Histological Examination of the Rat Ovarian Follicle Wall Prior to Ovation

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The histological changes occurring in rat ovarian follicles prior to ovulation were studied by light and electron microscopy. Follicles were collected at 0, 5, 10, and 11 h after the administration of HCG to stimulate ovulation, and also at what was judged to be the instant before rupture from ovaries that were exteriorized in a plastic chamber for direct observation. The earliest morphological change detected was the leakage of erythrocytes from blood vessels, and thus their presence, presumably along with plasma, in the intercellular spaces of the follicle walls. Somewhat later, occasional theca cells died and degenerated, and slight signs of cytolysis appeared at the surface of all cell types. The tunica albuginea often became loose, with a large increase in the extent of extracellular space between cells. A conspicuous feature of follicle walls near the time of ovulation was the rounding up and detachment from the follicle wall of apparently healthy granulosa and theca cells; at ovulation the granulosa layer was absent from the stigma. Clumps of fibrin were commonly found in the extracellular space of the follicle wall and in follicular fluid, as was debris from degenerating cells. The final change noted before ovulation was the degeneration and loss of peritoneal epithelial cells. No collagen could be detected in follicle walls at any time.

The early leakage of erythrocytes from blood vessels and the common occurrence of fibrin in the intercellular space and follicular fluid suggested that an acute inflammatory reaction may be involved in the formation and rupture of the stigma. The anti-inflammatory drugs aspirin, salicylate, and indomethacin inhibited ovulation in immature rats when administered in anti-inflammatory dosages shortly before the expected time of ovulation. The inhibition was observed in animals superovulated by exogenous PMS and HCG, as well as in animals ovulating spontaneously after a small dose of PMS. It is concluded that a process which involves prostaglandin synthesis is involved in follicle rupture, and that this step may possibly be the early vascular phase of an inflammatory response.

The factors which cause the ovarian follicle wall to rupture at the time of ovulation are not well known. There have been only a few histological studies of the changes in follicle walls as ovulation nears, and none has been done on the rat. The present study was undertaken in the hope that the histological appearance of the follicle wall during its development toward ovulation would provide some additional clues to the basic mechanisms which lead to rupture.

MATERIALS AND METHODS

Histological Studies

Superovulation was induced in 25-day-old female Charles River rats by ip administration of 25 IU of PMS (Ayerst Labs) at 1700 h, followed 54 h later at 2300 h by 40 IU of HCG (Ayerst Labs). The ovulatory response was determined to begin about 11 h after injection of HCG, and to continue for several hours. At various times following the administration of HCG, animals were killed and the ovaries were rapidly removed and immersed intact in a 2.5% glutaraldehyde fixative solution (Parr, 1973). The time intervals chosen were 0, 5, 10, and 11 h after HCG. Well-developed preovulatory follicles were identified, and

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after 30 min of fixation that portion of the follicle wall that bulged out from the surface of the ovary was trimmed off and washed in Michaelis buffer. Subsequent treatment is described below.

In addition to follicles collected at the above mentioned intervals after HCG, some follicles were collected at essentially the instant of rupture. Beginning at 0900 h on the morning after HCG administration, ovaries were exteriorized into a plastic chamber so that ovulation could be observed, using the procedures described by Blandau (1955), and in more detail by Blandau (1971). After observing ovulation in this system a number of times to become familiar with the appearance of the follicle as it approached the time of ovulation, I collected for histological study several follicles which were very close to the time of rupture. The procedure finally adopted for collecting the follicles was as follows: When a well-developed stigma began to bulge to form the secondary cone, an overdose of nembutal was quickly injected ip; a 2.5% glutaraldehyde fixative solution was then added dropwise to the Hanks’ solution bathing the ovary in the chamber to strengthen the stigma. If the follicle did not rupture before the animal’s heart stopped beating (about 1 min), the remainder of the Hanks’ solution in the chamber was replaced with fixative; glutaraldehyde fixation continued for about 45 min at 37°C. That part of the follicle wall that bulged out from the surface of the ovary was then dissected off and trimmed in Michaelis buffer.

