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Estrogen Replacement in Ovariectomized Rats Results in Physiologically Significant Levels of Circulating Progesterone, and Co-Administration of Progesterone Markedly Reduces the Circulating Estrogen

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Estrogen or progesterone replacement in ovariectomized rats is an often-used experimental system for determination of the specific effects of these hormones. In this study, two different delivery systems and two different dosage levels of estrogen, progesterone or a combination of the two have been used. Estrogen and progesterone in the circulation have been measured in response to each treatment. It is reported that estrogen treatment (237.2 ± 49.2 pg/mL) results in physiologically significant levels of circulating progesterone (11.1 ± 1.3 ng/mL). Also, co-administration of progesterone (23.7 ± 2.0 ng/mL) with estrogen decreases the level of estrogen over that seen with estrogen alone (96.7 ± 19.2 pg/mL with progesterone vs 237.2 ± 49.2 pg/mL without progesterone). Thus, contrary to expectations, estrogen replacement therapy is not specific to estrogen and some of the antagonistic effects of progesterone are the result of a decrease in circulating estrogen, and not a specific effect on a target tissue. Whereas the mechanism of these effects has not been determined, obvious artifactitious phenomena have been excluded as being their cause. These results could have a major impact on the interpretation of past and future experiments of this kind.

Key Words: Estrogen; progesterone; ovariectomy; rat serum; combined therapy.

Introduction

Throughout the course of endocrinology, ovariectomy with or without the subsequent replacement of either estrogen, progesterone or both has been used as a means of determining the individual effects of these two steroids on specific cells or processes in the intact animal. As far as the authors have been able to ascertain, it is generally assumed that administration of estrogen to an ovariectomized animal raises only circulating estrogen, that administration of progesterone raises only circulating progesterone, and that administration of both results in levels of estrogen and progesterone equivalent to each alone. These too were the assumptions at the outset of a study, the results of which caused the question of their validity. It is now reported that estrogen administration to ovariectomized animals results in significant circulating progesterone and that co-administration of progesterone with estrogen decreases circulating estrogen over estrogen administration alone. Thus, on the one hand estrogen replacement cannot be considered to be replacement of estrogen alone and second, some of the antagonistic effects of progesterone, which have been interpreted as effects on the target tissue, may really be the result of a decrease in circulating estrogen levels. Whereas the mechanism of these effects has not been determined, obvious artifactitious phenomena have been excluded as being their cause. These results could have a major impact on past and future interpretations of these kinds of experiments.

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Results

Trial Set 1

In preliminary experiments, it was determined that the same results were obtained whether serum or plasma was assayed. Figures 1 and 2, therefore, combine results from separate experiments, two assaying serum and one assaying plasma. For each treatment, samples from six animals were analyzed in each of the experiments.

Figure 1 shows the prolactin (PRL), estradiol (E_2), and progesterone (P) levels after 1 wk of treatment with either a placebo control pellet, an E_2 pellet, a P pellet or, E_2 plus P pellets. PRL was assayed so as to have a totally separate measure, independent of steroid assays, of effective E_2 levels. As anticipated, E_2 treatment raised PRL levels (about sixfold) and resulted in a high circulating level of E_2 (827.5 ± 103.0 pg/mL). E_2 treatment, however, also resulted in a P level of 12.8 ± 4.1 ng/mL (about threefold the placebo level). P treatment, on the other hand, resulted in no statisti-

