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The Participation of the Cholinergic System in Regulating Progesterone Secretion Through the Ovarian–Adrenal Crosstalk Varies Along the Estrous Cycle

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This study was designed to analyze the acute effects of unilateral or bilateral ovariectomy or adrenalectomy, performed on different days of the estrous cycle, on progesterone (P_4) serum levels 1 h after surgery. The effects of blocking the cholinergic system by injecting atropine sulfate were also analyzed. Ether anesthesia treatment on diestrus 1 (D1) increased P_4 serum levels. Compared to right sham-operated animals, right ovariectomy (left ovary *in situ*) performed on diestrus 2 (D2) or proestrus (P), resulted in P_4 serum levels increase. Compared to animals with left sham surgery, left adrenalectomy (right adrenal *in situ*) performed on P day resulted in significantly lower P_4 concentrations. Bilateral adrenalectomy resulted in a significant drop of P_4 serum levels; the most remarkable drop was observed in animals treated on D2. Bilateral ovariectomy performed on D1 resulted in lower P_4 serum levels, and the same treatment performed on P resulted in a significant rise of P_4 serum levels. Injecting atropine sulfate to untouched (control group) rats resulted in significantly higher P_4 concentrations. Blocking the cholinergic system on D1 or P to rats with the right adrenal removed resulted in lower P_4 serum levels; while, in contrast, atropine sulfate treatment performed on D2 resulted in P_4 serum levels increase. The results support the hypothesis of asymmetry in the ovaries' and adrenals' capacities to secrete P_4; that this capacity varies along the estrous cycle; and that P_4 secretion by the ovaries and adrenals is regulated by the cholinergic system.

Key Words: Ovarian asymmetry; hormone secretion; estrous cycle; adrenal asymmetry; progesterone; cholinergic system.

Introduction

In the rat, the relationship between the hypothalamo–pituitary–gonadal (HPG) axis and the hypothalamo–pituitary–adrenal (HPA) axis is well documented. A negative coupling between the axis has been observed in both clinical and experimental studies; and when activated by stress, the HPA axis exerts an inhibitory effect on the female reproductive system *(1)*.

Progesterone (P_4) is mainly secreted by the ovaries and adrenals, and acts on both reproductive and nonreproductive organs. In the female rat, progesterone plasma levels vary along the estrous cycle, and present two peaks; the first immediately after ovulation and the second in the afternoon-night of D1 day *(2)*. De Geyter et al. *(3)* showed that in women P_4 is produced by the adrenal cortex during most of the follicular phase, and that prior to ovulation the source of P_4 shifts toward the ovaries. De Geyter et al. *(3)* also suggested that during the menstrual and estrous cycle hormonal levels are regulated by endocrine crosstalk between the ovaries and the adrenal cortex. According to Matzuk et al. *(4)*, transgenic mice deficient in inhibin α-subunit developed ovarian and testicular tumors, and that adrenocortical tumors developed after gonadectomy. In animals with polycystic ovary syndrome, ovarian wedge resection reduces basal progesterone concentrations after ACTH administration *(5)*.

Extirpating one of the paired organs is a widely accepted experimental manipulation for studying the mechanisms regulating their individual function. Considerable information has been accumulated on the effects of extirpating one ovary on gonadotropin and ovarian steroids hormones levels *(6,7)*; however, to the best of our knowledge, information on the effects of unilateral adrenalectomy on steroid hormones secretion has not yet been gathered.

Asymmetry in the ovaries' morphology, physiology, and regulatory structures is well established. Evidence suggesting that these asymmetries play an important functional role in hormone secretion and that the degree of asymmetry between gonads fluctuates along the estrous cycle has been published *(6)*. Ovarian functions are influenced by biochemical signals generated in the hypothalamus and through direct

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Table 1
Means ± SEM of Progesterone Serum Concentration in Control Rats, and Ether-Anesthesia Treated Animals, Performed at 13:00 h on D1, D2, or P, Sacrificed 1 h Later

Group	*n*	D1	*n*	D2	*n*	P
Control	18	21.8 ± 1.1	20	8.9 ± 1.1*	15	8.1 ± 1.1*
Anesthesia	9	26.3 ± 1.9#	11	14.8 ± 2.9#	11	16.9 ± 2.6#

*$p < 0.05$ vs D1; MANOVA followed by Tukey's test.
#$p < 0.05$ vs control; Student's *t* test.

