Progesterone Exacerbates Striatal Stroke Injury in Progesterone-Deficient Female Animals

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Background and Purpose—We have previously shown that female animals experience substantial protection from brain injury after reversible middle cerebral artery occlusion (MCAO) compared with their male or ovariectomized female counterparts. The reproductive steroid estrogen has been shown to provide neuroprotection from a variety of experimental insults, but the importance of progesterone as an anti-ischemic treatment has not been well explored. We evaluated histological outcomes after MCAO in ovariectomized female rats with or without acute or chronic progesterone replacement therapy.

Methods—Age-matched, adult female Wistar rats were ovariectomized and treated with 0, 30, or 60 mg/kg progesterone IP 30 minutes before ischemia (n = 12 to 14 per group) or with 30 mg/kg progesterone IP daily for 7 to 10 days before ischemia (n = 16). Each animal subsequently underwent 2 hours of MCAO with the intraluminal filament technique, followed by 22 hours of reperfusion. Ipsilateral parietal cortex perfusion was monitored with laser Doppler flowmetry throughout ischemia. Cortical, caudate-putamen, and hemispheric infarction volumes were determined with 2,3,5-triphenyltetrazolium chloride staining and digital image analysis.

Results—Intraischemic plasma progesterone levels were 5 ± 3, 102 ± 20,* 181 ± 28,* and 133 ± 25,* ng/mL in the 0, 30, and 60 mg/kg acute progesterone group and the 30 mg/kg chronic progesterone group, respectively (*P < 0.05 compared with 0 mg/kg). Caudate-putamen infarction volume (percent contralateral structure) was significantly increased by chronic progesterone treatment: 45.6 ± 5.1%* in the 30 mg/kg chronic progesterone group and 29.2 ± 5.3%, 35.8 ± 5.1%, and 42.0 ± 5.0% in the 0, 30, and 60 mg/kg acute progesterone groups, respectively (*P < 0.05 compared with 0 mg/kg). Cortical and total hemispheric infarction volumes (percent contralateral structure) were unchanged by progesterone treatment.

Conclusions—Exogenous progesterone therapy does not ameliorate histological injury after MCAO in previously ovariectomized, adult female rats. Furthermore, chronic progesterone administration can exacerbate infarction in subcortical regions. (Stroke. 2000;31:1173-1178.)

Key Words: cerebral ischemia ■ hormone replacement therapy ■ progesterone ■ stroke, experimental ■ rats

Stroke risk is lower in premenopausal women than in men of the same age, but vascular events increase in frequency after menopause. However, the benefit of hormone replacement therapy for stroke is not clearly defined. We have previously shown that female animals sustain less brain injury from middle cerebral artery occlusion (MCAO) than do age-matched male animals and that this advantage is abolished with the loss of endogenous female sex steroids through ovariectomy. Hence, both estrogen and progesterone must be considered as neuroprotective candidates. Ongoing inquiries in various laboratories have been centered on estrogen, yet little is known about progesterone and ischemic injury in the female. Furthermore, current hormone replacement therapy for postmenopausal women incorporates progestin combined with estrogen, so it is important to evaluate the efficacy of progestin compounds in stroke. The purpose of the present study was to determine whether there are differences in infarction volume in progesterone-treated, previously ovariectomized female rats compared with hormone-deficient female rats.

