Plasma protein and blood volume restitution after hemorrhage in conscious pregnant and ovarian steroid-replaced rats

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Blair, Martha L., and Deanne Mickelsen. Plasma protein and blood volume restitution after hemorrhage in conscious pregnant and ovarian steroid-replaced rats. Am J Physiol Regul Integr Comp Physiol 290: R425–R434, 2006. First published September 15, 2005; doi:10.1152/ajpregu.00011.2005.—We have previously shown that both plasma protein restitution and plasma volume restitution are significantly enhanced in female rats hemorrhaged during the proestrus phase of the estrous cycle. Estradiol and progesterone levels are markedly elevated during proestrus and also increase during pregnancy. The present studies were therefore designed to determine whether the ability to restore plasma protein and blood volume after hemorrhage is augmented during pregnancy and by chronically elevated estradiol levels. The response to moderate hemorrhage (22–23% blood loss) was evaluated in conscious pregnant rats during early and midgestation and compared with that of virgin female rats studied during metestrus. At 22 h posthemorrhage, plasma volume had increased to greater than basal levels, and blood volume was restored to 93 ± 1% (metestrus), 91 ± 2% (early pregnancy), and 98 ± 2% (midgestation) of control (P > 0.05). Animals hemorrhaged during metestrus or early pregnancy restored the same amount of protein to the plasma as had been removed, whereas those hemorrhaged during midgestation restored nearly 50% more plasma protein than had been removed (P < 0.01). In ovariectomized animals with chronic steroid replacement that maintained plasma progesterone at metestrus levels (15 ± 2 ng/ml) but raised plasma estradiol to twofold that of midgestation (22 ± 3 pg/ml), the blood volume and plasma protein restitution responses to hemorrhage did not differ from those of ovariectomized animals with no steroid replacement. In summary, posthemorrhage restoration of plasma protein content is significantly augmented during midgestation, but not during early pregnancy. This augmented response cannot be attributed to chronic elevation of plasma estradiol levels alone.

Plasma protein; blood pressure; estrogen; progesterone; plasma osmolality

HUMAN PREGNANCY INITIATES a marked increase in maternal blood volume that begins during the first trimester of pregnancy. This increase in blood volume is supported by expansion of the entire extracellular fluid volume and requires that plasma protein content be sufficient to maintain appropriate distribution of the expanded fluid volume to the blood (6, 15, 16, 45). In addition, there is a substantial decrease in maternal systemic vascular resistance that also begins early in pregnancy (9). Increased maternal blood volume is therefore essential for appropriate perfusion of the vasodilated maternal circulation as well as the uteroplacental circulation. The importance of gestational blood volume expansion is highlighted by the significant positive correlation between maternal plasma volume and fetal growth. Conversely, reduced maternal plasma volume is an early harbinger both of intrauterine growth retardation and of eclampsia (6, 14, 15).

Under conditions of maternal blood loss due to hemorrhage, it is therefore critical for both maternal and fetal health that plasma protein content and blood volume be rapidly restored back to the appropriate level. Studies performed in nonpregnant animals show that following blood loss, blood volume is restored by fluid shifts from the interstitial spaces into the plasma. The rapid early phase of vascular refilling is initiated by compensatory vasoconstriction that reduces capillary hydrostatic pressure and thereby promotes transcapillary influx of fluid that is nearly protein free. A second, slower phase of vascular refilling begins 2–3 h posthemorrhage and continues over the following 18–24 h. Vascular refilling during this second phase is facilitated by the gradual addition of protein to the plasma. This increase in plasma protein content is essential for sustained blood volume restoration because it permits continued reabsorption of interstitial fluid into the plasma by augmenting plasma oncotic pressure (7, 13, 32).

The process of plasma protein and volume restitution depends on a complex profile of neuroendocrine, metabolic, and vascular responses (7, 13, 32). The multiple endocrine and cardiorenal adaptations of pregnancy (3, 15, 21, 24, 25, 40) could have significant impact on this process. For example, the vasoconstrictor response to angiotensin and norepinephrine is blunted during pregnancy (20). The basal vasodilatation and reduced response to vasoactive hormones released in response to blood loss might be expected to impede vascular refilling by blunting the posthemorrhage reduction in capillary hydrostatic pressure. On the other hand, we have previously shown that the plasma volume and plasma protein restitution responses to hemorrhage are augmented during the proestrus phase of the rat estrous cycle compared with females hemorrhaged during the metestrus phase or with males (37). Metestrus is characterized by low plasma estradiol and moderately elevated plasma progesterone concentrations, whereas both estradiol and progesterone rise to markedly elevated levels during proestrus. Both estradiol and progesterone also are elevated during pregnancy. This suggests that during pregnancy the ability to restore plasma volume and plasma protein after blood loss may instead be enhanced, just as it is during proestrus.

The present study was therefore designed to determine whether pregnancy alters the ability to restore blood volume after a blood loss and to test the hypothesis that plasma protein restitution is augmented during pregnancy. The response to a moderate hemorrhage was evaluated in conscious unrestrained pregnant and ovarian steroid-replaced rats.
pregnant rats during early and midgestation and compared with that of virgin female rats studied during metestrus. Plasma volume is first significantly elevated on day 6 of the 22-day rat gestation period (1) and continues to increase for the duration of pregnancy. Plasma progesterone levels begin to increase by day 3 of rat gestation, whereas estradiol does not increase until the second half of pregnancy (18). Gestation days 6-9 (early pregnancy) and days 12-14 (midgestation) were therefore selected for study because they represent differing stages in the process of plasma volume expansion and also differ in their endocrine profiles.

Additional experiments were performed to test the hypothesis that posthemorrhage restoration of plasma protein is augmented by estradiol. In these experiments, ovarioctomized animals received chronic steroid replacement that raised serum estradiol to levels double those of midgestation and within the midrange of estradiol levels reported for the day of proestrus (38). Because estradiol is not normally present in the absence of progesterone, these animals also received chronic progesterone replacement in a low dosage that maintained progesterone at the same level as during metestrus.

