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# Contribution of estrogen receptors alpha and beta in the brain response to traumatic brain injury

## Laboratory investigation

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*Object*. Although there is evidence that estradiol has neuroprotective effects after traumatic brain injury (TBI) in female rats, it is unclear which estrogen receptor (ER) subtype, ERα or ERβ, mediates this effect. The authors therefore examined the roles of the different ERs in this effect. Here the authors used the ERα selective agonist propyl pyrazole triol (PPT) and the ERβ selective agonist diarylpropionitrile (DPN) alone and in combination in female rats to investigate this question.

*Methods*. Before the ovariectomized animals were injured using the Marmarou TBI technique, they were randomly divided into the following 9 groups: control, sham, TBI, vehicle, E1 (physiological dose of 17-β estradiol), E2 (pharmacological dose of 17-β estradiol), PPT, DPN, and PPT+DPN. Levels of blood-brain barrier (BBB) disruption (5 hours) and water content (24 hours) were evaluated after TBI. In groups receiving drugs or vehicle, treatment was administered as a single dose intraperitoneally 30 minutes after induction of TBI.

*Results*. Results showed that brain edema or brain water content after TBI was lower ($p < 0.001$) in the E2, PPT, DPN, and PPT+DPN groups than it was in the vehicle group. After trauma, the Evans blue dye content or BBB permeability was significantly higher in the TBI and vehicle groups ($p < 0.001$) than in the E2, PPT, DPN, and PPT+DPN groups. The inhibitory effects of PPT+DPN on brain water content, neurological scores, and Evans blue dye content were the highest for all groups. Although both PPT and DPN increased neurological scores after TBI, PPT appears to be more effective in increasing neurological scores.

*Conclusions*. Neuroprotective effects of estradiol on brain edema, BBB permeability, and neurological scores are mediated through both ERα and ERβ. This may suggest a therapeutic potential in the brain trauma for ER-specific agonists.

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**Key Words** • **traumatic brain injury** • **propyl pyrazole triol** • **diarylpropionitrile** • **estrogen receptor** • **neuroprotection**

Traumatic brain injury is defined as a physiological disruption in brain function that results from transportation accidents, falls, assault, and sports. Traumatic brain injury mainly affects young individuals and is the leading cause of death and disability in the population younger than 50 years of age.[10] Every year, approximately 1.5 million people die and at least 10 million people are killed or hospitalized for TBI.[23]

Despite substantial efforts, no new specific treatment for TBI has entered clinical practice for more than 30 years, and most therapeutic strategies have failed in translation from bench to bedside.[6] Attention is now turning to drugs that act on multiple pathways to enhance survival and functional outcomes. Estradiol has been found to be beneficial in different models of brain injury in several animal species. Epidemiological studies have shown that the clinical prognosis after TBI is better in female than in male patients.[17] This holds promise as a treatment for TBI. Traumatic brain injury produces a marked inflammatory reaction along with the influx of neutrophils and macrophages and heavy gliosis surrounding the zone of injury. Estradiol may regulate microglia and astrocyte functions related to inflammation. Estradiol is a potent antiinflammatory agent that works by inhibiting cytokine release and immune cell activation and migration. The key mechanism is inhibiting proinflammatory cytokines, most notably IL-

*Abbreviations used in this paper:* BBB = blood-brain barrier; DMSO = dimethyl sulfoxide; DPN = diarylpropionitrile; E1 = physiological dose of 17-β estradiol; E2 = pharmacological dose of 17-β estradiol; ER = estrogen receptor; IL = interleukin; MMP-9 = matrix metalloproteinase–9; PPT = propyl pyrazole triol; TBI = traumatic brain injury; TNF = tumor necrosis factor; VCS = veterinary coma scale.

1β, IL-6, and TNF.[14,17,39] Estradiol regulates the expression of 16 neuroinflammatory genes in the cortex of female rats, including downregulation of complement C3 and C4b.[41] In the rat brain, estradiol suppresses activation of microglia, recruitment of blood-derived monocytes, and expression of the C3 receptor and MMP-9.[55] Estradiol inhibits vasogenic brain edema and improves neurological scores[42] and disruption to the BBB after TBI in female rats.[20] Inflammation is a significant contributor to secondary injury, and secondary brain injury is believed to contribute to BBB injury; therefore, control of the inflammatory response benefits the immediate zone of injury and more distal sites, even other organs.[51]

The effects of estradiol are primarily mediated by ERα and ERβ, which are members of the nuclear receptor superfamily of ligand-activated transcription factors.[7] These 2 estrogen receptors have partially distinct tissue distributions, thereby providing some tissue selectivity using selective ER modifiers. The 2 receptors act synergistically in some tissues, whereas they act antagonistically in others. These tissue-specific differences in biological outcomes are thought to be due to tissue-specific differences in transcription factors, which become activated upon the binding of each ER by a ligand.[42,53] The 2 receptors regulate gene expression through multiple mechanisms. In the cortex, estradiol may alter gene transcription directly via ERs in astrocytes and microglia.[5] Estradiol regulates neuroinflammatory genes via ERα and ERβ in the frontal cortex of female rats.[41] Despite the fact that ERβ has been shown to be expressed widely in the CNS in adult mice,[33] in most neurological disease models, the protective effect of estrogen treatment has been shown to be mediated through ERα and has been associated with antiinflammatory effects.[51] It was found that ERα ligand treatment reduced CNS inflammation, whereas ERβ ligand treatment did not.[41] Interestingly, treatment with either the ERα or ERβ ligand was neuroprotective. Therefore, Tiwari-Woodruff and Voskuhl[52] have dissociated the antiinflammatory effect from the neuroprotective effect of estrogen treatment. Thus, both ERα and ERβ are located in areas that could influence estradiol's neuroprotective effects on TBI.

