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RNA and Protein Synthesis in Control and Follicularly-Aged Rat Oocytes (38339)

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Delayed ovulation, whether naturally occurring or artificially induced, results in the follicular aging of the oocyte and the subsequent production of embryonic abnormalities (1–3). An increased incidence of morphological and chromosomal defects, as well as a decrease in the fertilization and implantation rates, has been associated with a 48-hr delay in ovulation (1, 4). Previous studies have demonstrated that delayed ovulation not only alters the oocytes such that they are less conducive to normal embryological development, but also changes the intrauterine environment (5). These changes in the intrauterine environment resulted in a decrease in implantation rate. Freeman *et al.* (6) have shown that during the follicular aging process, the preovulatory oocyte remains at a constant size and in meiotic arrest, while the follicle continues to enlarge. Ultrastructural analysis has demonstrated changes in the cortical granules which could explain the increased incidence of polyspermy in the follicularly-aged oocyte. These studies also have described changes in the mitochondrial morphology which suggest alterations in the metabolism of the follicularly-aged oocyte (7). Since metabolic alterations within the follicularly-aged oocyte could be responsible for the developmental anomalies, the present study was designed to determine if differences in RNA or protein synthesis exist between control and follicularly-aged tubal oocytes.

Methods and Materials. Mature female Sprague–Dawley rats were housed under controlled conditions of temperature, humidity and light. The lights were on from 5:45 AM to 6:15 PM. Vaginal smears were taken daily between 8 and 10 AM and only those animals which had three consecutive 4-day estrous cycles were assigned to an experiment. Ovulation was delayed for 48 hr by intraperitoneal injections of sodium pentobarbital (Nembutal) at 1:50 PM during proestrus and 1:50 PM and 3:30 PM during the following day (1). Each injection of Nembutal was 31.5 mg/kg body wt.

RNA synthesis in oocytes. Oocytes were collected from control and delayed-ovulatory rats at 8 AM on the morning of estrus by flushing the oviduct with physiological saline. The oocytes in their cumulus mass were rinsed in saline and then transferred in 10–15 μl of saline to 100 μl of Brinster's Ova Culture Media (BMOC-3) to which ^{3}H-5-uridine (50 μCi/ml; sp. act. = 4 Ci/mmole) had been added. The oocytes were then incubated under oil for 4 hr at 37° in a humidified atmosphere of 5% CO_2 and air. After incubation, the oocytes and cumulus mass were embedded in agar, rapidly dehydrated in a graded series of alcohol and stored in methylbenzoate for 1–7 days. Subsequently, the oocytes were embedded in paraffin and serially sectioned at 7 μm. Alternate sections were mounted with gelatin on separate slides. After the paraffin was removed, half of the slides were placed in phosphate buffer at 37° for 1 hr, while the remaining slides were placed in phosphate buffer which contained RNase (3 units/ml) to digest the RNA. After the RNase or buffer treatment, the slides were placed in 5% trichloroacetic acid (TCA) at 4° for 10–15 min to remove the unincorporated uridine (8). The slides were then air dried, dipped in NTB-2 emulsion and exposed for 18 days. Subsequently, the slides were developed and stained with hematoxylin and eosin.

RNA synthesis in cumulus cells. Oocytes and cumulus cells were collected and incubated as previously described above except that the

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^{3}H-uridine concentration was reduced to 25 μCi/ml. After 4 hr of incubation, the oocytes and cumulus cells were washed twice in BMOC-3 and then exposed to hyaluronidase (340 units/ml) for 5–10 min to disperse the cumulus cells. The cumulus cells were then transferred in 2–4 μl of media to a chilled slide which had been flooded with Carnoy's solution. Ten to 15 min later, the slides were washed with distilled water and air dried. The slides were subjected to either RNase or buffer treatment. After the slides were dipped in NTB-2 emulsion, they were exposed for 7 days, developed and stained with hematoxylin and eosin.

Protein synthesis in oocytes. The oocytes and cumulus mass were collected from control and delayed-ovulatory animals at 8 AM on the morning of estrus. The oocytes and cumulus cells were incubated for 1 hr in BMOC-3 which had been supplemented with ^{3}H-4,5-leucine (7 μCi/ml; sp. act.=58 Ci/mmole). After incubation the oocytes were washed and fixed in Bouin's solution. After 30 min of fixation, the Bouin's solution was replaced with fresh Bouin's and fixation continued for an additional 22 hr. The oocytes were then prepared for autoradiographic analysis.

