Human Reproduction vol.12 no.5 pp.1028–1031, 1997

Follicular density in ovarian biopsy of infertile women: a novel method to assess ovarian reserve

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The ageing ovary appears to be characterized by depletion of primordial follicles. The relationship between infertility and the number of follicles in the ovarian cortex is not known. Moreover, there are no accurate markers or clinical methods to predict the decline in ovarian reserve. This study investigates the correlation between early follicular follicle stimulating hormone, ovarian size and follicular density in 60 infertile women aged 19–45 years (mean = 34.4 ± 5.5). An ovarian biopsy was taken from each patient while performing diagnostic laparoscopy (n = 28) or laparotomy for tubal surgery or myomectomy (n = 32). The median number of follicles was similar in tubal and unexplained infertility patients (9.5 versus 5.5). Increasing age showed a significant negative correlation with follicular density and ovarian volume (r = –0.46, P = 0.0003; r = –0.43, P = 0.0016, respectively). In women ⩾35 years of age the ovarian volume showed a strong correlation with follicular density (r = 0.71, P < 0.0001). Our results indicate that infertile women in their late thirties and over have a decreased ovarian reserve which could possibly be predicted by ovarian volume measurement. Ovarian biopsy may have a place as part of infertility evaluation in older women.

Key words: age/follicle/ovarian biopsy/ovarian reserve/ovarian volume

Introduction

The relationship between increased female age and decreased fertility is well established (Menken *et al.*, 1986; Speroff, 1994) and is apparently due to reduced numbers of primordial follicles: from >250 000 at menarche only a few hundreds or thousands remain at the end of reproductive life (Block, 1952; Baker, 1963). This loss accelerates around the age of 37 years and precedes the menopause by 10–12 years (Richardson *et al.*, 1987; Faddy and Gosden, 1995, 1996). There is variation in the number and rate of depletion of follicles. In previous studies, follicles were counted in whole ovarian specimens obtained in autopsies from the general population. There would be considerable clinical value in being able to predict whether a given infertile patient still has significant ovarian reserve to justify fertility treatment in the later stage of reproductive life. However, there are no data about the distribution and number of primordial follicles in infertile women, nor any markers or methods to predict that depletion. Age and regularity of menses alone are unreliable ways of predicting ovarian reserve. Follicular phase follicle stimulating hormone (FSH) concentrations, although presumably raised in older women, do not fully indicate ovarian function (Scott and Hofmann, 1995; Wallach, 1995): usually by the time FSH concentrations are elevated, the ovaries have already become refractory. We recently showed that decrease in ovarian volume measured on transvaginal scan (TVS) is an earlier sign of ovarian depletion and is associated with poor response to ovulation induction (Lass *et al.*, 1997). In this study we counted the number of early follicles in ovarian biopsies from infertile women and assessed whether a combination of age, FSH concentrations and ovarian volume measurements would be useful in the prediction of ovarian reserve.

Materials and methods

Subjects

A total of 60 menstruating women aged 19–45 years (mean ± SD, 34.4 ± 5.5) undergoing infertility evaluation at Hammersmith Hospital was studied prospectively. These women had no clinical signs of the menopause and had had no hormonal treatment in the 3 months preceding the study. All patients gave written informed consent to participate in the study. A group of 28 women underwent diagnostic laparoscopy and ovarian tissue was obtained by biopsy, while 32 women had a laparotomy for tubal surgery or myomectomy at which a scalpel biopsy was taken. All procedures were uneventful with no bleeding from the biopsy site.

The operation was performed mostly in the early luteal phase; therefore FSH concentrations and ovarian volume measurements were taken in the early proliferative phase either before the operation or immediately afterwards in the following cycle.

Ovarian volume measurement

A Kretz Combison 410 ultrasound scanner (Kretz Technik, Vienna, Austria) with 5 or 7.5 MHz transvaginal transducers was used. Each ovary was measured in three planes and the ovarian volume (V) calculated using the prolate ellipsoid formula:

$$V = D1 \times D2 \times D3 \times 0.523$$

where $D1$, $D2$ and $D3$ are the three maximal longitudinal, anteroposterior and transverse diameters respectively. Mean ovarian volume was the mean volume calculated for both ovaries in the same individual.