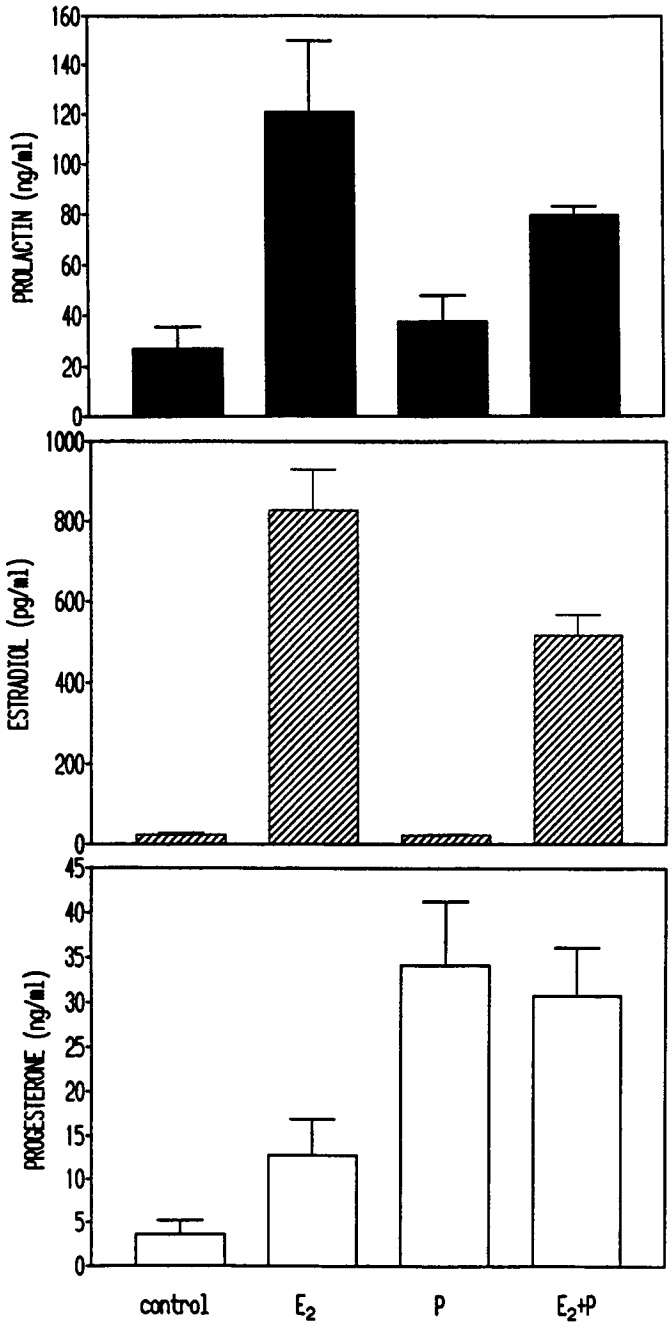

Fig. 1. PRL, E_2, and P levels after 1 wk of treatment in trial 1. The concentration of PRL in the E_2-treated group was higher than in the E_2 + P-treated group ($p < 0.05$). The E_2 levels in the E_2-treated group were 1.6-fold, the E_2 + P group with an absolute difference of 300 pg/mL ($p < 0.01$). The P levels between the P alone and E_2 + P group were not significantly different, but the P level in the E_2-treated group was significantly higher than the placebo control group (threefold) ($p < 0.05$). $n = 18$ animals for each bar.