Materials and Methods

The present study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research, and the protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University. All methods are as previously described. Briefly, age-matched, sexually mature female Wistar rats (200 to 225 g) were ovariectomized 1 to 2 weeks before progesterone treatments (50 mg/mL progesterone USP; Schein) and MCAO. Ovariectomized female rats received either no hormone (P0, n = 14), 30 mg/kg progesterone (P30, n = 14), or 60 mg/kg progesterone (P60, n = 12) IP 30 minutes before MCAO. In an...
additional cohort, ovariectomized female rats were administered 30 mg/kg progesterone (chronic P30, n=16) IP daily for 7 to 10 days before MCAO. For MCAO, the animal was anesthetized with halothane (1.25% to 1.5% delivered via mask in O2-enriched air), and a femoral artery catheter was placed for the continuous monitoring of mean arterial blood pressure and the measurement of arterial blood gases. Rectal and temporalis muscle temperatures were monitored with a Mon-a-therm system (model 6510; Mallinckrodt Medical, Inc) and maintained with a heat lamp. The effectiveness of vascular occlusion was determined by the reduction in the laser Doppler flow signal (LDF) (model MBF3D; Moor Instruments Ltd) obtained with probe placement 6 mm lateral and 2 mm posterior to bregma.2,3 Focal cerebral ischemia was accomplished with a modified intraluminal filament technique as previously described.4 Briefly, the right common carotid and external carotid arteries were exposed and ligated. The right occipital artery was cauterized, and then the right pterygopalatine artery was ligated. After the baseline LDF was determined, a 4–0 nylon monofilament surgical suture with a heat-blunted tip was introduced via the right common and external carotid arteries. After the baseline recording averages were analyzed for each time point. The animal was killed 22 hours after the 2-hour MCAO period, and the brain was harvested for staining with a 1% solution of 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co.) and subsequently fixed in 10% neutral buffered formalin. Infarction volumes in cerebral cortex, caudate-putamen complex, and hemisphere were determined with digital image analysis (SigmaScan Pro; Jandel) and expressed either in mm³ or indirectly as a percent of the contralateral structure (cortex, caudate-putamen, or hemisphere). Direct infarction volumes were determined through subtraction of the ipsilateral noninfarcted volume of the structure of interest from the volume of the contralateral structure. This value was then divided by the volume of the contralateral structure and multiplied by 100 to yield the infarction volume as a percent of the contralateral structure. Blood was obtained 1 hour into ischemia and at 22 hours' reperfusion for steroid analysis. Plasma 17β-estradiol and progesterone levels were measured in duplicate with radioimmunoassay (Coat-a-count; Diagnostic Products Corp), as previously described.5

All values are reported as mean±SEM unless otherwise indicated. Physiological parameters and LDF were subjected to 2-way ANOVA with post hoc Newman-Keuls test for multiple comparisons. Plasma hormone levels were analyzed with t test with Bonferroni's correction. Differences in mean ischemic LDF, cortical, and hemispheric infarction volumes were determined with 1-way ANOVA. If significant differences were observed with 1-way ANOVA, a post hoc Tukey’s test was performed. Striatal infarction volume was analyzed with t test with Bonferroni’s correction. The criterion for statistical significance was P<0.05.

Results
Baseline, intraschismic, and reperfusion arterial blood pressure and blood gases, glucose, and hemoglobin measurements were equivalent among experimental groups (Table). However, arterial CO₂ tension was higher at 60-minute MCAO in all progesterone-treated rats and at preischemia and reperfusion in the chronic P30 group relative to the P0 group, although all values remained within normal limits for the rat. Intraschismic rectal and temporalis muscle temperatures were controlled in all groups at 38.1° to 38.8°C and 36.3° to 37.0°C, respectively. Intraschismic plasma estradiol was not different among groups: 0±0.0, 0±0.1, 1±1, and 5±2 pg/mL in P0, P30, P60, and chronic P30 groups, respectively. Progesterone levels were elevated relative to untreated, ovariectomized animals within the ischemic period (Table) but fell by 22 hours of reperfusion: 5±2, 10±3, 33±11, and 8±1 ng/mL in P0, P30, P60, and chronic P30 groups, respectively. Figure 1 illustrates the lack of effect of progesterone on cortical infarction volume at either acute dose tested compared with the P0 female rats. Similarly, acute treatment did not improve caudate-putamen injury (percent of contralateral caudate-putamen): 29.2±5.3% for P0 versus 35.8±5.1% and
42.0\pm5.0\% for P30 and P60, respectively. However, striatal damage was increased by chronic administration (45.6\pm5.1\%, P0.05 compared with P0). Figure 2 summarizes residual intraischemic LDF (expressed as a percent of baseline signal) and emphasizes the lack of difference in reduction of LDF signal among treatment groups. Mean ischemic LDF in ovariectomized rats with progesterone replacement (24.4\pm2.7\% in P30, 25.7\pm2.3\% in P60, 27.4\pm2.5\% in chronic P30) was not different from that observed in P0 (26.9\pm2.2\%).

Discussion
The present study demonstrates 2 important findings. First, exogenous progesterone treatment over a range of physiological and supraphysiological peak plasma levels did not improve brain injury after vascular occlusion in young adult, ovariectomized female rats. Second, chronic hormone exposure at high doses can exacerbate infarction volume after reversible MCAO. These findings suggest that progesterone is not neuroprotective in the ischemic female rat brain when administered as a single injection and that exogenous steroid has a dose- or exposure-dependent, deleterious action in experimental stroke.