**MATERIALS AND METHODS**

Experiments were performed in female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 200–225 g at the time of arrival at the University of Rochester vivarium. The rats were housed in individual cages in the University of Rochester vivarium with a 12:12-h light-dark cycle (lights on 06.00–18.00 h) with standard laboratory chow and tap water available ad libitum. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Animal preparation.** All surgical procedures were performed while the animals were anesthetized with Equithesin anesthesia (pentobarbital 25 mg/kg ip with chloral hydrate 128 mg/kg) using aseptic conditions.

For rats to be studied during pregnancy, a male breeder rat was placed in the cage with each female during the proestrus phase of the estrous cycle, and the presence of sperm in the vaginal smear on the following day indicated day 0 of pregnancy. The estrous cycle phase was determined from the cytology of vaginal smears obtained daily between 0900 and 1130 h. For virgin females (n = 11), hemorrhage experiments were performed on the morning of the metestrus phase of the estrous cycle with the estrous phase documented for at least one complete cycle before experiment. Femoral arterial and venous catheters were surgically implanted 4–7 days before the hemorrhage experiment and no earlier than day 2 of pregnancy. Catheters were implanted as previously described (37). Hemorrhage experiments were performed on pregnancy days 6-9 (early pregnancy; n = 9) or days 12-14 (midgestation; n = 8). Complete data sets were obtained for nine metestrus virgin females, five animals that were hemorrhaged during early pregnancy, and six animals that were hemorrhaged during midgestation. Hemorrhage experiments were not performed in animals during late pregnancy in view of the confounding effects of the impaired blood pressure regulation observed in near-term pregnancy (5, 10, 21) on interpretation of the blood volume restitution data.

Animals studied in the ovarian steroid replacement protocol were ovarioctomized through bilateral flank incisions. Chronic steroid replacement was achieved by subcutaneous implantation of one slow-release estradiol pellet (0.1 mg 17-β-estradiol/3 wk; Innovative Research) inserted through the left flank and either one slow-release progesterone pellet (Innovative Research; 100 mg/3 wk) or three progesterone capsules inserted through the right flank incision at the time of ovarioctomy. Because initial experiments showed variable serum progesterone levels in animals using the commercially available progesterone pellets (n = 7; range 6–18 ng/ml), the remaining steroid-replaced animals (n = 3) were implanted with progesterone capsules assembled by filling 30-mm lengths of Silastic tubing (0.125-in. outer diameter, 0.078-in. inner diameter; Dow-Corning) with crystalline progesterone (11). The final data analysis included only those animals for which the serum progesterone levels exceeded 10 ng/ml (4 of 7 pellet-implanted animals plus 3 capsule-implanted animals). The control group of ovarioctomized females received no steroid replacement (n = 7). Femoral arterial and venous catheters were surgically implanted 1 wk later, and hemorrhage experiments were performed 2 wk after ovarioctomy.

**Experimental procedures.** During hemorrhage experiments, each rat was placed in a recording cage (37). Food and water were not available while the rat was in the recording cage. The catheters were connected to tubing extensions that permitted blood to be withdrawn and arterial blood pressure to be recorded without restraining or otherwise disturbing the rat. The rat was adapted to the experimental conditions by being placed in the recording cage for 1–2 h on at least 1 day before the experiment and was permitted an additional 30-min adaptation period after connecting the arterial catheter to the blood pressure recording apparatus on the morning of the experiment before beginning the control measurements. Blood pressure was recorded using an Ohmeda TC-BEC disposable transducer and Beckman Sensormedics R611 Dynograph or Gould 2600 recorder. Mean arterial pressure was obtained by electronic integration of the arterial pressure signal. All animals were weighed on the morning of the hemorrhage procedure and at 22 h posthemorrhage. Body weight was slightly less at 22 h posthemorrhage than before hemorrhage in most animals (average body weight decrease of 1–2% at 22 h posthemorrhage in pregnant and intact virgin females; 3–4% in ovarioctomized animals).

Hemorrhage experiments were performed in the morning, beginning at 0800–1100 h. Sodium heparin (100 U in 100 μl) was injected into the arterial catheter 15 min before beginning the hemorrhage procedure to prevent clotting during blood withdrawal. The hemorrhage procedure consisted of two sequential blood withdrawals. Blood was initially withdrawn from the arterial catheter at a rate of 6.5 ml/kg over 4 min for intact virgin and pregnant animals, or 9.5 ml/kg body wt over 4 min for ovarioctomized animals. After a 10-min observation period, a second hemorrhage was performed at a rate of 6.5 ml/kg body wt over 4 min, resulting in a total blood loss during hemorrhage of 13 ml/kg over 18 min for intact virgin and pregnant animals and 16 ml/kg body wt over 18 min for ovarioctomized animals. Because ovarioctomized animals had a slightly higher blood volume relative to body weight than did intact virgin or pregnant animals the hemorrhage procedures resulted in the same percent blood loss (22–23% of initial blood volume) for all experimental groups.

Blood volume measurements were performed by the Evans blue dye method. The first blood volume determination was performed at the beginning of the hemorrhage procedure. At 8 min before hemorrhage, a 0.8-ml blood sample was collected for use as a plasma blank in the Evans blue dye spectrophotometric analysis. Three minutes later, 0.3-ml sterile Evans blue dye (0.5 mg/ml in saline; Harvey Labs, Philadelphia, PA) was infused into the venous catheter and flushed in with 0.5-ml sterile isotonic saline. The hemorrhage blood withdrawal procedure began 5 min after infusing Evans blue dye. Blood withdrawn at the beginning of the hemorrhage period was used for measurement of Evans blue dye concentration. All blood samples were withdrawn through the arterial catheter. The venous catheter was used solely for infusion of Evans blue dye.

Additional 0.7-ml blood samples (0.4 ml for plasma osmolality and protein determinations, 0.3 ml for triplicate microhematocrit samples) were collected at 90 min and 3 h after initiation of the hemorrhage procedure, and the volume was replaced with sterile isotonic saline to avoid a hypotensive response to the blood sampling procedure. The
animal was then returned to housing overnight with food and water freely available. On the morning following the hemorrhage procedure, the animal was again placed in the recording cage, and blood pressure was monitored for 25 min. The final blood volume measurement was performed at 22 h posthemorrhage. A 0.8-ml blood sample was first collected for use in the Evans blue dye analysis. Three minutes later, 0.3 ml of Evans blue dye (0.5 mg/ml) was infused through the venous catheter and flushed in with 0.5 ml of sterile isotonic saline. The final blood sample (2.8 ml) was withdrawn through the arterial catheter 5 min after the Evans blue dye infusion. Each animal was euthanized by an overdose of Equithesin anesthesia immediately following collection of the final blood sample. Uterine weight was determined at autopsy in all animals, and pregnancy was confirmed by the presence of multiple fetuses.