All of the follicle walls examined in this study were, at this point, postfixed in 1% OsO₄ and prepared for microscopy as previously described (Parr, 1973). During embedding, the follicle walls were oriented so that sections could be cut parallel to the radius of the follicle; 1 μm sections for light microscopy and thin sections for electron microscopy were cut at intervals.

**Pharmacological Studies**

Two methods were used to induce ovulation. In the first, superovulation was induced in 25-day-old female Charles River rats by sc administration of 25 IU of PMS (Ayerst Labs) at 2200 h, followed by 40 IU of HCG (Ayerst Labs) 48 h later. Ovulation occurred about 11–13 h after the administration of HCG. Anti-inflammatory drugs were administered at 8 h, and the rats were killed at 15 h after HCG. In the second method, ovulation was induced in 28-day-old rats by sc injection of 4 IU of PMS at 0900 h (Ying and Meyer, 1969). Ovulation, presumably in response to the release of endogenous LH, occurred between 2400–0200 h of the third night following the PMS. Anti-inflammatory drugs were given at 2300 h, just prior to the onset of ovulation, and the animals were killed the following morning. In order to count ova, the oviducts were pressed between two microscope slides and examined with a low power objective.

**Anti-Inflammatory Drugs**

Aspirin, sodium salicylate, and indomethacin were suspended in isotonic saline and injected subcutaneously at the times indicated above. The average weight of the immature rats was about 75 g.

**RESULTS**

**No HCG Stimulation**

Three preovulatory follicle walls stimulated only by PMS (0 h after HCG) were studied. Their appearance was uniform and is illustrated by Fig. 1. Peritoneal epithelium, tunica albuginea, theca, and granulosa layers were all present. The theca layer was not clearly divided into an interna and an externa. Cells of the tunica albuginea, theca, and granulosa layers were closely packed, with little extracellular space. No collagen fibers could be found anywhere in the follicle wall. There was little or no evidence of cell degeneration at this time, and there were no extravascular erythrocytes.

**5-Hour HCG Stimulation**

Four preovulatory follicle walls from 5 h after HCG administration were examined. Their appearance was uniform and is illustrated by Fig. 2. All four layers of the follicle wall were still present, but a slight loosening of the cells had occurred as compared to the 0-h HCG group; there was now more extracellular space between the cells. The thickness of the follicle wall remained about the same as earlier, and there were no collagen fibers or erythrocytes in the intercellular space.

**10-Hour HCG Stimulation**

Eight preovulatory follicle walls from 10 h after HCG administration were studied. Figure 3 shows a light micrograph of one of them. The peritoneal epithelium, tunica albuginea, and varying numbers of thecal...
Fig. 1. Preovulatory rat ovarian follicle wall after 54 h of PMS stimulation. The peritoneal epithelium (PE), tunica albuginea (TA), theca (T), and granulosa (G) layers are all present. There is no clear division of the theca into an interna and externa. The cells of all layers are closely packed, with little extracellular space, and there is little cell degeneration. Collagen fibers are lacking, and erythrocytes are confined within blood vessels. ×1950.
Fig. 2. This follicle, at 5 h after HCG stimulation, retains all four of its layers, but the cells appear more loosely packed than in the previous group. No collagen can be detected, and erythrocytes are still confined to blood vessels. Theca cells (T) show an increase in lipid droplets (L). ×1950.
cells were present in all cases, but in three specimens there were no longer any granulosa cells present in the thinnest region of the follicle wall. The tunica albuginea had become markedly loosened in three of the eight specimens, and the cells appeared more attenuated, as is illustrated in Figs. 3 and 4. The theca layer also often appeared loosened and contained relatively increased extracellular space (Fig. 4). The granulosa layer was loosened in one of the specimens (Fig. 3) where it was still present in the thinnest region of the follicle wall, and cells appeared to be detaching from the granulosa and migrating into the follicular fluid. Extravascular erythrocytes were observed in all eight of the specimens, indicating that blood vessels in the follicle wall became extremely leaky by this time, permitting diapedesis or extravasation. Cellular degeneration was observed in five of the samples; in two of these cases the peritoneal epithelium was involved (Fig. 4). Cell debris was a common feature of the follicle walls at this time. Fibrin was detected in or near the follicle wall in one case out of the eight. Osmiophilic lipid droplets, usually poorly fixed, were present in the peritoneal epithelium, tunica albuginea, theca, and granulosa layers. Theca cells showed an increase in the volume of cytoplasm and considerable proliferation of smooth endoplasmic reticulum (Fig. 4). These developments may be related to the increased secretion of progestins by the nonluteinized ovary just prior to ovulation (Cortes et al., 1971). The basement membrane of the granulosa is shown in Fig. 5; it consists only of a typical basement lamina.

