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Apoptosis Induced by Antigestagen RU486 in Rat Corpus Luteum of Pregnancy

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Administration of RU486 to late pregnant rats results in preterm delivery 24 h after treatment and the induction of a luteolytic process after labor. We investigated whether functional changes occurring within the corpora lutea after RU486 treatment were associated with morphologic features of apoptotic cell death. Rats on d 18 of pregnancy were treated with RU486 (5 mg/kg) at 10:00 AM and killed 72 h after. We studied the number of apoptotic cells in paraffin sections of the corpora lutea by routine hematoxylin and eosin (H&E) staining, and by *in situ* 3' end labeling (TdT-mediated dUTP nick-end labeling [TUNEL]). The corpora lutea were also processed for electron microscopy to study ultrastructural changes after RU486 treatment. The number of cells showing apoptotic nuclei in H&E-stained sections was higher in RU486-treated animals than in controls (vehicle-treated rats). The quantification of the number of apoptotic nuclei within the corpora lutea performed by TUNEL confirmed the higher number of apoptotic nuclei in animals receiving the antigestagen compared with controls. Ultrastructurally, the luteal cells undergoing apoptosis presented a highly deteriorated cytoplasmic organization The nuclei, in an initial step of regression, displayed condensation of the chromatin, a prominent nucleolus, and a perinuclear space. In an advanced step of degeneration, the nuclei showed evidence of large irregular aggregates of condensed chromatin. Prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$), which mediates the luteolytic action of RU486, mimicked the effect of the antigestagen on the induction of apoptosis when administered to rats on d 18 of pregnancy (100 µg at 9:00 AM and 1:00 PM), which were killed 72 after the last injection. In conclusion, the present results indicate that functional luteolysis in rats is associated with structural luteal regression with the morphologic features of apoptotic cell death, as demonstrated by studying the luteolytic process induced by the administration of the antigestagen RU486.

Key Words: Corpus luteum; luteolysis; apoptosis; RU486; rat.

Introduction

Luteal regression is characterized by a decrease in progesterone production (functional luteolysis) and a reduction in luteal mass (structural luteolysis); however, the exact mechanisms controlling functional and structural regression of the corpora lutea in mammals are not fully understood. Functional luteolysis is characterized by the rapid and massive luteal expression of the progesterone-catabolizing enzyme, 20α-hydroxysteroid dehydrogenase (20αHSD) *(1)*, which is associated with an increase in 20α-dihydroprogesterone (20αDHP) secretion and a fall in circulating progesterone *(2,3)*. We have previously shown that during functional luteal regression, the decrease in circulating progesterone parallels a decrease in the luteal activity of the enzyme that converts pregnenolone to progesterone, 3β-hydroxysteroid dehydrogenase (3βHSD). Such decreases occur before any detectable increase in the activity of 20αHSD *(4,5)*. These results indicate that the reduction in 3βHSD activity, preceding the luteal activation of 20αHSD, may be considered an early marker of functional luteolysis.

It is well documented that the synthetic steroid RU486 binds to uterine progesterone receptors (PRs) acting as a progesterone antagonist *(6)* and that it induces preterm delivery and a subsequent regression of the corpora lutea when administered to late pregnant rats *(4,7,8)*. We have previously characterized the RU486-induced functional luteal regression in rats at d 18 of pregnancy. This luteal regression occurs after the induced preterm delivery, mimicking what happens in humans, where parturition occurs in the presence of high levels of circulating progesterone. We have previously shown evidence that the functional luteolysis induced by RU486 after preterm delivery is mediated by the production of prostaglandin *(4)*. This particular luteolytic process involves a progressive decrease in circulating progesterone associated with a decrease in the luteal

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3βHSD activity, beginning at 48 h and reaching a maximum reduction 72 h after RU486 treatment *(4)*. In these RU486-treated rats, the activity of 20αHSD in the corpora lutea increased only after 58 h and reached a maximum 72 h after treatment *(4)*. The administration of a cyclooxygenase inhibitor to RU486-treated rats delayed abortion, prolonged duration of delivery, and prevented the decrease in 3βHSD and increase in 20αHSD luteal activities as well as the decrease in serum progesterone levels observed 58 h after treatment *(4)*.

Apoptosis, an intracellular genetic program resulting in cell death *(9–12)*, is a common pathway involved in luteal regression in several mammals *(13–17)*. In the rat, the process of structural luteolysis has been associated with apoptosis during luteal regression induced by prolactin (PRL) *(18)* or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) *(19)*, as well as during the physiologic involution of the corpora lutea that takes place after parturition *(20)*. The purpose of the present investigation was to study the morphologic changes that the luteal cells undergo (structural luteolysis), and that are associated with the functional regression of the corpora lutea. We were especially interested in determining whether the morphometric changes associated with luteolysis have the characteristics of apoptotic cell death. We took advantage of an experimental approach previously characterized by us *(4)* in which functional luteolysis is induced in late pregnant rats by the administration of the antigestagen RU486.