Table 2
Means ± SEM of Progesterone Serum Concentration in Rats with Unilateral or Bilateral Sham Operation (PP), Unilateral or Bilateral Ovariectomy (Ovx), or Adrenalectomy (ADX) Performed at 13:00 h on D1, D2, or P, Sacrificed 1 h After Surgery

Group	*n*	D1	*n*	D2	*n*	P
Anesthesia	9	26.3 ± 1.9	11	14.8 ± 2.9	11	16.9 ± 2.6
Right PP	11	25.6 ± 1.8	9	13.5 ± 2.7	10	15.6 ± 1.5†
Right Ovx	12	28.4 ± 2.0	11	21.7 ± 2.0*	10	20.2 ± 1.6
Right ADX	10	26.5 ± 2.2	11	13.4 ± 1.2	11	15.2 ± 1.4†
Left PP	8	22.9 ± 2.8	10	14.5 ± 2.3	9	21.4 ± 2.6
Left Ovx	10	26.8 ± 1.3	10	18.1 ± 2.6	9	22.3 ± 2.1
Left ADX	10	24.8 ± 1.4	10	17.6 ± 2.3	9	12.8 ± 1.7*
Bilateral PP	11	26.5 ± 1.1	11	21.7 ± 2.1	11	16.3 ± 1.6
Bilateral Ovx	10	22.0 ± 1.6*	10	19.7 ± 2.3	9	23.1 ± 1.7*
Bilateral ADX	10	14.8 ± 0.6*#	12	5.4 ± 1.5*#	9	5.7 ± 1.5*#

*$p < 0.05$ vs group with sham operation; #$p < 0.05$ vs Ovx group; MANOVA followed by Tukey's test. †$p < 0.04$ vs Ovx group; MANOVA.

neural pathways between the central nervous system and the ovaries; and both trigger the secretion of hormones by the pituitary *(6)*. The feedback from the ovaries to their regulatory mechanism is achieved by hormonal and/or neural pathways *(6–8)*.

The cholinergic system participates in regulating the secretion of hypothalamic and pituitary hormones and the reactivity of the peripheral endocrine organs to trophic pituitary hormones. Previously, we showed that performing acute hemiovariectomy to female cyclic rats on the day of estrus affects serum concentrations of P_4, testosterone (T), and estradiol (E_2); and that the extent of the effects depend on which ovary, left or right, remains *in situ (9)*. In the same study, we showed that unilaterally perforating the peritoneum results in hormone serum level changes, and that these changes depend on which side of the peritoneum is perforated *(9)*.

Because the ovaries and adrenals response to neuroendocrine control varies along the estrous cycle, the present study analyzed the acute effects of unilateral or bilateral ovariectomy or adrenalectomy, performed on diestrus 1 (D1), diestrus 2 (D2), or proestrus (P), on P_4 serum levels. The participation of the cholinergic system in regulating the response to each treatment was evaluated by administrating atropine sulfate 1 h before surgery was performed.

Results

Effects of Ether Anesthesia and Sham Operation

In the untouched control group, animals sacrificed on D1 showed significantly higher P_4 serum concentration than animals sacrificed on D2 or P (D1: 21.8 ± 1.1 ng/mL vs D2: 8.9 ± 1.1, P: 8.1 ± 1.1, $p < 0.05$ MANOVA followed by Tukey's test).

Compared to the control group, in all days of the estrous cycle, ether anesthesia treatment resulted in significant P_4 level increases (Table 1), and the highest increment was observed when anesthesia treatment was performed on P (D1 = 120%; D2 = 166%; p = 208%).

P_4 serum levels between ether-anesthetized animals and animals with unilateral or bilateral sham-surgery were not significantly different, regardless of the day of the estrous cycle when treatment was performed (Tables 1 and 2). Comparing the effects of unilateral vs bilateral sham-surgery, animals with bilateral sham-surgery performed on D2 had higher P_4 levels than animals with unilateral sham-surgery. Such differences were not observed in animals treated on D1 or P (Table 2).