Protein synthesis in cumulus cells. Oocytes in their cumulus mass were collected from control and delayed-ovulatory rats at 9 AM on the morning of estrus, rinsed in saline and then incubated with ^{3}H-4-5 leucine (3.5 μCi/ml; sp. act.=58 Ci/mmole). After 1 hr incubation, the oocytes and cumulus cells were washed in leucine-free media and exposed to a 2.5% trypsin solution. After the cumulus cells were removed from the oocyte by the trypsin, cumulus cell smears were prepared as described above.

Analysis of autoradiographs. The relative amount of RNA synthesis was calculated by counting the total number of silver grains/1000 μm^2 over the section of an oocyte with the largest diameter which had been subjected to buffer and TCA treatment. Background was estimated by determining the number of grains/1000 μm^2 ooplasm in an RNase-treated section of the same oocyte. The difference in these two counts represented the relative amount of RNA synthesized. The amount of protein synthesis was estimated by counting the number of grains in a predetermined 400 μm^2 area. Since previous studies have shown the Bouin's fixation removed unincorporated amino acids (9), this measurement was considered to represent the relative amount of protein synthesized.

In order to estimate RNA synthesis in the cumulus mass, cumulus cells from cell smear preparation were classified on the basis of their relative grain densities. When cells were incubated with ^{3}H-uridine and then subjected to RNase treatment, 98% of the cells had 15 grains or less. On the basis of the RNA digestion studies, cells incubated with ^{3}H-uridine and then exposed to buffer treatment were considered to have synthesized RNA only if they had 15 grains or more. Because many of these cells had high grain densities which could not be accurately counted, they were classified as either lightly labeled (Rank value=3), moderately labeled (Rank value=4) or heavily labeled (Rank value=5). After the cells from an individual animal were classified, the percentage of cells in each class was calculated. The percentage of cells in each class was multiplied by their respective rank value and their products summed. This sum was considered to represent the relative amount of RNA synthesized in the cumulus mass of an individual animal.

Assessment of protein synthesis in the cumulus cell mass also was based on relative grain densities. Ninety-eight percent of the cells which were not exposed to ^{3}H-leucine had either zero or 1 grain/cell. Therefore, cells incubated with ^{3}H-leucine that possessed two or more grains were considered to have synthesized protein and were classified as either lightly labeled (Rank value=2) moderately labeled (Rank value=3) or heavily labeled (Rank value=4). After determining the percentage of cells in each rank, the relative amount of protein synthesis in the cumulus mass of an individual animal was calculated as described for the studies which estimated RNA synthesis in the cumulus cell mass. The percentage of cumulus cells which synthesized either RNA or protein and the relative amounts of RNA or protein synthesized in the oocytes and cumulus mass were statistically evaluated by either chi-square or *t* test.

Results. ^{3}H-Uridine was incorporated into RNA by both control and aged oocytes (Fig. 1). The silver grains, which localize the incorporated ^{3}H-uridine, were distributed evenly throughout the ooplasm. Although labeled RNA remained evenly distributed in the ooplasm, the follicularly-aged oocyte showed a significant reduction in RNA synthesis (97 $\pm$ 11 in controls

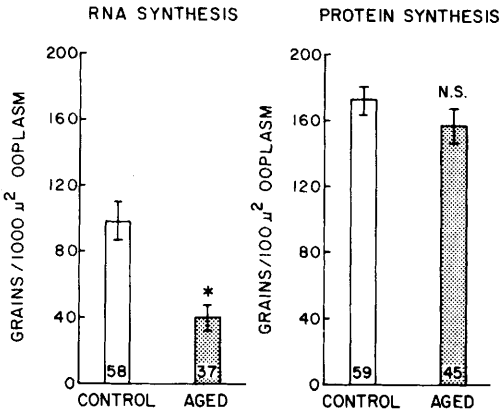

FIG. 1. RNA and protein synthesis in control and follicularly-aged oocytes. The number of oocytes examined is at the base of the bar. Values are expressed as a mean ± SE. *$P < 0.001$; N.S. = not significantly different.

vs 40 ± 8 grains/1000 μm^2 ooplasm in the aged oocyte) (Fig. 1). When the grain counts for control oocytes were ranked in ascending order, a break in the distribution was found between 3 and 28 grains/1000 μm^2 ooplasm. Seven percent of the control and 54% of the aged oocytes had fewer than 28 grains/1000 μm^2 ooplasm. Chi-square analysis has shown this difference to be highly significant ($P < 0.005$).

