

Pre- and post-estrogen administration in global cerebral ischemia reduces blood-brain barrier breakdown in ovariectomized rats

G Uzum¹, N Bahçekapılı¹, AK Baltacı², R Mogulkoc², YZ Ziyilan¹

¹Department of Physiology, Istanbul Medical School Istanbul University, Istanbul, Turkey

²Department of Physiology, Medical School, Selcuk University, Konya, Turkey

Received: February 20, 2014

Accepted after revision: August 11, 2014

The aim of present study was to determine the effect of estrogen treatment on blood-brain barrier permeability in rats with induced global cerebral ischemia. The study included six-month-old female Sprague–Dawley rats which were divided into the following groups: Control-Ischemia-Reperfusion (C + I-R); Ovariectomy-Ischemia-Reperfusion (Ovx + I-R); Ovariectomy + Estrogen + Ischemia-Reperfusion (Ovx + E + I-R); Ovariectomy + Ischemia-Reperfusion + Estrogen (Ovx + I-R + E). Ischemia-reperfusion was induced by clamping two carotid arteries, then opening the clamp. Blood-brain barrier permeability was visualized by Evans Blue extravasation and quantified by spectrophotometry. Our results indicate that following ischemia-reperfusion the BBB permeability is increased in ovariectomized rats (Evans Blue extravasation) compared to the control group in the cortex, thalamus, hippocampus, cerebellum and brain stem, while in the midbrain no significant increase was detected. In contrast, BBB permeability in the groups treated with estrogen, administered either before or after ischemia-reperfusion, was significantly lower than in ovariectomized animals. In conclusion, the increase in BBB permeability resulting from experimentally induced cerebral ischemia was prevented by exogenous estrogen treatment. The study results indicate that estrogen may be used for therapeutic purposes in ischemia-reperfusion.

Keywords: blood-brain barrier (BBB), ovariectomy, estrogen treatment, Evans Blue, rat

Blood-brain barrier (BBB), a physical and metabolic barrier between the central nervous system and peripheral circulation, is critical in the protection of brain homeostasis. The original structure of BBB is a highly specialized tight junction (17). The integrated function of the cerebral microvasculature, tight junction proteins, brain microvascular endothelial cells, cellular transport pathways, and enzymatic machinery jointly contribute to normal BBB integrity. Aging, systemic diseases, and ischemic injury can disrupt these processes, resulting in a decline in overall BBB function and integrity (22). Alterations to hormone levels at the onset of menopause and as a result of estrogen treatment may cause further changes in BBB functions (17). Besides, there are data suggesting that aging and hormonal alterations cause changes in the BBB tight junction integrity (2, 3, 9). It has been reported that replacement with physiological levels of estradiol protects against stroke-related injury in young and aging female rats and older animals remain responsive to the protective actions of estradiol (10). It was assumed that changes in estrogen levels or receptor activity may impair the tight junction in the BBB with aging. The two main types of estrogen receptors, ER α and ER β , through which estrogen exerts its effect, are found commonly in microvascular

Corresponding author: Dr. Rasim Mogulkoc

Department of Physiology, Medical School, Selcuk University, Konya, Turkey

Phone: +90 (332) 224 38 57; Fax: +90 (332) 241 21 84; E-mail: rasimmogulkoc@yahoo.com

beds (14, 20). It was reported in previous studies that 17β -estradiol had favorable effects on cerebral circulation and it exerted these effects on the vascular tone, inflammation, and reactive oxygen types (11, 13).

The objective of the present study was to determine the effect of estrogen administration to 6-month-old ovariectomized rats before and after cerebral ischemia-reperfusion on BBB permeability in different areas of the brain such as cortex, thalamus, hippocampus, corpus striatum, midbrain, cerebellum, and brain stem.

Materials and Methods

This study was carried out at the Selcuk University Experimental Medicine Research and Application Center and at the Istanbul University, Istanbul Medical School, Department of Physiology. It was approved by the local ethical committee of the Selcuk University Experimental Medicine Research and Application Center, where the ovariectomy procedure was performed. Cerebral ischemia-reperfusion and BBB experiments were carried out at Istanbul University, Istanbul Medical School, Department of Physiology and approved by the local ethical committee. The study included 30 adult female rats of Sprague–Dawley strain, which were 6 months old and weighed 200–250 g. The rats were kept at 18–21 °C (room temperature), under 12 hours light/dark cycle (07.00 am–07.00 pm) and their food was provided in special steel bowls and their water (normal tap water) in glass feeding bottles. The animals were given about 10 g food per 100 g of body weight daily. Our study was performed using experimental groups as follows. Ischemia and reperfusion procedure and estradiol supplementation were performed 3 weeks after ovariectomy. The experimental groups of our study were as follows:

1. Control-Ischemia-Reperfusion (C + I-R; $n = 7$): Without any other procedure, the animals in this group were subjected to ischemia for 20 minutes and then to reperfusion for another 20 minutes, and decapitated. Brain tissues of the decapitated animals were removed without delay. Animals in this group were in the proestrus phase of the cycle.