Blood sample analysis. Blood samples to be used for measurement of Evans blue dye concentration, plasma protein concentration, or plasma osmolality were collected in heparin-wetted syringes. Plasma from the first 1.3 ml of blood withdrawn during the first hemorrhage and from the final blood sample drawn at 22 h posthemorrhage were used for measurement of Evans blue dye concentration, plasma protein concentration, and plasma osmolality. Protein concentration and osmolality were also measured in plasma from the first 0.4 ml of blood withdrawn during the second hemorrhage and from samples withdrawn at 90 min and 3 h posthemorrhage. Serum progesterone and estradiol values were determined from the remaining blood pooled from the hemorrhage procedure. Triplicate hemocrit samples (80–100 µl each) were collected directly from the catheter extension into capillary tubes at the time of each hemorrhage and the 90-min, 3-h, and final 22-h blood sampling procedures.

Plasma protein concentration was determined by the colorimetric Bio-Rad DC Protein Assay kit. Spectrophotometric absorbance measurements were performed at a wavelength of 750 nm and were unaffected by the presence of Evans blue dye in the plasma. The interassay coefficient of variation for plasma protein concentration was 6% (n = 15). Plasma osmolality was determined by freezing point or vapor pressure osmometer (Precision Systems, Natick, MA). Serum estradiol and progesterone concentrations were determined in duplicate on unextracted samples using commercially available radioimmunoassay kits (DPC double antibody estradiol kit and DPC Coat-a-Count progesterone kit; Diagnostics Products, Los Angeles, CA). Interassay coefficients of variation for these determinations were 14% (n = 4) for the estradiol assay and 8% (n = 5) for the progesterone assay.

Evans blue dye concentration was measured from duplicate 75-µl plasma samples using a Shimadzu UV-160A spectrophotometer with microcuvettes. Absorbance measurements for unknowns (plasma from samples collected immediately before and 5 min after Evans blue dye infusion on both experiment days) were performed at wavelengths of 720, 610, 575, and 535 nm with clear plasma placed in the reference cuvette. Evans blue dye concentration was determined from the 610-nm absorbance values. Samples with significant turbidity (detected by elevated absorbance values at 720 nm) or hemolysis (demonstrated as absorbance peaks at 535 and 575 nm) were excluded from data analysis (17). Standard curves were constructed by linear regression analysis of the 610-nm absorbance values for solutions of Evans blue dye (0.001–0.02 mg/ml) dissolved in 0.9% saline containing 1% rat plasma. The interassay coefficient of variation for measurement of plasma Evans blue dye concentration was 7.6% (n = 5).

Calculations for plasma volume, blood volume, and total plasma protein content. For plasma volume calculations, the 610-nm absorbance measured in the plasma sample collected immediately before dye injection was subtracted from the absorbance measured from the plasma sample collected 5 min after Evans blue dye was injected. This procedure corrected for any residual dye that remained in the blood at 22 h posthemorrhage. The amount of Evans blue dye remaining in the plasma at 22 h was 13 ± 1% of the amount present at the start of the hemorrhage procedure (n = 21) and did not differ between groups. Plasma volume was then calculated as (milligrams dye injected/ (corrected plasma dye concentration). For blood volume calculations, percent hematocrit values were multiplied by an Fcv/Sv ratio (total body hematocrit/large vessel hematocrit) of 0.9 (44). Blood volume was therefore calculated as (plasma volume)/[1 – (0.009 hematocrit)].

Total plasma protein content at the beginning of each experiment (TPPcontrol) and at 22 h posthemorrhage (TPP22h) was calculated as the product of plasma protein concentration and plasma volume. Both plasma protein content values were corrected for the small amount of protein lost in the 0.8-ml blood sample withdrawn immediately before each Evans blue dye infusion. The amount of protein restored to the plasma was then computed as TPP22h – (TPPcontrol – TPPlost), where TPPlost was computed as the sum of the plasma protein contained in all blood withdrawals during the first 3 h of the experiment. The amount of protein in each blood withdrawal was computed as (plasma protein concentration) × (1 – 0.01 hematocrit) × (volume withdrawn). Plasma volume, blood volume, and total plasma protein content were normalized to the body weight obtained immediately before hemorrhage.

Statistical analysis. Statistical analyses were performed by SPSS SigmaStat 3.0 software. Between-group comparisons of values for serum progesterone and estradiol levels, body weight, uterine weight, and all other basal values were performed by t-test (ovariectomized animals) or by single-factor ANOVA (pregnancy series) followed by individual comparisons by the Holm-Sidak t-test when the ANOVA showed significant effects (P < 0.05) of pregnancy. The effect of pregnancy or steroid replacement on responses to hemorrhage for blood pressure, heart rate, and all other measurements was statistically evaluated by multifactorial repeated-measures (RM) ANOVA. When the multifactorial RM ANOVA showed significant effects of hemorrhage, individual Holm-Sidak comparisons of the initial control value with each subsequent sampling time were performed for each group of animals, using the pooled variance estimate computed by single-factor RM ANOVA for that group. Individual Holm-Sidak comparisons across groups at each time point, using the pooled variance from one-way ANOVA at each time, were performed when the multifactorial RM ANOVA showed significant effects of pregnancy or steroid treatment or a significant interaction effect. The null hypothesis for individual comparisons was rejected only when the unadjusted P value for that comparison was less than the critical level determined by the Holm-Sidak method (19), using an overall significance level of P < 0.05. All data are expressed as the means ± SE.