Our findings are not explained by differences in physiological parameters among treatment groups. Arterial CO\textsubscript{2} tension was statistically higher at 60-minute MCAO in all progesterone-treated rats and at all time points in the chronic P30 group compared with the P0 group. It seems unlikely that progesterone would increase CO\textsubscript{2} production. However, many members of the progestin neurosteroid family have mild anesthetic properties. It may be possible that progesterone at high doses provided an additional anesthetic effect with halothane inhalation and resulted in a slightly reduced ventilatory rate and thus retained CO\textsubscript{2}. However, these small increases in arterial CO\textsubscript{2} are unlikely to account for the deleterious effect of progesterone on infarction volume due to changes in tissue pH or altered cerebral perfusion. On the contrary, a relative hypercapnia would increase cerebral blood flow, not depress perfusion. Furthermore, the LDF signal was reduced by a similar percentage of baseline values in all groups, suggesting that the ischemic insult was equivalent among all groups. Differences in the recovery of cerebral flow during early reperfusion cannot be excluded with our measurements, and further studies are needed to quantify absolute cerebral blood flow in progesterone-treated animals. Although there is little evidence that progesterone is vasoactive in the cerebral circulation, large doses of the...
steroid induce in vitro coronary relaxation\textsuperscript{6} and enhance endothelium-dependent relaxation to agonists.\textsuperscript{7}

In the present study, progesterone treatment was associated with a larger infarct in the striatum but no changes in cortical infarct in young ovarioctomized female rats. This is in contrast to our previous findings in reproductively senescent female rats, in which progesterone (10 mg) reduced cortical injury\textsuperscript{8} and produced no deleterious histological outcome. However, it is not known whether mechanisms of ischemic injury and neuroprotection are similar in young adult versus reproductively senescent female rats. The lack of neuroprotection and exacerbation of striatal injury are also inconsistent with the evidence from the literature that reports beneficial effects of progesterone\textsuperscript{9} therapy in young animals. In models of traumatic brain injury, exogenous progesterone (4 mg/kg) in male rats has been shown to reduce secondary neuronal loss and to attenuate brain edema, independent of estrogen,\textsuperscript{10–13} via mechanisms linked to the reduction in free radical–induced lipid peroxidation.\textsuperscript{9,14} In male rats, pretreatment with progesterone significantly reduced brain edema during the early stages of ischemia.\textsuperscript{15,16} The administration of progesterone (4 to 10 mg/kg) before or after the onset of transient focal cerebral ischemia in male rats\textsuperscript{17} or of global ischemia in ovarioctomized cats\textsuperscript{18} greatly reduced ischemic cell damage and neurological deficits. One explanation for the divergence of the present data from previous reports could be linked to the dose and duration of progesterone used, as well as to differential effects linked to gender or age.

Although variations in plasma progesterone reflected individual differences in intraperitoneal absorption and metabolism of the injected drug, the chronic 30 mg/kg progesterone group demonstrated an average plasma progesterone level of 133 ng/mL compared with 102 and 181 ng/mL for the 30 and 60 mg/kg acute progesterone doses, respectively. In rat, plasma progesterone ranges from basal levels of 2 to 18 ng/mL to \( \approx 120 \) to 130 ng/mL in pregnancy, with intermediate values of 40 to 90 ng/mL during late proestrus.\textsuperscript{19–22} The low 30-mg treatment was intended to achieve robust elevation of intraschismic plasma progesterone within the physiological range, whereas the 60 mg/kg injection resulted in pharmacological levels. All treatment groups showed low levels by 22 hours of reperfusion, ranging from 6 to 33 ng/mL on average, or absolute decreases from ischemic levels, ranging from 92 to 148 ng/mL in progesterone treatment groups. Although previous studies in neurotrauma and cerebral ischemia provide only scattered reporting of hormone levels, it is likely that peak levels were lower than in the present study. We therefore cannot rule out the possibility that lower progesterone doses that yield basal plasma levels could be neuroprotective in female rats, as has been shown in male rats. Furthermore, only our chronic dosing regimen resulted in increased striatal injury, suggesting that exposure duration may be important, as well as peak steroid levels. In combination with the data in the literature, the present data suggest that the therapeutic window for progesterone may be narrow in both its efficacious actions\textsuperscript{8,17,18} and potential for exacerbation of ischemic injury. The mechanism for this exacerbation in not yet known.

It may be that the abrupt and large drop in plasma progesterone from ischemic values to those levels observed at 22 hours of reperfusion mimicked a withdrawal syndrome and contributed to striatal injury. Our previous study in reproductively senescent female rats, which demonstrated cortical protection with progesterone, used long-term steroid delivery via a subcutaneous hormone implant\textsuperscript{8} rather than a daily injection protocol. This method of hormone administration ensured sustained plasma progesterone levels during the reperfusion period rather than the declining levels we observed.

