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Original article

Proliferation of follicular cells and the effect of FSH on the onset of follicular growth in the ovary of 30-day old rabbits studied by continuous labelling with ^{3}H-thymidine

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Summary — The ovary of the 30-d-old rabbit contains only small follicles with, at most, 3 or 4 layers of cells. We have estimated the labelling index of the follicular cells only from follicles having at least one labelled cell and using a zero truncated binomial distribution. The labelling index of the follicular cells of such follicles was weak and enlarged with the number of cells. It never exceeded 20%. Repeated injections of tritiated thymidine up to 8 times entailed a significant increase of the labelling index even for the smallest follicles. The labelling index of follicles was significantly increased 30 h after 1 injection of FSH (2 mg P-FSH). These results confirmed that the fraction of follicular cells that proliferate was low in the young rabbit ovary and the doubling time of the cells was large and that at this age, FSH increased the proliferation of cells.

rabbit / follicular growth / FSH / follicular cells / small follicle

Résumé — Étude de la prolifération des cellules folliculeuses des ovaires de lapines âgées de 30 jours par la méthode du marquage continu à la thymidine tritiée. *L'ovaire de lapine âgée de 30 jours contient seulement des petits follicules avec au plus 3 ou 4 couches de cellules folliculeuses. Nous avons estimé l'index de marquage des cellules folliculeuses à partir des follicules ayant au moins une cellule marquée et en ajustant l'ensemble des résultats pour chaque classe de follicules par une distribution binomiale tronquée à zéro. L'index de marquage des cellules folliculeuses de tels follicles est faible et croît avec le nombre de cellules. Il ne dépasse jamais 20%. Des injections répétées de thymidine tritiée jusqu'à 8 fois entraînent un accroissement significatif de l'index de marquage même pour les très petits follicules. L'index de marquage des follicules est significativement accru 30 h après une injection de 2 mg de P.FSH. Ces résultats confirment que : 1) la fraction des cellules folliculeuses qui prolifèrent est très faible dans l'ovaire de la jeune lapine et que le temps de doublement des cellules est très grand; 2- FSH agit sur la prolifération cellulaire.*

lapin / croissance folliculaire / FSH / cellules folliculeuses / petits follicules

INTRODUCTION

Previous studies suggest that the generation time (Tc) of granulosa cells in the smallest follicles is long. In the adult cyclic rat (Mariana, 1978) and in the young rabbit (30-d-old: the time of primordial formation) the labelling index of follicular cells of small follicles is very low (Mariana and de Pol, 1986). It was estimated, through the gain halving method, that Tc was at least 1 week.

The estimate of the rate of follicular growth was based upon the assumption that the growth fraction of follicular cells was 100%. If this assumption were incorrect, the estimate of follicular growth would be inaccurate; for example, if it takes 100 h to double 1 population of cells when the growth fractions is 100%, it would take 130 h when it is 70%. The relation between the generation time Tc and the doubling time Td of an exponentially growing population of cells with growth fraction G.F is Td Ln (G.F + 1) = Tc Ln2; in our example 130 h = 100 x 0.6931/0.5306 where Ln is a Neperian logarithm (Valleron, 1971).

The objective of this study was to verify through the continuous labelling method that the growth fraction of very small follicles is 1.

MATERIALS AND METHODS

A random sample of 9 young 30-d rabbits of the Californian breed received either one: (n = 2 rabbits), 2 (n = 2), 4 (n = 2), 6 (n = 1) or 8 (n = 2) injections of [^{3}H] thymidine (^{3}HTdr; Specific activity: 925 cBq/mmol) – 0.2 µCi/gp) – IP at intervals of 6 h.

Another group of 8 rabbits taken at random received an injection of 2 mg P-FSH (IP) with the first injection of [^{3}H] Tdr. They also received either one (n = 2), 2 (n = 1), 4 (n = 1), 6 (n = 2) or 8 (n = 2) injections of [^{3}H] Tdr under the same conditions as the first group. In both groups, animals were randomly allocated to a treatment group. The ovaries were collected 1 h after the last thymidine injection, fixed in Bouin–Hollande, embedded in paraffin and serially sectioned at 5 µm.

All sections were stained with Feulgen before the slides were dipped in Ilford K_2 emulsion and then exposed for 1 month at 4 °C in the dark according to standard procedures (Pedersen, 1969).

