Ovariectomy Exacerbates and Estrogen Replacement Attenuates Photothrombotic Focal Ischemic Brain Injury in Rats

Kenji Fukuda, MD; Hiroshi Yao, MD; Setsuro Ibayashi, MD; Tatsuo Nakahara, PhD; Hideyuki Uchimura, MD; Masatoshi Fujishima, MD

Background and Purpose—We previously reported the infarct volumes in female spontaneously hypertensive rats (SHR) to be significantly smaller than those in male SHR. The purpose of the present study was to determine whether estrogen is responsible for the sex difference in ischemic vulnerability in SHR.

Methods—In experiment 1, 1 week (short-term) or 4 weeks (long-term) after the ovariectomy (OVX), female SHR (5 months old) were randomly subjected to photothrombotic occlusion of the middle cerebral artery, and the infarct volumes were determined. In experiment 2, the rats were randomly assigned to 3 groups (ie, the sham-ovariectomized, ovariectomized, and estrogen replacement groups). In the replacement group, estradiol valerate (200 μg/kg) was subcutaneously injected once a week after the OVX. Four weeks after the OVX or sham-OVX, all rats were subjected to middle cerebral artery occlusion. Changes in regional cerebral blood flow were determined by laser-Doppler flowmetry.

Results—In experiment 1, the infarct volume produced 1 week after the OVX was not different from that of the sham-ovariectomized group. In contrast, the infarct volume produced 4 weeks after the OVX was significantly larger than that of the sham-ovariectomized group (82.4 ± 11.6 versus 54.5 ± 16.0 mm³, P = 0.0058). In experiment 2, estradiol replacement after the OVX was observed to attenuate the infarct volume compared with the ovariectomized group (55.6 ± 18.8 versus 78.5 ± 21.0 mm³, P = 0.0321). The degrees of regional cerebral blood flow reduction did not differ among the sham-ovariectomized, ovariectomized, and estrogen replacement groups.

Conclusions—Chronic estrogen depletion was thus found to increase the infarct size, which was attenuated by estradiol replacement. These findings indicate that estrogen contributes to the sex difference in ischemic vulnerability and that endogenous estrogen also has a neuroprotective effect against ischemic brain damage. (Stroke. 2000;31:155-160.)

Key Words: stroke ■ estradiol ■ photochemistry ■ spontaneously hypertensive rats

In experimental stroke, female animals tend to show more tolerance to ischemic injury of the brain than males.1–5 For example, female gerbils displayed a lower incidence of overt neurological signs and less neuronal loss after unilateral carotid occlusion.3 We previously reported that the infarct size after middle cerebral artery (MCA) occlusion was significantly smaller in female spontaneously hypertensive rats (SHR) than in male SHR.5 But it remains unclear whether the sex difference in infarct size is a consequence of lower blood pressure levels in female SHR or is due to a neuroprotective or anti-ischemic effect of the gonadal hormones (eg, estrogen).

Recent studies suggest that estrogen protects against ischemic injury in vivo. In a study of MCA occlusion, ovariectomy (OVX) resulted in an increased infarct size in female rats, comparable to that in male rats.6 Administration of estrogen after MCA occlusion reduced mortality and infarct volumes.7 Estrogen pretreatment reduced ischemic injury in OVX female rats8 and showed a similar reduction in male rats.9 In these studies, however, the effects of estrogen on regional cerebral blood flow (CBF) during ischemia are controversial.3,6,8–11 Estrogen has been shown to be a vasoactive steroid,12–14 and Alkayed et al6 demonstrated the intraischemic cortical flow in female rats to be higher than in males. In contrast, Dubal et al9 showed that ischemic regional CBF did not differ between the estradiol-pretreated and oil-pretreated control rats.

Hypertensive rats are relevant to stroke research and are widely used for studies of hypertension-related cerebrovas-
cular complications. Thrombotic brain infarction is a major type of human stroke. Postmenopausal women are at risk for various cardiovascular events, including thrombotic stroke.\textsuperscript{15–18} We therefore investigated the effects of OVX and estrogen replacement in this thrombotic focal ischemia model of SHR.\textsuperscript{19,20} The purpose of the present study was to determine whether or not estrogen is responsible for the sex difference in ischemic vulnerability in SHR.

**Materials and Methods**

All procedures were done in accordance with the Animal Care Guidelines of Kyushu University.