In women with premenstrual syndrome, anxiety, and increased seizure susceptibility are associated with sharp declines in circulating progesterone levels. Rats undergoing progesterone withdrawal exhibit greater seizure-like activity than do control animals.\textsuperscript{26} Abrupt progesterone withdrawal has been shown to markedly decrease \( \gamma \)-aminobutyric acid (GABA) current decay time, consequently decreasing inhibitory function and increasing seizure susceptibility.\textsuperscript{27} The withdrawal of progesterone also increased expression of the GABA\(_{\alpha} 4\) receptor \( \alpha 4\) subunit.\textsuperscript{27} Blockade of the \( \alpha 4\) gene transcript via antisense oligonucleotide administration prevented the previously observed decreases in GABA current decay time after progesterone withdrawal.\textsuperscript{27} It may be that the increase in striatal injury is due to both GABA current modulation and alterations in GABA\(_{\alpha} 4\) receptor \( \alpha 4\) subunit levels as a result of sharply declining plasma progesterone levels after ischemia. Glutamate also could be a target of hormonal neuromodulation with deleterious consequences. Naturally occurring sulfate esters of pregnenolone have distinct excitatory properties that are due in part to augmentation of \( N \)-methyl-D-aspartate receptor activation by the excitatory amino acid glutamate.\textsuperscript{28} Marginal effects on cortical injury by progesterone may be related to differences in regional expression of GABA, glutamate, and progesterone receptor subunits/subtypes or to different ischemic mechanisms in the striatum versus the cortex.

Potential cooperation in stroke mechanisms between progesterone and estrogen must also be considered. From an endocrinological standpoint, it is well known that the background steroid environment of the brain alters the ability of progesterone or estrogen to alter reproductive signaling and behavior.\textsuperscript{29,30} Our previous work and that of others indicate that estrogen does not require normal plasma progesterone levels to reduce stroke injury.\textsuperscript{2,3,8,31–34} However, estrogen priming is requisite for many progesterone-mediated actions in normal brain.\textsuperscript{29,30,35} We also know that these 2 steroids interact at the receptor level, because estrogen, alone or in combination with progesterone, has been shown to upregulate progesterone receptor expression in brain.\textsuperscript{36} Therefore, it may be that progesterone is most efficacious in an ischemic brain environment that has been estrogen primed.

These data have potential implications for clinical progestin-containing hormone replacement regimens and for an understanding of the relative importance of female sex steroids in stroke. It is currently uncertain whether combined estrogen-progestin therapy constitutes major cerebrovascular risks or benefits for women. Furthermore, unlike the estrogens, potential neuroprotective properties of progesterone have not been well investigated in cerebral ischemia or vascular disease in women.
Although cardioprotection has been reported with postmeno-
pausal estrogen or estrogen-progestin replacement, a limited
umber of epidemiological studies have evaluated progestins and stroke risk in humans.

In conclusion, we have demonstrated that in contrast to the
protection against ischemic injury observed in estrogen-
treated ovariectomized animals, chronic progesterone therapy
before ischemia at pharmacological doses exacerbates ischemic
striatal injury in ovariectomized female rats. The mechanism
remains to be determined, and further studies are
required to define dose-response relationships in experimental
stroke.

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References
27. Deleted in proof.
A host of experimental studies in both animals and humans have demonstrated that estrogen has definite cardiovascular protective effects, most notably an advantageous plasma lipid profile and increased activity of endothelium-derived vasodilators. Several recent studies have also clearly demonstrated that estrogen protects against brain injury in animal models of stroke. However, the cardiovascular protective promise of hormone replacement therapy (HRT) has not been borne out by recent prospective human studies. For example, the HERS study\(^1\) found no protective effect of HRT in women with preexisting coronary artery disease. A number of possible explanations for this lack of effect have been suggested; among these is the possibility that progestins, in particular medroxyprogesterone, may have untoward effects.\(^2\) It is in this context that findings of the current study need to be considered. In ovariectomized female rats, acute administration of progesterone had no effect on infarction volume after middle cerebral artery occlusion, but striatal damage was increased by chronic administration of progesterone. As the authors point out, other reports in the literature have found different effects, which could be explained by differences in gender, age of the animal, or progesterone dose or regimen of administration. Another potential area of importance is the possibility that progesterone and estrogen interact; because animals in the present study were ovariectomized, this would be the subject of future investigation. Nevertheless, the finding that administration of progesterone can actually exacerbate stroke injury underscores the importance of a more fundamental understanding of the mechanisms of action of gonadal steroids. Only with such basic insight together with results from carefully controlled clinical studies will it be possible to provide more rational therapy for postmenopausal women.

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**References**


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