**11-Hour HCG Stimulation**

Seven preovulatory follicle walls from 11 h after HCG were studied. Figure 6...
Fig. 4. In this follicle at 10 h after HCG administration, the granulosa layer is absent. The theca layer (T) is loosened, and many erythrocytes (e) are present in the extracellular space. Theca cells show an extensive accumulation of smooth endoplasmic reticulum and lipid droplets (L). Blebs (B) are forming on some tunica albuginea and theca cells, indicating cytolysis. The tunica albuginea (TA) is markedly loosened, with an increase in the extracellular space (ECS), and the cells are more attenuated, as though they have been stretched. Cellular debris is common throughout the theca and tunica albuginea layers. The peritoneal epithelium (PE) contains many large vacuoles (V), suggesting degeneration. The cells of all layers, and even endothelial cells, contain lipid droplets. A platelet (p) is present in the venule. ×5000.
shows the light microscopic appearance of two of them. The most striking feature of these follicle walls was that cells along the inner border adjacent to the follicular fluid were becoming detached from the wall and were migrating into the follicular fluid. This was observed in five of the seven follicle walls examined. Both granulosa cells and theca cells were involved. The detaching cells appeared to be viable, as can be seen in Fig. 7. In only three of the seven specimens did granulosa cells remain in the thinnest portion of the follicle wall. Theca cells were still present in varying numbers in all cases. The peritoneal epithelium and tunica albuginea were also present in all cases, but the latter had become loose in five of the seven specimens. Evidence of cell degeneration was clearly present in six cases and dying cells were common (Fig. 8). Figures 7 and 8 show several examples of more subtle deterioration, such as the formation of blebs from the surface membrane of cells in the theca, tunica albuginea, and peritoneal epithelium. Extravascular erythrocytes were observed in five specimens, and fibrin was present in four cases (Fig. 9).

**Ovulation Imminent**

Three follicle walls were collected at what was judged to be the instant before rupture. One of them is shown in Fig. 10. Sections through each of the three follicle walls are shown in Fig. 11. The follicle wall of Fig. 11a is seen to have a narrow pathway through which the follicular fluid is in continuity with fluid in the periovarian bursa. Nevertheless, the expression of fluid associated with ovulation had not yet occurred, and a discrete boundary is still visible between the follicular and the periovarian fluid. The follicle wall adjacent to the gap has lost its peritoneal epithelial layer and its granulosa cell layer. The tunica albuginea has been reduced to a layer only.

**Fig. 5.** In this follicle at 10 h after HCG stimulation there are still some granulosa cells (G) present. The basement membrane of the granulosa layer is seen to consist only of a typical basement lamina (arrows). ×8250.
one or two cells thick. The remainder of the cells are theca cells that are loosened, disorganized, and often rounded up. The accumulation of erythrocytes in the follicle wall is probably due mainly to their natural escape from capillaries, as noted above, but the extent of their accumulation may have been influenced to some degree by the addition of glutaraldehyde to the chamber while blood was still flowing in the ovary. We have observed that in ovaries immersed in glutaraldehyde while still connected to a functioning circulatory system the blood vessels were packed with erythrocytes.

Figure 12 shows the cells adjacent to the gap in the follicle wall of Fig. 11a. It is again apparent that the peritoneal epithelial layer is lacking, that the cells of the theca layer are loosened and disorganized, and that erythrocytes are present in the extracellular space. A small clump of what is probably fibrin is labeled in the upper center of the figure.