This study used the ER subtype selective agonists PPT (an ERα agonist) and DPN (an ERβ agonist). We previously showed that estradiol has a neuroprotective effect against TBI.[20,41] The purpose of the present research was 1) to identify which ERs mediate neuroprotective effects of 17-β estradiol after TBI in ovariectomized female rats and 2) to determine whether both ER subtypes have comodulatory effects in estradiol-induced neuroprotection after treatment with ER-specific agonists.

## Methods

### *Animals*

This study was conducted according to the guidelines for animal experimental protocols of Kerman University of Medical Sciences. The protocol was approved by the ethics committee of this university, in accordance with the internationally accepted principles for laboratory animal use and care, as found in the European Commission guidelines (EEC Directive of 1986; 86/609/EEC) or US guidelines (NIH publication #85-23, revised in 1985). The rats (mature female albino N-Mary rats, weighing 200–250 g) were housed in an air-conditioned room at 22°C–25°C, with a 12-hour light/12-hour dark cycle and free access to food and water.

### *Bilateral Ovariectomy Procedure*

The female rats were first anesthetized intraperitoneally by injection of 60 mg/kg thiopental. The subabdominal area was then shaved, and a 2-cm incision was made. The skin, fascia, and abdominal muscles were opened. Fat and intestine were sheared off until the uterus and its tubes were exposed. Catgut 4 thread was then twisted around the tube of the uterus and vascular base of the ovaries in the proximal area, and the ovaries were cut from the distal area. Saline (1–2 ml) was then poured into the abdomen, and the muscles and skin were replaced. The incision was stitched using 2-0 silk thread, and the wound was washed with Betadine solution. To avoid interference due to the estrus cycle, all experimental animals were ovariectomized 2 weeks before the experiments.[58]

### *Experimental Protocols*

Before the ovariectomized animals were injured using the TBI technique, they were randomly divided into 9 groups (7 rats/group) as follows: 1) control group, intact animals that were neither ovariectomized nor given any drugs; 2) sham group, animals that underwent ovariectomy 2 weeks before the start of the experiment and underwent false brain trauma under anesthesia but did not receive hormones or vehicle; and 7 TBI ovariectomized groups consisting of the following: 3) TBI group, brain injury was induced 2 weeks after ovariectomy; 4) vehicle group, rats received an injection of an equal volume (0.1 ml) of the vehicle (DMSO), which was used as estrogen or ER agonist solvent; 5) E1-treated group, rats received an injection of a physiological dose of 17-β estradiol (33.3 μg/kg); 6) E2-treated group, rats received an injection of a pharmacological dose of 17-β estradiol (1 mg/kg);[22,38,47] 7) PPT group, ovariectomized rats received an injection of the ERα agonist PPT (2.5 mg/kg); 8) DPN group, animals received an injection of the ERβ agonist DPN (2.5 mg/kg); and 9) PPT+DPN group, animals received an injection of a PPT and DPN combination dose (2.5 mg/kg PPT + 2.5 mg/kg DPN/0.1 ml DMSO). In its effects, PPT is a potent ERα agonist that does not activate ERβ,[48] whereas DPN is a full ERβ agonist.[31] These doses of PPT and DPN were chosen based on prior studies investigating female sexual behavior and neurogenesis.[15,30,44,48] In groups receiving drugs or vehicle, treatment was administered as a single dose intraperitoneally 30 minutes after induction of brain trauma. Since the estrogen level shows diurnal rhythm in rodents, all injections were given between 11:00 a.m. and 12:00 a.m. The 17-β estradiol and ER agonists were dissolved in 0.1 ml DMSO. The 17-β estradiol was purchased from Aburaihan Pharmaceutical; PPT and DPN were purchased from Tocris Bioscience; and DMSO was purchased from Sigma-Aldrich.

The 17-β estradiol was measured in all groups up to 24 hours after TBI using an enzyme-linked immunosorbent assay kit (American BMS), following the manufacturer's protocols.

*Model of Diffuse TBI*

All animals were intubated before TBI. The TBI method was the diffuse type, induced by the Marmarou method,[29] using a TBI induction device made by the Department of Physiology, Kerman University of Medical Sciences. The protocol was as follows: a 300-g weight was dropped from a 2-m height onto the head of the anesthetized rat (halothane in 70% $N_2O$ and 30% $O_2$ gas mixture) while a metal disc (stainless steel, 10 mm in diameter, 3 mm thick) was attached to the animal's skull. After induction of the trauma, the rats were immediately connected to a respiratory pump (TSA animal respiratory compact). After spontaneous breathing had been restored, the endotracheal tube was removed, and after recovery, the rats were placed in individual cages. This model induces diffuse cellular and axonal injury in forebrain structures, such as the sensorimotor cortex and hippocampus, but limits brainstem and cerebellar damage.