Results

Administration of RU486 (5 mg/kg subcutaneously) to rats on d 18 of pregnancy induced preterm delivery 24.8 ± 0.52 h after treatment ($n = 10$), confirming previous results using a similar experimental protocol in which the antiprogesterone was administered at the dose of 2 mg/kg *(4)*. None of the control rats had given birth before sacrifice.

Under the light microscope, the healthy steroidogenic cells within the corpora lutea consisted primarily of large nucleated polyhedral cells with abundant cytoplasm and regular, circular nuclei (Fig. 1, arrowheads in D, G, H, and K). Some degree of apoptosis was evident in cells within the corpora lutea collected from control rats on d 21 of pregnancy, as well as in rats receiving RU486 and killed 72 h after treatment. The images of apoptosis included cells with a single, small densely stained nucleus (pyknotic appearance) (Fig. 1, large arrows in D and G); nuclei containing marginated chromatin (Fig. 1, arrow in C); and cells containing multiple, smaller, densely stained nuclear fragments (Fig. 1, small arrows in D, G, H, K, and L). The nuclear fragments observed in the corpora lutea were similar to those observed in the granulosa cell layer of an atretic follicle (Fig. 1, arrows in A). After quantification, the number of cells showing fragmented nuclei in the corpora lutea obtained from animals treated with RU486 for 72 h was significantly higher ($p < 0.01$) than that of control rats sacrificed on d 21 of pregnancy (Fig. 2A). In the same animals used to quantify luteal apoptosis, serum progesterone concentrations were determined. Confirming previous results *(4)*, serum progesterone levels were significantly decreased by RU486 treatment when compared with animals receiving vehicle alone (Fig. 2B; $p < 0.001$).

In experiment 1, the number of cells undergoing apoptosis was expressed as the percentage of cells present in the corpora lutea. However, the animals treated with RU486 had a higher number of cells per unit area: 37.3 ± 0.81 cells per high-power field ($n = 130$) vs 24.1 ± 0.51 cells per high-power field in vehicle-treated rats ($n = 173$) ($p < 0.001$). This could be owing either to the shrinkage of cells undergoing apoptosis or to an invasion of blood cells during the structural luteolysis. Therefore, we decided to express the results in number of apoptotic cells on a per-corpus-luteum basis, in order to standardize the counts.

In experiment 2, apoptosis expressed as the number of apoptotic nuclei per corpus luteum was higher in animals treated with RU486 than in vehicle-treated rats when counting cells in hematoxylin and eosin (H&E)-stained sections (Fig. 3A; $p < 0.001$). The use of an *in situ* apoptosis detection system (a TdT-mediated dUTP nick-end labeling [TUNEL] variant) validated the apoptotic nature of the cells displaying fragmented nuclei by routine staining. Nuclei undergoing apoptosis could be observed stained in brown, whereas healthy luteal cells were stained only by the hematoxylin used as counterstainer (Fig. 1, arrows in E, F, and I). Nuclei after fragmentation also stained positive (Fig. 1, arrows in J, M, and N). An atretic follicle was used as positive control for the *in situ* apoptosis technique; thus, in Fig. 1B, extensive apoptosis can be observed in the granulosa cell layer of the follicle, particularly in the cells neighboring the follicular antrum.

After quantification of the cells undergoing apoptosis as detected by TUNEL, the animals receiving RU486 presented a higher degree of apoptosis in their corpora lutea than the control animals (Fig. 3B, $p < 0.001$). The morphologic changes observed by optic microscopy were further studied by performing electron microscopy in corpora lutea from RU486-treated animals. Ultrastructurally, the healthy luteal cells contained a preserved nucleus, large and round with dispersed chromatin, and one or two clear nucleoli. The healthy cells also presented a prominent Golgi complex with dense granules in their vicinity, moderate amount of rough endoplasmic reticulum and polyribosomes, abundant smooth endoplasmic reticulum, and numerous pleomorphic mitochondria characteristic of steroidogenic cells (Fig. 4, A–C). In the cells undergoing apoptosis, cytoplasmic organization was heavily deteriorated (Fig. 4D) owing to the distention and fragmentation of the smooth endoplasmic reticulum. The nuclei, in an initial step of luteal regression, displayed condensation of the chromatin, a prominent nucleolus, and a perinuclear space (Fig. 4D). In an advanced step of degeneration, the nuclei evidenced large

