ± 5 years (mean ± SD; range: 23–46 years). Fourteen of the patients (30.4%) were <30 years of age, 25 (54.3%) were between 30 and 39 years old and seven (15.2%) were >40 years. There was no significant correlation between the incidence of aneuploidy and the age of patients, although the number of aneuploidies tended to decline with increasing age (Table V).

Table II. Frequency of follicles with diploid, aneuploid and multiploid granulosa cells

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Follicles (n)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicles containing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only diploid granulosa</td>
<td>78</td>
<td>48.8</td>
</tr>
<tr>
<td>Aneuploid granulosa</td>
<td>73</td>
<td>45.6</td>
</tr>
<tr>
<td>Multiploid granulosa</td>
<td>9</td>
<td>5.6</td>
</tr>
<tr>
<td>Total number of follicles</td>
<td>160</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table III. Frequency of patients with follicles containing aneuploid granulosa cells

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Patients* (n)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only follicles with diploid granulosa</td>
<td>12</td>
<td>27</td>
</tr>
<tr>
<td>At least one follicle with aneuploid</td>
<td>26</td>
<td>58</td>
</tr>
<tr>
<td>granulosa cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At least one follicle with multiploid</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>granulosa cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At least one follicle with multiploid or aneuploid</td>
<td>33</td>
<td>74</td>
</tr>
</tbody>
</table>

*It was not possible to identify the categories from which pregnant patients came.
Figure 4. Follicular fluid hormone concentrations of progesterone, luteinizing hormone (LH), testosterone and dehydroepiandrosterone sulphate (DHEA-S) in follicles with only diploid and with at least one aneuploid granulosa cell population (AN = follicles with at least one aneuploid granulosa cell population, DIP = follicles with only diploid granulosa cells).

Table IV. Percentages of cells in G1-phase, S-phase, G2/M-phase and proliferative index in aneuploid and diploid granulosa lutein cell populations

<table>
<thead>
<tr>
<th>% cells in cycle phase</th>
<th>Aneuploid cell populations</th>
<th>Diploid cell populations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
</tr>
<tr>
<td>G1</td>
<td>34</td>
<td>8.1</td>
</tr>
<tr>
<td>S</td>
<td>34</td>
<td>12.3</td>
</tr>
<tr>
<td>G2/M</td>
<td>34</td>
<td>4.59</td>
</tr>
<tr>
<td>S + G2/M</td>
<td>34</td>
<td>16.9</td>
</tr>
</tbody>
</table>

Max = maximum, Min = minimum, Mann–Whitney U-test.

Table V. Frequency of follicles with diploid, aneuploid and multiploid granulosa cells correlated to the age of the patients

<table>
<thead>
<tr>
<th>Ploidy of the follicle</th>
<th>Aneuploid/multiploid granulosa cells</th>
<th>Only diploid granulosa cells</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>20–29</td>
<td>21</td>
<td>25.6</td>
<td>14</td>
</tr>
<tr>
<td>30–39</td>
<td>50</td>
<td>60.9</td>
<td>51</td>
</tr>
<tr>
<td>40–49</td>
<td>11</td>
<td>13.4</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>100</td>
<td>78</td>
</tr>
</tbody>
</table>

n = no. of follicles.
The concentrations of intrafollicular androgens, testosterone and DHEA-S, were significantly lower in follicles with aneuploid granulosa lutein cells (testosterone: \( P < 0.01; \) DHEAS: \( P < 0.05 \)). Intrafollicular progesterone concentrations were also significantly lower in follicles with aneuploid granulosa cells (\( P < 0.05 \)). In contrast, LH concentrations were higher in follicles with aneuploid granulosa cells (\( P < 0.05 \)) (Figure 4, Table VI).

Intrafollicular oestradiol and FSH concentrations did not show any significant difference when follicles with aneuploid granulosa lutein cells were compared to diploid ones.

### Pregnancies

A total of six singleton pregnancies resulted from IVF treatment. One of these ended in an early miscarriage before the 12th week of gestation. In all patients, three embryos were transferred. In this part of the investigation, it was not documented whether the transferred embryos were obtained from follicles with aneuploid or diploid granulosa cells. Thus it was not possible to correlate the outcome of IVF treatment to the results from DNA flow cytometry.

### Discussion

In \( >50\% \) of the follicles and in \( >70\% \) of the IVF patients examined, aneuploid granulosa cells were found in follicular fluids. Though not all follicles of individual patients could be assessed, these data show clearly that aneuploidy of luteinized granulosa cells in gonadotrophin-releasing hormone (GnRH)-down-regulated gonadotrophin-stimulated IVF patients is a frequent phenomenon.