2. Ovariectomy-Ischemia-Reperfusion (Ovx + I-R; $n = 7$): This group included the animals which were ovariectomized and then subjected to ischemia and reperfusion, for 20 minutes each, like the control group, and decapitated. After decapitation, relevant brain tissues were removed.

3. Ovariectomy + Estrogen + Ischemia-Reperfusion (Ovx + E + I-R; $n = 8$): The animals in the group were ovariectomized and then injected with 10 $\mu\text{g}/100$ g body weight intraperitoneal 17β -estradiol before ischemia-reperfusion (2 hours before ischemia). The injection was followed by ischemia-reperfusion.

4. Ovariectomy + Ischemia-Reperfusion + Estrogen (Ovx + I-R + E; $n = 8$): The ovariectomized rats in this group were given 17β -estradiol treatment (10 $\mu\text{g}/100$ g body weight) 1 hour after ischemia-reperfusion.

Ovariectomy

Ovariectomy was performed in rats under general anesthesia with intramuscular ketamine 60 mg/kg [Ketamin hydrochloride, Eczacibasi, Turkey, and Xylazine 5 mg/kg (Rompun, Bayer, Germany)]. The hair on the back of the rats was shaved for ovariectomy. Following appropriate asepsis and antisepsis with Betadine (Sanofi), the rats were placed in the ventral position and the skin was incised from 1/3 upper point of the stretch between the medial part

of the back and tail. After subcutaneous tissues were freed, peritoneal cavity was accessed through abdomen on back wall muscles. Ovaries were taken out together with lipid tissue. The ovaries were cleared of the lipid tissue, and then clamped, ligated and cut. After controlling the hemorrhage, other organs were replaced into the peritoneal cavity and the muscle was sutured with 2/0 chrome catgut and the skin with 2/0 silk (21).

Induction of ischemia-reperfusion

In order to induce global cerebral ischemia, the animals were put under general anesthesia with pentobarbital sodium. After clamping both carotid arteries for 20 minutes, 3 to 4 ml blood was taken, and blood pressure was dropped to 50 mmHg. All rats were anesthetized with pentobarbital sodium 35 mg/kg intraperitoneally (i.p.) and catheters filled with 100 IU of heparin in isotonic saline were inserted into bilateral femoral arteries and veins. One of the femoral artery was used for blood withdrawal the other for continuous monitoring (polygraph system-Nihon-Kohden, Tokyo, Japan) of mean arterial blood pressure. Reperfusion was induced by opening the clamp and the withdrawn blood in the syringe was re-infused slowly. Reperfusion was lasted for 20 min. The animals in all groups were sacrificed by decapitation 2 hours after the procedures and brain tissue samples were collected without any delay. After the anesthesia, 3 ml×kg⁻¹ 2% Evans Blue (EB) solution was injected intravenously for visual examination of blood brain barrier disruption and for spectrophotometric measurements.

Changes in blood-brain barrier permeability

Spectrophotometric Evans Blue quantification

EB distribution was determined by spectrophotometric method. The perfused brains were removed and different brain areas (cortex, thalamus, hippocampus, corpus striatum, midbrain, cerebellum, and brain stem) were separated. Tissues taken from each brain area were kept in aluminum foil until the time of analysis. At the time of analysis, the samples were homogenized with 50% trichloroacetic acid and centrifuged at 15000 rpm for 20 minutes. The absorbance values were measured at 615 nm. EB values were calculated as $\mu\text{g dye}\times\text{g}^{-1}$ wet weight (1, 16).

Statistics

Values are expressed as the mean EB value in $\mu\text{g}\cdot\text{g}^{-1}$. The data were analyzed using a one-way ANOVA test. The criterion for statistical significance was ($p < 0.05$) in all statistical evaluations.