RESULTS

Pregnancy effects on body weight, progesterone, and estrogen. Pregnant animals showed a progressive increase in body weight and uterine weight (Table 1). Serum progesterone levels were significantly higher during early pregnancy (6–9 days of pregnancy) than in virgin females studied in the metestrus phase of the estrous cycle and increased further to fivefold metestrus levels by midpregnancy (12–14 days of pregnancy) (Table 1; P < 0.001). Serum estradiol levels were not significantly increased during early pregnancy but reached 2.5-fold metestrus levels by midpregnancy (P < 0.05). This profile of hormone values is consistent with reports from other laboratories (1, 18) indicating that progesterone is significantly increased early in rat gestation and shows an additional increase during midpregnancy, whereas estradiol does not increase until the second half of pregnancy.

Basal values in pregnant and intact virgin females. Plasma volume, blood volume, and total plasma protein content (the product of plasma volume and plasma protein concentration) increased progressively with pregnancy (Table 1; P < 0.01 for
The increase in these values was proportionate to the increase in body weight. Consequently, plasma volume, blood volume, and total plasma protein content values did not differ between pregnant and nonpregnant females when normalized to body weight. Neither hematocrit nor plasma protein concentration differed significantly between pregnant and virgin females, whereas plasma osmolality was significantly decreased in midpregnant animals (P < 0.001).

Blood pressure and heart rate response to hemorrhage in pregnant and intact virgin females. The hemorrhage procedure (13 ml/kg over 16 min) resulted in a total blood loss of 23 ± 1% of initial blood volume in virgin females, 22 ± 1% in early pregnancy and 22 ± 1% in midpregnancy. Resting mean arterial blood pressure and heart rate were not significantly different between groups (Fig. 1). In both pregnant and virgin females, mean arterial pressure remained near normotensive levels following 6.5 ml/kg blood loss, decreased by 45–55 mmHg immediately after 13 ml/kg blood loss, and then gradually returned toward control levels during the posthemorrhage recovery period (RM ANOVA, pregnancy-hemorrhage interaction effect, P < 0.001). Blood pressure tended to be lower in pregnant than in virgin females (pregnancy effect, P < 0.01; pregnancy-hemorrhage interaction effect, P > 0.20). This difference was most prominent during the posthemorrhage recovery period (Fig. 1; 40 to 90 min), during which time mean arterial pressure was an average of 14 mmHg less in midpregnant animals than in virgin females and was 9 mmHg less in early pregnant animals than in virgin females (Holm–Šidák comparison; P < 0.01 for midpregnant vs. virgin, P < 0.05 for early pregnant vs. virgin). Blood pressure had returned to normotensive levels in all three groups by 22 h posthemorrhage.

All three groups of animals exhibited bradycardia immediately following 13 ml/kg blood loss and showed a gradual reversal of the bradycardia during the first 45 min posthemorrhage (Fig. 1, RM ANOVA for hemorrhage effect, P < 0.001). In contrast to the effect on blood pressure, there was no statistically significant effect of pregnancy on the heart rate response to blood loss (RM ANOVA for pregnancy and pregnancy-hemorrhage interaction effects, P > 0.20 for each). This suggests that the lower posthemorrhage blood pressure observed in pregnant animals was primarily caused by differences in vascular tone rather than by differences in neural control of cardiac function.

Hematocrit, plasma protein concentration, and plasma osmolality in pregnant and intact virgin females. Hematocrit decreases after hemorrhage because the vascular refilling brought about by transcapillary fluid influx results in hemodilution. The magnitude of decrease in hematocrit therefore provides a useful index of the magnitude of plasma volume restoration. In all three groups of animals, hematocrit showed a gradual decrease during the first 3 h posthemorrhage and decreased further between 3 and 22 h posthemorrhage. Hematocrit was lower in animals hemorrhaged during midgestation than in virgin females at 90 min after initiation of the hemorrhage procedure (Fig. 2; RM ANOVA, pregnancy-hemorrhage interaction effect, P < 0.01). Calculations of posthemorrhage plasma volume derived from hematocrit values (calculations as previously described in Ref. 37) indicate that whereas virgin and early pregnant animals had both restored 6 ± 1 ml/kg of fluid to the blood by 90 min posthemorrhage, animals hemorrhaged during midgestation had restored 9 ± 1 ml/kg (P < 0.05). However, the overall magnitude of decrease in percent

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**Table 1. Basal values for pregnant and virgin female rats**

<table>
<thead>
<tr>
<th></th>
<th>Virgin Metestrus</th>
<th>Early Pregnancy, days 6–9</th>
<th>Midgestation days 12–14</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10–11</td>
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<tr>
<td>Body weight, gm</td>
<td>269±5</td>
<td>288±3*</td>
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<td>Uterine weight, mg</td>
<td>0.7±0.1</td>
<td>1.8±0.3*</td>
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<tr>
<td>Serum estradiol, pg/ml</td>
<td>3.7±0.4</td>
<td>6.0±0.7</td>
<td>9.1±2.4*</td>
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<td>Serum progesterone, ng/ml</td>
<td>17.6±3.6</td>
<td>49.2±6.7†</td>
<td>89.6±6.2‡</td>
</tr>
<tr>
<td>Plasma osmolality, mOsmol/kg</td>
<td>283±1</td>
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<td>276±1†‡</td>
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<tr>
<td>Hematocrit, %</td>
<td>36.8±0.6</td>
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<tr>
<td>Plasma protein concentration, gm/l</td>
<td>64.0±1.4</td>
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<td>Plasma volume, ml</td>
<td>10.3±0.3</td>
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<td>Plasma volume, ml/kg</td>
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<td>Blood volume, ml</td>
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<td>Blood volume, ml/kg</td>
<td>57.4±1.3</td>
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<td>Total plasma protein content, gm</td>
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<td>Total plasma protein content, gm/kg</td>
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Data are means ± SE; n is number of rats. * P < 0.05, P < 0.01 vs. virgin female in metestrus phase of estrous cycle; † P < 0.05, P < 0.01 vs. days 6–9 of pregnancy.