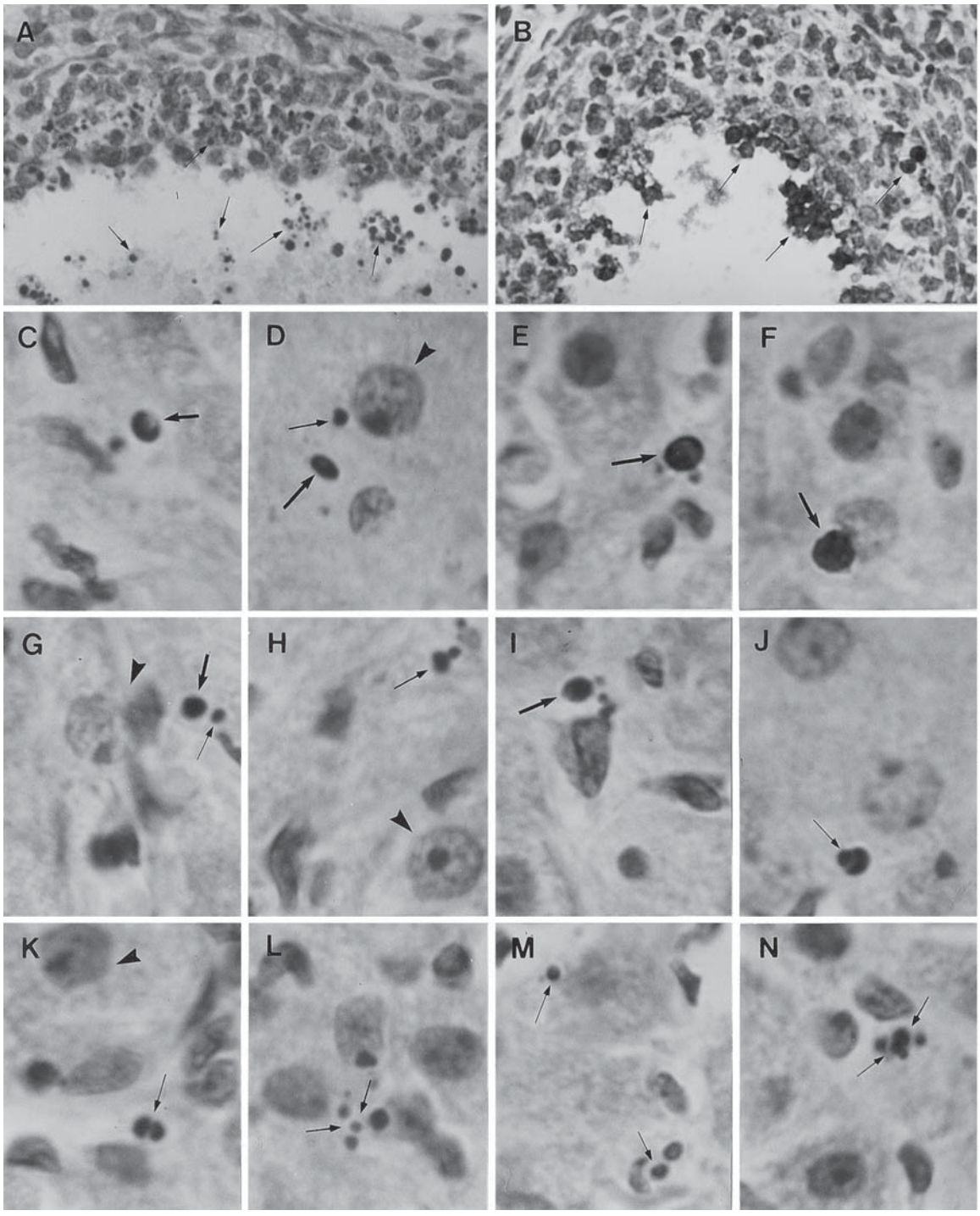

Fig. 1. Micrographs of corpora lutea obtained from rats sacrificed 72 h after treatment with RU486, stained with H&E (**C**, **D, G, H, K, L**), or *in situ* 3' end labeling (TUNEL) (**E**, **F, I, J, M, N**). The apoptotic cells included cells with a single small densely stained nucleus (large arrows in [D] and [G]), nuclei with marginated chromatin (arrow in [C]), and cells containing multiple smaller nuclear fragments (small arrows in [D], [G], [H], [K], and [L]). The nuclear fragments observed in the corpora lutea were similar to that observed in the granulosa cell layer of an atretic follicle used as positive control of apoptosis (small arrows in [**A**]). Arrowheads (D, G, H, and K) indicate nuclei of healthy luteal cells. Nuclei undergoing apoptosis could be observed stained in brown after in situ 3' end labeling (arrows in [E], [F], and [I]), whereas nuclei of healthy luteal cells were stained only by the hematoxylin used as counterstainer. Nuclei after fragmentation also stained positive (small arrows in [J], [M], and [N]). An atretic follicle was again used as a positive control of apoptosis for the TUNEL technique (small arrows in [**B**]). A,B: ×1800; C–N: ×4600.