Results

EB values in different brain areas of the study groups are presented in Table I. When EB values in the brain cortex area were examined, the Ov_x + I-R group was found to have the highest values compared to other groups ($p < 0.05$). Control group EB levels were higher than those in the treated groups. An examination of the EB levels in thalamus area showed that the highest levels were found in the Ov_x + I-R group ($p < 0.05$) and the lowest levels were in the Ov_x + I-R + E group ($p < 0.05$). Similarly, hippocampus EB levels were the highest in the Ov_x-I-R group and the lowest in the Ov_x + E + I-R group ($p < 0.05$). Concerning the EB permeability values in corpus striatum area, it was found that the Ov_x + I-R group had the highest permeability and the Ov_x + I-R + E group had the lowest permeability ($p < 0.05$). EB permeability of the midbrain in the control and Ov_x - I groups was higher than that in the groups receiving estrogen ($p < 0.05$). As for cerebellum EB permeability values, the Ov_x +

I-R group had the highest and the Ovx + I-R + E group had the lowest values ($p < 0.05$). When the EB permeability values in the brain stem were addressed, it was seen that the Ovx + I-R group had higher permeability values than all the other groups. This parameter was higher in the control group than in estrogen treatment groups. The lowest EB permeability values were found in the Ovx + I-R + E group ($p < 0.05$).

Table I. Evans Blue permeability levels in different brain areas ($\mu\text{g dye} \times \text{g}^{-1}$ wet tissue)

Groups	Cortex	Thalamus	Hippocampus	Corpus striatum	Midbrain	Cerebellum	Brain stem
C + I-R	0.04±0.01b	0.15±0.09b	0.31±0.11b	0.36±0.07b	0.17±0.07a	0.12±0.06b	0.21±0.09b
Ovx + I-R	0.57±0.01a	0.36±0.02a	0.94±0.04a	0.58±0.02a	0.19±0.01a	0.21±0.07a	0.30±0.01a
Ovx + E+ I-R	0.02±0.03b	0.12±0.05b	0.10±0.05d	0.13±0.09c	0.10±0.04b	0.10±0.06b	0.10±0.03c
Ovx + I-R + E	0.03±0.00b	0.03±0.00c	0.14±0.11c	0.11±0.17c	0.08±0.00b	0.04±0.00c	0.03±0.00d

Different letters in same column stand for significant differences ($P < 0.05$)

(a > b >) for **cortex** significant difference between Ovx + I-R and C + I-R, Ovx + E + I-R, Ovx + I-R + E

(a > b > c >) for **thalamus and cerebellum**, significant difference between Ovx + I-R and C + I-R, Ovx + E + I-R, Ovx + I-R + E; significant difference between C + I-R, Ovx + E + I-R, and Ovx + I-R + E

(a > b > c > d) for **hippocampus** significant difference between Ovx + I-R and C + I-R, Ovx + E + I-R, Ovx + I-R + E; significant difference between C + I-R and Ovx + E + I-R, Ovx + I-R + E; significant difference between Ovx + I-R + E and Ovx + E + I-R

(a > b > c >) for **corpus striatum** significant difference between Ovx + I-R and C + I-R, Ovx + E + I-R, Ovx + I-R + E; significant difference between C + I-R and Ovx + E + I-R, and Ovx + I-R + E

(a > b >) **midbrain** significant difference between C + I-R, Ovx + I-R and Ovx + E + I-R, Ovx + I-R

(a > b > c > d) for **brain stem** significant difference between Ovx + I-R and C + I-R, Ovx + E + I-R, Ovx + I-R + E; significant difference between C + I-R and Ovx + E + I-R, Ovx + I-R + E; significant difference between Ovx + I-R + E and Ovx + E + I-R

C-I-R (Control-Ischemia-Reperfusion);

Ovx-I-R (Ovariectomized + Ischemia-Reperfusion);

Ovx + E + I-R (Ovariectomized + Estrogen + Ischemia-Reperfusion);

Ovx + I-R + E (Ovariectomized + Ischemia-Reperfusion + Estrogen)

Discussion

The first result of the present study is that ischemia induced in 6-month-old ovariectomized rats significantly increased the permeability of the BBB. However, estrogen treatment before and after ischemia-reperfusion following ovariectomy markedly restored the impairments in the blood-brain barrier. Still, while estrogen treatment before ischemia reperfusion produced more favorable effects in the blood-brain barrier in some brain areas, estrogen treatment after ischemia-reperfusion brought about greater improvements in the BBB function in other brain areas.

