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FOLLICULAR GROWTH AND ATRESIA IN THE MOUSE[1]

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DISCLAIMER

FOLLICULAR GROWTH AND ATRESIA IN THE MOUSE

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Running head: GROWTH AND ATRESIA OF FOLLICLES

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SUMMARY

Follicles were classified on the basis of number of layers of folicle cells, presence and degree of development of the zona pellucida, and presence of an antrum. Formation of an antrum in follicles with less than 7–8 cell layers and/or presence of necrotic cells was considered indicative of degeneration. When classified in this manner, the data suggest that follicles and their contained oocytes are committed to either normal development or atresia by the time a third layer of granulosa cells is formed.

Key words: mouse; oocytes; follicles; atresia; ovary.

INTRODUCTION

Data on the dynamics of follicle and oocyte growth in adult mammals have only recently become available (1–3). The chronology of follicular development in fetal, newborn, and juvenile animals has been recorded (4–7), and it is known that female mice become fertile at 5–7 weeks of age, depending upon strain. The stimulus to obtain definitive data on oocyte growth in the adult mouse came from the observation of Russell (8, 9) that there is a dramatic change in mutational response of the oocyte with the time elapsed between irradiation and mating. What is the biological basis for this change in radiation response of the growing oocyte? The initial task is to identify the stage of oocyte development at which the change in mutational response occurs in order that underlying mechanisms can be investigated. These results also bear significantly on the use of data for the mouse in estimating genetic hazards to women.

Timing of follicular growth requires an objective classification of the different follicle stages, and this has been provided by

Pedersen and Peters (10). This scheme, utilized in conjunction with ^{3}H-thymidine labeling, indicated that a stage 3 follicle of ∿20 cells reached maturity and was ovulated in about 19 days (1). Meanwhile, we were attempting to label the zona pellucida as a technique for timing follicular growth and achieved success with N-[^{3}H]acetyl-D-glucosamine (2). Subsequently we also have used D-[1-^{3}H]glucosamine·HCl and L-]1-^{3}H]fucose with identical results. Our present best estimate, based on zonal labeling, indicates that a stage 3b follicle requires slightly more than 6 weeks to mature instead of 19 days, as estimated by Pedersen (1).

The above results of timing oocyte development, together with counts of oocytes after 50 R, indicated that there only were enough normal surviving follicles to explain observed reproductive performance. There were few oocytes to be spared for atresia, and it became clear that the main problem was identification of degenerating follicles. Little advance has been made on this subject since the review of

Ingram (11). A more recent review is given by Greenwald (6). Criteria using frequency of necrotic cells (12, 13), change in lipids (14), cavities in small follicles (12), or surface ultrastructural changes (15) usually refer to advanced stages of atresia instead of very early changes needed for our purposes. The criteria for follicular degeneration presented here still are not as objective as one would like, but, when combined with a total oocyte count, they reveal a new concept of follicular development and atresia in the mouse.

MATERIALS AND METHODS

Nine-month-old 101 X C3H females that had been pretested for possible fertility effects of N-[^{3}H]acetyl-D-glucosamine, were used for oocyte counts at different stages of the estrus cycle. N-[^{3}H]acetyl-D-glucosamine had no effect on their breeding performance. The ovaries appeared normal in every respect, and there is no evidence that labeling several months earlier had any residual effect on ovarian histology. All tissues were fixed in Zenker-formol, and serial 7-μm sections were prepared. Slides were stained by the PAS technique and counterstained with Ehrlich's hematoxylin.

Oocytes were classified according to size, number of follicle layers, and antrum formation according to the criteria given in Table 1. (T-1) This approximates but differs in some respects from the classification of Pedersen and Peters (10). Their stages 1 and 2 are both combined in our stage 1, and our stage 2 is intermediate between their stages 2 and 3. Also, we differentiate stages 3a and 3b on the basis of

initiation of zona formation and have divided their stage 4 into stages 4a and 4b. Stage 1 and 2 oocytes were counted in every 10th section; all other stages were counted in every section. Adjacent sections were checked to preclude scoring the same cell twice.