All tissue sections were examined under the light microscope. Follicles were allocated to classes according to the number of follicular cells in the cross section containing the nucleus of the oocyte. All follicles with < 80 cells and at least 1 labelled cell in the section were selected for analysis. Four hundred (range: 168–520) follicles were counted on average, for each ovary. It was assumed that granulosa cells divide independently from others in a given follicle. The distribution of the number of labelled cells would thus be expected to follow a binomial distribution. Therefore, the labelling index (LI) of granulosa cells is a function of the probability P of any granulosa cell being labelled. It is estimated by:

$$\hat{p} = \frac{1}{s.N} \sum_{r=0}^{s} r.n_r$$

where r is the number of labelled cells, n_r is the number of follicles with r labelled cells, s is the total number of cells in one follicle, and N is the number of follicles with s cells.

In this case, the frequency distribution of the number of labelled cells in a follicle is a truncated binomial distribution since follicles with zero labelled cells were ignored. $\hat{p}$ overestimates the LI, especially, for follicles with small numbers of cells, s, and a low probability of being labelled. For small s and p the probability P_0 that follicles will be unlabelled equal to $(1-p)^s$ and will be very important. For example, the probability P_0 of a follicle with 10 cells, and $p < 0.10$ of not being labelled is larger than 0.5 (fig 1a).

To correct this phenomenon, the labelling index $\hat{p}_e$ adjusted for missing unlabelled follicles was estimated by an iterative method proposed by Finney (1949) and discussed by Rider (1955); $\hat{p}$ and $\hat{p}_e$ were calculated for each rabbit and for each class of follicles. Figure 1b illustrates the effect of the correction. The difference between $\hat{p}$ and $\hat{p}_e$ decreases as the number of

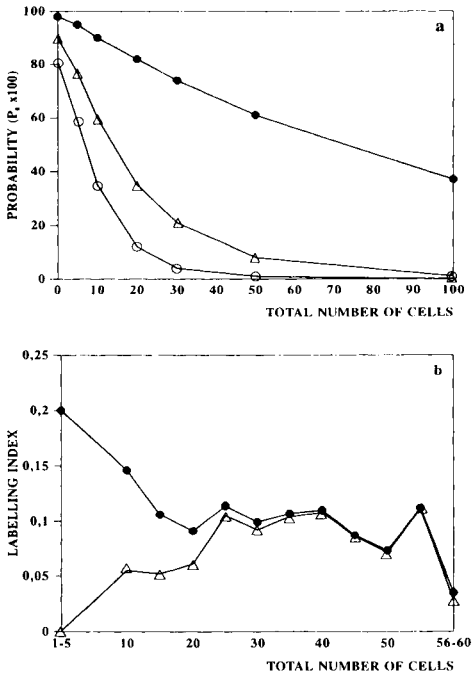

Fig 1. a. Theoretical example to illustrate the binomial distribution of probability P_0 (x 100) of having no labelled cells as a function of the total number of cells. Each curve corresponds to the different level of probability p that a cell may be labelled (-●- p = 0.01; -Δ- p = 0.05; -O- p = 0.10). **b.** Distribution of adjusted p_e (-Δ-) and observed p (-●-) values of the labelling index LI according to the number of follicular cells in one case taken as an example: one animal receiving 6 thymidine injections without FSH.

granulosa cells increases. All rabbits exhibited the same pattern.

The main effects analyzed were:

i) time after FSH injection confounded with the number of thymidine injections (5 modalities); ii) FSH effect characterized by presence or absence of FSH injection. We showed a significant interaction between the main effects: the pattern of thymidine incorporation is different between the two groups with or without FSH (fig 2). Due to the non-additivity of the main effects, we chose to analyze the effects of thymidine injections and size of follicles on the adjusted labelling according to a split plot design for unbalanced data and repeated measures on one factor (size of follicles) (Searle, 1987; Winer *et al*, 1991). The labelling indexes were adjusted by weighting them by the inverse of the estimate (Finney, 1949; SAS Stat 1987).

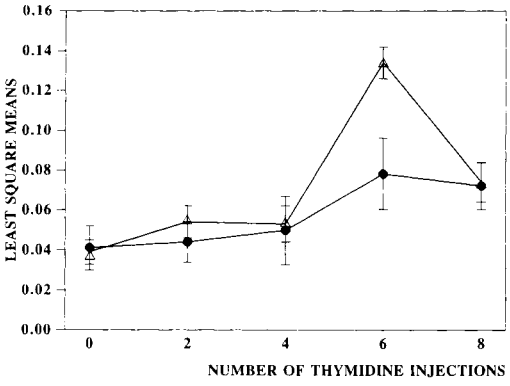

Fig 2. Distribution of the least square means (ILS means ± SE) of the adjusted labelling index $\hat{p}_e$ according to the number of thymidine injections, with -●- or without -Δ- FSH.