*Determination of Brain Water Content*

The brain edema of each animal was assessed by measuring brain water content. Anesthetized animals were killed by cervical dislocation 24 hours after TBI, the brain was removed, and brain samples were placed in preweighed glass vials and then weighed (wet weight). The lids were removed, and the vials were placed in an incubator (Memmert) at 60°C for 72 hours and then reweighed (dry weight). The percentage of water in each sample was then calculated using a previously published formula:[36,41] (100 × [(wet weight – dry weight)/wet weight]).

*Determination of BBB Disruption*

The degree of BBB disruption was assessed by measuring Evans blue dye leakage according to the O'Connor protocol,[35] with a slight modification. Briefly, Evans blue dye was dissolved in 0.01 mol/L phosphate-buffered saline at a concentration of 2% and injected (2 ml/kg) into the tail vein 4 hours after TBI as a BBB permeability tracer. Animals were then reanesthetized at 5 hours with halothane and were transcardially perfused with 200 ml heparinized saline through the left ventricle to remove the intravascular Evans blue dye. The brains were removed and homogenized in 1 ml of 0.1 mol/L phosphate-buffered saline; 0.7 ml of 100% (weight/volume) trichloroacetic acid was added; and the mixture was then centrifuged. After centrifugation for 30 minutes at 1000*g*, the absorbance of Evans blue dye in supernatant was measured at 610 nm using a spectrophotometer (Pharmacia Biotech). The amount of extravasated Evans blue dye was quantified as micrograms per gram brain tissue.

*Evaluation of Neurological Outcomes*

The neurological outcomes were measured according to the VCS and were expressed as a score from 3 to 15 that was the sum of 3 parts: motor function (score range 1–8), eye function (score range 1–4), and respiration (score range 1–3).[2,19,22] According to VCS criteria,[21] higher scores indicate better neurological outcomes, and lower scores indicate worse neurological outcomes. In the present study, the outcomes were measured 1 hour before trauma induction and immediately after trauma (Time 0), and measurements were continued at 1, 4, and 24 hours posttrauma.

*Statistical Analysis*

Quantitative data are expressed as the mean ± SEM. The data were analyzed using ANOVA or independent t-test. The Tukey HSD (honestly significant difference) test was used for the ANOVA post hoc analysis. The criterion for statistical significance was set at $p < 0.05$.

## Results

*Brain Water Content*

Changes in the brain water content of the control, sham, and ovariectomized rats 24 hours after TBI without and with different doses of estrogen (E1 and E2) and ER agonist injections are shown in Fig. 1. Figure 1A shows that a marked increase in the water content of the TBI groups (83.63% ± 0.13%) was observed compared with the control and sham groups (73.3% ± 0.42%); this damage was not significantly affected by the administration of the vehicle solution (82.48% ± 0.3%). The increase in the water content due to TBI was significantly inhibited by treatment with E2 (78.88% ± 1.06%, $p < 0.001$) but not by treatment with E1 (81.64% ± 0.15%). In addition, the water content in the E2-treated group was statistically different from the other treated groups (vehicle or E1, $p < 0.001$). Figure 1B shows the effects of the ERα agonist PPT and the ERβ agonist DPN on brain water content compared with the sham and vehicle-treated groups. The increase in the water content in the vehicle-treated group was significantly inhibited by the treatment with PPT (76.4% ± 0.9%, $p < 0.001$) or DPN (75.17% ± 1.7%, $p < 0.001$). A marked reduction in the water content in the PPT+DPN group (73.74% ± 0.8%, $p < 0.001$) was observed compared with the PPT and DPN groups. The percentage changes in brain water content compared with vehicle are shown in Fig. 1C. The percentage change in water content in the PPT+DPN group was significantly higher than those in the E2-, PPT-, or DPN-treated groups ($p < 0.001$). In addition, the percentage change in water content in the E2-treated group was significantly lower than that in the PPT ($p < 0.001$) or DPN ($p < 0.001$) group.

*Extravasation of Evans Blue Dye Content*

Changes in the brain content of Evans blue dye in the control, sham, and ovariectomized rats 5 hours after TBI without and with different doses of estrogen (E1 and E2) and ER agonist injections are shown in Fig. 2. Figure 2A shows that the Evans blue dye content in the TBI rats (35.04 ± 0.3 μg/g tissue, $p < 0.001$) was significantly higher than the content in the control or sham groups (26.0.9 ± 0.5 μg/g tissue), and this damage was not significantly affected by the administration of the vehicle solution (34.49 ± 0.46 μg/g tissue). Disruption of the BBB after TBI was