The morphology of normal follicles is illustrated by Figs. 1A–H; 2A,C; 3A,C; and 4A. Stage 1 is characterized by a few follicle cell nuclei which appear within the basement membrane. Owing to the small number of follicle cells, their nuclei may not be apparent in some sections. As the follicle cells divide, they form a continuous ring (Fig. 1B,C), and the mitotic activity leading to development of stage 1 into stage 2 may be explosive (Fig. 1B). As the follicle continues to grow, the cells become cuboidal (Fig. 1C); then zonal material begins to collect in the interstices between oocyte and follicle cells in stage 3b (Fig. 1E). By stage 4a (Fig. 1F), the zona forms a complete, but thin, layer around the oocyte with some clumps of zonal material, and the formation of a second granulosa layer begins. A stage 4b

F 1–4

follicle is defined (Table 1) as having 1-1/4–2-1/4 layers of cells (Fig. 1G,H). It is at this point that degenerative changes first can be seen in the follicle, and Fig. 1I illustrates precocious antrum formation and separation of the follicle cell layers by a space filled with what appears to be follicular fluid. This also occurs in stage 5a, where a normal follicle is shown in Fig. 2A and an abnormal one in Fig. 2B. A normal stage 5b is illustrated in Fig. 2C. Note that there is no indication of follicular fluid accumulation and no antrum formation even though many cell layers are present. The entire series of sections for these follicles must be examined in order to be certain that there are no cavities. Precocious antrum formation also occurs in stage 5b (Fig. 2D) and may occur with no associated necrosis; other degenerating 5b follicles show many degenerating cells (Fig. 2E). Some of the follicles degenerating at this stage appear to shrink, and eventually they will disappear except for the zona pellucida, which persists for as long

as 10 weeks or more (2). True antrum formation occurs in stage 6 (Fig. 3A) where small accumulations of follicular fluid appear between the granulosa cells and enlarge to form small antra. No cell degeneration is associated with this process. The degenerating stage 6 follicle in Fig. 3B, however, shows many necrotic cells, and this is characteristic of all atretic antral follicles. In stage 7 (Fig. 3C), the antra are larger and by late stage 7 unite to form a single cavity. Degenerating follicles are characterized by many necrotic cells and a poorly developed theca (Fig. 3D). Stage 8 (Fig. 4A) occurs only in proestrus, and most of the follicles reaching this stage will be ovulated. Atresia could have begun in the large follicle shown in Fig. 4B at either stage 7 or 8. Oocytes in many of the large atretic follicles are in meiotic division (Fig. 4C). Also, note the large number of pyknotic nuclei and some normal mitoses among the granulosa cells.

Number of normal oocytes scored for each stage of development are given in Table 2. Since there were only two females scored for

T-2

each stage of the estrus cycle and variability between females was large, statistical analyses were not performed. However, it is clear that stage of the cycle has little effect before a follicle attains three or more cell layers. An effect is seen only in estrus where stages 5b and 6 were higher than the general mean. Early stage 7 follicles appeared in metestrus 1 and progressively matured until only late stage 7 and stage 8 follicles were seen at proestrus.

The most interesting observation from Table 2 is that the number of normal follicles at stages 5a–8 is surprisingly constant; and, while they are sufficient to explain fertility, their numbers are too low to permit significant contribution to the degenerative pathway.

DISCUSSION

The small, arrested oocyte is surrounded by a basement membrane, within which a few follicle cell nuclei usually can be seen (Fig. 1A). Follicle cell cytoplasm completely surrounds the oocyte (16), and, as observed by Brambell (17), these cells form the granulosa. Growth into a stage 2 follicle with a single layer of flattened cells occurs by mitosis (Fig. 1B,C) and not by apposition of stromal cells to the oocyte, as often has been postulated. This mitotic activity can be explosive (Fig. 1B); and since it affects only a few follicles at a given moment, it is easy to understand why the importance of these rare divisions has been underestimated. Once a follicle begins its growth phase (stage 3a), it is programmed either to degenerate or to be ovulated.