**Materials**

Female SHR (5 months old, 190 to 230 g body weight) were maintained in the Kyushu University Animal Center under a 12:12-hour light-dark cycle with unrestricted access to food and water. The rats were bilaterally ovariectomized or sham-ovariectomized under amobarbital anesthesia (100 mg/kg IP). In experiment 1, 1 week (short-term) or 4 weeks (long-term) after the OVX or sham-OVX, the rats were subjected to MCA occlusion. In experiment 2, the rats were randomly assigned to 3 groups, as follows. The first group of rats received sham-OVX, and the second and third groups were ovariectomized. The rats in the third group were subcutaneously injected with a depot preparation of estradiol valerate (200 mg/kg; Mochida Pharmaceutical Co) suspended in sesame oil once a week until 3 weeks commencing 1 week after OVX (estrogen replacement group, n=8). The rats in the first group (sham-OVX group, n=8) and second group (OVX group, n=8) were injected with the same amount of sesame oil as a vehicle once a week. All rats were subjected to MCA occlusion 4 weeks after either OVX or sham-OVX.

**TABLE 2. Infarct Volume in Experiment 1**

<table>
<thead>
<tr>
<th>Infarct volume, mm(^3)</th>
<th>1 Week</th>
<th>4 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-OVX</td>
<td>61.1±16.2</td>
<td>54.5±16.0</td>
</tr>
<tr>
<td>OVX</td>
<td>46.8±10.5</td>
<td>54.5±16.0</td>
</tr>
</tbody>
</table>

*Values are mean±SD (n=5). *P<0.0058 vs sham-OVX (4 weeks) by ANOVA followed by Fisher’s protected least significant difference test.

**Surgical Procedures**

The rats were anesthetized with halothane (4% for induction; 1.5% during the surgical preparation, with a face mask; 0.75% after intubation; and 0.5% for maintenance) in a mixture of 70% nitrous oxide and 30% oxygen. The right femoral artery and vein were cannulated with PE 50 tubing. The rats were endotracheally intubated with PE 205 tubing. Pancuronium bromide (an initial dose of 0.3 mg followed by 0.1 mg every 30 minutes) was injected intravenously, and the rats were mechanically ventilated. The mean arterial blood pressure was monitored continuously, and physiological variables were determined before and after distal MCA occlusion. The rectal and head temperatures were maintained at 37°C and 36°C, respectively, by means of a warming lamp.

The rats were mounted on a stereotaxic head holder in a prone position, and a 2-cm incision was made vertically midway between the right orbit and the right external auditory canal. The temporalis muscle was separated and retracted while a burr hole 3 mm in diameter was made 1 mm rostral to the anterior junction of the zygoma and squamosal bones (under an operation microscope), thus revealing the distal segment of the MCA above the rhinal fissure. The dura was left intact.

**Photothrombotic Distal MCA Occlusion**

A krypton laser operating at 568 nm (Innova 301, Coherent Inc) was used to irradiatorate the distal MCA at a power of 20 mW. The laser beam was focused with a cylindrical lens with a 30-cm focal length (CKX 300, Newport Corp) and positioned with a mirror onto the distal MCA. The photosensitizing dye rose bengal (15 mg/mL in 0.9% saline; Wako Pure Chemical Industries Ltd) was administered intravenously at a dose of 20 mg/kg over 90 seconds simultaneously with 4 minutes of laser irradiation. One hour after distal MCA occlusion, the head wound was closed, and the catheters were removed. The rats were carefully weaned from the respirator and returned to the home cage after regaining the ability to breath independently.

**Measurement of the Regional CBF**

In experiment 2, the regional CBVs were determined by laser-Doppler flowmetry. Through a craniotomy, a laser-Doppler flowmetry probe was laterally scanned, and CBF was measured at 5 points (1 mm posterior to 2.0, 2.5, 3.0, 3.5, and 4.0 mm lateral to the bregma).\textsuperscript{21} Because visible light interferes with laser-Doppler flowmetry, the heating lamp was temporarily turned off during measurements of CBF. Changes in CBF were expressed as a percentage of the average of 3 baseline values.
Quantification of the Infarct Volume

After 3 days, the rat was decapitated, and the brain was rapidly removed. The entire brain was cooled in ice-cold saline for 10 minutes and cut into 2-mm-thick coronal sections in a cutting block; the brain slices were then immersed in 2% 2,3,5-triphenyltetrazolium chloride (Wako Pure Chemical Industries Ltd.) at 37°C for 30 minutes in the dark. The posterior surface of each section was photographed, and the infarct areas, indicated by a lack of staining, were determined with NIH Image software (version 1.56). The infarct volume of each rat was calculated according to the trapezoidal rule.22

Plasma Estradiol Assays

In a separate experiment, 1 week after the last of 3 injections, plasma 17β-estradiol samples were obtained from the femoral artery and centrifuged and then were frozen until the time of assay. Samples (0.5 mL) were analyzed for estradiol by a radioimmunoassay after ether extraction.

Statistical Analysis

The values were expressed as the mean±SD. Differences in physiological variables, infarct volume, and changes in CBF were analyzed with ANOVA followed by Fisher’s protected least significant difference test. The levels of significance were set at \( P<0.05 \).