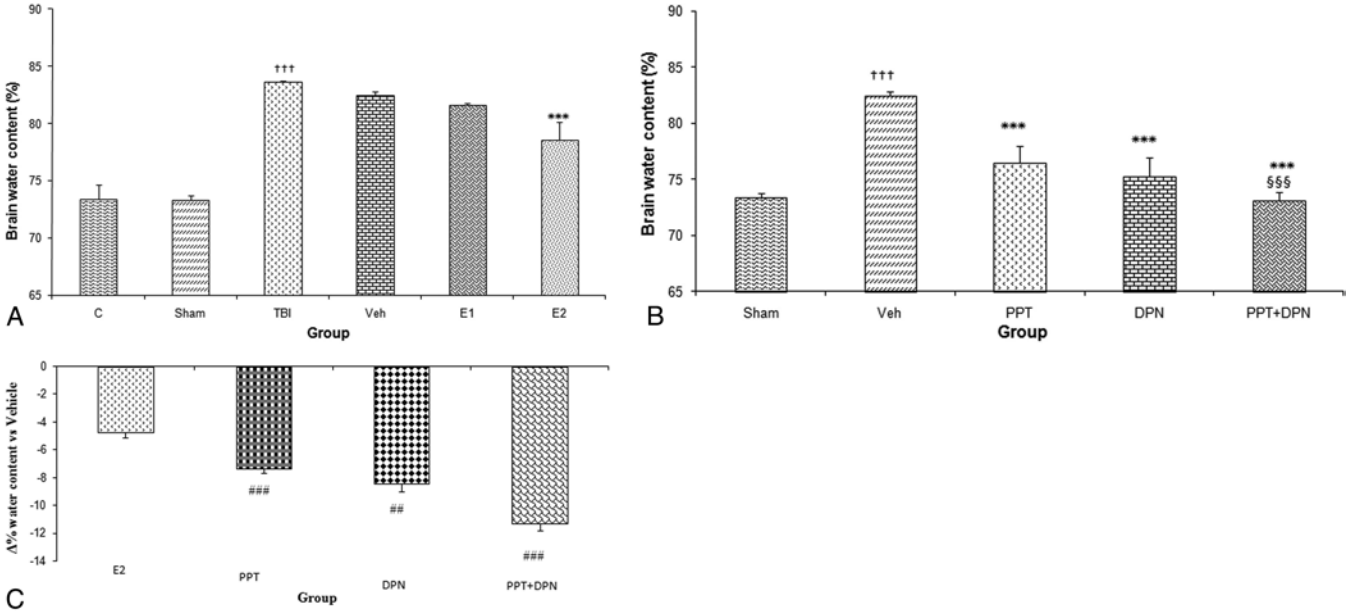


**Fig. 1. A and B:** Effects of the acute administration of 2 doses of estrogen (A) and selective agonists of ERs (PPT and DPN) on brain water content (%) after TBI in ovariectomized rats (B). **C:** Changes in percentage of water content after TBI compared with vehicle. There were 7 rats per group. ***p < 0.001 compared with vehicle. †††p < 0.001 compared with sham. §§§p < 0.001 compared with PPT and DPN. ##p < 0.01 higher than E2. ###p < 0.001 higher than E2, PPT, and DPN. Values represent the mean ± SEM. C = control; Veh = vehicle.

significantly inhibited by treatment with E2 (30.14 ± 1.2 μg/g tissue, p < 0.001) but not by E1 treatment (33.14 ± 0.41 μg/g tissue). In addition, the Evans blue dye content in the E2-treated group was significantly different from the Evans blue dye content in other treated groups (vehicle and E1, p < 0.001).

Figure 2B shows the effects of the ERα agonist PPT and the ERβ agonist DPN on the Evans blue dye content compared with the sham and vehicle-treated groups. The increase in the Evans blue dye content in the vehicle-treated group was significantly inhibited by treatment with PPT (27.67 ± 0.9 μg/g tissue, p < 0.001) and DPN