Effects of Unilateral Ovariectomy or Adrenalectomy

Right ovariectomy (left ovary *in situ*) performed on D2 resulted in significantly higher P_4 serum levels than of ani-

Table 3
Means ± SEM of Progesterone Serum Concentration in Control Rats and Animals Injected with Atropine Sulfate (ATR) at 12:00 h on D1, D2, or P; the Animals Were Sacrificed at 14:00 h

Group	*n*	D1	*n*	D2	*n*	P
Control	18	21.8 ± 1.1	20	8.9 ± 1.1	15	8.1 ± 1.1
ATR	9	26.2 ± 2.2	8	21.3 ± 3.5*	8	22.0 ± 3.2*
Anesthesia	9	26.3 ± 1.9	11	14.8 ± 2.9	11	16.9 ± 2.6
ATR+Anesthesia	8	30.4 ± 1.7	6	24.4 ± 2.1*	10	20.0 ± 2.0

*$p < 0.05$ vs non-ATR treated groups, Student's *t* test.

Table 4
Means ± SEM of Progesterone Serum Concentration in Rats Injected with Atropine Sulfate (ATR) at 12:00 h on D1, D2, or P, and of Animals Injected with ATR at 12:00 h and Submitted 1 h Later to Ether Anesthesia, Sham Surgery in the Right Side (RPP), Right Ovariectomy (R-Ovx), or Adrenalectomy (R-ADX); Animals Were Sacrificed at 14:00 h

Group	*n*	D1	*n*	D2	*n*	P
Right PP	11	25.6 ± 1.8	9	13.5 ± 2.7	10	15.6 ± 1.5
ATR+Right PP	8	22.9 ± 1.8	6	20.1 ± 1.6*	10	18.9 ± 2.1
R-Ovx	12	28.4 ± 2.0	11	21.7 ± 2.0	10	20.2 ± 1.6
ATR+R-Ovx	8	23.1 ± 1.8	8	26.1 ± 2.9	10	13.0 ± 3.0*
R-ADX	10	26.5 ± 2.2	11	13.4 ± 1.2	11	15.2 ± 1.4
ATR+R-ADX	10	18.6 ± 1.8*	10	20.8 ± 3.3*	10	9.6 ± 1.4*

*$p < 0.05$ vs non-ATR treated group, Student's *t* test.

mals with right sham operation, while right adrenalectomy (left adrenal *in situ)* did not modify P_4 serum levels (Table 2). P_4 levels in animals with right ovariectomy surgery performed on the other days of the estrous were similar to P_4 levels of animals exposed to the equivalent sham surgery.

Animals with left ovariectomy (right ovary *in situ*), performed on D1, D2, or P, showed similar P_4 serum levels as animals with left sham surgery, while left adrenalectomy (right adrenal *in situ*), performed on P day resulted in significantly lower P_4 serum levels (Table 2).

Effects of Bilateral Adrenalectomy or Ovariectomy

Bilateral ovariectomy performed on D1 resulted in lower P_4 serum levels; but when performed on P, P_4 serum levels rose significantly (Table 2). Compared to animals with a bilateral sham operation, bilateral adrenalectomy resulted in a significant drop of P_4 serum levels (Table 2). The highest drop was observed in animals treated on D2 (D1: drop 45%; D2: drop 75%; P drop 65%).

Effects of the Blockade of the Cholinergic System

Injecting ATR resulted in a significant increase of P_4 serum levels (Table 3). The highest increase was observed in animals treated on P (D1: 20%; D2: 139%; P 171%).

The effects of ether anesthesia on P_4 serum levels were increased when ATR treatment was performed on D2 (Table 3). Similarly, injecting ATR on D2 to rats with unilateral or bilateral sham surgery resulted in significant P_4 serum levels increase (Tables 4–6).

The effects of blocking the cholinergic system of unilaterally ovariectomized or adrenalectomized rats depended on which ovary or adrenal was extirpated and the day of the cycle when surgery was performed.

