Original Article

The Effect of Angiotensin Receptor Type 2 Inhibition and Estrogen on Experimental Traumatic Brain Injury

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Abstract

Background: Estrogen interferes with renin-angiotensin system (RAS). Increasing evidence suggests that estrogen interferes with the RAS such as decreasing angiotensin receptor in the brain. **Objectives:** This study aimed at investigating the mutual interaction between estrogen and candesartan (an angiotensin receptor blocker) to inhibit or amplify each other's neuroprotective effects after traumatic brain injury (TBI). **Materials and Methods:** Female rats were divided into 11 groups and the ovaries were removed in nine groups. Study groups included sham, TBI, oil, vehicle (Veh), a low dose (LC) and a high dose (HC) of candesartan, estrogen (E2), Veh + Veh, and a combination of estrogen with a low dose (E2 + LC) and a high dose (E2 + HC) of candesartan. TBI was induced by the Marmarou's method. Brain edema and integrity of blood–brain barrier (BBB) were assayed by calculating brain water content (BWC) and Evans blue content, respectively. The neurological outcome was evaluated using the veterinary coma scale (VCS). **Results:** The results showed that the BWC in the E2 group was less than that of the oil group ($P < 0.01$) and in the HC group was also less than that of the Veh group ($P < 0.05$). Posttraumatic Evans blue content in the TBI, oil, and Veh groups was higher than that in the E2 ($P < 0.001$) and HC ($P < 0.001$) groups. Although there was no significant difference in the above indicators between the LC and Veh groups, both the BWC and Evans blue content in the E2 + LC group were lower compared to the oil + Veh group ($P < 0.001$). In addition, the VCS increased in the E2, HC, and combined groups after TBI ($P < 0.01$). **Conclusion:** Prescribing estrogen alone and a high dose of candesartan and a low dose of candesartan with estrogen has a neuroprotective effect on brain edema, permeability of BBB, and neurological scores. This may suggest that estrogen and candesartan (especially in a low dose) act via similar paths.

Keywords: Blood–brain barrier, candesartan, cerebral edema, estrogen, neurologic score, traumatic brain injury

Background

Every year, ten millions of people around the world suffer from traumatic brain injury (TBI), leading to their disability and death. The TBI is a major social, economic, and health problem.[1] The World Health Organization has estimated that by 2020, TBI will become a common cause of death in comparison with other major important diseases.[2] Furthermore, TBI often occurs in young people (aged between 15 and 45 years) and 75% of the victims are men.[3]

At present, there is a group of treatments with more than 30 years of clinical work, yet they have become flawed. Therefore, there is an urgent need to find new neuroprotective factors that reduce the complications of TBI.[4]

Investigating the performance of angiotensin 2 (Ang II) and angiotensin receptor type 1 (AT1R) and AT2R in the brain and after the stroke has shown that AT1R is vastly expressed in mature people and causes many of Ang II activities, such

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as inflammation and vasoconstriction. A2TR expression is less in mature individuals, yet it increases due to diseases, and its impacts are against AT1R. In other words, it has anti-inflammatory, vasodilator, antioxidant, antiapoptotic, angiogenesis, and neurogenesis impacts.[5] The balance between the two receptors can determine the usefulness of Ang II. The consumption of AT1R blockers or the stimulation of AT2R causes this balance.[5] It is reported that angiotensin receptor blockers (ARBs) can account for such an effect against cerebral ischemia.[6]

The decreased expression of transcription factors involved in apoptosis[7] and the decreased oxidative stress[8] have been reported following estrogen administration after TBI similar to AT1R blockers. Reduced brain edema[9] and permeability of the blood–brain barrier (BBB)[10] and improved neurologic outcomes[11] by estrogen have also been found in our previous studies. Other studies have also confirmed reduced cell death and increased nerve regeneration and neurotrophic support by estrogen following TBI.[12]

On the other hand, considerable evidence suggests that estrogen can interfere with renin-angiotensin system (RAS), which includes reducing the production of angiotensinogen[13] and decreasing angiotensin-converting enzyme (ACE).[13] It has been reported that estrogen alpha receptor leads to the activation of neuroprotective genes and reduction in the brain Ang II.[14] A research shows that estrogen reduces AT1R in the brain.[15]

Objectives

Few studies have been done on efficiency and anti-inflammatory mechanisms of ARBs alone or in combination with estrogen in TBI damages. Therefore, in the present study, we assumed that changes in the elements of RAS and activation of AT1R after TBI may play a role in damage caused by TBI as well as anti-inflammatory effects of estrogen. Therefore, this study was designed to assess the effect of candesartan doses ineffective on blood pressure as well as the combined effect of estrogen and candesartan on brain edema, permeability of the BBB, and neurological outcome following TBI in ovariectomized animals.