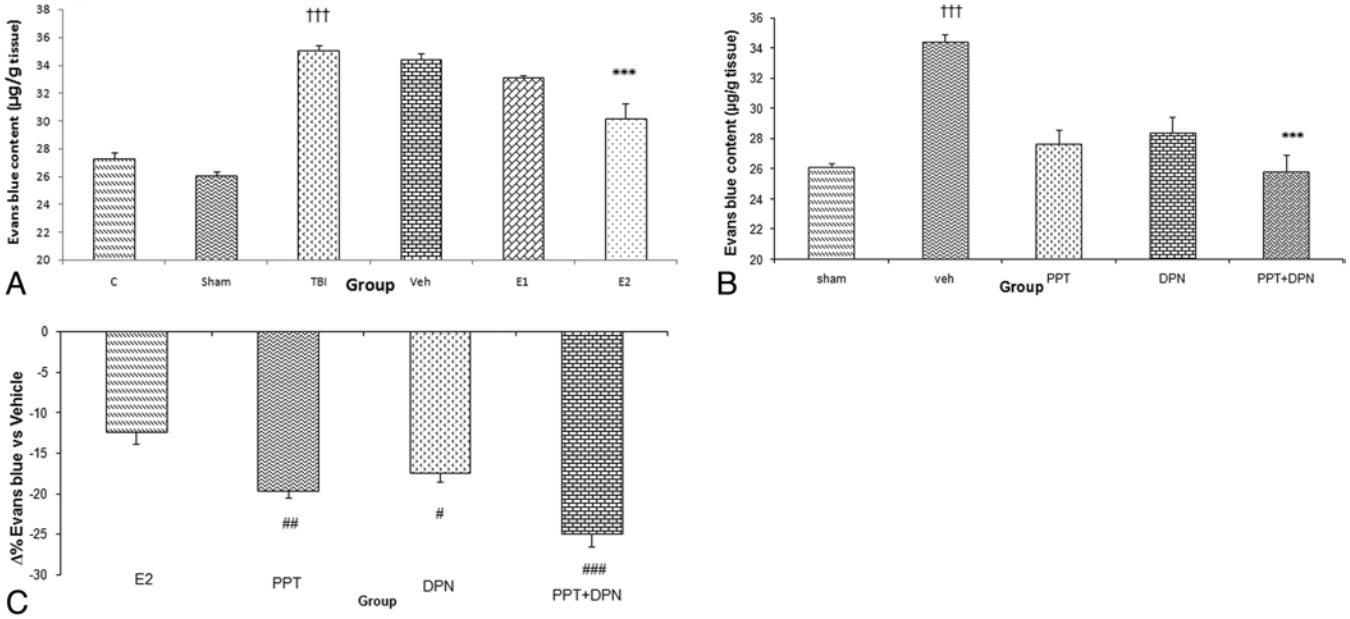


**Fig. 2. A and B:** Effects of the acute administration of 2 doses of estrogen (A) and selective agonists of ERs (PPT and DPN) on Evans blue dye content (μg/g tissue) (%) after TBI in ovariectomized rats (B). **C:** Changes in percentage of Evans blue content after TBI compared with vehicle. There were 7 rats per group. ***p < 0.001 compared with vehicle. †††p < 0.001 compared with sham. #p < 0.05 higher than E2. ##p < 0.01 higher than E2. ###p < 0.001 higher than E2, PPT, and DPN. Values represent the mean ± SEM.

(28.41 ± 1.5 μg/g tissue, $p < 0.001$). The PPT+DPN group (25.84 ± 1.7 μg/g tissue, $p < 0.001$) showed a marked reduction in Evans blue dye content compared with the vehicle-treated group. The percentage changes in the Evans blue dye content compared with the vehicle are shown in Fig. 2C. The percentage change in Evans blue dye content in the PPT+DPN group was significantly higher than that in the E2-, PPT-, or DPN-treated groups ($p < 0.001$). In addition, the percentage change in content in the E2-treated group was significantly lower than that in the PPT ($p < 0.001$) or DPN ($p < 0.05$) group.

*Veterinary Coma Scale*

Changes in the neurological scores of the control, sham, and differently treated ovariectomized rats at different times after TBI are shown in Fig. 3. The VCS scores changed over time after TBI in all groups, but the VCS scores in TBI and vehicle-treated rats were significantly lower than the VCS scores in the other groups at each time point. Figure 3A shows that a marked decrease in VCS score in the TBI group (3.9 ± 0.3) was observed in comparison with the control and sham groups (15 ± 0.0) immediately after TBI. This damage was not significantly affected by the administration of the vehicle solution (3.8 ± 0.2), which was not significantly different with different doses of estrogen. However, the difference between all treated groups and the sham group was significant ($p < 0.001$). One hour after TBI, the decrease in the VCS score was significantly inhibited by treatment with E2 (4.8 ± 0.4, $p < 0.001$) but not by treatment with E1 (4.1 ± 0.46). Four hours after TBI, the difference between the E2 (11 ± 0.81) and vehicle (7.28 ± 0.48) groups was still significant ($p < 0.001$). At this time, rats treated with E1 (9 ± 0.71) had an improved VCS score compared with those in the vehicle group ($p < 0.001$). Twenty-four hours after TBI, the E1 (11.8 ± 0.37) and E2 (13.14 ± 0.69) groups had improved VCS scores compared with those in the vehicle group ($p < 0.001$), but the VCS scores in the 4 groups were significantly lower than those of the control or sham groups (15 ± 0.0). In addition, the VCS score in the E2-treated group was statistically different from the E1-treated group at 1, 4, and 24 hours after TBI ($p < 0.001$).

Figure 3B shows the effects of PPT and DPN on the VCS compared with the sham- and vehicle-treated groups. One, 4, and 24 hours after TBI, a decrease in VCS score in the vehicle-treated group was significantly inhibited by treatment with PPT (5.57 ± 0.53, 11.42 ± 0.78, and 13.57 ± 0.53 at 1, 4, and 24 hours, respectively, $p < 0.001$) or DPN (5 ± 0.81, 10 ± 0.72, and 11.85 ± 0.69 at 1, 4, and 24 hours, respectively, $p < 0.001$). The PPT+DPN group (6.5 ± 0.53, 12.28 ± 0.7, and 13.57 ± 0.97 at 1, 4, and 24 hours, respectively) exhibited a marked increase in VCS score compared with the vehicle-treated group ($p < 0.001$). The percentage changes in VCS score compared with vehicle are shown in Fig. 3C. One hour after TBI, the percentage change in VCS score in the PPT+DPN group was significantly higher than in all other groups ($p < 0.001$), whereas the percentage changes in the E1 group were significantly