The section in Fig. 11b was taken from the follicle shown in Fig. 10. Its peritoneal epithelial layer is mostly absent, although some cells do remain at the periphery. The granulosa layer is also absent from the thinnest portion of the wall. The tunica albuginea has become thinned to a tenuous layer about two cells thick between the follicular fluid and the periovarian space. Much of the theca layer is infiltrated with follicular fluid in which some of the cells
This follicle, collected at 11 h after the administration of HCG, shows a theca cell (T) rounding up as it detaches from the theca layer. The cell appears to be viable. Nothing remains of the granulosa layer at this time. Several cells are undergoing cytolysis, and numerous blebs (B) can be seen. There is membrane debris (d) in the follicular fluid. ×5025.

The follicle seen in Fig. 11c was also fixed before it could rupture, but the appearance of the stigma indicates that ovulation was very near. The follicle at the stigma is little more than a broad gap filled with follicular fluid and a little blood. A few nucleated cells that presumably came from the follicle wall in this region are now suspended freely in the stigma. With the cellular components of the follicle wall being absent from such a broad area in the stigma, it is perhaps surprising that this follicle had not yet burst. The border region between follicular fluid and periovarian fluid near the center of the stigma is shown in Fig. 14. Nothing remains of the follicle wall to form a boundary between follicular fluid and periovarian fluid; the two appear to be in intimate contact over a considerable distance. Apparently, the greater viscosity of the follicular fluid is sufficient to maintain it in its place for the...
Fig. 8. This shows a follicle wall from 11 h after HCG administration. The granulosa layer is absent, and cells from the theca are detaching and migrating into the follicular fluid. The tunica albuginea has become loose, and a dense material, possibly plasma, is present in the extracellular space. Some of the tunica albuginea and theca appear to be degenerating (D), and in several places the cells show blebs (B) of membrane debris, indicating cytolysis. ×6600.
Fig. 9. This follicle, from 11 h after HCG stimulation, has lost its granulosa layer, but retains the theca (T), tunica albuginea (TA), and peritoneal epithelium (PE). Strands of fibrin have formed in the follicular fluid at its junction with the theca. ×1350.
Fig. 10. A rat ovarian follicle at what was judged to be the instant before rupture. In (a), the stigma is seen to bulge from the surface of the follicle, surrounded by a ring of blood vessels. The ovary was photographed in glutaraldehyde solution at $\times 16$. In (b), the follicle wall has been dissected away from the ovary and pinned to a sheet of wax while washing in buffer. The stigma is visible as a translucent bulge. $\times 45$. 
Fig. 11. Sections through three follicle walls collected at what was judged to be the instant before rupture. Each shows accumulations of dark-staining erythrocytes. The peritoneal epithelium and granulosa are absent in each case from the thinnest portion of the follicle wall. In (a), the theca (T) and tunica albuginea (TA) are thinned and separated to form a narrow gap (g) uniting follicular fluid and periovarian space. Figure (b) is a section through the follicle shown in Fig. 13. The theca and tunica albuginea layers are disrupted, and follicular fluid is present between the layers. The crack through the follicular fluid is an artifact of preparation. In (c), little remains of the follicle wall in the stigma, and follicular fluid appears to be held back for the moment only by its own viscosity. ×190.
moment. Figure 15 shows a region still farther away from the center of the stigma. A germinal epithelial cell, several tunica albuginea cells, and a theca cell can be seen. The organization of the follicle wall is quite disordered, with expanded extracellular space, cell debris, and extravasated erythrocytes intervening between tunica albuginea and theca.

**Anti-Inflammatory Drugs**

All three of the follicles collected at the instant of rupture, as well as many of the earlier follicles, showed evidence that the blood clotting reactions had been activated. Clumps of fibrin and aggregations of platelets were present, as shown in Fig. 16. The activation of the clotting reactions, along with the early extravasation of erythrocytes, suggested that the early vascular phase of an inflammatory reaction might be occurring in follicles near the time of ovulation. The effects of anti-inflammatory drugs on ovulation are shown in Fig. 17. The upper half of the figure shows results from rats which ovulated in response to a small priming dose of PMS. When administered at 2300 h, about an hour prior to expected ovulation, 500 mg/kg of salicylate was moderately inhibitory, but aspirin had only a minor effect. A 1 mg dose of indomethacin significantly decreased the number of oocytes found in the oviducts of the ovulating animals compared to controls, and also tended to decrease the proportion of rats ovulating. A more marked inhibition of ovulation might have been observed had the anti-inflammatory drugs been administered somewhat earlier. The lower half of Fig. 17 shows the results obtained using superovulated rats. The administration of 200 mg/kg of aspirin or salicylate at 8 h
after HCG had little effect on ovulation, but 500 mg/kg of either substance produced significant inhibition. Indomethacin in doses of 0.2 mg/rat had little effect, but 1 and 5 mg almost completely suppressed ovulation.