^{3}H-Leucine was incorporated into protein by both control and follicularly-aged oocytes. The amount of ^{3}H-leucine incorporation in the aged oocytes was not significantly different from the controls (158 ± 7 grains/100 μm^2 in the aged vs 172 ± 6 grains/100 μm^2 ooplasm in the controls) (Fig. 1).

Evaluation of RNA and protein synthesis in the cumulus cell mass which surrounds the tubal oocyte demonstrated that neither the percentage of cells labeled nor the amount of RNA or protein synthesized in the cumulus cells is altered during the follicular aging process (Table I). Under the dissecting microscope, the cumulus cells appear to be loosely attached to the follicularly-aged oocyte. Histological preparations of oocytes, after the 4-hr incubation period, revealed a similar dispersement of cumulus cells around the aged oocyte.

Discussion. Control and follicularly-aged oocytes incorporate ^{3}H-uridine into RNA. However, 54% of the aged oocytes have a reduced ability to synthesize this nucleic acid. Although follicular aging alters the ability of the tubal oocyte to synthesize RNA, the capability of aged oocytes to incorporate ^{3}H-leucine into protein is not impaired. Presumably, protein synthesis in both the control and aged oocyte is under the direction of RNA which was synthesized prior to ovulation (10). As preimplantation development precedes, protein synthesis comes under the direction of RNA which is synthesized after ovulation (11). RNA, synthesized as early as the 1-cell stage, has been shown to be necessary for the normal embryological development of the mouse (12). In the preimplantation embryos derived from the fertilization of aged oocytes, quantitative and qualitative changes in the proteins synthesized could result from the reduced synthesis of RNA by the follicularly-aged oocytes. Thus, those aged oocytes which have a reduced capacity to synthesize RNA, could account for a large percentage of developmental anomalies.

TABLE I. RNA and Protein Synthesis in Control and Follicularly-Aged Cumulus Cells.

Type of cumulus cell	RNA Synthesis[a]		Protein Synthesis[b]	
	No. cells labeled / No. cell examined	RNA synthesized[c] (relative units)	No. cells labeled / No. cells examined	Protein synthesized[c] (relative units)
Control	1444/1596 (91%)	333 ± 22	846/846 (100%)	335 ± 2
Follicularly-Aged	897/975 (92%)[d]	326 ± 21[d]	1433/1434 (100%)[d]	345 ± 19[d]

[a] Collected from six control and five delayed-ovulatory rats.
[b] Collected from three control and two delayed-ovulatory rats.
[c] Mean ± SE.
[d] Values are not significantly different from respective control ($p > 0.05$).

The role that cumulus cells play in the fertilization and development of the tubal oocyte has not been clearly defined. Donahue and Stern (13) have demonstrated that the cumulus cells support *in vitro* oocyte maturation by making pyruvate available to the oocyte. Although follicular aging does not alter either the amount of RNA and protein synthesized or the percentage of cumulus cells which synthesize these macromolecules, follicular aging does result in an increased dispersement of the cumulus cells. If the cumulus cells also support the tubal oocyte by making substrates available, then the loose association of the cumulus cells to the aged oocyte could play an important role in the production of developmental anomalies.

Summary. Autoradiographic analysis revealed that follicularly-aged oocytes show a significant reduction in RNA synthesis, but the capacity to synthesize protein was unaltered. Abnormally low amounts of RNA were synthesized by 54% of the oocytes obtained from delayed-ovulatory rats and 7% of the control oocytes. The reduced RNA synthesis characteristic of the aged oocyte could be associated with metabolic changes which are responsible for the developmental anomalies.

Evaluation of RNA and protein synthesis in the cumulus cell mass, which surrounds the tubal oocyte, demonstrated that neither the percentage of cells labeled, nor the amount of RNA or protein synthesized by the cumulus cells, is altered during the follicular aging process. However, the cumulus cells which enclose the oocyte are more loosely associated with the aged oocyte.

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