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The mineralocorticoid, aldosterone, has recently been shown to play a role in the renin-angiotensin system, which is involved in cardiovascular regulation. The renin-angiotensin system is activated in response to decreases in blood pressure or increased plasma volume expansion. This activation stimulates the secretion of aldosterone, which has a powerful vasoconstrictor effect. In pregnant females, aldosterone levels are significantly increased compared to nonpregnant females. This increased aldosterone production in pregnant females may contribute to the increased blood pressure observed in pregnancy. Aldosterone has a direct effect on vascular smooth muscle, causing vasoconstriction and narrowing of the blood vessels.

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**Fig. 1. Effect of hemorrhage on mean arterial pressure (MAP) and heart rate (HR) in conscious pregnant (Preg) and metestrus virgin female rats. Two 6.5 ml/kg blood samples were withdrawn during the time periods shown by the cross-hatched bars, to a cumulative blood loss of 13 ml/kg over 18 min. The average MAP during the 70-min posthemorrhage recovery period was significantly lower in pregnant animals than in virgin females (ANOVA, P < 0.01), whereas there was no statistically significant effect of pregnancy on the heart rate response to hemorrhage. Solid square, virgin female hemorrhaged during the metestrus phase of the estrous cycle (n = 11); open triangle, early pregnancy (gestation days 6–9; n = 9); shaded circle, midpregnancy (gestation days 12–14; n = 8). * P < 0.05 for midpregnant vs. metestrus virgin; † P < 0.05 vs. –2 min for metestrus virgin; ‡ P < 0.05 vs. –2 min for early pregnant; § P < 0.05 vs. –2 min for midpregnant. Data are means ± SE.**

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Data are means ± SE; n is number of rats. * P < 0.05, P < 0.01 vs. virgin female in metestrus phase of estrous cycle; † P < 0.05, P < 0.01 vs. days 6–9 of pregnancy.
Plasma volume, blood volume, and total plasma protein content in pregnant and intact virgin females. Plasma volume measured as the 5-min volume of distribution of Evans blue dye was higher at 22 h posthemorrhage than before hemorrhage (Fig. 3; RM ANOVA, \( P < 0.001 \)). The extent of vascular refilling was sufficient not only to replace all of the plasma volume lost in hemorrhage, but to restore total blood volume nearly to basal levels within 22 h posthemorrhage, to 93 ± 1, 91 ± 2, and 98 ± 2% of basal blood volume in virgin, early pregnant, and midpregnant females, respectively (ANOVA, \( P = 0.085 \)). The magnitude of plasma volume and blood volume restitution was not significantly altered by pregnancy (RM ANOVA of values expressed as ml/kg; pregnancy and pregnancy-hemorrhage interaction effects, \( P > 0.10 \) for all).

In contrast, plasma protein restitution was significantly augmented during midpregnancy. By 22 h posthemorrhage, total plasma protein content was restored to basal levels in virgin and early pregnant females but exceeded basal levels in midpregnant females (Fig. 3; RM ANOVA pregnancy-hemorrhage interaction effect, \( P < 0.01 \)). The amount of protein restored to the plasma in midpregnant animals exceeded the amount removed during hemorrhage and subsequent blood sampling procedures by 46 ± 12% and was significantly greater than in either early pregnant or virgin females (Fig. 4; RM ANOVA pregnancy-hemorrhage interaction effect, \( P < 0.01 \)).

Progesterone, estrogen, and basal values in ovariectomized animals. In ovariectomized animals without steroid replacement, serum estradiol levels were below the detectable limits of the assay (<2.5 pg/ml), and serum progesterone concentra-

hematocrit over the 22-h posthemorrhage observation period did not differ between groups (virgin, 14 ± 1; early pregnant, 14 ± 1; midpregnant, 13 ± 1), indicating that pregnant and virgin females ultimately restored their plasma volumes to a similar extent.

Plasma protein concentration also decreased during the first 3 h after initiation of the hemorrhage but subsequently increased between 3 and 22 h posthemorrhage, reaching significantly higher levels in animals hemorrhaged during midgestation than in early pregnant or virgin females (Fig. 2; RM ANOVA for hemorrhage effect, \( P < 0.001 \); pregnancy-hemorrhage interaction effect, \( P < 0.01 \)). Plasma protein concentration was fully restored to control levels at 22 h posthemorrhage in midpregnant animals but remained significantly lower than initial control levels in virgin and early pregnant animals.

Plasma osmolality remained significantly lower in animals hemorrhaged during midgestation than in those hemorrhaged during early pregnancy or metestrus for the duration of the posthemorrhage observation period (\( P < 0.001 \)). There was no change in plasma osmolality following blood loss in either pregnant or virgin females (Fig. 2).
tion was 3.2 ± 0.5 ng/ml. Chronic steroid hormone replacement raised the serum concentration of estradiol to 21.6 ± 3.3 pg/ml, which is approximately twice that of midgestation. The serum progesterone concentration in steroid-replaced animals was similar to that of metestrus and substantially lower than either early or midpregnancy (15.4 ± 1.5 ng/ml). The biological efficacy of the steroid replacement regimen was confirmed by the increased uterine weight of animals with chronic estrogen-progesterone replacement compared with those without steroid replacement (0.76 ± 0.02 vs. 0.39 ± 0.04 g; P < 0.001). Ovariectomized animals without steroid replacement gained weight during the 2-wk period following surgery, whereas animals with chronic steroid replacement did not and consequently weighed less than animals without replacement on the day of hemorrhage (258 ± 5 vs. 294 ± 10 g; P < 0.01).

Basal plasma volume measured at the start of the hemorrhage procedure was nearly identical in ovariectomized animals with and without steroid replacement when expressed as milliliters of total volume (not normalized to body weight; Table 2), and basal plasma protein concentration was not significantly different between groups (70.3 ± 3.3 g/l with steroid replacement; 63.4 ± 1.6 g/l without replacement; P = 0.08). However, because animals with steroid replacement weighed less, basal values for both plasma volume and total plasma protein content were significantly greater in animals with steroid replacement than in those without replacement when normalized to body weight (Table 2). Animals with chronic steroid replacement also had a reduced basal hematocrit (32 ± 1%) compared with those without replacement (38 ± 1%; P < 0.001). Consequently, basal blood volume (expressed as ml/kg) did not differ between animals with and without steroid replacement.

Basal plasma osmolality was the same in both groups (288 ± 2 mOsmol/kg with steroid replacement; 288 ± 1 mOsmol/kg with no steroid replacement) and did not change in response to blood loss.