The origin of the zona has been the subject of many investigations, and Kang (18), in a study of the rat, concluded that it was formed by the oocyte. Likewise, Haddad and Nagai (19) came to the same

conclusion using tritiated L-fucose to label the zona of the mouse oocyte. The present study, where the PAS technique was used as a mucopolysaccharide stain, shows that the material fills spaces between the follicle cells, outlining them and running together to form local masses between the oocyte and follicle cells (Fig. 1E,F). These foci then join. As pointed out by Chiquoine (20), this is suggestive of the participation of many cells. Furthermore, the follicle cells change from cuboidal to low columnar in form (Fig. 1D-F), suggesting secretory activity. The suggestion of Chiquoine (20) that monomers secreted by the follicle cells are polymerized on the oocyte appears to be the most plausible hypothesis of zona formation.

Degeneration of follicles (and oocytes) in stages 1-4a is rare, but it becomes common when a second and third follicular layer appears (Table 3). By stage 5a, 78% of the follicles were classified as degenerating, and there was no effect of stage of estrus (Table 3). Only stage 6, where antrum formation begins, shows an effect of stage

T-3

of cycle with only 32% degenerating follicles in estrus and 80% in metestrus 1. This differs from the results of Engle (13), who demonstrated a more pronounced effect of stage of the cycle. However, our data are difficult to compare with his owing to different staging of the estrus cycle, but our estimate of 32–82% degenerating follicles agrees well with his range of 26– 86%.

The classification of follicles as normal or atretic has in the past been highly subjective, and I had hoped that an objective system of scoring could be developed. However, there always will be follicles that are difficult to classify, and the only hope is to reduce subjectivity to a minimal value. Precocious antrum formation (Figs. 1I, 2B) is the primary form of degeneration at stages 4b and 5a. By stage 5b, the occurrence of necrotic cells also is common; and though Fig. 2D shows only one degenerating cell, other 5b follicles have numerous pyknotic cells. The follicle in Fig. 2D is classified as abnormal because the antrum is too large for a follicle of this size,

whereas a few areas of PAS-positive material between follicle cells (without antrum formation) would be considered normal. Is the follicle in Fig. 2D derived from those in stage 4b and 5a that show precocious antrum formation? Certainly many of them go no farther than the stage in which regression is initiated, as shown by Fig. 2E; but often there is little evidence of necrosis and high mitotic activity in follicles with precocious antra. Do they go on to form abnormal stage 7 and 8 follicles, especially those that have antra that are disproportionately large for the size of the follicle? In stages 6, 7, and 8 presence of necrotic cells is characteristic of atretic follicles (Figs. 3B,D; 4B,C). Precocious meiotic division (Fig. 4C) also is typical of oocytes in degenerating follicles.

The classification described above, combined with enumeration of all follicles, leads to a new concept of the process of follicular growth and degeneration. The data of Table 2 show numbers of normal follicles by stage of development and by phase of the estrus cycle.

It is clear that the fate of most follicles is determined by the time they reach stage 5a [a follicle with 2-1/4–3-1/4 layers of follicle cells (Table 1; Fig. 2A)]. Thereafter, the number of normal oocytes is adequate to provide only those that will be ovulated (Table 2). This assumes, however, that each of the stages listed (5a–8) requires approximately one estrus cycle for development, as suggested by our timing of follicular growth, i.e., the 28 days required for a 4a–4b follicle to reach ovulation (2) would provide oocytes for six estrus cycles of ~4-1/2 days each (Table 2). However, the excess stage 5b and 6 oocytes in estrus (Table 3) must be accounted for. The most likely explanation is that early degenerative changes in many follicles were not recognized, and that they were misclassified as normal. This interpretation is supported by the high proportion of degenerating stage 6 follicles at metestrus 1. Emphasis is placed on the overall pattern showing a low number of normal follicles at stages 5a–8 suggesting that by the time the follicles have three layers of granulosa cells most are committed to either an atretic

or normal pathway. This is different from the model presented by Chiras and Greenwald (21) for the hamster, where they propose that 50% of the follicles degenerate at each stage. However, since it takes about 4 days for a follicle to become atretic (12) and since they probably are recognizable for a longer interval, a model based on numbers of normal and atretic follicles at any one time could be misleading. Furthermore, it is difficult to compare our data with those of Chiras and Greenwald (21) because we have used different systems of follicle classification.