DISCUSSION

Previous histological studies of the ovarian follicle wall of mammals near the time of ovulation have been conducted only on mice and rabbits. Moricard and Gothie (1946) observed ovulation in exteriorized ovaries of living anesthetized mice and studied serial sections of follicle walls. They noted that the granulosa layer, still present at 7–8 h after HCG, had disappeared by the time of ovulation. They postulated that enzyme digestion might be involved in ovulation. More recently, Byskov (1969) studied the forming stigma in the mouse ovarian follicle with the electron microscope and concluded that the stigma forms by successive degeneration of the cell layers, except the granulosa layer, starting with the outermost layer, the peritoneal epithelium. The last event which occurred before ovulation was the separation of the cells in the granulosa layer, which remained as the only barrier between follicular fluid and periovarian space. Byskov concluded that cell degeneration is also an important factor in the thinning of the follicle wall. Unaccountably, the descriptions of follicle wall thinning given by Moricard and Gothie and by Byskov are quite different;
Fig. 14. These are electron micrographs of the follicle shown in Fig. 11c, showing the border region between follicular fluid and the periovian space near the center of the stigma. Essentially nothing remains of the follicle wall at the center of the stigma, as shown in (b). Figure (a) shows a lone tunica albuginea cell and the conspicuous cell debris at the periphery of the stigma. Figure (a) at ×10,700 and Fig. (b) at ×8400.

our results with the rat are similar to those of Moricard and Gothie. Espey (1967) studied the formation of the stigma in rabbits. He found that the tissue began a progressive phase of disintegration about 2 h before ovulation in the general area where the stigma normally develops. Follicles near to ovulation had, at most, only fragmentary remnants of the granulosa layer. There was a separation of the cells, and collagen fibrils became very sparse. Our observations on the rat are thus in general agreement with those of Espey on the rabbit, except that collagen is not a significant component of the follicle wall in the rat. Our observations are also in general agreement with those of Blandau (1970), which are based on light microscopy.

The earliest change we noted was the leakage of erythrocytes from blood vessels into the intercellular space of the follicle wall. This phenomenon was noted in 13 of the 15 follicle walls we examined from 10 h and 11 h after HCG. We assume that blood plasma is also present in the intercellular space at this time. It is possible that the formation of the stigma, which involves local changes in the vascular system, may be related in some way to the extravasation of erythrocytes, but the connection is not clear from the present work. Somewhat after the appearance of extravascular erythrocytes, the tunica albuginea often became loosened, in that the cells were separated by greater expanses of extracellular space than formerly. This extra-
Fig. 15. This shows a region further from the center of the stigma in the follicle of Fig. 14c. The germinal epithelium, tunica albuginea, and a part of the theca are present. The tunica albuginea layer, especially, is disordered, with expanded extracellular space, cell debris, and extravasated erythrocytes intervening between tunica albuginea and theca. ×2880.
Fig. 16. Figure (a) shows a tunica albuginea cell near the center of the stigma in the follicle of Fig. 11c. A strand of fibrin is present in the follicular fluid. ×18,000. Figure (b) shows a clump of fibrin at higher magnification; the typical banding pattern is present. ×50,000. Figure (c) shows the lumen of a small vein in the follicle of Fig. 11b. Two lymphocytes adhere to the endothelium, and several platelets are present. ×3100. Figure (d) shows a group of platelets in another small vein in that follicle. ×2880.
Fig. 17. Inhibition of ovulation by anti-inflammatory drugs. The results shown in the upper half of the figure were obtained from 28-day-old rats in which ovulation was induced by 4 IU of PMS given at 0900 h. The ovulatory response occurred between 2400–0200 h on the third night after hormone injection; however, not all animals ovulated. The average number of ovulations per group is based on the animals that ovulated. The number at the base of each block gives the proportion of animals ovulating in that group, and the bar at the top gives the standard error of the mean. Animals killed at 2300 h on the third night after hormone injection had no ova in the oviducts. Anti-inflammatory drugs were administered at 2300 h, about 1 h before the onset of ovulation. In the lower half of the figure, superovulation was induced by gonadotropins administered at 2200 h, 48 h apart. The ovulatory response to HCG occurred about 11–13 h after injection of the gonadotropin. However, in 16 rats killed at 8 h after HCG there were 6.5 ± 0.9 ova already present in the oviducts, possibly due to some release of endogenous LH. Anti-inflammatory drugs were administered at 8 h after HCG, so the proper baseline for these studies is 6.5, and not 0. The number of rats per group is given at the base of each block, and the standard error of the mean is given by the bar at the top.
cellular space appeared to be filled with plasma, or follicular fluid, or a mixture of the two. The cells of the tunica albuginea were then always found to have greatly elongated cell processes; these processes may be drawn out as the cells are pulled apart while remaining in contact at small junctional regions.

