Maturation and apoptosis of human oocytes in vitro are age-related

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Objective: To study the morphological changes of apoptotic oocytes, and rates of in vitro maturation and apoptosis of human oocytes in relation to age.

Design: Prospective comparative study.

Setting: Reproductive medicine center.

Patient(s): Women undergoing surgery for ovarian cysts.

Intervention(s): Oocytes were incubated in Ham’s F-10 medium with 15% fetal cord serum (FCS) for 32 to 120 hours and were examined under inverted microscope every 6 to 8 hours.

Main Outcome Measure(s): Oocyte maturation and apoptosis, Fas antigen.

Result(s): The morphologic changes characteristic of apoptosis oocytes were shrinkage, or the occurrence of cytoplasmic condensation, membrane blebbing, fragmentation of the oocyte into “apoptotic” bodies of unequal size, or internucleosomal DNA cleavage as shown by TUNEL. The maturation rates of oocytes were highest in those from women aged 21 to 30 years, and lowest in those aged 41 to 50 years. Apoptosis occurred in 17.1% (age group 21 to 30 years), 37.7% (31 to 40 years), and 52.3% (41 to 50 years). The rate of apoptosis of human immature oocytes cultured in vitro was significantly higher in those from older women who were 41 to 50 years old than in those women 21 to 40 years old. Fas antigen was found to be present on apoptotic oocyte membranes.

Conclusion(s): The developmental potential of oocytes from older women decreased in vitro in a manner similar to that seen in vivo. DNA fragmentation in oocytes associated with apoptotic death might be one of the reasons for poor oocyte quality and lower fertility in aged mice (9). However, the process of in vitro maturation and apoptosis of human oocytes in relation to age has not been studied. Our aim was to study the morphologic changes of apoptotic oocytes and the rates of maturation and apoptosis of human oocytes in relation to age, and to determine the expression of the Fas antigen in human oocytes.

Key Words: Oocyte, maturation, apoptosis, Fas antigen, relation to age

The relationship between the progressive decline in fertility and age has been recognized. Sharma et al. (1) and Hughes et al. (2) reported that aging resulted in a progressive decline in the response of ovaries to treatment with hMG for IVF in terms of number of oocytes and treatment cycle cancellations. The stockpile of germ cells, depleted throughout prepubertal and adult life, is near exhaustion by the time of menopause (3–6). However, fertility is markedly compromised several years before menopause, suggesting that factors other than complete exhaustion of the follicle pool play a role in the loss of oocyte competency in aging women (1, 2, 7, 8).

It was reported that DNA fragmentation in oocytes associated with apoptotic death might be one of the reasons for poor oocyte quality and lower fertility in aged mice (9). However, the process of in vitro maturation and apoptosis of human oocytes in relation to age has not been studied. Our aim was to study the morphologic changes of apoptotic oocytes and the rates of maturation and apoptosis of human oocytes in relation to age, and to determine the expression of the Fas antigen in human oocytes.

MATERIALS AND METHODS

Collection of Immature Oocytes

Ovarian tissue was obtained from three age groups of 59 patients (group one, 21 to 30 years old; group two, 31 to 40 years old; group three, 41 to 50 years old) who had had surgery for ovarian cysts on days 9 to 12 after their last menstrual period. Patients had essentially reg-
ular menstrual cycles, ranging from 27 to 34 days, and had not received hormones for 3 months before the surgery. The ovarian tissue was washed with 37°C saline to remove adhering blood clots. Then 216 oocytes were aspirated from antral follicles (4 to 6 mm in diameter) from the ovarian tissue. Institutional review board approval was obtained for this study.

Culture of Immature Oocytes

Oocytes of the same grade and stage of maturation and health status from the patients were organized by age group. The oocytes were placed in four-well culture dishes (Nunclon, Roskilde, Denmark), containing 500 μL per well culture medium, and were cultured at 37°C in 5% CO₂ in air for 32 to 120 hours. The culture medium consisted of Ham’s F10 (Flow Laboratories, Irvine, Scotland, UK) with 15% fetal cord serum (FCS). The oocytes were examined under an inverted microscope every 6 to 8 hours to monitor the process of maturation as well as the process of apoptosis. An oocyte was considered mature when a polar body was present. Apoptosis was evident when morphologic changes appeared, such as shrinkage, cytoplasmic condensation, membrane blebbing, and/or fragmentation of the oocyte into apoptotic bodies of unequal size (10).