We need more data on timing of the individual stages of follicular growth and more objective criteria of classifying normal versus degenerating follicles. However, the procedures and results presented here already lead to a new concept of the dynamics of oocyte and follicle growth. It now should be possible to make more objective studies of the effects of pregnancy, irradiation, and other experimental procedures on the ovary.

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TABLE 1

CLASSIFICATION OF FOLLICULES IN THE ADULT MOUSE OVARY

Follicle Stage	CHARACTERISTICS	Illustration (Fig.)
1	0 to 3 or 4 follicle cells	1A
2	1 complete layer of flattened follicle cells.	1B,C
3a	Single layer of cuboidal cells, no zona.	1D
3b	Single layer of cuboidal or low columnar cells, zona formation beginning.	1E
4a	1–1-1/4 layers of follicle cells, zona complete but thin.	1F
4b	1-1/4–2-1/4 layers of cells, zona completely surrounds oocyte but still not fully developed.	1G,H,I
5a	2-1/4–3-1/4 follicle cell layers.	2A,B

(Table 1 continued)

5b	>3-1/4 follicle cell layers, oocyte at mature size, zona fully developed.	2C,D,E,
6	Many layers of follicle cells, antrum formation initiated.	3A,B
7	Large follicle, one or two small antra.	3C,D
8	Mature Graafian follicle with large antrum and thin wall. Normal stage 8 follicles occur only in proestrus.	4A,B,C

TABLE 2

MEAN NUMBER OF NORMAL FOLLICLES AT DIFFERENT ESTRUS STAGES

Follicle Stage	Proestrus	Estrus	Metestrus 1	Metestrus 2	Diestrus	General Mean
1	1040	1300	1525	815	1005	1137
2	665	885	905	755	670	776
3a	206	222	263.5	184	195.5	214.2
3b	201	186.5	215	190.5	171	192.8
4a	118	92	116	115	92	106.6
4b	90.5	120	118.5	79	99	101.4
5a	13	12.5	13	12	10.5	12.2
5b	16	22.5	17	9.5	12.5	15.5
6	18	31.5	12	12	9	16.5
7 & 8	15.5	10.5	11	12	15.5	12.9

TABLE 3

PERCENTAGE OF DEGENERATING FOLLICLES AT DIFFERENT STAGES OF THE ESTRUS CYCLE

Follicle Stage	Proestrus	Estrus	Metestrus 1	Metestrus 2	Diestrus	General Mean
1–3b	0	0	0	0	0	0
4a	0	0	2.5	0	0	0.5
4b	56.8	42.6	41.8	49.5	44.5	47.04
5a	78.9	80.8	80.0	72.7	77.7	78.02
5b	78.4	69.8	74.0	82.2	74.2	75.72
6	69.2	32.3	80.5	70.7	77.5	66.04
7 & 8	49.2	50.0	61.4	53.8	50.8	53.04

FIGURE LEGENDS

Fig. 1. Photomicrographs of follicle stages 1–4G. A, Stage 1 oocyte with two follicle cell nuclei inside basement membrane. B, Early stage 2 follicle, 6 late telophase–early interphase and 1 metaphase follicle nuclei. C, Stage 2 follicle. D, Stage 3a with cuboidal follicle cells, no zona. E, Stage 3b, with zona material accumulating between follicle cells and oocyte (arrows). F, Stage 4a, zona is a complete ring. Note how PAS-positive material outlines follicle cells (arrows). G, Stage 4b, with almost two complete cell layers. H, Stage 4b with complete second layer of cells. Note that no separation is present. I, Degenerating 4b follicle as evidenced by separation of follicle cells to form a cavity (arrow). A–E, X970; F, X700; H,I, X400.

Fig. 2. Photomicrographs of follicle stages 5a and 5b. A, Normal 5a follicle with 3 layers of cells. B, Degenerating stage 5a with precocious antra (arrow). C, Normal 5b with 5–6 cell layers, no

antrum. D, Degenerating 5b follicle with antrum, rare necrotic cells, and normal mitoses. E, Advanced stage of degenerating 5b follicle with many pyknotic cells around abnormal antrum. X400.