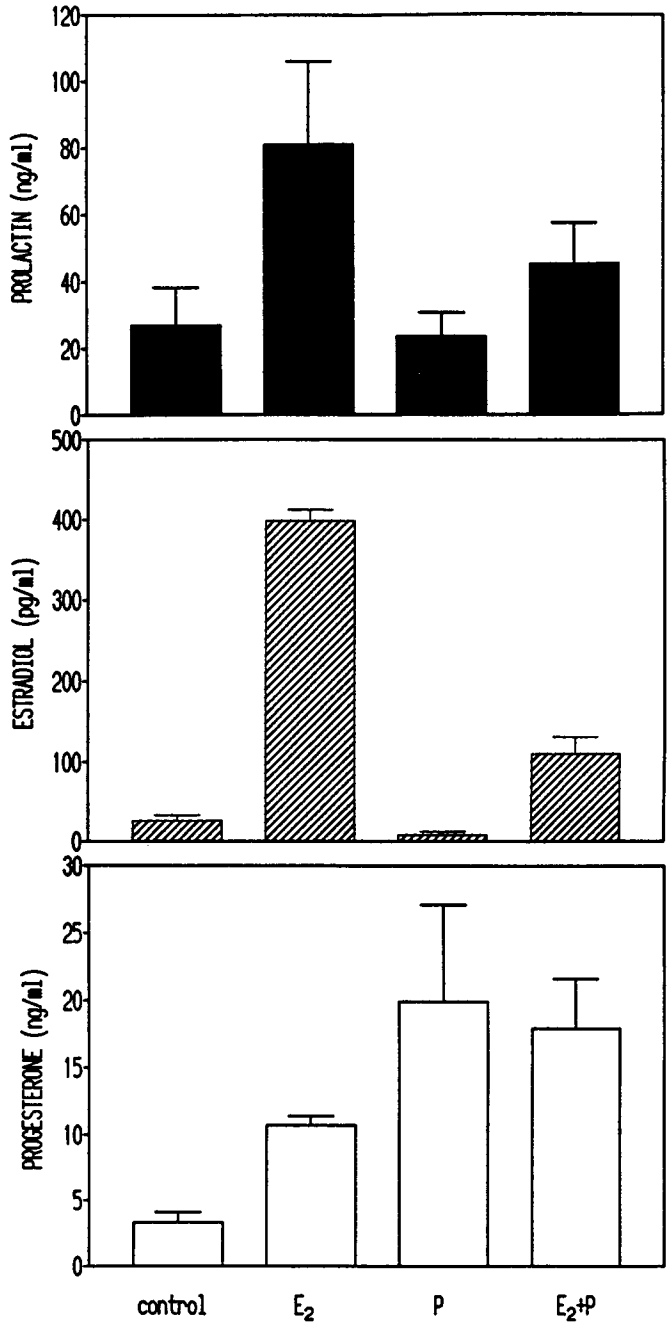

Fig. 2. PRL, E_2, and P levels after 2 wk of treatment in trial 1. The E_2 level in the E_2-treated group was again higher (3.6-fold) than the E_2 + P treated group with an absolute difference of 290 pg/mL ($p < 0.001$). The P level was again higher than in the placebo control group (3.6-fold) ($p < 0.01$). $n = 12$ animals for each bar.

cally significant change in PRL over the placebo and no measurable change in E_2 levels over the placebo and, as expected, in substantial levels of P (34.2 ± 7.1 ng/mL). P treatment in combination with E_2 treatment, however, reduced the amount of circulating PRL vs E_2 alone (120.9 ± 28.9 to 79.5 ± 3.6 ng/mL) and reduced the amount of circulating E_2 vs E_2 alone (827.5 ± 103.0 to 517.5 ± 50.5 pg/mL). The combination, however, had no statistically significant effect on circulating P. The measurable levels of P were determined to be not statistically significantly different from each other whether 150 mg, 2 × 100 mg, or 2 × 150 mg pellets were used. After analysis for possible different dose effects of the three P levels on measured parameters by two-way analysis of variance (ANOVA), it was concluded that all three trials could be combined for presentation and analysis. Figure 1 therefore reflects all three trials.

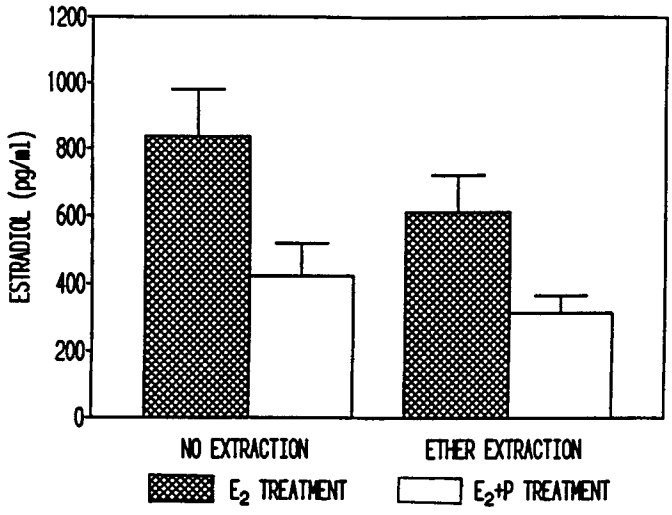

Fig. 3. Effect of ether extraction on the levels of E_2 with or without concurrent administration of P. There was no significant difference in E_2 levels with or without ether extraction, but the difference between E_2 administration alone and E_2 + P administration was maintained in the ether extracted samples ($p < 0.01$). $n = 6$ samples for each bar. The same samples were assayed with and without extraction.