The brain and its vascular structures are strong targets of estrogen. Therefore, age-related decreases in estrogen levels and changes in estrogen receptors damage the integrity of the blood-brain barrier as a result of which toxic products pass into the brain and harmful effects may arise (3). Similarly, estrogen was reported to perform a protective action against

brain injuries inflicted in different conditions and the resulting impairments to the blood-brain barrier (5, 6). Age-related increases in the blood-brain barrier permeability were attributed to the loss of sex hormones in the serum (22). It was reported that ovariectomy caused an almost 2.2-fold increase in the Evans Blue permeability of the blood-brain barrier (22). In our study, BBB permeability significantly increased in all brain areas after ovariectomy, relative to the control group, and this result is parallel to the result of the above-cited studies. It was suggested in previous studies that acute estrogen administration prevented the impairment to the BBB in ischemic rats (15). Estrogen levels may affect BBB structures and brain homeostasis with aging and lead to pathological conditions. In fact, Sandoval and Witt (18) examined the effects of age and 17β -estradiol on BBB tight junction permeability and estrogen receptor proteins. It was found in the concerned study that estrogen treatment influenced different tight junction proteins in different age groups (occludin in the young and claudin in the middle-aged), but did not have any impact on functional paracellular permeability. In our study, however, BBB permeability increased significantly in the ovariectomized ischemic group, and this result is different from the results of the above-cited study. In a study by Cipolla et al. (8) estrogen treatment to ovariectomized rats was found to significantly lower the permeability of the blood-brain barrier. Chi et al. (7) investigated the effect of 17β -estradiol on BBB permeability during focal cerebral ischemia. They found that ischemia and 17β -estradiol produced different effects on young (3-month-old) and old (24-month-old) Fischer rats. Ischemia did not affect the blood-brain permeability of young rats, whereas increased BBB permeability was found in both the ischemic cortex and contralateral cortex of old rats. However, no such effect was observed in young rats. Our study was conducted on 6-month-old rats and significant ischemia-related increases were found in cortical Evans Blue values which were studied as an indicator of BBB permeability. Thus, the result we obtained is in consistency with the results established in old rats in the previous study.

Chi et al. (6) found in their study that prior treatment of ovariectomized rats with 21-day estrogen pellets before ischemia significantly restored the destruction caused by vascular endothelial growth factor in the BBB and this finding attests to the protective effect of estrogen.

In our study, ovariectomy was found to significantly increase BBB permeability in the hippocampus. However, estrogen treatment before and after ischemia-reperfusion following ovariectomy was seen to markedly restore the impairment to the BBB. In the same vein, previous studies demonstrated that estrogen treatment played a protective role against the hippocampus damage inflicted during global ischemia, and this result lends support to ours (4, 12).

We established in our study that Evans Blue permeability values in different brain areas such as corpus striatum, midbrain, brain stem, and cerebellum increased significantly after ovariectomy. In a similar, previous study, increased blood-brain barrier permeability was found in structures like frontal, temporal, parietal, and occipital cortices, as well as cerebellum and brain stem 1 week after ovariectomy (17). In our study, BBB, Evans Blue permeability values in experimental animals were determined in ischemia-reperfusion induced 3 weeks after ovariectomy and significantly elevated EB levels were established in different brain areas, while estrogen treatment before and after ischemia-reperfusion markedly restored the impairment to the structure of the BBB. These findings are parallel to results reported previously (19).

An overall assessment of the study results shows that cerebral ischemia in 6-month-old ovariectomized rats causes a significant increase in the BBB permeability. However, estrogen treatment before and after ischemia-reperfusion in experimental animals significantly prevented the increase in the blood-brain barrier permeability resulting from ovariectomy. In fact, estrogen treatment administered after ischemia was more effective in impeding the destruction in the brain areas other than the hippocampus. These results suggest that estrogen can be utilized for therapeutic purposes in pathological conditions which cause neuronal damage like ischemia-reperfusion.

