Sequential effects of FSH on the first stages of ovarian follicular development in normal and dwarf Snell mice

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Abstract. The population of small growing ovarian follicles was divided into 4 classes according to the number of granulosa cells (from 15 to 95) surrounding the oocyte, and a comparison was made of normal and dwarf mice. Follicular cell proliferation was estimated by tritiated thymidine incorporation. In normal mice, most follicles in classes 1 (15 to 35 granulosa cells in their largest cross-section) and 2 (36 to 55 cells) were labelled (86 and 95%, respectively); FSH treatment increased the labelling index (L.I.) in all follicle classes. In dwarf mice, only 38 and 76% of follicles in classes 1 and 2, respectively, were labelled. However, FSH treatment increased the percentage of labelled follicles and the L.I. to levels which were similar to those in the ovaries of untreated, normal animals. FSH stimulation of the percentage of labelled follicles and L.I. was obvious as early as 3 h after injection. There was a major increase of the L.I. 24 h after FSH stimulation, specially in dwarf mice; several hypotheses are proposed to explain this finding. We conclude that FSH is necessary for the development of the population of small growing follicles in the mouse ovary.

The time interval between the initiation of ovarian follicular growth and ovulation has been determined for the mouse (Pedersen 1970b; Peters & Levy 1966; Hoage & Cameron 1976) and the rat (Hage et al. 1978). However, the stage when the follicle begins continuous growth has not been precisely defined: is it from the size of 15 to 20 cells (one layer of follicular cells) (Pedersen 1969; Mariana 1978) or from 100 cells and upwards (2 or 3 layers of granulosa cells) (Peters & Levy 1966)? Similarly, the stage during which follicular growth becomes dependent on gonadotropic hormones has not been clearly defined. According to several authors, FSH treatment in vivo (Pedersen 1970a) and in vitro (Ryle 1972) or after hypophysectomy (de Reviers & Mauléon 1973) stimulates growth in small preantral follicles, as measured by increased incorporation of tritiated thymidine into granulosa cells. However, other authors have concluded that gonadotropins are not necessary for the development of small follicles as demonstrated by in vitro (Fainstat 1968) or in vivo studies using hypophysectomized animals (Paesi 1949; Nakano et al. 1975).

The present study was undertaken to answer the following questions: what is the pattern of growth of small follicles containing 15 to 95 granulosa cells, and is it affected by FSH? A Snell strain of mice carrying the dwarf gene was used: the homozygous dwarf animals (dw/dw) have low pituitary and plasma levels of gonadotropins (de Reviers et al. 1984) as compared with littermates with a normal phenotype. Multiplication of granulosa cells in the ovary was measured by tritiated thymidine incorporation at different times after FSH stimulation. The conditions were similar to those used by Pedersen (1970a) for the study of FSH effect on follicular growth.

Material and Methods

Animals
Female mice aged 24 to 29 days of a Snell strain carrying the dwarf gene (Prof Falconer, Edinburgh) were used.
Mice with normal phenotype (+/+ or +/dw = N) (body weight: 12.6 g ± 0.4) and their homozygous dwarf littermates (dw/dw) (body weight: 5.4 g ± 0.2) were used. From each phenotype 24 females were treated under conditions similar to those used by Pedersen (1970a) giving a high labelling index: two injections (sc) of 1 µg of FSH (rFSH R6 SX = 28 x FSH NIH S10) in 0.2 ml of saline at an 8 h interval.

Animals were decapitated 3, 6, 12 or 24 h after the last FSH injection. One hour before sacrifice, mice received an injection (ip) of tritiated thymidine (3H]methyl thymidine, CEA, France): 2 µCi/g body weight).

**Histological treatment of ovaries**
The ovaries were removed, fixed in Bouin Hollande fluid and embedded in paraffin. One ovary of each animal was serially cut in 5 µm sections. Nuclei were stained by Feulgen reagent, and sections were covered with Ilford K2 emulsion. Autoradiographs were developed after 4 weeks of exposure.

**Classification of follicles**
Follicles containing 15 granulosa cells in their largest cross-section were considered as growing (Mariana 1978). Healthy follicles were classified, according to their number of granulosa cells surrounding the oocyte in their largest cross-section, into groups of 20 cells from 15 to 95 cells. Accordingly four classes were established: class 1: 15 to 35; class 2: 36 to 55; class 3: 56 to 75, and class 4: 76 to 95.

These four classes correspond roughly to the follicles of types 3a, 3b and 4 (20 to 100 cells) described by Pedersen & Peters (1968). As such oocyte have not reached their full size, these follicles are referred to as small follicles.

Follicles with 2 oocytes, with pycnotic bodies among granulosa cells, or with a basal membrane not clearly delimited were discarded. These anomalies were mainly found in dwarf mice ovaries.