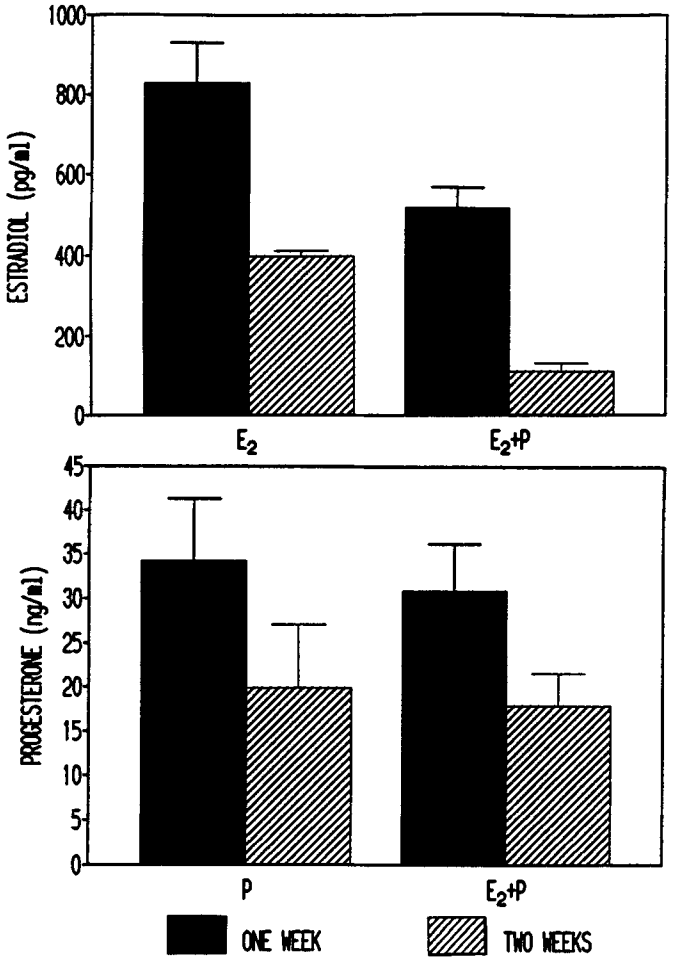

Fig. 4. Levels of E_2, P, and the combination at wk 1 and 2 of trial 1. E_2 levels at wk 2 were 300 pg/mL less than at wk 1 in both the E_2- and E_2 plus P-treated animals ($p < 0.005$). $n = 18$ animals for the wk 1 samples and $n = 12$ animals for the wk 2 samples.