Detection of DNA Fragmentation Change

Each oocyte that appeared apoptotic was removed from the culture medium and washed in several changes of Dulbecco’s phosphate-buffered saline (DPBS, Gibco/Life Technologies, Beijing, China) for 5 to 10 minutes. After washing, the oocyte was transferred with a minimal amount of DPBS to a microdrop (1–2 μL) of 0.01 N HCl/0.1% Tween 20 in bidistilled water on a microscope slide. During the procedure the oocyte was watched constantly under an inverted microscope. After 30 seconds to 2 minutes the zona pellucida began to disintegrate, followed by the oocyte membrane. The remainder was attached to the glass slide, and the slide was air-dried and washed with DPBS (11). After air drying, the slide was stored at −70°C until further use.

After thawing, the TUNEL assay was carried out on the oocyte. Briefly the oocyte was treated with proteinase K and labeled with biotin-11-dUTP (Sigma Chemical, St. Louis, MO) by incubation with terminal deoxynucleotidyl transferase (TDT, Promega, Madison, WI). Afterwards, it was incubated with avidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA). Diaminobenzidine-H₂O₂ was used for staining (12).

The slide was counterstained with Mayer’s hematoxylin. Negative controls were made as above, but terminal deoxynucleotidyl transferase was omitted.

Detection of the Fas Antigen

The oocyte was placed in a mouse uterus to facilitate histologic handling. Immunohistochemical staining was performed as described by Maruo and Mochizuki (13). A rabbit polyclonal antibody against the human Fas antigen (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody. The antibody was diluted to 1:100 before use. Secondary antibody (IgG, Vector Laboratories) which was biotinylated was diluted to 1:200, and avidin-horseradish peroxidase was diluted to 1:500, before use. As negative controls, slides were processed as described above except...
that the appropriate normal serum was substituted for the primary antibody.

**Statistical Analysis**

The Student’s t-test and $\chi^2$ test were used for statistics. $P$ values of $<0.05$ were considered significant.

**RESULTS**

As shown in Table 1, the maturation rates of oocytes were highest in those from women aged 21 to 30 years, and the lowest rates were in oocytes from women aged 41 to 50 years ($P<0.005$, Fig. 1A). In contrast, apoptosis occurred in 17.1% in the 21 to 30 age group, 37.7% in the 31 to 40 age group, and 52.3% in the 41 to 50 age group. The rate of apoptosis of the human immature oocytes cultured in vitro was significantly higher in older women (ages 41 to 50 years) than those from younger women (aged 21 to 40 years) ($P<0.005$). However, there was no significant difference in time of in vitro maturation of oocytes among the different age groups (mean time: 43.4 ± 12.5 hours for ages 21 to 30 years, 43.9 ± 11.6 hours for ages 31 to 40 years, and 44.8 ± 10.7 hours for ages 41 to 50 years). Furthermore, some oocytes that matured in vitro underwent apoptosis (number: six for ages 21 to 30 years, three for ages 31 to 40 years, and two for ages 41 to 50 years).

The morphologic changes characteristic of apoptosis oocytes were shrinkage, or the occurrence of cytoplasmic condensation (as indicated by retraction of the oolemma from the zona pellucida), membrane blebbing, fragmentation of the oocyte into “apoptotic” bodies of unequal size (see Fig. 1B, C), and internucleosomal DNA cleavage as shown by TUNEL (Fig. 2A, B).

The morphologic process of apoptosis revealed that the oocyte went through oocyte shrinkage (one of the first signs) and DNA fragmentation or apoptotic body formation (the end point in the process of apoptosis). The time of the process was from oocyte shrinkage to apoptotic body formation. As shown in Table 2, the time was the shortest in oocytes from women aged 41 to 50 years, and the longest in those from women aged 21 to 30 years.