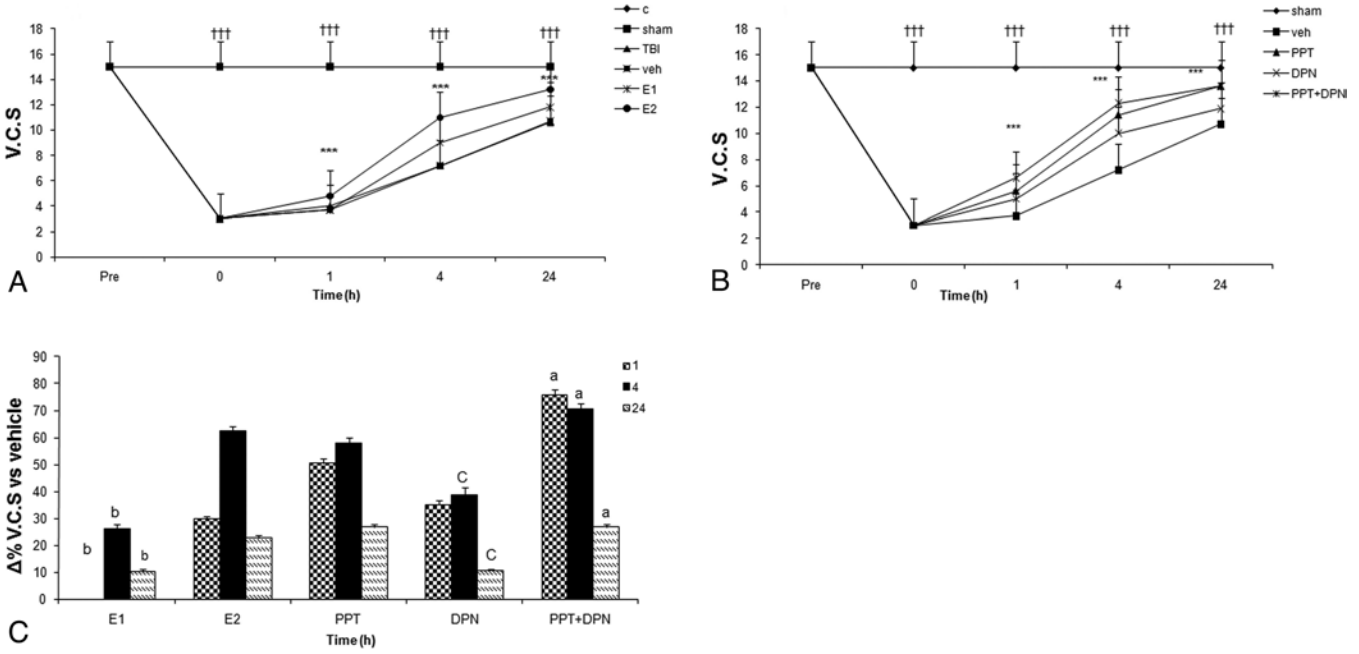


**Fig. 3. A and B:** Comparison of the effects of acute administration of 2 doses of estrogen (A) and selective agonists of ERs (PPT and DPN) on neurological consequences as measured by the VCS at different time points after TBI (B) in ovariectomized rats. Higher scores (consisting of motor function, eye function, and respiration) correlated with better neurological outcomes. **C:** Changes in percentage of VCS scores after TBI compared with vehicle. ***$p < 0.001$ at 1, 4, and 24 hours after TBI for E2, PPT, DPN, and PPT+DPN compared with vehicle and at 4 and 24 hours for E1 compared with vehicle. †††$p < 0.001$ for all time points after TBI for sham compared with all other groups. [a]Higher than all other groups (E1, E2, PPT, and DPN) at all times after TBI ($p < 0.001$). [b]Less than all other groups (E2, PPT, DPN, and PPT+DPN) at 1 hour after TBI and less than E2, PPT, and PPT+DPN at 4 and 24 hours after TBI ($p < 0.001$). [C]Less than PPT at 4 and 24 hours after TBI and less than E2 and PPT at 24 hours after TBI ($p < 0.001$). Values represent the mean ± SEM.

lower than in the E2, PPT, or DPN groups ($p < 0.001$). Four hours after TBI, the percentage changes in VCS score were significantly different in the PPT+DPN group compared with E1, E2, DPN, and PPT+DPN ($p < 0.001$), whereas the changes following PPT treatment were similar to DPN. Twenty-four hours after TBI, the percentage changes of VCS in the PPT+DPN group were significantly higher than those in the E1 or DPN groups ($p < 0.001$). At this time there was no difference between the E2, PPT, and PPT+DPN groups.

*Changes of Estrogen Level in Brain*

Figure 4 illustrates changes in the brain level of 17-β estradiol in different study groups at 24 hours after TBI. The highest 17-β estradiol level in the brain was observed as a high dose of estrogen (608.7 ± 40.3 pg/ml) after TBI; that is, it was higher than in the vehicle and E1 groups (333.4 ± 10.6 and 365 ± 5.2 pg/ml, respectively, $p < 0.001$).