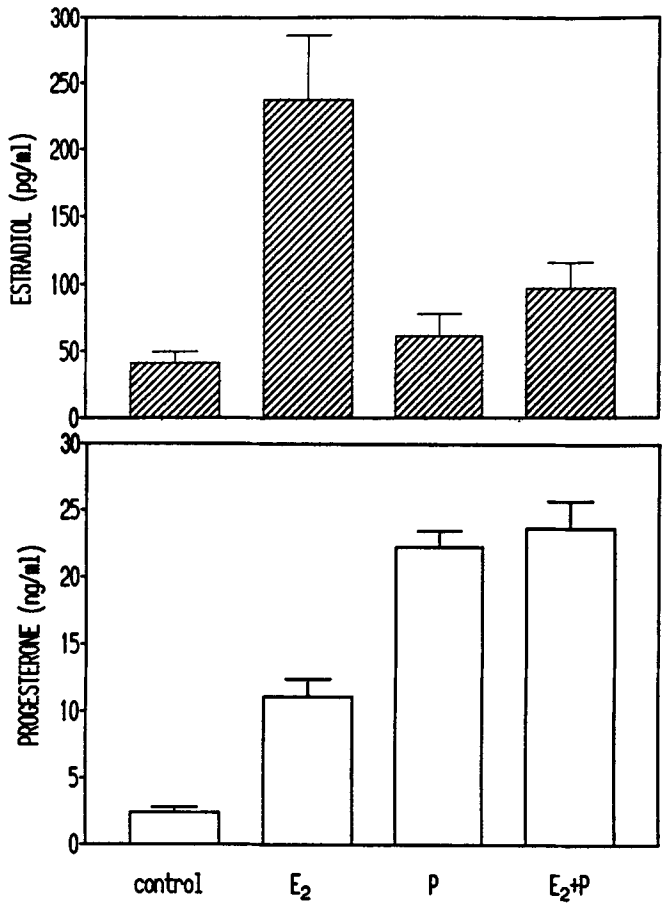

Fig. 5. E_2 and P levels after 2 wk of treatment in trial 2. The E_2 levels in the E_2-treated group were 2.4-fold the E_2 + P treated group ($p < 0.05$). The P level in the E_2-treated group was 4.6-fold the control ($p < 0.01$). $n = 8$ animals for each bar.

After 2 wk of treatment, circulating levels of the hormones had decreased, but the effects of co-implantation of E_2 and P described above were the same (Fig. 2). In addition, at both wk 1 and wk 2, co-implantation of P with E_2 lowered measurable E_2 levels by the same amount, ~300 pg/mL.

In Fig. 3, the effects of ether extraction on the measurable levels of E_2 are illustrated. Although E_2 levels were lower with extraction, co-administration of P with the E_2, reduced the measurable E_2 by about 50% whether the sample was extracted or not.

In Fig. 4, E_2 and P levels are compared in the samples at wk 1 and 2. Administration of E_2 in a constant release pellet form resulted in levels of E_2 of 827.5 ± 103.0 pg/mL at wk 1 and 398.0 ± 14.1 pg/mL at wk 2. The E_2 plus P combination gave E_2 levels of 517.5 ± 50.5 pg/mL at wk 1 and 109.9 ± 21.5 pg/mL at wk 2. Likewise, P administration gave P levels of 34.2 ± 7.1 ng/mL at wk 1 and 19.9 ± 7.2 ng/mL at wk 2.

Trial Set 2

Administration of E_2 at a lower level (237.2 ± 49.2 pg/mL), and using a silastic tubing capsule also resulted in a significant level of P (11.1 ± 1.3 ng/mL) in the circulation (Fig. 5). As before, P administration, together with the E_2,

Table 1
Specificity of the Progesterone Antiserum used for Progesterone RIA

Compound	Percent cross-reactivity
Progesterone	100%
Androstenediol	ND
Corticosterone	0.4%
Cortisol	ND
Danazol	ND
11-Deoxycorticosterone	1.7%
11-Deoxycortisol	2.4%
20α-Dihydroprogesterone	2.0%
Estradiol	ND
17α-Hydroxyprogesterone	0.3%
Medroxyprogesterone	ND
Pregnane	ND
5β-Pregnan-3α-ol-20-one	0.2%
5α-Pregnan-3,20-dione	0.8%
5β-Pregnan-3,20-dione	1.3%
Pregnenolone	ND
Testosterone	ND

ND indicates nondetectable.

reduced the amount of circulating E_2 over E_2 administration alone (96.7 ± 19.2 pg/mL with P vs 237.2 ± 49.2 pg/mL without P). Thus, using lower levels of hormone and a different means of delivery did not change these two results.

Discussion

The results of this set of experiments bring to light three important findings. The first is that E_2 treatment of ovariectomized rats results in significant levels of circulating P. The second is that co-implantation of P with E_2 reduces the amount of circulating E_2 compared to implantation of E_2 alone. The third is that implantation of constant release pellets at the doses used in trial 1 does not result in constant circulating levels of either E_2 or P.