Histology

In this study we define the number of follicles per unit volume of cortical ovarian tissue. In a previous study we showed that the mean diameter of primordial follicles was 39.5 ± 7.6 μm (mean ± SD) while the largest follicle was 49 μm (Hovatta *et al.*, 1996). Each biopsy was fixed in Bouin's solution, embedded in paraffin wax and serially cut in 10 step sections at 50 μm intervals perpendicular to the ovarian capsule and stained with haematoxylin and eosin. The cutting ensured that no follicle was counted twice. The primordial, primary and secondary follicles were classified (Gougeon, 1986) and counted in each section using a Zeiss microscope at ×400 magnification (Carl Zeiss Ltd., Jena, Germany). Eosinophilia of the ooplasm, contraction and clumping of the chromatin material and wrinkling of the nuclear membrane of the oocytes were regarded as signs of atresia (Gougeon, 1986).

Image analysis

Each tissue section was digitized via a Sun VideoPix card (Sun Microsystems Inc., Mountain View, CA, USA) in conjunction with a Sony CCD XC-77CE video camera (Sony Corporation, Tokyo, Japan) and a Panasonic WV-5470 video monitor (Panasonic Technologies Inc., Santa Barbara, CA, USA). The area of the biopsies was determined by delineation of the tissue boundary using a computer program OpenStereo (Abrams *et al.*, 1994). The sections were digitized into 512×512 eight-bit images, and stored on disk. The size of the biopsy varied between patients, but all primary and primordial follicles were located no deeper than 2 mm from the ovarian surface. This area was measured to prevent false readings of decreased follicular density in larger biopsies. Tissue sections with a width >2 mm were displayed on the video monitor and a transparency sheet was superimposed on the image. Using the computer mouse, the external surface of the cortex was delineated. Using a ruler and magnification factor, a parallel line 2 mm from the surface of the cortex was drawn on the transparency sheet using a non-indelible pen. This provided a contour that could be used to trace a line in the digital image that was 2 mm from the external surface of the ovarian cortex. The two parallel lines were then joined to create an enclosure. This method ensured that the area of tissue measured did not extend deeper than 2 mm into the cortex. Tissue sections with a width <2 mm had their whole profile traced to produce the enclosure. In both cases, the enclosure was filled using the appropriate option in the computer software. The area of the filled profile was determined and converted to mm^2. The volume of the analysed ovarian tissue (V) was then calculated by the formula:

$$V\ (mm^3) = S(A1, A10) \times 0.05$$

where S(A1, A10) is the sum of the 10 section areas and 0.05 is the interval between the sections (mm). Tissue volumes ranged from 0.3 to 17 mm^3 with a median volume of 2 mm^3. The density of primordial and primary follicles in the ovarian biopsy was calculated as the total number of follicles divided by the volume and expressed as the number of follicles/mm^3 of ovarian tissue.

The χ^2-test, Student's t-test and multiple logistic regression analysis were used for statistical comparisons.

Results

The follicular density in ovarian biopsies obtained from 60 infertile women ranged from 0 to 160 follicles/mm^3 with a median of 8 follicles/mm^3. The majority of the follicles was primordial (88%) with very few primary or secondary follicles (8 and 4% respectively). Only 3% of the early follicles were atretic.

Table I. Relationship between the cause and duration of infertility, age, follicle stimulating hormone (FSH), mean ovarian volume and follicular density in ovarian biopsy[a]

	Tubal disease	Unexplained infertility
No. of patients	37	17
Age (years)	33.3 ± 5.7	36.0 ± 5.0
Primary infertility (%)	14 (37.8)	12 (70.6)
Duration of infertility (years)	4.4 ± 3.3	4.4 ± 4.3
FSH (mIU/ml)	7.1 ± 2.4	6.8 ± 2.2
Mean ovarian volume (cm^3)	8.3 ± 4.2	6.9 ± 3.2
Follicular density (no./mm^3)	9.5 (0–160)	5.5 (0–131)

[a]Values are means ± SD except those of follicular density, which are medians (range).
There were no significant differences between the two groups.