Animals with the left organ (ovary or adrenal) *in situ*. When the right ovary of ATR-treated rats was extirpated in D1 or D2, P_4 serum levels were similar to animals without ATR treatment, while animals with ATR treatment performed on P showed significantly lower P_4 serum levels (Table 4). When ATR-injected rats had the right adrenal extirpated in D1 or P, P_4 serum levels were significantly lower than right-adrenalectomized rats, while the same treatment performed on D2 resulted in significantly higher P_4 serum levels (Table 4).

Animals with the right organ (ovary or adrenal) *in situ*. ATR injection before left ovariectomy resulted in the same changes observed in animals with the left ovary *in situ*, i.e., low P_4 serum levels in rats treated on D1 or P, and

Table 5
Means ± SEM of Progesterone Serum Concentration in Rats Injected with Atropine Sulfate (ATR) at 12:00 h on D1, D2, or P, and Animals Injected with ATR at 12:00 h Submitted 1 h Later to Ether Anesthesia, Sham Operation in the Left Side (LPP), Right Ovariectomy (L-Ovx), or Adrenalectomy (LADX); the Animals Were Sacrificed at 14:00 h

Group	*n*	D1	*n*	D2	*n*	P
L-PP	8	22.9 ± 2.8	10	14.5 ± 2.3	9	21.4 ± 2.6
ATR+L-PP	11	22.9 ± 2.1	6	24.6 ± 3.0*	10	20.0 ± 1.7
L-Ovx	10	26.8 ± 1.3	10	18.1 ± 2.6	9	22.3 ± 2.1
ATR+L-Ovx	9	19.5 ± 2.6*	9	25.5 ± 2.3*	9	14.5 ± 1.5*
L-ADX	10	24.8 ± 1.4	10	17.6 ± 2.3	9	12.8 ± 1.7
ATR+L-ADX	10	18.6 ± 0.7*	10	19.2 ± 2.3	11	10.6 ± 1.9

*$p < 0.05$ vs non-ATR treated group, Student's *t* test.

Table 6
Means ± SEM of Progesterone Serum Concentration in Rats Injected with Atropine Sulfate (ATR) at 12:00 h on D1, D2, or P, and Animals Injected with ATR at 12:00 h Submitted 1 h Later to Ether Anesthesia, Bilateral Sham Operation (BPP), Bilateral Ovariectomy (Ovx), or Adrenalectomy (ADX); the Animals Were Sacrificed at 14:00 h

Group	*n*	D1	*n*	D2	*n*	P
B-PP	11	26.5 ± 1.1	11	21.7 ± 2.1	11	16.3 ± 1.6
ATR+B-PP	9	25.4 ± 2.6	7	32.4 ± 2.6*	11	17.5 ± 1.6
Ovx	10	22.0 ± 1.6	10	19.7 ± 2.3	9	23.1 ± 1.7
ATR+ Ovx	9	17.1 ± 3.1	8	20.2 ± 0.9	10	12.2 ± 2.6*
ADX	10	14.8 ± 0.6	12	5.4 ± 1.5	9	5.7 ± 1.5
ATR+ ADX	10	10.4 ± 0.7*	10	4.4 ± 0.8	10	2.2 ± 0.4*

*$p < 0.05$ vs non-ATR treated group, Student's *t* test.

higher hormone levels in animals treated on D2 (Table 5). Blocking the cholinergic system with ATR treatment on D1 to animals with the left adrenal removed resulted in lower P_4 serum levels (Table 5).

Bilateral ovariectomized or adrenalectomized animals. In bilaterally ovariectomized rats, blocking the cholinergic system on P resulted in lower P_4 serum levels. In bilaterally adrenalectomized animals, ATR treatment on D1 or P resulted in lower P_4 serum levels (Table 4).