Results

Experiment 1

There were no significant differences in mean blood pressure, head temperature, rectal temperature, blood gases, hematocrit, and blood glucose among the groups in experiment 1 (Table 1). These physiological variables were not significantly different at 5, 30, and 60 minutes after MCA occlusion (data not shown). Body weight in the rats 4 weeks after OVX was higher than that in the other 3 groups. The infarct volume produced 1 week after OVX was 46.8±10.5 mm³, which was not different from the 61.1±16.2 mm³ observed in the sham-ovariectomized group. In contrast, the infarct volume produced 4 weeks after OVX was 82.4±11.6 mm³, which was significantly larger than the 54.5±16.0 mm³ in the sham-ovariectomized group (\( P=0.0058 \)) (Table 2).

Experiment 2

Table 3 demonstrates the physiological variables in experiment 2, which showed no significant differences among the groups. OVX increased the body weight gain to the same...
degree as experiment 1, and estrogen replacement reversed this effect (Figure 1). There are no significant correlations between body weight and infarct volume either in the sham-OVX (Pearson’s correlation coefficient, $r = 0.147$, $n = 18$) or in the OVX group ($r = 0.030$, $n = 13$). Figure 2 demonstrates infarct volumes in the sham-OVX, OVX, and estrogen replacement groups. The OVX group showed a larger infarction (78.5 ± 21.0 mm$^3$) than the sham-OVX group (49.9 ± 19.8 mm$^3$), and the difference was significant ($P = 0.0091$). Estrogen replacement after OVX attenuated the infarct volume (55.6 ± 18.8 mm$^3$) compared with the OVX group ($P = 0.0321$). The average size of the cortical infarction in each group is presented in Figure 3.

Figure 4 demonstrates the changes in the regional CBF measured by laser-Doppler flowmetry 30 minutes after MCA occlusion. The degree of regional CBF reductions did not differ among the sham-OVX, OVX, and estrogen replacement (Replace) groups at any regions (2.0 mm to 4.0 mm from midline). No differences were observed among the 3 groups 10 and 60 minutes after MCA occlusion (data not shown).

The plasma estradiol levels determined 1 week after the last injection were 8.1 ± 6.9, 3.8 ± 1.0, and 31.1 ± 19.4 pg/mL in the sham-OVX, OVX, and estrogen replacement groups, respectively ($n = 5$). The estradiol level in the estrogen replacement group remained within the physiological level, but it was slightly higher than the basal circulating estradiol level in female rats during the estrous cycle and was close to the proestrous estradiol level.23,24

**Discussion**

In this study, we demonstrated that OVX increased the infarct size after photothrombotic focal ischemia by 50%, whereas estrogen replacement reversed this effect. This finding suggests that estrogen may be responsible for the observed sex-linked differences in susceptibility to cerebral ischemia in experimental stroke. Because OVX rats had decreased plasma levels of gonadal hormones at 24 hours after OVX,24,25 the different infarct size between the short-term and long-term OVX groups might have resulted from the different durations of gonadal hormone depletion.

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**Figure 1.** Changes of body weight in sham-OVX, OVX, and estrogen replacement (Replace) groups in experiment 2. Values are mean ± SD ($n = 8$). *$P = 0.0015$, **$P = 0.0002$ vs sham-OVX by unpaired t test; $P < 0.005$ was considered significant according to Bonferroni principle.

**Figure 2.** Infarct volume in sham-OVX, OVX, and estrogen replacement (Replace) groups in experiment 2. Values are mean ± SD ($n = 8$). *$P = 0.0091$ vs sham-OVX, **$P = 0.0321$ vs OVX by ANOVA followed by Fisher’s protected least significant difference test.

**Figure 3.** Schematic of cortical infarction in the sham-OVX, OVX, and estrogen replacement (Replace) groups in experiment 2.

**Figure 4.** Degrees of regional CBF (rCBF) reduction were compared among sham-OVX, OVX, and estrogen replacement (Replace) groups in experiment 2. rCBF was measured by laser-Doppler flowmetry at 5 points (1 mm posterior and 2.0, 2.5, 3.0, 3.5, and 4.0 mm lateral to the bregma) 30 minutes after MCA occlusion. Values are mean ± SD ($n = 8$).
The effects of estrogen on regional CBF reductions during ischemia remain controversial in previous studies. The degree of regional CBF reductions did not differ among the sham-OVX, OVX, and estrogen replacement groups in our present study. Because OVX and estradiol pretreatment were shown not to alter the baseline CBF, our results thus suggested that blood flow–independent neuroprotective mechanisms are responsible for the differences in infarct size in our model of photothrombotic MCA occlusion.