To consider the first result as accurate, it is important to weigh all alternative explanations that could explain the data. What comes to mind immediately is the possibility that E_2 cross reacts in the P assay. Not only is such cross reactivity undetectable in the P assay (Table 1), but given that E_2 was administered as a 0.5 mg pellet to give levels of ~800 pg/mL at 1 wk and the P levels measured at 1 wk were ~12 ng/mL (an order of magnitude higher), it is not possible for this result to be a result of cross-reactivity in the assay. Likewise for the same reasons, it does not seem likely that contamination of the E_2 pellet with P could be the explanation since it takes a 150 mg pellet of P to produce ~35 ng/mL of P and only 0.5-mg pellets of E_2 were implanted. In addition, for trial 2, the silastic implants were manufactured in house and every caution was taken to minimize any possible contamination.

The most reasonable proposal at present is that E_2 alters adrenal steroid metabolism increasing the formation of P. In the 1960s, Kitay reported that E_2 enhanced basal corticosteroid secretion by rat adrenal slices *(1–4)*. The rate-limiting step in the formation of corticosterone, which goes from cholesterol to corticosterone via P, is the conversion of cholesterol to pregnenolone *(5)*. E_2 stimulates this first step both by direct effects on the adrenal cortex *(3,4,6,7)* and via increased release of adrenocorticotrophic hormone (ACTH) *(5)*. Several studies have shown that the adrenal can be a source of significant amounts of progesterone in the rat *(8–11)*. As demonstrated by Feder and Ruf *(12)*, the amount of progesterone after E_2 treatment is sufficient in quantity to facilitate female receptivity.

For co-implantation of P with E_2 to lower E_2 levels vs those with E_2 alone, the first artifactitious possibility considered was interference of P in the E_2 assay. However, even when administered at 100,000 ng/mL, P did not interact with the E_2 antiserum (Table 2). The second possibility was physical interference during dissolution of the pellet in the animal. Not only was an attempt made to eliminate this by the separate placement of the pellets, but it seems unlikely that the difference between effects of E_2 + P and effects of E_2 alone would be the same at wk 1 and wk 2 under these circumstances because of the different sizes of the pellets at wk 1 vs wk 2. In addition, the results of trial 2, using implants with very different geometries, were the same as those in trial 1.

There remain two physiological explanations, the first of which is the induction of E_2 binding proteins by P. It is not believed that this is the likely explanation since this phenomenon was observed with or without prior ether extraction of the samples. Since extraction allows us to measure total E_2, the phenomenon should have disappeared in the extracted samples and it was not even diminished. It is suggested, therefore, that P alters clearance of E_2 and that the equivalent reduction in circulating E_2 caused by P treatment at wk 1 and 2 (~300 pg/mL) may be the maximum induced clearance. Actual lowering of circulating E_2 is supported by the PRL results that show a 30% decrease in response to the combination vs E_2 alone, a change not dissimilar in magnitude to the reduction in E_2 (38%). According to the work of Tapper and Brown-Grant *(13)*, estrogen clearance is most significant in the livers of rats since functional nephrectomy does not change the plasma metabolic clearance rate, but subtotal hepatectomy does. Also, it would seem that P upregulates its own clearance since administration of 200 or 300 mg gave no greater amount of circulating P than 150 mg. When a single P silastic capsule was implanted, circulating levels of P of 11 ng/mL were obtained and maintained for 2 wk (data not shown). Thus with lower levels of P, no evidence was found of induced clearance. As far as could be ascertained, there have been no studies of induced clearance in either E_2- or P-treated ovariectomized rats. One study of E_2 clearance at different