### Cell types

Contamination of the follicular fluid with red blood cells was negligible, because they do not have DNA which could influence the results of the DNA measurements. Cell clumps also cannot influence the results, because in the standardized protocol (Otto et al., 1981) all cells membranes were dissolved with pepsin solution prior to measurement. So there was no need to dissociate clumps of granulosa cells. This is one of the advantages of the DAPI staining method, which has a high resolution for DNA and is known as a very stable method.

### DNA measurements of follicular fluid

The idea of measuring the DNA content through flow cytometry of the follicular fluid of IVF patients was first published by Westergaard and coworkers (1982). Many groups tried to find a relationship between DNA content and granulosa cell proliferation and oocyte maturation, fertilization and other parameters (Westergaard et al., 1982; Marrone and Crissman, 1988; Whitman et al., 1991; Seifer et al., 1992, 1993a,b; De Neubourg et al., 1996). Except for Ben Rafael et al. (1987), methods that were applied for that purpose did not have the sensitivity to detect DNA aneuploidies with a DNA Index close to 1. Such a high resolution is warranted by the DAPI staining method (Otto et al., 1994).

Westergaard et al. (1982) showed a significant association between S-phase and intrafollicular oestradiol concentrations. More than \( 85\% \) of the follicles with an S-phase above \( 16\% \) had intrafollicular oestradiol concentrations of \( >200 \) ng/ml and a low oestradiol:androstendione ratio, as a marker for atresia.

There are only two studies (Greenebaum et al., 1994; Agorastos et al., 1996) which found aneuploidies in the follicular fluid of IVF patients. Both used DNA image analysis.
which has an important disadvantage compared to the flow cytometry of being able to examine only ~100 cells compared to ~10,000 cells with flow cytometry. Image analysis compared to flow cytometry has also a much higher variability with regard to repeated assessments and different researchers.

Greenebaum et al. (1994) examined ovarian and adnexal fluids (functional and paraovarian cysts) (55 benign, three borderline and six malignant aspirates), but also follicles from women after hormonal stimulation for IVF. In two out of 19 women, aneuploid granulosa cell lines were found, which could be expressed as 10.5% of aneuploidies. She suggests that the presence of aneuploidy in aspirates of ovarian or adnexal cysts should arouse suspicion (Greenebaum et al., 1994).

The study of Agorastos et al. (1996) found 1% of aneuploid granulosa cells in follicular fluids of IVF patients; 1.12% in women who became pregnant and 0.92% in women who did not conceive. Overall, he examined 59 follicles of 17 patients, who were included in an IVF programme for tubal reasons.

**Ovarian cancers and ovarian stimulation with gonadotrophins**

In a highly debated paper, Willemsen et al. (1993) pointed to a possible association between ovarian stimulation and an increased incidence of granulosa cell tumours. He reported 12 patients who developed granulosa cell tumours after ovarian stimulation therapy. The authors offered three hypotheses. Firstly, a pre-existing latent granulosa cell tumour might become manifest through stimulation therapy; secondly, increased FSH concentrations might exert an oncogenic effect on granulosa cells; and lastly it could not be excluded that ovarian stimulation and the occurrence of granulosa cell tumours was simply a coincidence. According to Kolstad and Beecham, the incidence of granulosa cell tumours varies between 0.05 and 1.7 per 100,000 women (Kolstad and Beecham, 1975). These tumours occur most frequently between the 50th and 59th year of age, though cases have been published between the ages of 2 and 90 years (Ohel et al., 1983). Epidemiologically rare events of granulosa cell tumours was simply a coincidence. According to Kolstad and Beecham, the incidence of granulosa cell tumours varies between 0.05 and 1.7 per 100,000 women (Kolstad and Beecham, 1975). These tumours occur most frequently between the 50th and 59th year of age, though cases have been published between the ages of 2 and 90 years (Ohel et al., 1983). Epidemiologically rare events of granulosa cell tumours could not be related to the large number of gonadotrophin treatments.

The studies by Whittemore and colleagues (Whittemore et al., 1989, 1992; Harris et al., 1992; Whittemore, 1993) on an increased risk of ovarian neoplasia following the use of fertility drugs were put in doubt by an international team of experts (Cohen et al., 1993) and also by the author. Other epidemiological studies (Ron et al., 1987; Lunenfeld, 1995; Venn et al., 1995) did not show an increased risk of ovarian cancer following the use of gonadotrophins or clomiphene. Infertility itself is a risk factor for ovarian cancer (Harlow et al., 1988; Booth et al., 1989; Hartge et al., 1989). On the basis of current knowledge, a slightly increased risk of ovarian cancer after ovarian stimulation therapy cannot be excluded entirely; though, because of the low total incidence of ovarian cancer, the absolute risk is small. However, successful infertility treatment leading to the birth of a child and subsequent breastfeeding are protective factors with regard to ovarian carcinoma.