At about the time the tunica albuginea loosened, a few theca cells died and began to degenerate. Blebs were also found at the surface of cells in all layers. The blebs may be a sign of degenerative changes, leading to cytolyis and death. Cellular debris and fibrin were often abundant in the intercellular space and follicular fluid. Another intriguing change associated with the development of follicles toward ovulation was the detachment of cells from the granulosa layer. Granulosa cells bordering on the follicular fluid became rounded up; they then detached and migrated deeper into the follicle. Before ovulation, the entire granulosa layer was removed from the follicle wall in the region of the stigma; it appears likely that a few theca cells may also be lost from the wall in this way. Finally, shortly before ovulation, the peritoneal epithelial cells showed degenerative changes. Possibly, they degenerate further and slough off; these cells appear to be absent from the stigma at the time of ovulation.

In each of the follicles collected just before rupture, as well as in many earlier ones, there were clumps of fibrin or platelet aggregates. Since the work of Jancso (1961), it has become well accepted that the blood clotting reactions are activated during the earliest phase of an inflammatory process (Wiseman and Chang, 1968); Michal and Firkin, 1969; Willoughby and DiRosa, 1971; Glenn and Chandrasekar, 1971). Thus, the observation of fibrin and platelet aggregates, along with increased vascular permeability, suggest that the early phase of an inflammatory process occurs in the rat follicle wall near the time of ovulation. Previous studies have shown that the anti-inflammatory drug, indomethacine, can inhibit ovulation in rats by a direct effect on the ovary (Armstrong and Grinwich, 1972; Tsafiri et al., 1972; Tsafiri et al., 1973). The present study confirms this observation and shows that salicylate and aspirin have a similar effect. The inhibition of ovulation produced by aspirin and salicylate was quantitatively similar to the effects of the same doses of these drugs on model systems of inflammation in rats, where doses of 500 mg/kg produce on the order of 50% inhibition of the inflammatory responses (Domenjoz, 1966). Indomethacin inhibited ovulation more completely.

Since the original observations of Piper and Vane and their colleagues, it has become increasingly clear that prostaglandins are intimately involved in the inflammatory process; possibly they are the primary mediators of the response (Vane, 1971). Recent evidence directly implicates prostaglandins in the rupture of the ovarian follicle. Tsafiri et al. (1972) found that the administration of exogenous prostaglandin E2 could induce ovulation in rats in which the preovulatory surge of LH was blocked by nembutal, and also in nembutal-blocked rats treated with indomethacin. Furthermore, LeMaire et al. (1973) found that the concentration and content of prostaglandins E and F in rabbit Graafian follicles increased strikingly in response to the administration of HCG. The source of the increased prostaglandins in the follicle appears to be local secretion (Armstrong et al., 1974) by granulosa cells (Challice et al., 1974). The observations mentioned above suggest that prostaglandins, and/or the inflammatory response they mediate, are necessary for the rupture of the ovarian follicle at ovulation. It is also well established that the tensile strength of the follicle wall decreases prior to ovulation (Rondell, 1964; Harvey and Rondell, 1970; Espey, 1967). The connection between prostaglandins or acute inflammation and the weakening of follicular tissue remains obscure.
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REFERENCES


RECOMMENDED REVIEW