Table 2
Specificity of the Estradiol Antiserum used for Estradiol RIA

Compound	ng/mL Added	Percent cross-reactivity
d-Equilenin	3.6	4.4%
Equilinin	3.6	0.80%
17α-Estradiol	100	0.017%
17β-Estradiol-3-D-glucuronide	5	0.69%
β-Estradiol-17β-D-glucuronide	50	ND
17β-Estradiol-3-glucuronide-17-sulfate	50	ND
17β-Estradiol-3-monosulfate	50	0.29%
β-Estradiol-3-sulfate-17-glucuronide	50	ND
Estriol	20	0.32%
Estriol-3-glucuronide	1000	0.012%
Estriol-3-sulfate	10,000	0.002%
5(10)-Estren-17α-ethanyl-17β-ol-3-one	50	0.021%
1,3,5(10)-Estratrien-17α-methyl-3,17β-diol -3-methyl ether	50	0.47%
1,3,5(10)-Estratrien-17α-vinyl-3,17β-diol	100	0.024%
1,3,5(10)-Estratrien-17α-ethinyl-3,17β-diol-3-methyl ether	100	0.02%
4-Estren-17β-ol-3-one	100	0.25%
4-Pregnen-6β,11β,17α,21-tetrol-3,20-dione	100	0.008%
5β-Pregnan-3α-ol-20-one	100	0.009%
5β-Pregnan-3α,17α,20α-triol	100	0.009%
Estriol-16α-glucuronide	10,000	0.001%
Estrone	3.6	10.0%
Estrone-β-D-glucuronide	3.6	1.8%
Estrone-3-sulfate	3.6	0.58%
Aldosterone	160	ND
Androstenedione	100,000	ND
Androsterone	100,000	ND
Corticosterone	100,000	ND
Cortisol	100,000	ND
Cortisone	4000	ND
11-Deoxycortisol	4000	ND
DHEA	4000	0.001%
DHEA-sulfate	5000	ND
Progesterone	100,000	ND
11β-Hydroxytestosterone	4000	ND
5α-Dihydrotestosterone	100,000	0.004%
Testosterone	100,000	0.001%
19-Hydroxyandrostenedione	1000	0.006%

ND indicates nondetectable.

stages of the estrous cycle showed no differences according to stage *(13)*, a result that may imply that it takes more than a day or so of increased hormone levels to induce increased clearance. A study of rhesus monkeys given high doses of synthetic, combination E_2/P-based oral contraceptives, however, has shown that contraceptive administration markedly increased clearance of subsequently administered ^{14}C-E_2 *(14)*.

The size of the E_2 pellet used in the trial 1 experiments was based on data provided by the manufacturer showing that a 0.5-mg pellet will give blood levels of about 250 pg/mL. This estimate, however, was for intact animals. Perhaps the predicted 250 pg/mL vs the actual ~800 pg/mL in the ovariectomized animals is further evidence that P, this time normally present in the animal, lowers circulating E_2 by increasing liver clearance.

Because the 800 pg/mL of E_2 was supraphysiological, a second trial was conducted, during which more physiological levels were achieved. By obtaining the same results, their physiological relevance was demonstrated.

In summary it has, therefore, been demonstrated that E_2 treatment of ovariectomized rats results in significant P levels in the blood. Second, it was demonstrated that P treatment lowers the amount of circulating E_2. Third, it was demonstrated that administration of either E_2 or P or both as constant release pellets at the doses used in trial 1 does not result in constant circulating levels of these steroids.

Materials and Methods

Animals and Steroid Administration

Trial Set 1

Ovariectomized Sprague-Dawley rats (approx 250 g) were obtained from Taconic (Germantown, NY). Ten days after ovariectomy, 21-d constant release pellets were implanted subcutaneously under local anesthesia in all animals. When more than one pellet was implanted per animal, they were placed in different areas, at least 2 cm apart, so that one pellet did not physically interfere with the release properties of the other. The constant release pellets were obtained from Innovative Research of America (Sarasota, FL). The control placebo pellet was 150 mg of binder base. Estradiol pellets were 0.5 mg. Progesterone implants of 150, 200, and 300 mg were achieved by the implantation of one or two 150 mg pellets, or two 100 mg pellets.