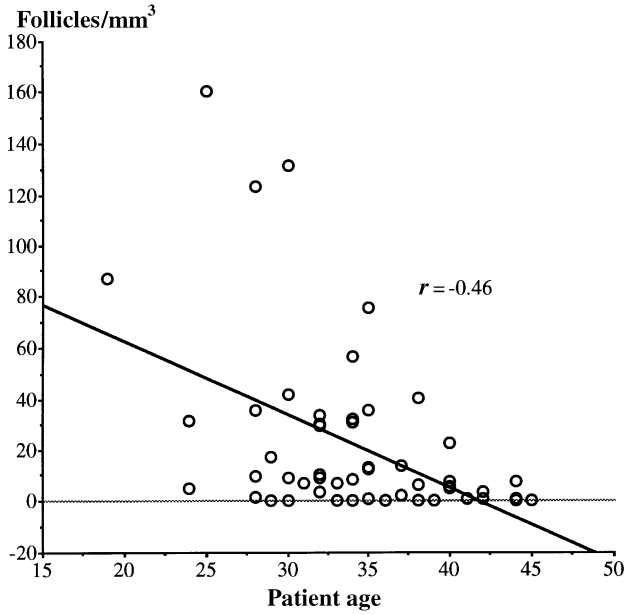

Figure 1. Correlation between follicular density and patient's age (r = –0.46, P = 0.003).

A comparison was made between 37 women with tubal disease and 17 women with unexplained infertility. They were similar in age, duration of infertility, number of previous pregnancies and deliveries, as well as FSH concentrations and mean ovarian volume. Although the follicular density was lower in women with unexplained infertility (5.5 versus 9.5 follicles/mm^3), this difference was not significant (Table I). Three women had endometriosis and three others had polycystic ovaries (PCO), but these numbers were too small for statistical comparison. Increasing age showed a strong negative correlation with follicular density (Figure 1) and ovarian volume (r = –0.46, P = 0.0003; r = –0.43, P = 0.0016, respectively).

Mean ovarian volume was significantly less in women ⩾35 years than in women <35 years (Table II). Multiple logistic regression analysis showed that in the younger group of patients, the ovarian volume was not correlated with folliclar density (r = 0.04). In women >35 years of age the ovarian volume was strongly correlated with follicular density (r = 0.71, P < 0.0001).

Table II. Correlation between follicle stimulating hormone (FSH), mean ovarian volume and follicular density in different age groups

	Women <35 years	Women ⩾35 years	*P* value
No. of patients	33	27	
FSH (mIU/ml)	6.7 ± 1.9	7.5 ± 2.6	NS
Mean ovarian volume (cm^3)	10.4 ± 4.7	6.1 ± 3.1	0.0004
Follicular density (no./mm^3)	17.0 (0–160)	5.2 (0–76)	0.01

Values are means ± SD except those of follicular density, which are medians (range).
NS = not significant.

Table III. Comparison between women with <1 follicle/mm^3 to women with ⩾1 follicle/mm^3 in their biopsies

	<1 follicle/mm^3	⩾1 follicle/mm^3	*P* value
No. of patients	14	46	
Age (years)	37.5 ± 5.1	33.2 ± 5.3	0.01
FSH (mIU/ml)	8.1 ± 3.6	6.7 ± 1.7	NS
Mean ovarian volume (cm^3)	7.3 ± 3.7	8.8 ± 4.8	NS
Biopsy size (mm^3)	3.0	3.0	NS

All values are means ± SD.
FSH = follicle stimulating hormone; NS = not significant.

All values of early follicular phase FSH concentrations were within what is usually considered to be the normal range (median 6.5, range 2.5–13.4 mIU/ml); FSH concentrations were not correlated with follicular density in the biopsies.

Some women (n = 14; 23.3%) had <1 follicle/mm^3. They were significantly older than women who had more follicles (37.5 versus 33.2 years, P = 0.01). FSH concentrations, biopsy size and mean ovarian volume were similar in the two groups (Table III).