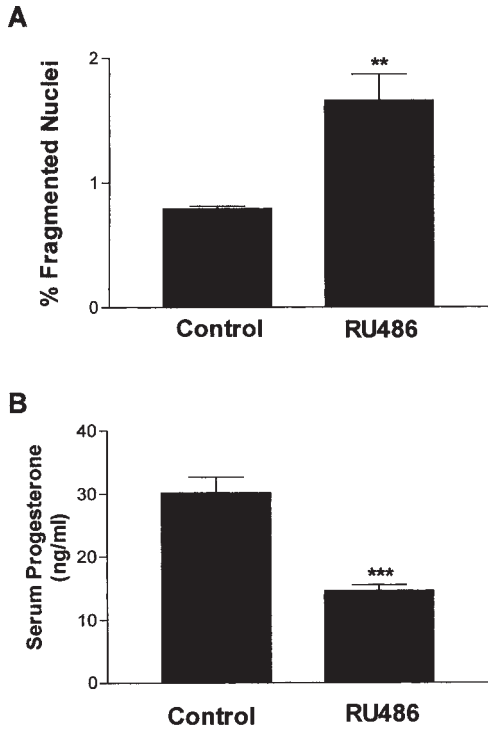

Fig. 2. Percentage of luteal cells showing fragmented nuclei in sections of whole ovaries obtained from rats sacrificed 72 h after treatment with vehicle (Control) or RU486 (**A**), and serum progesterone concentrations in the same groups of animals (**B**). Data are the mean ± SEM for $n = 6$ (Control) and $n = 7$ (RU486) rats. **, Significant difference ($p < 0.01$, Mann-Whitney U test); ***, significant difference ($p < 0.001$, student's t-test).

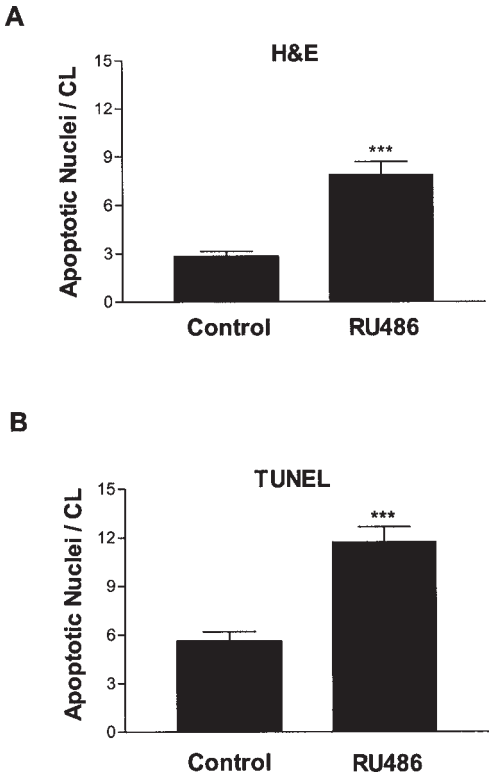

Fig. 3. Number of apoptotic cells per corpus luteum (CL) in ovaries obtained from rats killed 72 h after treatment with vehicle (Control) or RU486. Data are the mean ± SEM for n = 20 corpora lutea from four control rats, and $n = 27$ corpora lutea from five RU486-treated rats. ***, Significant differences ($p < 0.001$, Mann-Whitney U test).

irregular aggregates of condensed chromatin (Fig. 4E, arrowhead). After fragmentation of the nucleus, chromatin aggregates could be observed among cytoplasmic residues, and the whole structure surrounded by a membrane (Fig. 4F, arrowheads). The vast majority of luteal cells displaying chromatin condensation and fragmentation were steroidogenic, as evidenced by their abundant smooth endoplasmic reticulum and pleomorphic mitochondria.

The administration of $PGF_{2\alpha}$ to rats on d 18 of pregnancy (two doses of 100 µg intraperitoneally) at 9:00 AM and 1:00 PM, mimicked the effect of RU486 on the induction of apoptosis measured 72 h after treatment, showing a higher number of apoptotic cells when compared with vehicle-treated controls sacrificed on d 21 of gestation (Fig. 5A; $p < 0.001$). Confirming previous results, $PGF_{2\alpha}$-treated rats delivered prematurely after 36–48 h of administration *(21)* and presented a significant decrease in serum progesterone levels when compared with controls 72 h after treatment (Fig. 5B; $p < 0.001$).

Discussion

This study describes and quantifies morphologic changes occurring during luteal regression induced after the administration of an abortifacient dose of RU486 to rats in late pregnancy. A significant amount of apoptotic cells have been identified in the regressing corpora lutea of the RU486-treated rats by utilizing morphologic criteria. The abortifacient effect of RU486 in the rat is well documented. This synthetic steroid binds to the uterine PRs acting as an antagonist *(4,7,8,22)*. The effect of RU486 on labor induction is mediated by prostaglandins, as demonstrated by Cabrol et al. *(22)* and confirmed by us *(4)*. Less information is available regarding luteal function following treatment with the antihormone. However, in a previous study *(4)*, we described the effect of RU486 on luteal function in late pregnant rats. The antigestagen triggers a luteolytic process that occurs after preterm delivery that is characterized by a decrease in luteal 3βHSD and in circulating progesterone, followed by an increase in luteal 20αHSD activity *(4)*. The luteolytic cascade induced by RU486 is mediated by prostaglandin synthesis. This was previously demonstrated by the administration of a cyclooxygenase inhibitor that prevented the decrease in luteal 3βHSD activity and in circulating progesterone, and the increase in luteal 20αHSD activity induced by the administration of RU486 *(4)*. In the present study, we report that the functional luteolysis induced by RU486, and documented through the abrupt decline in circulating