## Discussion

In this study, we made 3 important findings. First, consistent with the previous literature, we found that a high dose (pharmacological dose) of estradiol attenuates brain edema and BBB permeability, whereas both doses of estradiol were able to improve VCS scores.[20,39] Second, neuroprotective effects of estradiol on brain edema, BBB permeability, and VCS score are mediated through both ERα and ERβ. Third, administration of the agonists PPT and DPN in combination demonstrated greater inhibition of brain edema and BBB permeability and a greater increase in VCS score in ovariectomized rats after TBI.

Estradiol has neuroprotective effects due to its ability to modulate brain edema, BBB permeability, and neurological outcomes. There have been a number of reports supporting a neuroprotective role for estrogen after various insults to the CNS. Ovariectomy increases ischemic brain damage in female animals, and estradiol exerts neuroprotective and antiinflammatory action against the ischemic brain when administered immediately upon ovariectomy.[50] Pharmacological concentrations of 17-β estradiol attenuate the extent of injury and decrease cell death in the brain.[59] The BBB permeability decreased in E2-treated mice.[7] Recently, our laboratory showed that hormone loss facilitates, whereas administration of a different dose of estradiol

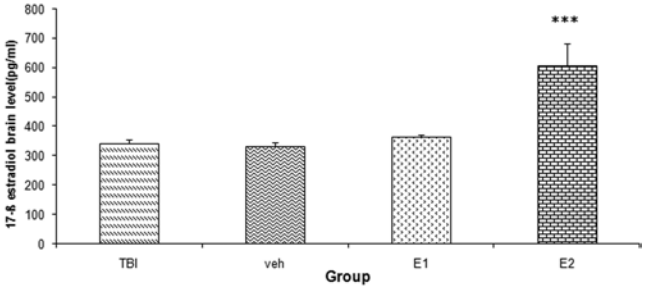


**Fig. 4.** Effects of estrogen administration on brain level of 17β-estradiol after TBI in ovariectomized rats (7 rats/group). 17-β estradiol was measured at 24 hours after injury. ***$p < 0.001$ compared with vehicle or E1. Values represent the mean ± SEM.

could prevent, edema formation[41] and destruction of the BBB after TBI.[20]

Estrogen provides powerful protection against formation of brain edema and prevents damage to the BBB, especially by reducing inflammatory responses. Estrogen reduces inflammatory responses, such as suppression of the production of several proinflammatory mediators including protein expression and the levels of IL-1β, IL-6, and TNFα.[20,24,39] Estrogen reduces damage to the BBB by inhibiting the expression of MMP-9 and cyclooxygenase[24] and increases cerebral blood flow by maintaining brain perfusion, reducing oxidative stress,[26] and inhibiting the activation of microglia.[27] In addition, estrogen might decrease brain edema, but it may also prevent an increase in local inflammation by acting on the BBB. The useful effects of steroids are not only limited to the nerve cells but also expand to the endothelial cells. Protective effects of estrogen on endothelial cells may enable them to protect the BBB against TBI.

The greatest effect of the pharmacological dose of estrogen compared with the physiological dose and vehicle might be related to the highest brain level of 17-β estradiol (Fig. 4). These findings verify that 17-β estradiol at a low dose is possibly unable to cross the BBB and achieve a concentration in brain tissue sufficient to produce neuroprotective responses mediated by estrogen.

To the best of our knowledge, the treatment of TBI with both ERα and ERβ agonists has not been reported thus far. In the present study, we showed that development of brain edema and permeability of the BBB was significantly inhibited by both PPT and DPN. Therefore, no significant differences were observed between the use of DPN or PPT on attenuation of brain edema and BBB permeability, suggesting that both ERs are critical links in estradiol-mediated protection against TBI. In this study, we demonstrated that a single dose of agonists is sufficient to produce such an effect. The inhibitory effects of PPT and DPN on many neurological disorders have been confirmed by other studies.[7,18,32,45,53] The receptors ERα and ERβ are essential for E2-mediated regulation of BBB permeability, antiinflammatory properties, the production of interleukins in the brain, and regulation of neutrophil recruitment into the brain.[7] Both ER agonists reduce the inflammatory response[45] and take part in the control of inflammation by estradiol,[18] neuromodulation, and neuroprotection processes after brain injury.[27,34,53]

There are several probable mechanisms by which ERα and ERβ attenuate BBB permeability and brain edema after TBI that result from the inflammatory response: upregulation of defensin genes, IL-6, MMP-9, MHC (major histocompatibility complex) antigen genes, and some of phagocytic receptors;[40,54] protection of cortical cells against oxidative glutamate toxicity;[59] neuroprotection of estrogen against activated microglia;[4] inhibition of the cytoplasmic transport of NF-κB (a transcription factor for inflammatory genes);[54,56] induction of activity of the phosphoinositol-3 kinase and Akt;[11,55] reduction of cytokines levels, T cells, and macrophages;[49,50,53] and downregulation of both astroglial proliferation and activation of astrocytes.[5]