**Calculation of the labelling index of granulosa cells**
All sections were examined. Round nuclei of granulosa cells were counted in the section where the nucleolus of the oocyte nucleus was located; this section was considered as the largest one. The number of labelled nuclei (> 10 silver grains) was determined.

For each class of follicle and for each animal, the percentage of labelled cells or labelling index (L.I.) in the largest cross-section was calculated, taking into account only follicles with at least one labelled cell. All small follicles were examined in order to obtain a high number of cells and an accurate L.I.:

\[
\text{L.I.} = \frac{\text{number of labelled cells}}{\text{total number of cells}} \times 100.
\]

For each follicle the labelling index was calculated and distributed into light (1–10%), medium (11–20%), and heavy (>20%) L.I.

**Statistical analysis**
The non parametrical test of Mann-Whitney (U-test) was used for the comparison of follicle numbers and L.I. in the experimental groups; and a χ²-test was used to compare the percentage of labelled follicles.

**Results**

**Effects of FSH on the number of small follicles**
The total number of small follicles did not differ significantly in ovaries of control animals of the two phenotypes (N and dw/dw). However, the total number of labelled follicles was significantly lower (−45%) (P < 0.05) in dwarf mice than in normal mice. This number differed in class 1 (−70%) (P < 0.01) and class 3 (−50%) (P < 0.05) (Table 1).

In normal mice, FSH stimulation had no significant effect on the total number of small follicles and on the total number of labelled follicles (Table 1).

In dwarf mice, the total number of labelled follicles increased 24 h after FSH stimulation (P < 0.05). This was essentially due to class 1 which evidenced an increase of 25 and 40%, 12 and 24 h, respectively, after FSH stimulation (P < 0.05) (Table 1).

**Effects of FSH on the percentage of labelled follicles**
In normal mice, most follicles were labelled (Table 2). The percentage of labelled follicles in classes 1 and 2 was significantly lower (respectively −50 and −20%) (P < 0.001) in dwarf mice than in normal mice (Table 2).

In dwarf mice stimulated with FSH, the percentage of labelled follicles in class 1 increased compared with controls within 3 h (+18%) (P < 0.01) and 6 h (+33%) (P < 0.001) after administration. In class 2, the increase in the percentage of labelled follicles compared to controls was statistically significant 6 h (P < 0.05) as well as 12 and 24 h (P < 0.001) (Table 2) after FSH stimulation.

**Effects of FSH on the L.I.**
In normal mice, as in control dwarf mice, the L.I. declined gradually from class 1 to 4 (Table 3). The L.I. only differed between the two phenotypes for class 2 (P < 0.05). FSH stimulation increased the L.I. in nearly all follicular classes of the various groups of animals.
Table 1.
Mean number (± SEM) of labelled follicles in ovaries of normal or dwarf mice sacrificed at different times after FSH treatment.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Normal</th>
<th>Dwarf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (h after FSH)</td>
<td>Controls (5)</td>
<td>3 h (4)</td>
</tr>
<tr>
<td>Class of follicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30.4 ± 6.5</td>
<td>38.5 ± 4.5</td>
</tr>
<tr>
<td>2</td>
<td>20.5 ± 1.7</td>
<td>18.0 ± 3.7</td>
</tr>
<tr>
<td>3</td>
<td>26.2 ± 3.9</td>
<td>27.0 ± 5.0</td>
</tr>
<tr>
<td>4</td>
<td>14.6 ± 3.1</td>
<td>13.7 ± 3.3</td>
</tr>
<tr>
<td>No. of labelled follicles/animal</td>
<td>92 ± 12</td>
<td>97 ± 15</td>
</tr>
<tr>
<td>Total No. of follicles/animal</td>
<td>98 ± 12</td>
<td>102 ± 17</td>
</tr>
</tbody>
</table>

(1) No. of animals. 1: vs normal controls, 2: vs dwarf controls, a: P < 0.01, b: P < 0.05.
In normal mice, a significant increase in the L.I. occurred in class 2 ($P < 0.05$) 3 h after FSH stimulation, and in class 3 in all periods studied (Table 3).

In dwarf mice, the L.I. increased in class 2 ($P < 0.05$) 6 h after FSH stimulation and in classes 1, 2 ($P < 0.01$) and 3 ($P < 0.05$) 24 h later.

**Distribution of the L.I.**

In both phenotypes, the percentage of follicles with a light L.I. (1–10%) increased with the size of the follicles, whereas the percentage of follicles with a medium L.I. (11–20%) decreased (Fig. 1). In follicle classes 3 and 4, the percentage of follicles with a high L.I. was very low or nil. Three hours after FSH stimulation, the percentage of highly labelled follicles increased (12 vs 1%) ($P < 0.01$) in the class 2 follicles of normal mice (Fig. 1).