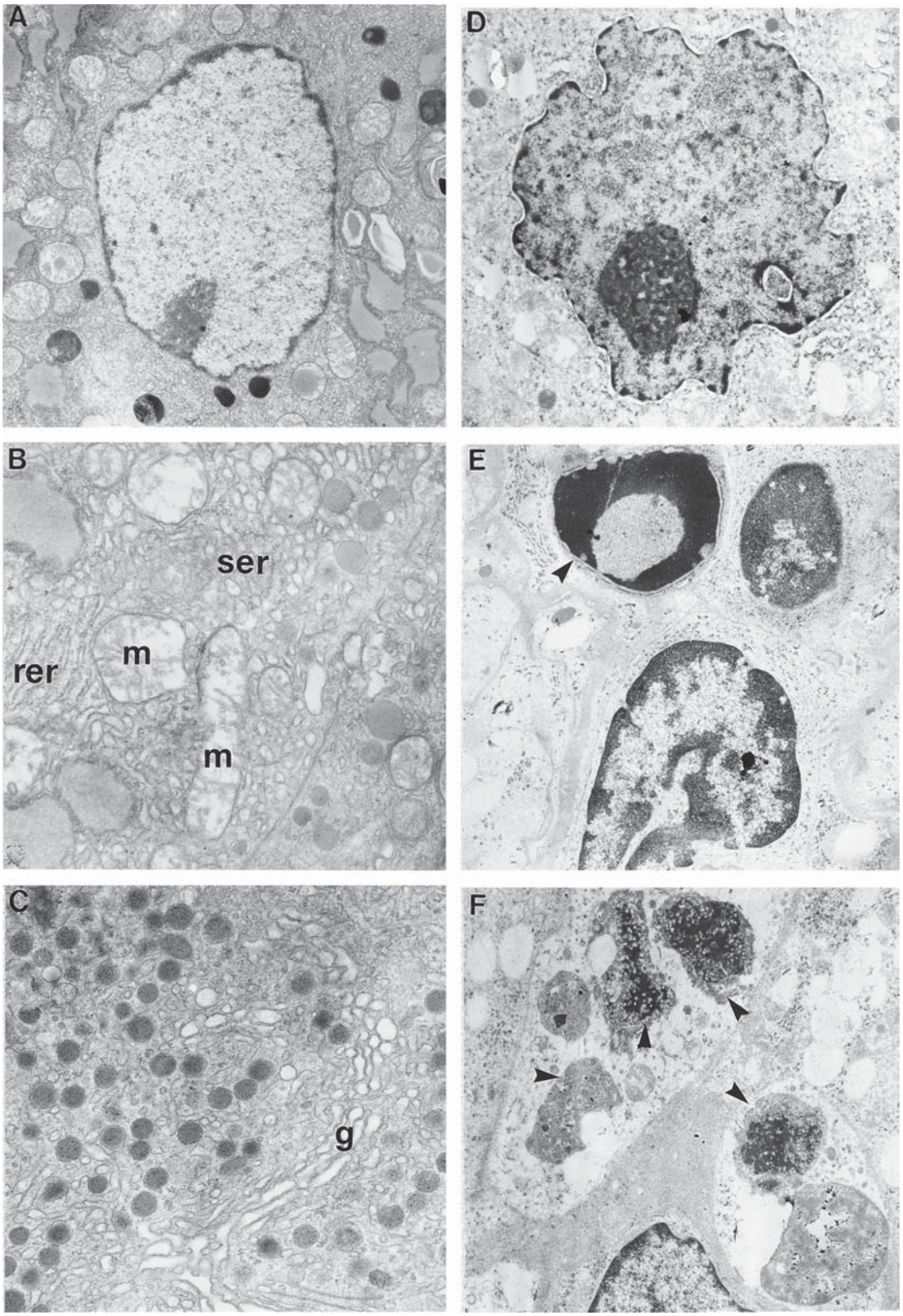

Fig. 4. Electron micrographs of corpora lutea obtained from rats sacrificed 72 h after treatment with RU486. Healthy steroidogenic luteal cell: (**A**), ×8500; (**B,C**) ×17,500). The nucleus is round and well-expanded (A); the cytoplasm shows stacked cisternae of the rough-surface endoplasmic reticulum (rer), abundant endoplasmic reticulum (ser), and pleomorphic mitochondria (m) (B), with a prominent Golgi region (g) (C). (**D–F**) Heavily deteriorated luteal cells (×8500). The nucleus is heterochromatic, with prominent nucleolus, and displays a perinuclear space (D); in and initial step of luteal regression, the nucleus presents its chromatin heavily condensed and marginated (E, arrowhead). In and advanced degree of apoptosis, the nucleus, as well as the cytoplasm, becomes fragmented (F, arrowheads).