Fig. 3. Photomicrographs of follicle stages 6 and 7. A, Normal stage 6 with antrum forming to right of oocyte (arrow), no necrotic cells, mitoses. B, Degenerating stage 6, many necrotic cells, no mitoses. C, Normal early stage 7 with two small antra (arrows), normal mitoses, no necrotic cells. D, Degenerating stage 7 with many necrotic cells. A,B, X300; C,D, X200.

Fig. 4. Photomicrographs of normal and degenerating stage 8 follicles. A, Normal stage 8 in proestrus. B, Degenerating stage 8. C, Degenerating stage 8 showing meiotic division, many necrotic granulosa cells, but some apparently normal mitoses. A,B, X200; C, X970.

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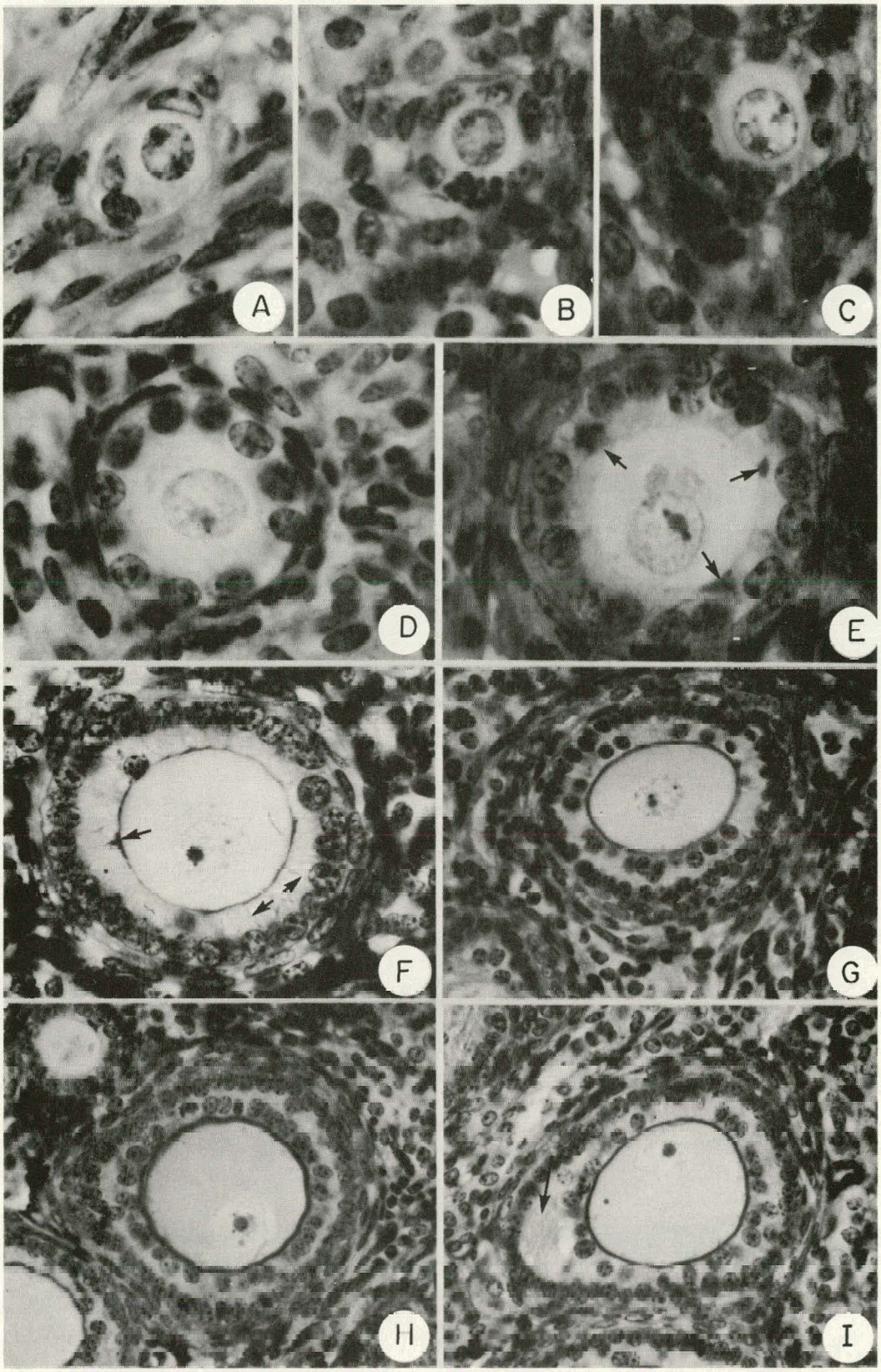