REFERENCES

1. Bahcekapili N, Uzüm G, Gökkusu C, Kuru A, Ziyilan YZ: The relationship between erythropoietin pretreatment with blood-brain barrier and lipid peroxidation after ischemia/reperfusion in rats. *Life Sci.* 80, 1245–1251 (2007)
2. Bake S, Friedman JA, Sohrabji F: Reproductive age-related changes in the blood brain barrier: expression of IgG and tight junction proteins. *Microvasc. Res.* 78, 413–424 (2009)
3. Bake S, Sohrabji F: 17beta-estradiol differentially regulates blood-brain barrier permeability in young and aging female rats. *Endocrinology* 145, 5471–5475 (2004)
4. Barouk S, Hintz T, Li P, Duffy AM, MacLusky NJ, Scharfman HE: 17 β -estradiol increases astrocytic vascular endothelial growth factor (VEGF) in adult female rat hippocampus. *Endocrinology* 152, 1745–1751 (2011)
5. Chen X, Zhang M, Jiang C, Guo W, Liu H, Wei S: Estrogen attenuates VEGF-initiated blood-retina barrier breakdown in male rats. *Horm. Metab. Res.* 43, 614–618 (2011)
6. Chi OZ, Barsoum S, Wen Y, Liu X, Weiss HR: 17beta-estradiol prevents blood-brain barrier disruption induced by VEGF. *Horm. Metab. Res.* 36, 272–276 (2004)
7. Chi OZ, Hunter C, Liu X, Weiss HR: Effects of 17beta-estradiol on blood-brain barrier disruption during focal cerebral ischemia in younger and older rats. *Horm. Metab. Res.* 38, 377–381 (2006)
8. Cipolla MJ, Godfrey JA, Wiegman MJ: The effect of ovariectomy and estrogen on penetrating brain arterioles and blood-brain barrier permeability. *Microcirculation* 16, 685–693 (2009)
9. Di Napoli VA, Huber JD, Houser K, Li X, Rosen CL: Early disruptions of the blood-brain barrier may contribute to exacerbated neuronal damage and prolonged functional recovery following stroke in aged rats. *Neurobiol. Aging* 29, 753–764 (2008)
10. Dubal DB, Wise PM: Neuroprotective effects of estradiol in middle-aged female rats. *Endocrinology* 142, 43–48 (2001)
11. Duckles SP, Krause DN: Cerebrovascular effects of oestrogen: multiplicity of action. *Clin. Exp. Pharmacol. Physiol.* 34, 801–808 (2007)
12. Durham JL, Jordan KA, Devos MJ, Williams EK, Sandstrom NJ: Estradiol protects against hippocampal damage and impairments in fear conditioning resulting from transient global ischemia in mice. *Brain Res.* 1443, 64–74 (2012)
13. Krause DN, Duckles SP, Pelligrino DA: Influence of sex steroid hormones on cerebrovascular function. *J Appl. Physiol.* 101, 1252–1261 (2006)
14. Levin ER: Plasma membrane estrogen receptors. *Trends Endocrinol. Metab.* 220, 477–482 (2009)
15. Li M, Zhang Z, Sun W, Koehler RC, Huang J: 17 β -estradiol attenuates breakdown of blood-brain barrier and hemorrhagic transformation induced by tissue plasminogen activator in cerebral ischemia. *Neurobiol. Dis.* 44, 277–283 (2011)
16. Rössner N, Tempel K: Quantitative Bestimmung der Permeabilität der sogenannten Blut-hirnschranke für Evans-blue (T 1824). *Medicina et Pharmacologia Experimentalis* 14, 169–182 (1996)
17. Saija A, Princi P, D'Amico N, De Pasquale R, Costa G: Aging and sex influence the permeability of the blood-brain barrier in the rat. *Life Sci.* 47, 2261–2267 (1990)
18. Sandoval KE, Witt KA: Age and 17 β -estradiol effects on blood-brain barrier tight junction and estrogen receptor proteins in ovariectomized rats. *Microvasc. Res.* 81, 198–205 (2011)
19. Simpkins JW, Rajakumar G, Zhang YQ, Simpkins CE, Greenwald D, Yu CJ, Bodor N, Day AL: Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. *J. Neurosurg.* 87, 724–730 (1997)

20. Stirone C, Boroujerdi A, Duckles SP, Krause DN: Estrogen receptor activation of phosphoinositide-3 kinase, akt, and nitric oxide signaling in cerebral blood vessels: rapid and long-term effects. *Mol. Pharmacol.* 67, 105–113 (2005)
21. Waynfort HB, Flecknell PA (1992): Ovariectomy In *Experimental and Surgical Technique in the Rat*. 2nd Ed. Academic Press, London, pp. 276–278.
22. Wilson AC, Clemente L, Liu T, Bowen RL, Meethal SV, Atwood CS: Reproductive hormones regulate the selective permeability of the blood-brain barrier. *Biochim. Biophys. Acta.* 1782, 401–407 (2008)
23. Zeevi N, Pachter J, McCullough LD, Wolfson L, Kuchel GA: The blood-brain barrier: geriatric relevance of a critical brain-body interface. *J. Am. Geriatr. Soc.* 58, 1749–1757 (2010)