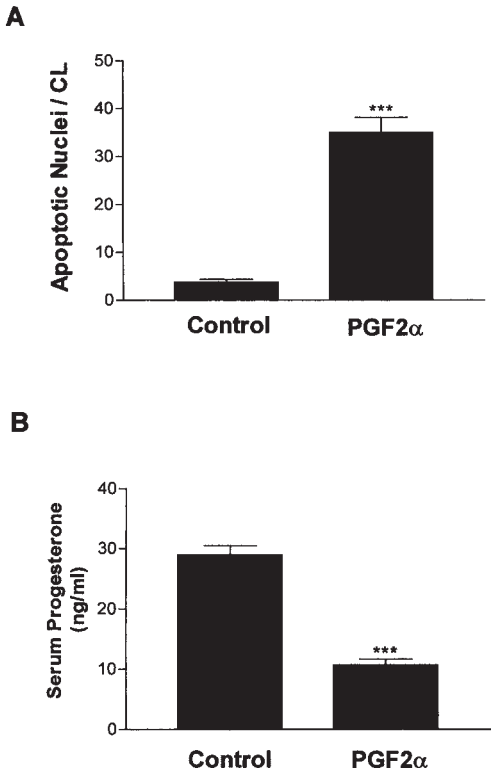

Fig. 5. (**A**) Number of apoptotic cells per corpus luteum (CL) in slides of ovaries stained with H&E and obtained from rats killed 72 h after treatment with vehicle (Control) or $PGF_{2\alpha}$. Data are the mean ± SEM for $n = 20$ corpora lutea from five control rats, and $n = 18$ corpora lutea from five RU486-treated rats. ***, Significant differences ($p < 0.001$, Mann-Whitney U test). (**B**) Serum progesterone concentrations in the same groups of animals as shown in (A). Data are the mean ± SEM. ***, Significant differences ($p < 0.001$, student's *t*-test).

progesterone, is accompanied by an increase in the number of luteal cells undergoing morphologic changes distinctive of programmed cell death (apoptosis).

The use of morphologic criteria in the routine staining of the slides (H&E) was sufficient to detect a significant difference in the degree of apoptosis between control and RU486-treated animals. Such a difference was even higher when *in situ* 3' end labeling (TUNEL) was used to validate the results. Yet, the degree of RU486-induced apoptosis detected was relatively low when compared with the high percentage of cells undergoing apoptosis observed within atretic follicles (present study) or on luteal tissue obtained 3 to 4 d after parturition *(20,23)*. Although the 1% increase in apoptotic cells after RU486 treatment appears to be quite low, it should not be considered as such, since apoptosis is a very dynamic process. A 1% increase means a two fold increase in apoptosis that may be correlated with the observed decrease in progesterone of a similar extent (50%). In the present study, only a single time point was considered. If we take into account that every single cell that undergoes apoptosis will be cleared by phagocytocis within a period of only 2 h, 2% of luteal cells undergoing apoptosis during a period of 24 h will account for a 20% removal of steroidogenic cells.

Despite the fact that during luteolysis the corpora lutea no longer produce progesterone, they are still steroidogenic since the induction of 20αHSD activity allows them to increase the secretion of 20αDHP into the circulation *(24)*. Interestingly, by the time the corpora lutea begin producing 20αDHP and functional luteolysis is established *(24)*, a considerable number of apoptotic cells can be seen within the involuting gland when compared with fully active progesterone-producing corpora lutea (present study). Therefore, functional and structural luteolysis should not be considered as separate events. On the contrary, luteolysis has to be contemplated as a process of regression in which several functional and structural changes coexist. For example, it has been shown in primary as well as in immortalized granulosa cells that organelles associated with steroidogenesis remain intact and highly organized during the first 24 h of induction of apoptosis. In the vast majority of those cells, evident condensation of chromatin and break-down of the nuclear membrane are displayed while high levels of progesterone are still being produced *(25,26)*. These data strongly suggest that compartmentalization of the steroidogenic organelles during the initial steps of apoptosis allows steroidogenesis to continue in the same cells that show progressive signs of apoptotic cell death, such as chromatin condensation and fragmentation.

Interestingly, $PGF_{2\alpha}$ was more effective than RU486 in inducing luteal apoptosis. This phenomenon can be clearly explained by the fact that the prostaglandin affects directly the luteal cells that express $PGF_{2\alpha}$ receptor *(27)*. In the process of luteolysis induced by RU486, the steroid would not act directly on the corpus luteum, owing to the absence of cognate PRs *(28,29)*. RU486 acts by blocking the uterine PRs, inducing prostaglandin synthesis and abortion *(4)*. Thus, the less extensive apoptotic effect of RU486 when compared with that of $PGF_{2\alpha}$ could be a consequence of either the indirect effect of the antigestagen on the corpus luteum or a reduced amount of uterine prostaglandin induced by RU486 as compared with the amount of $PGF_{2\alpha}$ injected.