**Aneuploidy and apoptosis**

A hypothetical and very speculative explanation could be that aneuploidy is one step towards planned cell death – apoptosis – leading to a selection of granulosa cells that predominantly secrete progesterone while oestrogen-producing cells – aromatizing cells – undergo programmed cell death. Breckwoldt et al. (1996) showed that apoptosis and progesterone secretion can take place in the same granulosa lutein cells. According to their data, steroid secretion remains high until total cell collapse. Our flow cytometry results of did not reveal the typical pattern of apoptotic cells where DNA fragments are expressed as a shoulder on the left part of the resulting graph (Figure 1). Nuclear fragments with variable DNA content would be elevated and scattered in the range between very little DNA content and the diploid peak. Flow cytometry on the other hand is not a very suitable method to detect apoptosis. Further data which explore the extent of apoptosis and aneuploidy in correlation to their steroidogenic activity are needed.

**Correlation between intrafollicular hormones and presence of aneuploidy**

Intrafollicular concentrations of androgens, DHEA-S and testosterone were significantly higher in follicles with only diploid granulosa lutein cells in comparison to the concentrations found in follicles with aneuploid granulosa cells. Also, progesterone, which is exclusively synthesized by luteinized granulosa cells, was found to be significantly higher in follicles with only diploid granulosa cells. In contrast follicular LH concentrations were significantly lower in follicles that contained only diploid granulosa cells, though their absolute concentrations were low. These findings are coupled to a significantly higher PI and S-phase of the aneuploid cell populations in comparison to the diploid ones indicating that aneuploid granulosa lutein cells have a higher mitotic activity than diploid luteinized granulosa cells.

Yong et al. (1992) showed that there is a inverse relationship between the production of progesterone and the degree of differentiation of granulosa cells. In-vitro studies suggest that proliferative activity and steroidogenesis are inversely correlated (Epstein-Almog and Orly, 1985). Maximal progesterone synthesis is associated with an almost complete cessation of DNA synthesis and cell proliferation. While granulosa cells in large pre-ovulatory follicles have high mitotic activity, there is a rapid decline in mitotic activity between the LH surge and ovulation (Delforge et al., 1972). In-vivo studies in rats demonstrated that steroid secretion began only after the arrest of DNA synthesis (Klinken and Stevenson, 1977; Naumoff and Stevenson, 1981).

After the onset of the LH surge, LH seems to have a strong stimulatory effect on steroidogenesis and a suppressive effect on DNA synthesis and cell proliferation. Cessation of cell division coincides with granulosa cell luteinization, which explains why the number of steroidogenic cells in the human corpus luteum no longer increases during the luteal phase (Fish et al., 1989). Since in GnRH down-regulated cycles LH concentrations were low both in serum and in the follicle, the lack of LH might lead to insufficient stimulation of...
steroidogenesis and a prolonged phase of proliferation of granulosa cells.

**Genesis of aneuploidy: a physiological event?**

Presented data demonstrate that changes of DNA as shown by DNA flow cytometry are paralleled by changes in the intrafollicular hormonal environment. With the demonstrated results it cannot be decided whether the occurrence of aneuploidy in granulosa cells is a physiological or a pathological phenomenon. The investigation of unstimulated follicles, as a control group, might give more information, though for technical and physiological reasons it is difficult to obtain follicles under similar conditions at this precise time of the menstrual cycle. During this very dynamic period of the menstrual cycle after the LH surge and immediately before ovulation, a shift from oestrogen dominance to progesterone dominance takes place. Since IVF/embryo transfer treatment in spontaneous cycles is not performed in our unit, we did not have the opportunity to obtain aspirates from unstimulated follicles. Furthermore, animal studies would be informative regarding occurrence of aneuploid granulosa cells.

At present it can only be speculated whether these observations are physiological or represent pathological changes within the ovaries. It could be postulated that since by gonadotrophin stimulation a larger number of follicles ovulate, thus a larger number of follicles will ovulate, thus a larger number of aneuploid granulosa cells. The investigation of unstimulated follicles, as a control group, might give more information, though for technical and physiological reasons it is difficult to obtain follicles under similar conditions at this precise time of the menstrual cycle. During this very dynamic period of the menstrual cycle after the LH surge and immediately before ovulation, a shift from oestrogen dominance to progesterone dominance takes place. Since IVF/embryo transfer treatment in spontaneous cycles is not performed in our unit, we did not have the opportunity to obtain aspirates from unstimulated follicles. Furthermore, animal studies would be informative regarding occurrence of aneuploid granulosa cells.

At present it can only be speculated whether these observations are physiological or represent pathological changes within the ovaries. It could be postulated that since by gonadotrophin stimulation a larger number of follicles ovulate, thus a larger number of aneuploid granulosa cells are removed from the ovary. However, the malignant potential that may be associated with aneuploidy must be kept in mind and should promote further research on this subject.

**References**


Aneuploidy in human granulosa lutein cells


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