Response to hemorrhage in ovariectomized animals. The 16 ml/kg hemorrhage resulted in the loss of 23 ± 1% of initial blood volume in both groups of ovariectomized animals. Ovarian steroid replacement did not alter the blood pressure response to hemorrhage. Mean arterial blood pressure decreased by 65 mmHg following blood loss and had returned toward normotensive levels within 40 min posthemorrhage in both groups of ovariectomized animals (RM ANOVA hemorrhage effect, P < 0.001; steroid and hemorrhage-steroid interaction effects, P > 0.20). The hypotensive response to blood loss was accompanied by significant bradycardia in both groups.

In both groups of ovariectomized animals, hematocrit began to decrease after the first blood withdrawal and continued to decrease over the following 22 h. Steroid-replaced animals had a lower hematocrit throughout the experimental period than animals without steroid replacement, but the rate of hematocrit decrease did not differ between groups (Fig. 5; RM ANOVA hemorrhage and steroid effects, P < 0.001; hemorrhage-steroid interaction effect, P > 0.20). The overall magnitude of decrease in percent hematocrit over the 22-h posthemorrhage observation period was 13 ± 1% for animals with steroid replacement and 14 ± 1% for those without replacement, indicating that the extent of vascular refilling was not altered by the presence or absence of estrogen and progesterone. This was confirmed by the plasma volume measurements that showed that both groups restored plasma volume back to 108% of basal levels (Table 2; RM ANOVA for ml/kg plasma volume; hemorrhage effect, P < 0.001; steroid effect, P < 0.05; hemorrhage-steroid interaction effect, P > 0.20). The extent of vascular filling was sufficient to return total blood volume to 91 ± 2 and 88 ± 2% of initial blood volume in animals with and without steroid replacement, respectively (P > 0.20; RM ANOVA for ml/kg blood volume: hemorrhage effect, P < 0.001; steroid and hemorrhage-steroid interaction effects, P > 0.20).

In contrast to the effect of pregnancy, plasma protein restitution was not altered by steroid replacement in ovariectomized animals. Plasma protein concentration initially decreased after hemorrhage and was partially restored during the remainder of the 22-h posthemorrhage recovery period (Fig. 5; RM ANOVA hemorrhage effect P < 0.001, hemorrhage and hemorrhage-steroid interaction effects, P > 0.10). Although total plasma protein content (normalized to body weight) was higher both before and after hemorrhage in animals with steroid replacement than in those without (Table 2), both groups restored approximately the same amount of plasma protein as was lost

Table 2. Plasma volume, blood volume and plasma protein content in hemorrhaged ovariectomized animals with and without ovarian steroid hormone replacement

<table>
<thead>
<tr>
<th>Time After Hemorrhage</th>
<th>No Steroid Replacement</th>
<th>With Steroid Replacement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 7</td>
<td>n = 7</td>
</tr>
<tr>
<td>Plasma volume, ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>12.6 ± 0.6</td>
<td>12.4 ± 0.4</td>
</tr>
<tr>
<td>+22 h</td>
<td>13.6 ± 0.6‡</td>
<td>13.4 ± 0.5‡</td>
</tr>
<tr>
<td>Plasma volume, ml/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>42.8 ± 1.8</td>
<td>47.9 ± 1.1*</td>
</tr>
<tr>
<td>+22 h</td>
<td>46.4 ± 2.3‡</td>
<td>51.2 ± 1.5§</td>
</tr>
<tr>
<td>Blood volume, ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>20.3 ± 1.0</td>
<td>18.2 ± 0.4</td>
</tr>
<tr>
<td>+22 h</td>
<td>17.8 ± 0.8‡</td>
<td>16.5 ± 0.5§</td>
</tr>
<tr>
<td>Blood volume, ml/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>68.9 ± 2.4</td>
<td>70.5 ± 1.1</td>
</tr>
<tr>
<td>+22 h</td>
<td>60.6 ± 3.08</td>
<td>64.0 ± 1.6§</td>
</tr>
<tr>
<td>Total plasma protein content, g/m</td>
<td>0.83 ± 0.03</td>
<td>0.90 ± 0.03</td>
</tr>
<tr>
<td>0 h</td>
<td>2.81 ± 0.08</td>
<td>3.51 ± 0.14†</td>
</tr>
<tr>
<td>+22 h</td>
<td>2.87 ± 0.16</td>
<td>3.40 ± 0.16*</td>
</tr>
</tbody>
</table>

Data are means ± SE; n is number of rats. *, †P < 0.05, P < 0.01 vs. ovariectomized without steroid replacement, at the same protocol time; ‡, §P < 0.05, P < 0.01 vs. time 0 for the same group of animals.
during the hemorrhage procedure (steroid replaced: 1.12 ± 0.04 g/kg lost, 1.02 ± 0.16 restored; no steroid: 0.89 ± 0.02 g/kg lost, 0.95 ± 0.16 restored). Consequently, both groups had restored total plasma protein content back to initial control levels by the end of the 22-h posthemorrhage recovery period (RM ANOVA for g/kg plasma protein content: steroid effect, P > 0.20 for each). Shaded bar, ovariectomized, no steroid replacement (n = 7); hatched bar, ovariectomized with steroid replacement (n = 7); *P < 0.001 vs. control (0 min) for same group of animals; *P < 0.05 vs. no steroid replacement at the same sampling time.

DISCUSSION

The expanded blood volume of pregnancy is vigorously and effectively defended after a hemorrhage. In animals hemorrhaged during midgestation, blood volume was restored back to control levels within 22 h. Their restitution response was accelerated compared with virgin females hemorrhaged during metestrus in two respects. Animals hemorrhaged during midgestation showed a more rapid rate of plasma volume replacement during the early phase of vascular refilling than virgin females, as evidenced by the greater decrease in hematocrit during the first 90 min after blood loss. The major effect of pregnancy, however, is that the restoration of plasma protein is significantly enhanced during midgestation. Whereas virgin females restored approximately the same amount of plasma protein as was removed during the hemorrhage and subsequent blood sampling procedures, animals hemorrhaged during midgestation restored nearly 50% more protein to the plasma than had been removed. By 22 h posthemorrhage, both virgin and midpregnant animals had restored plasma volume to greater than basal levels. Consequently, plasma protein concentration remained below basal levels in virgin females, whereas midpregnant animals fully restored plasma protein concentration back to basal levels by 22 h posthemorrhage.