Studies to date have demonstrated that luteolysis is a process highly regulated by hormones *(30,31)*. Therefore, because apoptosis is associated with luteolysis, it is assumed that apoptotic cell death within the corpora lutea is a hormonally regulated process. In pregnant rats, regression of the corpora lutea occurs as a physiologic event before parturition *(32)*. Nonetheless, it can also be induced by the administration of $PGF_{2\alpha}$ *(33,34)*, luteinizing hormone *(35)*, or the antigestagen RU486 *(4,36,37)*. In every case, the drop in circulating progesterone is a common feature associated with luteolysis, indicating that it is highly possible that luteal progesterone protects the corpora lutea from

becoming apoptotic as well as doing so in other tissues such as the uterine epithelium and mammary gland *(38,39)*. In fact, progesterone administered locally into the ovarian bursa of pregnant rats protects luteal function by preventing the induction of 20αHSD and the decrease in 3βHSD luteal activities induced by $PGF_{2\alpha}$ *(29)*. In addition, progesterone inhibits 20αHSD gene expression in a luteal cell line *(40)* and prevents the expression of luteal interleukin-6, a cytokine detrimental for steroidogenesis *(41)*. In a recent study, Kuranaga et al. *(42)* showed that progesterone suppresses apoptosis induced by prolactin (PRL) in luteal cells obtained on the day of proestrus and maintained in culture. In the present study, we have shown that animals treated with luteolytic doses of either RU486 or $PGF_{2\alpha}$ presented very low levels of circulating progesterone, together with an increase in luteal apoptosis when compared with vehicle-treated controls. Thus, the evaluation of a putative role of progesterone as a natural antiapoptotic factor within the corpus luteum of pregnant rats remains an attractive subject for further investigation.

Another hormone that is associated with luteolysis and apoptosis is PRL. However, this hormone induces luteolysis and apoptosis only in the corpora lutea of the estrual cycle *(18,43)*. During pregnancy, the lactogenic hormones, PRL and PRL-like proteins of placental origin, regulate luteal function by acting as luteotropins *(32,44)*. Furthermore, by the time the corpora lutea of the pregnant rat regress, PRL receptors are sharply downregulated within the luteal tissue *(24)*, making impossible any action of the lactogenic hormones that could be associated with apoptosis and luteolysis.

In conclusion, the present results indicate that functional luteolysis induced by the antigestagen RU486 at the end of pregnancy in rats is associated with luteal regression that has the morphologic features of apoptotic cell death. Our previous and present results support a protective role for progesterone on the function and survival of the corpus luteum.

Materials and Methods

Animals

Adult female rats bred in our laboratory (originally Wistar strain) and weighing 180–220 g were used. The rats were housed under controlled light (lights on from 6:00 AM to 8:00 PM) and temperature (22–24ºC) conditions and had free access to standard rat chow (Cargill, Córdoba, Argentina) and tap water. To induce pregnancy, female rats were caged individually with fertile males in the afternoon of proestrus. Positive mating was verified on the following morning by identifying sperm or copulation plugs in the vagina. This day was designated as d 0 of pregnancy. In our laboratory, rats usually give birth on d 22. Animals were handled according to the procedures approved in the *Guide for the Care and Use of Laboratory Animals*, National Institutes of Health, Bethesda, MD (publication number 86-23, revised 1985).

Experimental Procedure

The progesterone antagonist RU486 (17β-hydroxy-11β-[4-dimethyl-aminophenyl]-17α-[1-propynyl]-estra-4,9-diene-3-one; mifepristone, Roussel-Uclaf, Romainville, France) was dissolved in sunflower seed oil and injected subcutaneously into the rats at 10:00 AM on d 18 of pregnancy at a dose of 5 mg/kg, and the rats were killed by decapitation 72 h later. Rats receiving oil were used as controls and killed by decapitation at 10:00 AM on d 21 of pregnancy. In experiment 1, control and RU486-treated rats were decapitated, and trunk blood was collected to determine serum progesterone concentrations. One ovary from each control and the RU486-treated animals was removed and processed for routine optic microscopy (H&E staining), whereas the corpora lutea from the other ovary were dissected and processed for electron microscopy. In experiment 2, control and RU486-treated rats were decapitated, and the ovaries were fixed for *in situ* detection of apoptosis. In both experiments, the time of delivery after treatment with RU486 was recorded.