Discussion

Adequate ovarian reserve seems essential to maintain a woman's fertility. Loss of early follicles in the human ovary depends on age (Block, 1952). Follicular depletion is accelerated around age 37–38 years, when there are ~25 000 follicles; ~10% of the total number of follicles thought to be present at menarche (Richardson *et al.*, 1987; Faddy *et al.*, 1992). According to a mathematical model, 1000 follicles remain at the age of 51 years, the median age of menopause in Western society. Faddy *et al.* (1992) suggested that this number is the critical threshold needed to maintain menstrual cycles. Whether the depletion is caused mainly by entrance of small follicles into the growing pool in older women (Gougeon *et al.*, 1994; Gougeon, 1996), or by a high death rate among the smallest follicles (Faddy and Gosden, 1995) is not clear. In previous studies, ovaries were obtained from children or adults who had died suddenly, or from women undergoing oophorectomy for conditions not involving ovarian pathology. The subjects were part of the general population and not a selected group of infertile patients. The critical number of follicles required for maintenance of fertility is unknown.

In this study we developed a novel method to assess follicular density in ovarian biopsies. Contrary to Block (1952) and others who sliced the whole ovary and then counted the total number of follicles, our method avoided inaccurate observations caused by differences in size and shape of biopsies and we were able to unify the results. Follicular density was thus not dependent on the size of the ovarian biopsy. Although the density measurements we made may not entirely reflect the concentration of follicles in the ovarian cortex, an accurate estimation of the total follicle number can be obtained by sampling sections at regular intervals, as was also suggested by Richardson *et al.* (1987). Because follicles were located no deeper than 2 mm from the ovarian capsule, a shallow biopsy in a volume of ⩾5 mm seemed to be sufficient for reasonable assessment.

The use of OpenStereo provided a quick, flexible and robust mechanism for analysis. The accuracy and reliability of delineation of a given area for measurement is described by Abrams *et al.* (1994) and compares well with other non-computerized methods (Weibel, 1976). The method we described eliminates the need for micrographs, resulting in a substantial saving of labour, time and cost.

The vast majority of follicles were primordial whereas primary follicles were less frequently seen and secondary follicles were present only occasionally. This finding was similar to those seen in studies from a general population (Faddy *et al.*, 1992). The atresia rate (3%) was comparable to that found in Richardson's study (1987) on perimenopausal women.

Follicular density decreases significantly with increased age. Women >35 years of age have only a third of the concentration of follicles of younger women. Moreover, women who had practically no follicles in their biopsies were significantly older than those with follicles.

FSH concentrations were similar in the two age groups and were not correlated with follicular density. All our patients went through diagnostic laparoscopy or tubal surgery only if they had normal FSH concentrations. Although FSH concentrations rise more than a decade before the menopause this is probably a consequence of the declining number of follicles, and therefore a relatively late indication of ageing. There are also wide variations in FSH concentrations from cycle to cycle (Scott and Hofman, 1995).

Decreased ovarian volume seems a sign of ovarian ageing which can be observed earlier than a rise in FSH concentrations and can predict poor response to ovulation induction (Syrop *et al.*, 1995; Lass *et al.*, 1997). We have confirmed that increased age is associated with decreased ovarian volume. In our previous study, there was a poor chance of adequate ovarian function when ovarian volume was ⩽3 cm^3 (Lass *et al.*, 1997). In this study only two patients had ovaries in this size range and more data are needed to see whether such ovaries have a very low follicular density.

The median follicular density was apparently less in women with unexplained infertility than in women with tubal disease, although this difference was not significant. If this finding is confirmed by studies of more patients, it might suggest that lower follicular reserve has a role in the pathophysiology of their infertility.

In this study we established a method to quantify the number

of small follicles in ovarian biopsies. It is possible that studies of follicular density taken in conjunction with measurement of ovarian volume may help predict the basal fertility of women who desire infertility treatment. Ovarian biopsy may have a place as part of infertility evaluation in unexplained infertility and in women in the later part of their reproductive life.

Acknowledgements

We gratefully acknowledge Dr Geoffrey Trew, Dr Ronit Abir and Dr William Dennes for their invaluable assistance in collecting the biopsies.

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Received on December 5, 1996; accepted on March 4, 1997