Several possible mechanisms have been proposed to underlie the neuroprotective effects of estrogen. Previous studies demonstrated that glutamate toxicity was attenuated in cultured neurons pretreated with estrogen, suggesting that estrogen possesses antioxidant activities or that the neuroprotective action is mediated through classic estrogen receptors. Estradiol was shown to protect against $\mathcal{N}$-methyl-D-aspartate (NMDA)-induced neuronal cell death, thus directly inhibiting the NMDA receptor. These findings suggest that one of the mechanisms by which estrogen exerts its neuroprotective effects is by attenuating the glutamate excitotoxicity in ischemic injury. Recently, Garcia-Segura et al. reported that Bcl-2–immunoreactive neurons were decreased by OVX and also showed a dose-dependent increase after estradiol administration to OVX rats. Because the apoptotic process may be a good target for therapeutic drugs aimed at limiting stroke damage, it may be important to investigate the effects of estrogen on the apoptotic process in cerebral ischemia.

The present study produced infarction by photothrombotic MCA occlusion, in which prominent platelet aggregation results in vascular occlusion. Other studies, but not all, have demonstrated that estrogen inhibits platelet aggregation. The possible beneficial action of estrogen to suppress harmful platelet activation in ischemia should be addressed in future studies.

We injected the rats with 200 $\mu$g/kg of estradiol valerate once a week, and the plasma estradiol level, which was measured 1 week after the last injection, was 31.1±19.4 pg/mL, which was close to the physiological proestrous estradiol level. Both physiological high and physiological low doses (10 and 60 pg/mL of plasma estradiol concentration, respectively) were equally effective at reducing the size of ischemic injury. Therefore, the relatively lower dose of estradiol valerate used in the present study was considered to be adequate to analyze the neuroprotective role of estrogen against brain ischemia. In addition, the neuroprotective effects of progesterone still cannot be ruled out, because the administration of progesterone to male rats or female cats reduced the degree of ischemic damage.

In summary, we demonstrated here that chronic estrogen depletion increased the infarct size, which was thereafter attenuated by estradiol replacement. These findings indicate that estrogen contributes to the sex-linked difference in ischemic vulnerability, while endogenous estrogen also has a neuroprotective effect against ischemic damage.

Acknowledgments

This study was supported in part by the Social Insurance Agency Contract Fund commissioned by the Japanese Health Sciences Foundation. We thank M. Nozaki, MD, and T. Kitzazono, MD, for their valuable advice during the course of this study.

References

It has been nearly 10 years since my colleagues and I first demonstrated a significant gender difference in focal ischemic brain damage in the gerbil 3-hour unilateral carotid occlusion model. Using that model, we observed that females display significantly less 24-hour postreperfusion cortical and hippocampal CA1 neuronal necrosis compared with males, and that this finding is unrelated to cortical blood flow disparities in cerebral blood flow do not appear to be involved in the worsening of ischemic damage seen in ovariectomized females or in the benefits of estrogen replacement therapy on platelet aggregation and adenosine triphosphate release in postmenopausal women. Obstet Gynecol. 1993;81:261–264.


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It has been nearly 10 years since my colleagues and I first demonstrated a significant gender difference in focal ischemic brain damage in the gerbil 3-hour unilateral carotid occlusion model. Using that model, we observed that females display significantly less 24-hour postreperfusion cortical and hippocampal CA1 neuronal necrosis compared with males, and that this finding is unrelated to cortical blood flow differences before, during, or after ischemia. We also showed that 17β-estradiol can potently inhibit iron-catalyzed lipid peroxidation in brain tissue homogenates. This, taken together with our observation that postreperfusion depletion of brain vitamin E was much less in female gerbil brains than in those of males, led us to hypothesize that the gender difference in ischemic damage was due to an antioxidant effect of endogenous 17β-estradiol. Little follow-up study was undertaken either by my laboratory or others over the next several years.

However, multiple recent efforts, with rat focal ischemia paradigms, began the arduous task of defining the mechanistic bases of gender differences in ischemic vulnerability. These investigations have confirmed that 17β-estradiol is the key player, although the neuroprotective mechanisms associated with this female hormone are likely to be complex and multiple. The present investigation confirms the neuroprotective importance of 17β-estradiol by demonstrating in the clinically relevant rat photothrombotic occlusion model that ovariectomy leads to an increase in infarct size and that estrogen replacement restores the neuroprotective aspect of being female. Moreover, like the original gerbil investigation, disparities in cerebral blood flow do not appear to be involved in the worsening of ischemic damage seen in ovariectomized females or in the benefits of estrogen replacement. Much additional work is needed to determine the precise mechanism(s) of 17β-estradiol neuroprotection. However, the magnitude of estrogenic neuroprotection suggests that this is an important pursuit. Indeed, the unraveling of estrogenic neuroprotective actions may lead to a more complete understanding of ischemic pathophysiology and to the design of more effective neuroprotective agents that are presently available.

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Editorial Comment
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*Stroke.* 2000;31:155-160
doi: 10.1161/01.STR.31.1.155

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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