Rats were killed at 1 wk or 2 wk after implantation or were bled from the tail at 1 wk and killed at 2 wk. When killed, trunk blood was collected either as serum (by clotting on ice) or plasma (by EDTA inhibition of clotting in a coated tube). Tail blood in trial 1 was collected as plasma. Serum or plasma was stored at –20°C prior to RIA for E_2, P, and PRL. Samples were assayed in two separate RIAs, but common samples were assayed in each to control for interassay variation. The quoted interassay variations were determined using standard preparations of either E_2 or P. EDTA was determined to have no effect on the assay results.

Trial Set 2

In a completely separate trial, eight animals were ovariectomized in house under pentobarbital anesthesia and were implanted at the time of surgery with silastic capsules containing E_2. Two weeks later, the animals were bled from the tail vein and the capsules were removed and replaced with equivalent fresh E_2 capsules together with P capsules. Two weeks later, they were bled again and the capsules removed and replaced with only P capsules. At the next change, all capsules were removed, with the final bleed occurring 8 d later. Tail blood in trial 2 was collected as serum and stored at –70°C until assayed. All samples were measured together in a single P or E_2 RIA.

Silastic Implants

Silastic implants contained either crystalline E_2 or P (Sigma Chemical Co., St. Louis, MO) packed into silastic tubing (Dow Corning Corp., Midland, MI). The ends were plugged with silicone sealant. E_2 capsules were made by filling 1.57 mm id × 2.41 mm OD tubing with a 1.4 mm column of equal amounts of E_2 and cholesterol. P capsules were prepared using 1.47 mm id × 1.96 mm od tubing packed with a 40 mm column of P. Two P capsules per rat were needed to produce the desired levels. Prior to implantation, capsules were wiped with ethanol and incubated for 24 h in 0.01*M* phosphate-buffered saline (pH 7.4).

Assays

The E_2 and P assays were performed using kits from Diagnostic Products (Los Angeles, CA). E_2 was assayed either with or without extraction with ether. For samples subject to ether extraction, 200 µL of plasma was extracted into 2 mL ether, dried under nitrogen and concentrated twofold by subsequent dissolution in 100 µL of assay buffer. To correct for losses during storage and extraction, plasma from ovariectomized rats was spiked with standard concentrations of E_2 and taken through the same procedures to serve as the standard curve. Preliminary measurements showed that this curve was parallel to the one generated using the standards in the kit. In the present study, this assay has a detection limit of ~1.5 pg/mL, an intra-assay variation of <6%, and an interassay variation of 6.9%. All samples were assayed in duplicate.

According to Lye et al. *(15)*, the primary antiserum exhibits cross-reactivity of 6% with 17β-estradiol-3β-D glucuronide, 1.3% with estrone, and 0.235% with estriol with minimal or no cross-reactivity to other naturally occurring steroids. Because of the importance of this issue in the current study, the cross-reactivities provided by the manufacturer are presented as Table 2.

P was assayed without extraction. In the present study, this assay has a detection limit of 0.1 ng/mL, an intra-assay variation of 5.1% and an interassay variation of 7.8%. The cross-reactivities for this assay are presented as Table 2. All samples were assayed at least in duplicate.

PRL was assayed using materials provided by the National Pituitary and Hormone Program of the NIDDK. Rabbit antirat PRL IC5 was used as antiserum. The assay has a sensitivity of 0.5 ng/mL and an intra-assay variation of 7% and an interassay variation of 16%.

Statistical Analysis

Two-way ANOVA was utilized to determine the validity of combining the data from all three doses of progesterone used in trial 1. Two-way ANOVA was performed after $\log_{10}$ transformation of the raw data. One-way ANOVA was used to determine the level of significance in both trials, using Bonferroni corrections for multiple comparisons between groups. Data are presented throughout as mean ± SEM.

Approvals

All animal procedures were approved by the University of California, Riverside Committee on Laboratory Animal Care, and were in compliance with National Institutes of Health standards.

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