Fig 1

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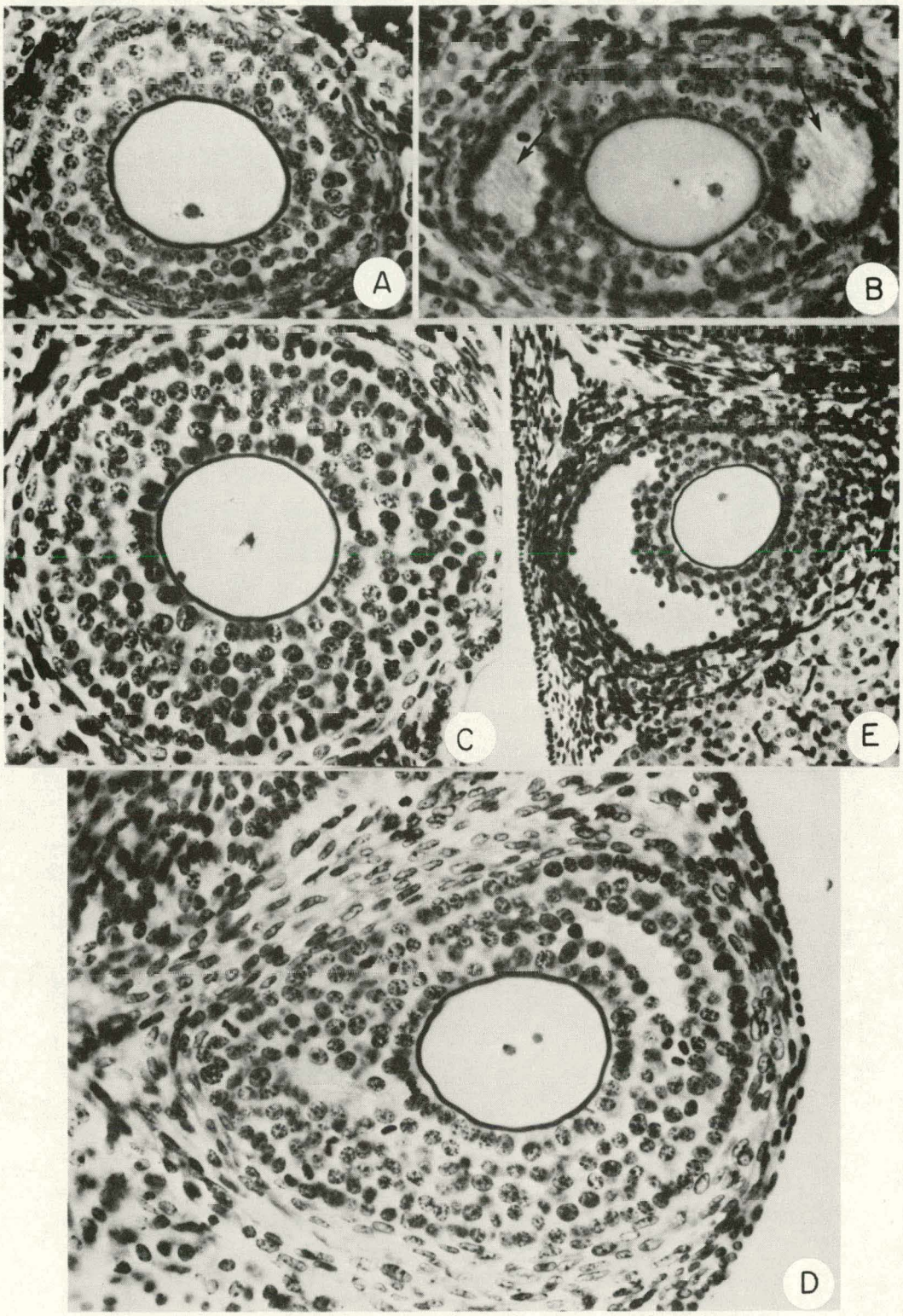