**Discussion**

The aim of this work was to study the growth pattern of follicles leaving the pool of non-growing, primordial follicles in the mouse ovary. Using
exogenous FSH and a genetic model deficient in FSH, we have demonstrated a role for this hormone in stimulating these first steps of follicular development.

Our results indicate that, in normal mice, small follicles are growing since most of them are labelled with thymidine. In the two phenotypes studied, we found a decreasing L.I. with increasing follicle size (from 15 to 95 cells). This decrease results from the decreasing number of cellular divisions necessary for the follicle to pass from one size class to the next one; the numbers of divisions are 2.44, 1.86, 1.55 and 1.43 for follicles with 20, 40, 60 and 80 cells, respectively, in their largest cross-section or 90, 220, 410 and 635, respectively, for the total number of cells (see Pedersen 1970b).

The distribution of the L.I. shows that there is a heterogenous population of small follicles in classes 1 and 2 (15 to 55 cells). Some follicles were found to be highly labelled and therefore presumed to be growing rapidly. The percentage of these highly labelled follicles increased after FSH stimulation. In classes 3 and 4, all follicles were slightly labelled, indicating slow growth. FSH had no effect on the percentage of highly labelled follicles.

In a previous study (Pedersen 1969), the transit time from one class to another was found to be much longer in follicles from types 3b and 4 (20 to 100 cells) than in larger ones. In the mature mouse, Peters & Levy (1966) observed that some follicles (20 to 60 cells) remained in an 'inactive' condition, since some heavily labelled cells were found 20 days after flash labelling, showing that they had not divided. Unfortunately no quantitative study was made.

We observed a high number of unlabelled follicles (15 to 55 cells) in the dwarf mice. This high number of 'inactive' follicles may be related to the low FSH levels found in these animals (de Reviers et al. 1984). The fact that FSH treatment stimulated the percentage of labelled follicles indicates that these 'inactive' follicles were, in fact, able to resume their growth.

Within 3 h of FSH stimulation, we observed an increased labelling index in nearly all groups of treated animals. This result is similar to that of Pedersen (1970a), except that we found a slight decrease of the L.I. 12 h after stimulation, followed by a rebound a 24 h after stimulation, particularly in the dwarf mice. This result may be attributed to 1) a local effect of oestrogens produced by FSH stimulation, possibly more marked in the dwarf mice ovaries where blood flow is low (Hochereau-de Reviers et al. 1987); 2) an increase in FSH receptors; 3) a synchronization of rapidly dividing cells after FSH stimulation giving rise to daughter cells in G1 phase within 24 h of treatment (Quastler & Sherman 1959), and/or 4) diurnal variations (Sahu 1985).

Very little information on the role of FSH in the first steps of follicular growth is available. Lantern-Moore (1977) reported an increased number of growing follicles in mice aged 1 to 5 days, with pregnant mare serum gonadotropin as a source of exogenous FSH.

While there is evidence that FSH plays a part in the cellular proliferation of follicles, it is worth noting that the low FSH levels in the dwarf mice are none the less capable of maintaining some follicular growth. These follicles never reach the pre-ovulatory stage as in the dwarf mouse (de Reviers et al. 1984) and in the hypogonadic mouse (Halpin et al. 1986). The L.I. does not differ markedly in the two phenotypes, but the number of developing follicles is greatly reduced.

We cannot assert that FSH is the stimulus that triggers follicles to migrate from the pool of primordial follicles, but our results corroborate...
Table 3.
L.I. (%) (± SEM) in ovaries of normal or dwarf mice sacrificed at different times after FSH treatment.

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<tr>
<td>Class of follicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12.5 ± 1.3 (3873)</td>
<td>14.5 ± 1.0 (3458)</td>
</tr>
<tr>
<td>2</td>
<td>9.0 ± 0.8 (5279)</td>
<td>13.4 ± 1.2 (3207)</td>
</tr>
<tr>
<td>3</td>
<td>5.6 ± 0.5 (9344)</td>
<td>1a (6992)</td>
</tr>
<tr>
<td>4</td>
<td>5.7 ± 0.4 (6406)</td>
<td>7.2 ± 1.0 (4613)</td>
</tr>
</tbody>
</table>

(Total No. of cells counted in labelled follicles). No. of animals as in Table 1.
1: vs class 1, 2: vs controls, 3: vs normal controls, a: P < 0.005, b: P < 0.01, c: P < 0.05.
and expand on previous findings concerning the effect of FSH on small growing follicles (Pedersen 1970a; Ryle 1972; de Reviers & Mauléon 1973). Our present results demonstrate, therefore, that FSH is necessary for the development of the population of small follicles.

Acknowledgments

The author wishes to thank Dr M. T. Hochereau-de Reviers and Dr. D. Monniaux for their constructive criticism of the manuscript.

References


Received May 6th, 1987.
Accepted September 22nd, 1987.

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