Discussion

It is well documented that ether anesthesia activates the hypothalamus–pituitary–adrenal (HPA) axis and the release of corticotopin-releasing factor (CRF) by the hypothalamus, ACTH and β-endorphin by the pituitary, and corticosterone, norepinephrine, and epinephrine by the adrenals *(12)*. The results presented herein suggest that stimulating the HPA axis with ether anesthesia on P day has evident effects on P_4 secretion, and no apparent effects when treatment was performed on D1 or D2. Because in most of the experimental groups of this study the superimposition of other stressors, such as the perforation of the peritoneum, did not result in further P_4 secretion increases, present results also suggest that the HPA axis' capacity to respond to stress, by increasing P_4 secretion, reaches its maximum peak with the effects of ether anesthesia. The increase in P_4 serum levels observed in animals with bilateral peritoneum perforation performed on D2 could be explained by the participation of other signals regulating P_4 different than those caused by stress.

According to Shors et al. *(13)*, the stress-induced secretion of ovarian steroid hormones varies with the type of stressor. Bilateral adrenalectomy resulted in a drastic decrease in P_4 serum levels, regardless of the day of the estrous cycle when treatment was performed, and, therefore, we suppose that the increase in P_4 serum levels observed after anesthesia treatment originates in the adrenals.

According to Bailey *(14)*, untouched adult female mice display diurnal oscillation patterns of P_4 serum levels on D and P days. In both days, maximum P_4 serum levels were observed at the end of the light period, without a conspicuous nadir recorded, and levels of P_4 remained relatively constant. The same study reports that adrenalectomized mice did not display such rhythm on D day, suggesting that the adrenals are responsible for any diurnal rhythm in peripheral plasma progesterone concentrations. Bailey *(14)* recorded maximum P_4 levels on P day, approximately five times greater than on D day. These differences in P_4 concentrations persisted in adrenalectomized mice, suggesting that the rhythm of adrenal secretion of P_4 is masked by ovarian secretion.

Bailey *(14)* concluded that his results indicate that the functional capacity of the adrenal cortex is constant during the estrous cycle phases. However, present results suggest that in the female rat the functional capacity of the adrenal cortex to secrete P_4 varies along the estrous cycle.

According to Brown et al. *(15)*, P_4 concentration in peripheral plasma of 4-d cyclic rats is about 6% of the P_4 levels observed in adrenal venous plasma; and that the rise in P_4 secretion noted on P day may be due to the influence of reproductive hormones on adrenocortical function. In a study performed with women, Judd et al. *(16)* concluded that neither the preceding corpus luteum nor the developing follicles are important contributors to the serum concentration of P_4 during the normal follicular phase.

According to De Geyter et al. *(3)*, in women the adrenals are the main source of circulating P_4 during the early follicular phase, the ovaries during the late follicular phase, and during the preovulatory phase the adrenal cortex is the major contributor of circulating P_4 levels.

Because ethinyl estradiol has a suppressive effect on both basal and ACTH-stimulated concentrations of P_4, De Geyter et al. *(3)* suggest that the ovaries mediate the P_4 contribution of the adrenal cortex. Similarly, Lobo et al. *(17)* reported that compared to regularly ovulating women, a significantly lower output of adrenal androgens was observed in ovarectomized women after ACTH stimulation. Present results show that bilateral adrenalectomy produced a significant drop in P_4 serum levels, with the lowest levels observed in animals treated on D2, a time of the estrous cycle that can be compared with the end of the follicular phase in women.

The decrease in P_4 serum levels observed in bilaterally ovariectomized rats on D1 suggests that in the rat the ovaries are a significant source of P_4; and because bilateral ovariectomy on P resulted in significant P_4 serum level increases, the adrenals seem the main source of P_4 near ovulation. These results agree with De Geyter et al. *(3)*, indicating that during the preovulatory phase the adrenal cortex is a major contributor of circulating P_4 levels.

The adrenals' participation in secreting P_4 is supported by our results, since the drop in P_4 serum levels in bilateral adrenalectomized rats is higher than in bilaterally ovariectomized rats.

The results presented herein support the hypothesis of asymmetry in the ovaries' abilities to secrete P_4 *(9)* and ovulation capacity *(11)*. The results also suggest that the adrenals' ability to secrete P_4 could be asymmetric and that such asymmetry varies along the estrous cycle.