Fig 2

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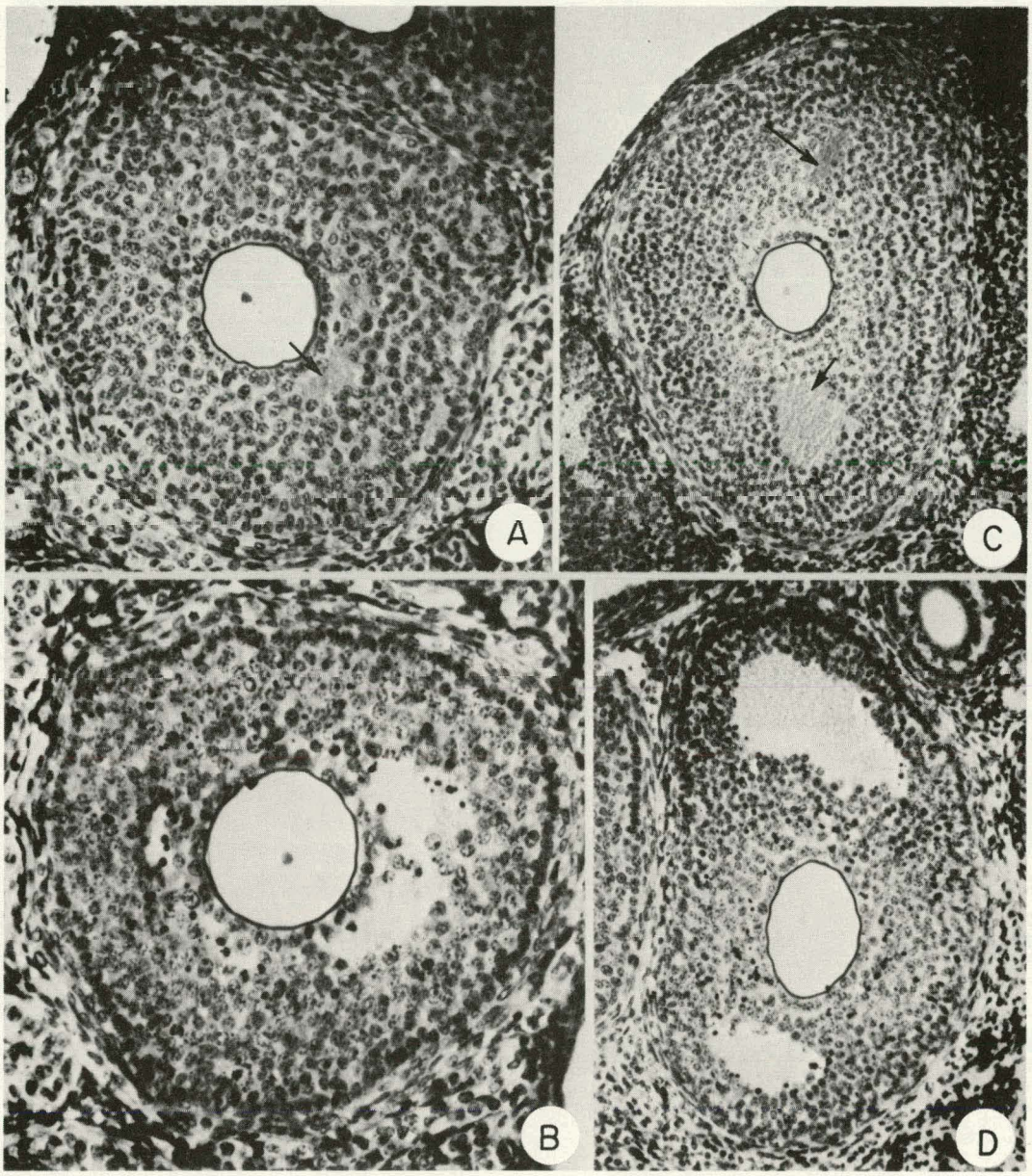