Plasma protein restitution plays a critical role in vascular refilling by maintaining the transcapillary oncotic pressure gradient high enough to favor continued fluid influx. Similarly, plasma protein content plays a key role in the progressive expansion of blood volume during pregnancy. Total plasma protein content increases during human pregnancy (45), and as shown in this study, also increases during pregnancy in the rat. The increase in plasma protein content serves to maintain plasma protein concentration and the resulting transcapillary oncotic pressure gradient high enough to support appropriate distribution of the expanded extracellular fluid volume between the plasma and interstitial fluid compartments (16). Thus the accelerated plasma protein restitution response of animals hemorrhaged during midgestation not only supports vascular refilling but also rapidly restores plasma protein concentration back to the levels needed for continued pregnancy-induced plasma volume expansion.

In contrast to animals hemorrhaged during midgestation, those hemorrhaged during early pregnancy showed restitution responses that did not differ from virgin females hemorrhaged during metestrus. Posthemorrhage plasma volume restoration was sufficient to restore blood volume nearly to control levels, and the magnitude of plasma volume replacement (normalized to body weight) at 22 h posthemorrhage did not differ between midpregnant, early pregnant, and virgin females. However, the amount of plasma protein replaced after hemorrhage was no greater in early pregnancy than in nonpregnant animals and was significantly less than in animals hemorrhaged during midgestation. Accordingly, plasma protein concentration remained below basal levels at 22 h posthemorrhage in animals hemorrhaged during early pregnancy just as was the case for metestrus females. This suggests that a hemorrhage that occurs early in pregnancy may pose a greater risk to fetal health than a hemorrhage during midgestation, because the slower restoration of plasma protein concentration jeopardizes the capacity for continued plasma volume expansion.

We have previously shown (37) that both plasma protein restitution and plasma volume restitution are significantly enhanced in female rats hemorrhaged during the proestrus phase of the estrous cycle. Recent studies from other laboratories have additionally shown that, compared with male rats, female subjects to resuscitated hemorrhagic shock (hypotensive hemorrhage with soft tissue trauma, followed by volume expansion with electrolyte solution) during proestrus show improved cardiac output and tissue perfusion (2, 22), decreased fluid loss into the interstitium (27), and reduced protein extravasation in the lung (8). Furthermore, females subjected to massive hemorrhagic splenic injury during proestrus have a significantly longer survival time than males or metestrus females (26). Thus the enhanced protein restitution observed in hemorrhaged proestrus females correlates with multiple indices of improved vascular protein and fluid retention, and with a significant survival advantage following traumatic hemorrhage. This raises the possibility that midgestation, similar to the proestrus phase, not only enhances the protein restitution response to blood loss but may also confer a significant maternal survival advantage.
Serum estradiol and progesterone levels are maximal during the proestrus phase compared with the remainder of the estrous cycle, suggesting that the advantages conferred to females hemorrhaged during the proestrus phase may be due to effects of the ovarian steroid hormones. Progesterone and estradiol levels are also increased during midgestation. It is unlikely, however, that the enhanced protein restitution observed in animals hemorrhaged during midgestation is a function solely of the effects of one of these steroid hormones. The fact that protein restitution is augmented during midgestation but not during early pregnancy provides evidence that conditions that enhance protein restitution develop slowly over gestation. The serum progesterone levels of animals hemorrhaged during early pregnancy were nearly threefold higher than those of metestrus females and were as high as the peak progesterone levels reported for animals in proestrus (35), indicating that elevated progesterone levels alone are not sufficient to significantly enhance protein restitution. Serum estradiol levels were moderately elevated in animals hemorrhaged during midgestation but not during early pregnancy, suggesting that enhanced plasma protein restitution may instead be mediated by estradiol. However, this hypothesis is not supported by results of our experiments performed in ovariectomized animals with and without steroid replacement. In animals with chronic ovarian steroid replacement that raised serum estradiol concentration to twofold that of midgestation but maintained serum progesterone at a level comparable to metestrus, posthemorrhage plasma protein restitution did not differ from that of ovariectomized animals with no steroid replacement. Furthermore, in a study performed in rabbits, which differ from both humans and rats in that there is no gestational increase in estradiol levels, there was, nonetheless, a modest enhancement of plasma protein restoration after a hemorrhage performed early in the second trimester of pregnancy (43). Thus elevated estradiol alone also is unlikely to account for augmented protein restitution during midpregnancy. Nonetheless, the failure of elevated levels of either progesterone or estradiol alone to augment posthemorrhage plasma protein restitution does not preclude the possibility that these hormones contribute to augmented protein restitution in the context of the complex endocrine and paracrine environment of midgestation.

The restoration of plasma protein content after blood loss is believed to result from redistribution of preformed plasma from the interstitium to the plasma (30). Posthemorrhage protein restitution may be largely accounted for by a decreased rate of protein escape from the vasculature (41), coupled with a sustained rate of protein return by the lymphatic system (29). Maximal lymphatic pumping ability is increased during pregnancy (42) and could potentially contribute to enhanced plasma protein restitution after hemorrhage. Other factors, such as fluid absorption from the lumen of the gut, can also contribute to late-phase protein and fluid restitution (12). Pregnancy causes multiple endocrine and paracrine changes that alter vascular tone and permeability and could thereby affect posthemorrhage plasma protein restitution by altering the rate of transcapillary protein flux. Endothelial nitric oxide production is markedly increased during pregnancy (20). In addition to its vasodilator properties, nitric oxide has complex effects on vascular permeability (34). Estradiol can affect vascular tone and permeability both directly and by interacting with other vasoactive agents (31). The vascular effects of estrogen are modified in the presence of progesterone (31). For example, the increase in vascular permeability caused by atrial natriuretic peptide is augmented by estrogen alone but is decreased by combined administration of estrogen plus progesterone (39). Thus the ratio of estrogen and progesterone concentrations, as well as the ratio of concentrations of these steroid hormones relative to those of other vasoactive endocrine and paracrine factors, appear to be critical determining factors for vascular permeability to protein. The enhanced posthemorrhage plasma protein restitution observed during midgestation may therefore reflect advantageous changes in vascular permeability to protein, brought about by interactions between the effects of vasoactive hormones and neurotransmitters released during hemorrhage (vasopressin, angiotensin, catecholamines; see Ref. 36) and the complex endocrine/paracrine environment of pregnancy.