The participation in regulating ovarian functions of various neurotransmitters arriving to the ovary through its innervation has been analyzed, mainly through in vitro studies. Vasointestinal peptide (VIP) increases the production of E_2 and P_4 from granulosa cells in immature rats *(18,19)*, while substance P does not have a significant effect on steroidogenesis of granulosa cells of immature rats *(20)*. There is evidence that in ovarian-cell cultures β_2 receptors are involved in regulating the release of P_4 and androgens *(21,22)*. After in vitro electric stimulation of the ovary, the release of tritiated norepinephrine [^{3}H]NE is higher when performed in P or E than during the other phases of the estrous cycle *(23)*. Depending on the animal's age, unilaterally sectioning the SON of prepubertal rats results in an increase or decrease of E_2 and P_4 levels *(24,25)*. There is evidence indicating that the central beta-adrenergic receptors play a significant role in regulating the ovarian release of P_4, and that the central beta-adrenergic neural input is almost entirely transmitted through the SON *(26)*. Stimulating the central adrenergic system on D1 increases ovarian P_4 release, and lowers it when treatment is performed on D2. According to De Bortolli et al. *(27,28)*, these results suggest that the neural input originating in the central adrenergic system conditions the ovarian response to LH on D2.

Studies on the participation of the cholinergic system in regulating P_4 ovarian secretion have shown that nicotine agonists inhibit the biosynthesis of P_4 induced by gonadotropins *(26)*. Acetylcholine treatment on D1 significantly inhibits P_4 release by the ovaries and results in a moderate stimulation when performed on E *(26)*. The results obtained in the present study suggest that the cholinergic system regulates ovarian P_4 secretion in both D1 and P; while P_4 secretion by the adrenals is under cholinergic control at 13.00 h of P, because injecting atropine sulfate to bilaterally ovariectomized animals resulted in lower P_4 serum levels.

Present results also suggest that the left ovary's ability to secrete P_4 on D2 and P is higher than in the right ovary. Such functional asymmetry could be explained by differences in the innervation received by each ovary *(6,29)*. In the present study, the observed changes in ovarian P_4 secretion could be attributed to signals coming from prevertebral celiac–superior mesenteric ganglia neurons, which are affected by the stimulation of the central adrenergic system**.** It is possible that the adrenals regulate ovarian steroid secretion through the adrenergic control of blood flow. There is evidence that adrenalectomy increases ovarian norepinephrine release, the content of α-adrenergic receptors, and basal androgen secretion. For instance, Galvez et al. *(30)* propose a tonic inhibition on ovarian nerve activity by the adrenal gland. In cultures of granulosa cells, epinephrine stimulates the secretion of P_4 *(22,31)*.

According to De Geyter et al. *(3)*, the adrenal function is modulated by an unknown ovarian factor, the molecular nature of which remains to be determined. Present results suggest that the cholinergic system could be one of the neural mechanisms participating in the cross talk between ovaries and adrenals. Both the ovaries and adrenals could be sharing neural information at the prevertebral celiac–superior mesenteric ganglia level. Another possibility is the existence of neural communication between the adrenals and the ovaries through the prevertebral celiac–superior mesenteric ganglia. Previously, we showed the existence of neural

communication between the ovaries and the prevertebral celiac–superior mesenteric ganglia *(32)*.

The neurons giving rise to the fibers reaching the ovary through the SON have two origins. Most originate in the celiac ganglion and a small number originate from the paravertebral ganglia and go through the celiac ganglion without establishing any contact with the ganglionar neurons *(33)*. The celiac plexus receives contributions from the major and minor splachnic nerves and the vagal nerves *(34)*. VIPergic fibers contained in the SON are more likely to be derived from the major and minor splachnic nerves, the vagal nerves and/or the suprarenal ganglion, which not only receives substantial contributions from the splachnic nerve, but also sends the bulk of its nerve terminals to the ovary via the suspensory ligament *(34)*.

Present results support the idea proposed by Klein and Burden *(35)* on the existence of a viscero–visceral reflex mechanism. This generalized component of the autonomic nervous system serves as a way to modulate the activity of the principal ganglion cell in the sympathetic ganglion, and, ultimately, the functional activity of specific visceral organs. To this idea, we add that such a viscero–visceral reflex mechanism, between the adrenals and ovaries, varies along the estrous cycle and is asymmetric.