fig 3

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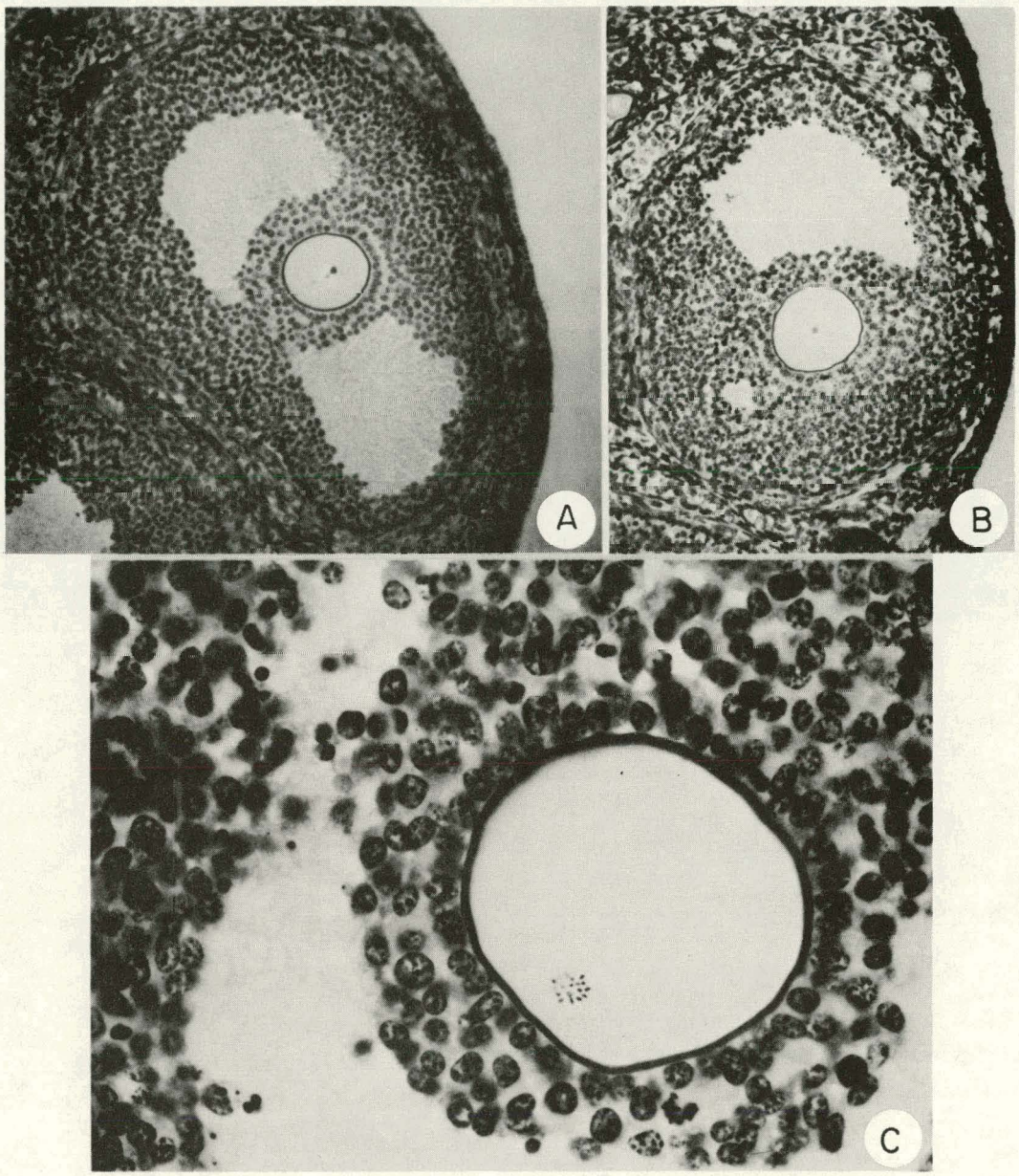

Fig. 4