In experiment 3, pregnant rats were injected intraperitoneally on d 18 of pregnancy with $PGF_{2\alpha}$ (Upjohn, Kalamazoo, MI) in two doses of 100 µg each on d 18 of pregnancy at 9:00 AM and 1:00 PM. The rats were killed 72 h after the last injection, trunk blood was collected to determine serum progesterone concentrations, and the ovaries were obtained and processed for routine optic microscopy (H&E staining). Control animals received saline and were sacrificed at 1:00 PM on d 21 of pregnancy. Time of parturition was recorded in the $PGF_{2\alpha}$-treated animals.

Optic and Electron Microscopies

The ovaries were immediately removed from each animal and cleaned of fat. For optic microscopy, the ovaries were placed overnight at room temperature in a solution of 10% phosphate-buffered neutral formalin, dehydrated in ethanol series, cleared in xylene, and embedded in paraffin. Serial 5-µm-thick paraffin sections were mounted on slides coated with 3-aminopropyltriethoxy-silane (Sigma, St. Louis, MO) and used for routine H&E staining. The tissue samples were observed and photographed with a Zeiss IM 35 microscope.

For electron microscopy, the corpora lutea were dissected from the ovaries and fixed for 1 h in 5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.2–7.4). Each corpus luteum was included in one block and postfixed with 1% osmium tetroxide and 1% potassium ferrocyanide in the same buffer for 4 h, dehydrated in a series of cold (4ºC) graded acetone, and embedded in Araldite. Thin sections, cut with a diamond knife in a Porter-Blum MT-2 ultramicrotome, were examined using a Siemmens Elmiskop 1A electron microscope.

Counting Apoptotic Cells

In experiment 1, apoptotic cells were recognized in H&E-stained tissue sections following the procedure described by Van der Schepop et al. *(45)* with slight modifications. Only cells with advanced signs of apoptosis (containing multiple nuclear fragments) were counted from a total of 2000 luteal cells per animal. A total of four corpora lutea per animal were analyzed, and approx 500 cells per corpus luteum were studied. Cells were counted at ×630 magnification, and the results were expressed as a percentage of fragmented nuclei. This morphometric method for the identification of apoptotic cells was validated by previous studies from other laboratories showing that all cells classified as apoptotic on the basis of morphologic criteria contained fragmented DNA *(46)*, and that no differences were found between counts in H&E-stained sections and *in situ* 3' end-labeled tissues *(14,43)*.

In experiment 2, apoptotic cells were counted on the basis of morphologic criteria as just explained, but the results were expressed as the number of apoptotic nuclei per corpus luteum. A microscope with a ×100 objective was used, and as many fields as possible were analyzed in each corpus luteum for the presence of fragmented nuclei. All the corpora lutea in the section were studied, and an average number of apoptotic nuclei per corpus luteum were obtained.

In Situ Detection of Apoptosis

Nuclei exhibiting DNA fragmentation were detected by using the DeathEnd Colorimetric Apoptosis System (Promega, Madison, WI), which end labels the fragmented DNA of apoptotic cells using a modified TUNEL assay. This kit was used according to the manufacturer's instructions with slight modifications. Paraffin-embedded sections were deparaffinized 5 min in xylene, washed in 100% ethanol for 5 min at room temperature, and rehydrated by sequentially immersing the slides through graded ethanol washes (95 and 70%) for 5 min each at room temperature. Slides were washed in distilled water for 5 min at room temperature and fixed in 10% phosphate-buffered neutral formalin for 15 min at room temperature. Sections were washed twice in Tris-buffered saline (TBS) for 5 min at room temperature and subjected to a proteinase K treatment (20 µg/mL) in TBS for 15 min. Tissue sections were washed in TBS for 5 min and refixed in 10% phosphate-buffered neutral formalin for 5 min. Sections were washed, incubated with an equilibration buffer for 10 min, and treated with the TUNEL reaction mixture, containing terminal deoxynucleotidyl transferase (TdT) and biotinylated nucleotides, for 60 min at 37ºC. The reaction was then terminated, the slides were washed twice in fresh TBS for 5 min at room temperature, and the endogenous peroxidases were blocked in 3% hydrogen peroxide for 20 min at room temperature. After another TBS wash, the sections were incubated with streptavidin conjugated with horseradish peroxidase (HRP) for 30 min at room temperature. Negative controls were incubated with a label solution lacking TdT. Then 3' end-bound streptavidin-HRP was visualized by incubating with diaminobenzidine according to the manufacturer's instructions. Finally, the sections were counterstained with hematoxylin, mounted, and analyzed with a light microscope.

Progesterone Assay

Progesterone was measured after the extraction of the sera with petroleum ether using a radioimmunoassay developed in our laboratory *(47)* that uses an antiserum raised in rabbits against progesterone-11-bovine serum albumin conjugate. The sensitivity, variability, and crossreactivity of this assay has been reported previously *(47,48)*.

Statistical Analyses

Comparisons among groups were carried out using the student's *t*-test. However, when variances were not homogeneous, the nonparametric Mann-Whitney test was used. Results were expressed as mean ± SEM. Differences of $p < 0.05$ were considered statistically significant.

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