It should be noted that the factors that elicit enhanced protein restitution may differ for proestrus and midgestation. In our previous study (37) of estrous cycle effects on blood volume restitution, female rats hemorrhaged during the proestrus phase showed a markedly enhanced increase in plasma osmolality compared either with males, or with females hemorrhaged during metestrus, in addition to enhanced plasma protein restitution. Increased extracellular fluid osmolality promotes intracellular-to-interstitial compartment fluid shifts, and it has been proposed that the resulting increase in interstitial fluid hydrostatic pressure accelerates the redistribution of both water and preformed protein from the interstitium to the blood during the late phase of restitution (7, 32). Accordingly, the enhanced plasma volume and protein restitution response observed in proestrus females may have been mediated, in part, by their enhanced osmotic response to hemorrhage. In the present study, basal plasma osmolality was 7 mOsmol/kg lower in animals studied during midgestation than in metestrus or early pregnancy. Decreased plasma osmolality during pregnancy is the consequence of a decrease in the threshold for osmotic stimulation of vasopressin release (28). Hemorrhage did not cause plasma osmolality to increase in any group of animals in the present study, probably because the magnitude of blood loss in the present study (22–23% of initial blood volume) was smaller than that in our earlier study (37) in proestrus females (33% of initial volume). The absence of an osmotic response to hemorrhage indicates that intracellular-to-extracellular fluid shifts are unlikely to contribute to the enhanced protein restitution response of midgestation.

Near-term pregnant animals have an impaired ability to maintain normotensive blood pressure during hemorrhage that may be accounted for, at least in part, by decreased baroreflex sensitivity in concert with attenuated reflex increases in vasopressin (5, 10, 21). The effect of pregnancy on baroreflex sensitivity is attributed to alterations in central nervous system processing of cardiovascular afferent signals brought about by the hormonal milieu of pregnancy. One prominent candidate for mediation of these effects is the neuroactive progesterone metabolite 3α-dihydroprogesterone (21, 40). Decreased baroreflex sensitivity and impaired ability to maintain normotensive blood pressure during progressive blood loss do not develop until late in pregnancy (5, 21, 33). Animals hemorrhaged during midgestation maintain normotensive blood pressure during progressive blood loss as well as virgin females (metestrus or proestrus). Animals hemorrhaged during mid-
gestation also increase plasma renin activity to the same extent as virgin females, and show the same increase in the number of Fos-immunoreactive cell nuclei in the supraoptic hypothalamic nucleus, suggesting that the vasopressin response to blood loss is also not impaired during midgestation (23). However, certain central nervous system responses to blood loss are already attenuated by midgestation. Animals hemorrhaged during midgestation showed reduced activation of the lateral parabrachial nucleus as indicated by reduced numbers of Fos-immunoreactive cell nuclei (23). The lateral parabrachial nucleus plays an essential role in mediating spontaneous blood pressure recovery after blood loss (4), suggesting that the ability to restore blood pressure after a hypotensive blood loss may be compromised during midgestation.

The present study confirms that the initial compensatory response to moderate hemorrhage is not significantly altered during early or midgestation; both pregnant and virgin females maintained blood pressure within ~15 mmHg of basal levels after a 6.5 ml/kg blood loss and developed hypotension (blood pressure decrease of 45–55 mmHg) immediately after 13 ml/kg blood loss. However, animals hemorrhaged during early or midgestation showed a sustained hypotensive response to 13 ml/kg blood loss that was greater in magnitude and duration than that of virgin females. This effect was most prominent in animals hemorrhaged during midgestation in whom blood pressure remained significantly lower than basal levels at 70 min after completion of the hemorrhage, whereas blood pressure had returned to the normotensive range in virgin females within 40 min posthemorrhage. This contrasts with results of a previous study (23) from our laboratory in which pregnant and virgin animals were exposed to a more severe, repetitive blood loss (16 ml/kg hemorrhage, followed by additional blood sampling). In that study, blood pressure remained significantly below basal levels throughout the posthemorrhage recovery period not only in animals hemorrhaged during midgestation but also in virgin females hemorrhaged during metestrus or proestrus. Taken together, these observations suggest that an impaired ability to compensate for blood loss begins to develop early in pregnancy, but initially affects only the posthemorrhage recovery phase, and is best demonstrated following a relatively modest fixed-volume blood loss.

In summary, the major effect of pregnancy on the restitution response to hemorrhage is that plasma protein restoration is substantially augmented during midgestation. Consequently, animals hemorrhaged during midgestation are able to fully restore plasma protein concentration back to basal levels within 22 h posthemorrhage. This is important because plasma protein provides the transcapillary oncotic pressure gradient essential for appropriate distribution of the expanded extracellular fluid between the plasma and interstitial fluid compartments, and thereby permits the continued plasma volume expansion essential for maternal and fetal well-being.

The augmented protein restitution response to blood loss develops slowly over the course of pregnancy and is not present during early pregnancy. Plasma progestrone levels are markedly elevated in early pregnancy, indicating that elevated progesterone alone is not sufficient to augment the protein restitution response. Results of our experiments in ovariectomized animals with steroid replacement indicate that elevated estradiol, in the presence of moderate progestrone levels comparable to those present during metestrus, also fails to augment posthemorrhage protein restitution. Thus the factors that mediate augmented protein restitution in animals hemorrhaged during midgestation are complex and cannot be attributed solely to the effects of increased levels of either estradiol or progesterone alone.

Finally, animals studied during both early pregnancy and midgestation are more hypotensive than virgin females during the posthemorrhage recovery period, suggesting that there is a deficit in the ability to restore blood pressure during hypovolemia that begins to develop early in pregnancy.

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GRANTS

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