**DISCUSSION**

In vivo oocyte maturation is induced by the preovulatory surge of multiple gonadotrophins, including LH. The maturation-inducing action of LH may be mediated by cumulus cells because oocytes do not have receptors for LH (14). The mechanism involves LH induction of the degradation of gap junctions that couple mural granulosa cells to cumulus cells. This would result in a reduction in the flow of meiosis-arresting substances from the mural granulosa cells to the oocyte by way of the cumulus cells, and resumption of meiosis would then commence. LH-induced maturation may also be mediated by the Ip3/Ca pathway (15). In the present study, immature oocytes obtained from surgically removed ovaries or ovarian tissue were incubated in Ham’s F10 with 15% fetal cord serum. The mechanism of oocyte maturation is probably the removal of the oocyte from the follicle, which terminates the flow of meiosis-arresting substances into the oocyte (16–19).

The decline in female fertility with age is a physiologic phenomenon. The age-dependent decrease in human female fertility begins by the late thirties, and by the late forties fertility is virtually lost. The age-dependent loss of reproductive potential is reflected in the lower pregnancy rates and higher early abortion rates observed in women within this age range. Previous investigations suggest that waning oocyte quality rather than declining uterine receptivity is the principal factor responsible for the age-related decline in female fertility potential (18).

Likewise, oocyte donation in older patients results in very similar implantation rates to those in younger patients (20). Furthermore, a study on oocyte quality in mares indicated that the developmental capacity of embryos was affected by maternal age (21). In the present study, the number of in vitro mature oocytes from older women was significantly lower than that from young women. This suggests that the

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**TABLE 1**

Maturation and apoptosis of human oocytes in different age groups.

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Number of oocytes</th>
<th>Mature oocytes (%)</th>
<th>Apoptotic oocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21–30</td>
<td>82</td>
<td>33 (40.2)*</td>
<td>14 (17.1)*</td>
</tr>
<tr>
<td>31–40</td>
<td>69</td>
<td>17 (24.9)</td>
<td>26 (37.7)</td>
</tr>
<tr>
<td>41–50</td>
<td>65</td>
<td>8 (12.3)*</td>
<td>34 (52.3)*</td>
</tr>
</tbody>
</table>

* $P<0.005$.


**TABLE 2**

Effect of age of woman on time of apoptosis process of human oocytes.

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Number of apoptotic oocytes</th>
<th>Time* (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21–30</td>
<td>14</td>
<td>39.4 ± 4.2*</td>
</tr>
<tr>
<td>31–40</td>
<td>26</td>
<td>30.3 ± 3.2</td>
</tr>
<tr>
<td>41–50</td>
<td>34</td>
<td>24.5 ± 4.2*</td>
</tr>
</tbody>
</table>

* $P<0.005$.


Fas antigen was found to be present in apoptotic oocyte membrane (see Fig. 2C, D).

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decreased developmental potential of oocytes from older women was similar in vitro to those in vivo.

Apoptosis is a morphologic process caused by a “cellular suicide” program, which is associated with physiologic or programmed cell death. We found that some oocytes that had matured in vitro underwent apoptosis. It is the same process that occurs in vivo, as oocytes that mature in vivo will degenerate if they are not fertilized. In the present study, the rate of apoptosis of human immature oocytes cultured in vitro is much higher in oocytes from older women (aged 41 to 51 years) than in those from younger women (aged 21 to 40 years). This result is in line with previous findings in mice (9). This suggests that DNA fragmentation in oocytes associated with apoptotic death might be one of the reasons for poor oocyte quality and lower fertility in older women.

Fas antigen in the oocyte presumably mediates apoptosis. In this study, Fas antigen was found to be present in the apoptotic oocyte membrane. Kondo et al. (22) reported that the expression of the Fas antigen in the oocyte was most abundant in primordial and primary follicles, especially in the shrunken oocyte in atretic follicles; it was less abundant in antral follicles, and negligible in preovulatory follicles. Additionally, previous investigations suggested that one of the first signs of atresia in primordial and primary follicles is the shrinkage of the oocyte (23).

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References