Materials and Methods

All experiments were carried out in strict accordance to the Guide for Care and Use of Laboratory Animals at the National Academy of Sciences. The Committee of the Facultad de Estudios Superiores Zaragoza approved the experimental protocols.

This study was performed with adult female rats from the CIIZ-V strain from our own stock (195–225 g body weight) that had shown at least two consecutive 4-d cycles, monitored by cytological examination of daily vaginal smears. All animals were housed in an artificial light–dark cycle (lights on from 05:00 to 19:00 h), with free access to food (Purina S.A., Mexico) and tap water *ad libitum*.

All surgeries were performed under ether anesthesia, between 13:00–13.15 h on D1, D2, or P, and the animals sacrificed between 14.00 and 14.15 the day of treatment.

Rats were randomly allotted to one of the experimental groups described below. Animals from different experimental groups were treated simultaneously and sacrificed 1 h after surgery. The number of animals used on each experimental group is presented in Tables 1–6.

Experimental Groups

Control: Nontreated cyclic rats sacrificed at 14:00 h on D1, D2, and P.

Ether anesthesia: Groups of rats anesthetized during 10 min on each day of the estrous cycle and sacrificed 1 h later.

Unilateral ovariectomy or adrenalectomy sham surgery: group with unilateral perforation of the peritoneum: A unilateral incision was performed 2-cm below the last rib, affecting skin, muscle, and peritoneum. Neither ovaries nor adrenals were injured or manipulated. After surgical procedures, the wound was sealed.

Bilateral ovariectomy or adrenalectomy sham surgery: animals with bilateral perforation of the peritoneum: A bilateral incision below the last rib was performed, including skin, muscle, and peritoneum. Neither ovaries nor adrenals were injured or manipulated. After surgical procedures, the wound was sealed.

Unilateral ovariectomy: A unilateral incision below the last rib was performed, including skin and muscle, and the right or left ovary was extirpated. The wound was sealed.

Unilateral adrenalectomy: A unilateral incision below the last rib was performed, including skin and muscle, and the right or left adrenal was extirpated. The wound was sealed.

Bilateral ovariectomy: A bilateral incision below the last rib was performed, including skin and muscle, and the ovaries removed. The wound was sealed.

Bilateral adrenalectomy: A bilateral incision below the last rib, including skin and muscle was performed, and the adrenals removed. The wound was sealed.

To analyze the effects of blocking the cholinergic system, groups of animals were injected subcutaneously with atropine sulfate (ATR) (Sigma Chem. Co., St. Louis, MO) 1 h before surgery. ATR was injected at 12:00 h at doses known to block ovulation: in D1, 100 mg/kg-body weight (bw); in D2, 300 mg/kg bw; and in P, 700 mg/kg bw *(10)*.

At 13:00 h (1 h after ATR injection), rats were randomly allotted to one of the experimental groups described above [ether anesthesia or one of the following surgeries: unilateral or bilateral sham operation (peritoneal perforation), unilateral or bilateral ovariectomy or adrenalectomy]. Animals were sacrificed 1 h after surgery.

Groups of untouched rats (control group) were injected with ATR on D1, D2, or P in the same dose as in each treatment group. The animals were sacrificed 2 h after the treatment.

Autopsy Procedures

Rats were sacrificed by decapitation; the trunk blood was collected, allowed to clot at room temperature for 30 min, and centrifuged at 2175*g* during 15 min. Serum was stored at –20°C, until P_4 concentrations were measured.

Hormone Assay

Concentrations of P_4 in serum were measured by radioimmunoassay (RIA), using kits purchased from Diagnostic Products (Los Angeles, CA). Results are expressed in ng/mL. The intra- and interassay variation coefficients were 5.3% and 9.87%.

Statistics

Data on hormonal concentrations in serum were analyzed using multivariate analysis of variance (MANOVA) followed by Tukey's test. Differences in serum hormone concentrations between two groups were analyzed by Stu-

dent's *t* test. A probability value of less than 5% was considered significant.

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