RESULTS AND DISCUSSION

There were few labelled follicles with less than 5 cells, and the estimate $\hat{p}_e$ was unreliable (fig 1b); most of the estimated values of $\hat{p}_e$ were less than 0.0011 and few were unduly large.$\hat{p}_e$ increased significantly with the number of follicular cells but never exceeded 0.20.

Effect of the number of thymidine injections

Group without FSH (fig 2)

There was no significant effect on the number of thymidine injections on the adjusted

labelling index even though we observed one slight increase in the adjusted labelling index values between 1 and 8 injections.

Groups with FSH (fig 2)

The labelling index after the sixth injection significantly differed from the 4 others which were themselves homogeneous ($P > 0.05$).

Effect of the size of follicles

With or without FSH, one slight but significant increase (5%) of the labelling index with the size of the follicle could be observed.

When values associated with the sixth injection are excluded from the calculation, the labeling index with or without FSH is similar in each class of size of follicles.

As Hirshfield (1989) suggested, little is known about the onset of follicular growth and there is a great deal of confusion about the terms 'primordial' or 'primary follicles' due to the fact that the definition of the initiation of follicular growth is not clearly defined.

In an earlier study it was estimated that the doubling time of granulosa cells in the smallest follicles was close to 8 d (Mariana and de Pol, 1986). Many follicular cells are in the G_0 state (non-proliferating pool of cells) in small follicles of the young rabbit and after 48 h of continuous labelling (6 injections) the labelling index is increased around 3-fold.

In this study, results obtained with repeated injections of [^{3}H] Tdr confirm that the generation time of granulosa cells is very long for all follicles present at this age but it is difficult to draw conclusions about the growth fraction (Aherne *et al*, 1977) since there are many pitfalls in the use of the continuous labelling technique; in particular, cellular death due to the continuous incorporation of radionucleides during many cellular cycles. Hirshfield (1989) administered [^{3}H] Tdr to cyclic rats *via* continuous infusion with osmotic minipumps for periods of up to 1 week. The LI of small follicles with less than 8 cells doubled after 7 d of infusion (*vs* 24 h of infusion). The labelling index of small follicles with 9–32 cells increased 4-fold and all follicles with more than 12 cells were 100% labelled at the end of the 7-d infusion period. If evidence of cell proliferation is taken as the criterion to distinguish growing from non growing follicles then, after a 7-day infusion, all follicles with more then 12 cells would be considered growing. After 48 h of continuous labelling, however, only 50% would be considered as growing and 50% would be mistakenly classified as non-growing.

Previous investigators have shown that the number of small follicles with less than 40 cells on the cross section was not modified in 7-d-old mice treated by an antiserum directed against FSH (Eshkol *et al*, 1970) although the cellular morphology was abnormal. Similarly, hypophysectomy of 22-d-old rats did not alter the size frequency of small follicles (Nakano *et al*, 1975). Furthermore the injection of PMSG to young 6-d-old mice did not modify the number of small follicles (Peters *et al*, 1973). These authors concluded that gonadotropins are not essential to the initiation of growth of primordial follicles. The significant statistical interaction, observed in our experiment, between FSH and the number of injections resulted from the increase of the labelling index at the 6th injection (30 h after the FSH injection). Pedersen (1969) also observed a slight increase in the labelling index of 21-d-old mice follicles with between 20 and 60 cells in the largest section, 11 h after an FSH injection. Despite the differences between the species, ages of the animals studied and the small num-

ber of animals in our study, the 2 results suggest that in small follicles a time lag occurs between FSH stimulation and the time when follicular cells leave the G_0 state to synthesized DNA.

Mariana and Hirshfield (1990, unpublished data) also observed that the labelling index of small follicles with less than 30 cells in one adult rabbit increased 200 hrs after hemicastration and a severe resection of the remaining ovary. This could be a direct consequence of the rise in gonadotropins that occurred after this severe depletion of ovarian tissue or it could mean that the combined changes in a wide variety of endogenous hormones and growth factors, following surgical trauma, were more effective for stimulating cell proliferation than the injection of FSH alone.

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