There is, however, some controversy. Many studies have reported a primary role for ERα as a regulator of the

antiinflammatory properties of estradiol, including the antiinflammatory and neuroprotective activity of estradiol,[3,9,55] neuroprotection after ischemia,[12,44,50] inhibition of microglial activation, and inhibition of high MMP-9 levels.[54] In addition, a growing number of studies have reported antiinflammatory roles for ERβ, such as neuroprotection after ischemia,[1,8,57] inhibition of microglial activity,[4] suppression of transcription, and decreased levels of IL-1β, IL-6, TNFα, and chemokine CCL2.[7,36] There are discrepancies in data among studies that used ER agonists versus genetically ER-deficient animals and studies that have used the agonists. Some reasons for the differences in these other results with those in our study might be differences in dose, formulation, the type and severity of injury, route of administration, time of using the agonists, and length of treatment.

Administration of the combined agonists (PPT+DPN) demonstrated the greatest inhibitory effect on brain edema and BBB permeability. Our results are consistent with the results of a number of other studies that showed that uterine weight was increased by PPT+DPN.[38] Based on the results of this part of the study, coadministration of PPT and DPN could be useful, indicating that activation of ERβ may modulate ERα activity.[13,25] It was found that ERα and ERβ expression increased after treatment with PPT or DPN, respectively;[46] however, estradiol had no effect on ERα level,[43] and possibly at these doses, both DPN and PPT have effects on either ER.[31,48] Moreover, the PPT+DPN-treated animals may have experienced pharmacological effects due to higher levels of steroids administered with the combined treatment.[15]

When administered alone, the agonists generated a neuroprotective effect more than estradiol. This finding is consistent with the results of a number of other non-TBI studies.[17,37,48] Several studies have reported that PPT and/or DPN might induce vasorelaxation[38] and phospho-Akt (pAkt)[17] and cause an increase in progesterone receptor levels in the brain[16] more than estrogen alone.

The results for neurological outcomes measured in this study showed that the PPT+DPN group had a higher score 1 hour after TBI; this difference also persisted at 4 hours after TBI. Of course, at all times after trauma, recovery in the E2, PPT, and DPN groups was better than that in the vehicle group, except at the 1-hour time point; recovery was also better for E1 than the control group. Another interesting observation was the reduced effect of DPN at longer time points, such that the neurological scores of this group at 4 and 24 hours after TBI were lower than those of the PPT or PPT+DPN group. Therefore, if an increase in VCS score is desired, the administration of DPN is not recommended. It is postulated that estrogen is effective in neurological recovery from the 1st hour after TBI.[41] The mechanism for the increased VCS score by steroids may also be an effect of estrogen, DPN, and PPT due to their role in the regulation of brain edema and BBB permeability, which was shown in the present study and our previous study.[41] Other possibilities include increased ERα expression after TBI[32] and an increase in the neurological score by lowering intracranial pressure.[28,41] Furthermore, the increase in progesterone and progesterone receptor levels[16] may mimic the effects of PPT and estrogen on VCS score. This increase in progesterone and progesterone receptor levels may be sufficient to mimic the effects of PPT and estrogen on VCS score.[41] Progesterone receptor levels in the PPT+DPN-treated group and the E2-treated group did not differ; perhaps this accounts for the similar effects of PPT+DPN-treated groups with estrogen on VCS score.

Although both PPT and DPN increased the VCS score after TBI, PPT appears to be more effective in increasing neurological scores. The data presented here suggest a different role for each ER subtype in targeting the mechanisms that occur in brain edema versus recovery after injury. The subtype ERα appears to play a predominant role in mediating the effects of estradiol in recovery after TBI, whereas activation of ERβ is not sufficient to increase the VCS score in the ovariectomized rat. This means that DPN is not an eligible candidate for the treatment of neurological behavior. The significant improvement in VCS score by the PPT for all time points after TBI may be consistent with evidence suggesting that ERα has a predominant role in the control of brain injury over ERβ. Indeed, in agreement with these findings, the combination of HPTE (2,2-bis[p-hydroxyphenyl]-1,1,1-trichloroethane, an ERα agonist) and DPN did not provide any further neuroprotection than HPTE alone;[3] this eliminates the possibility of a role for ERβ in conjunction with ERα on the VCS effect of PPT at 4 and 24 hours after TBI. The 2 receptors act synergistically in some tissues, whereas they act antagonistically in others.[42,53]

## Conclusions

Our study demonstrated a net effect mediated by both ERα and ERβ. These observations suggest that there may be a direct effect of estrogen on neurons, glia, endothelial cells, and ischemia-sensitive areas and that estradiol may mediate its neuroprotective effects on TBI directly. Our findings also suggest that the use of drugs that selectively inhibit ERα and ERβ might intensify the beneficial effects of estrogen. Future studies based on strategies that selectively block ERs will be important to determine whether ERα and ERβ signaling are both sufficient and necessary to decrease brain edema and BBB permeability and to increase neurological score in ovariectomized animals. In addition, the relative contribution of ERα versus ERβ on gene transcription and protein translation or degradation awaits further study.

### Disclosure

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