Materials and Methods

Animals

This study was performed according to license 280/93 K issued by the Ethics Committee of Kerman University of Medical Sciences. In this study, female rats (200–250 g) were housed at 20°C–22°C temperature and 12-h light/dark in the animal house of Faculty of Medical Sciences.

Drugs

The sesame oil and 17-beta-estradiol were obtained from Aburaihan Pharmaceutical Company (Tehran, Iran). Candesartan was purchased from the LKT Company in America.

Ovariectomy surgery

To eliminate the effect of estrous cycle and ovarian steroids, the animals were ovariectomized 2 weeks before TBI.[16] A combination of ketamine and xylosin was used intraperitoneally. Then, the lower abdominal area was shaved, and a small vertical incision was made in the skin of this area. The tissues and muscles were opened, and fat and intestines were pushed up so that the fallopian tubes could be observed. In each ovary, the fallopian tube was tied using a 0.4-mm catgut cord in the paroxysmal area, and it was disconnected from the distal area.

Finally, after injecting 2 mL of isotonic saline, the muscles and skin were stitched and the stitches were disinfected with iodine. The animals were under care until the end of the anesthesia.

Experiment protocols

The rats were divided into two groups. One group was sham-ovariectomy (OVX, animals whose ovaries were falsely removed, $n = 7$) and another group was OVX (the ovaries of these animals were removed 2 weeks before the TBI). Then, OVX rats were divided into 10 groups. One group experienced false TBI (sham), and the other nine groups had real TBI. These groups include (1) TBI, (2) oil (OVX rats that received estrogen vehicle [sesame oil] equal to the amount of the consumed estrogen, intraperitoneally 30 min after the TBI),[17] (3) E2 (OVX rats that received 1 mg/kg of estrogen intraperitoneally 30 min after the TBI),[17] (4) Vehicle (Veh, similar to oil group with the only difference that they received candesartan vehicle (sodium carbonate 0.1 N),[18] (5) LC (OVX rats that received 0.1 mg/kg of candesartan intraperitoneally 30 min after the TBI),[19] (6) HC (the same as LC only with the difference that they received 0.3 mg/kg of candesartan),[18] (7) oil + Veh (similar Veh only with the difference that they received estrogen vehicle + candesartan vehicle [sesame oil] + sodium carbonate 0.1 N), (8) E2 + LC (OVX rats that received 1 mg/kg of estrogen and 0.1 mg/kg of candesartan intraperitoneally 30 min after the TBI), (9) E2 + HC (similar to E2 + LC group with the difference that they received 1 mg/kg of estrogen and 0.3 mg/kg of candesartan).

Induction of diffuse traumatic brain injury

TBI was diffused and created using the Marmarou's method. After putting a metal disc (made of steel with a thickness of 3 mm and a diameter of 10 mm) on the skull of the animal (between Bregma and Lambda) using polyacrylamide glue, the anesthetized animal was put under the TBI device (developed in the Kerman Physiology Group). Two hundred and fifty grams weight was released from 2 m distance through a pipe and landed on the metal disc. After the injury, if necessary, the aspiration of the animals was immediately brought back by connecting them to the respiratory pumps.[20]

Determination of brain edema

Twenty-four hours after TBI, the brain of anesthetized animal was taken out, and after weighing (wet weight), it was kept for 72 h in an autoclave at a temperature of 60°C–70°C, and then, we weighed it again (dry weight).

Brain water content (BWC, an index of brain edema) was calculated using the following formula:[17,21]

Table 1: Changes in the brain water content in different groups (24 h after traumatic brain injury)

	Group				
	Sham-OVX	Sham + OVX	TBI	Oil	E2
Brain water content	0.14±77.78	0.11±77.87	0.12[a]±78.83	0.13[b]±78.69	0.14[c]±78.04

Data are presented as mean±SEM (n=7 in each group). [a]Versus sham-OVX and sham + OVX (P<0.001), [b]Versus Sham-OVX and Sham + OVX (P<0.001), [c]Versus Oil (P<0.01) and TBI (P<0.001). TBI: Traumatic brain injury, Oil: Vehicle of estrogen, E2: Estrogen, SEM: Standard error of the mean, OVX: Ovariectomized

Table 2: Changes in the brain water content in the group treated with candesartan and compound groups (24 h after traumatic brain injury)

	Group					
	Veh	LC	HC	Oil + Veh	E2 + LC	E2 + HC
Brain water content	0.13±78.83	0.2±78.59	0.15[a]±78.24	78.76±0.14	0.18[b]±78.30	0.18[b]±78.15

Data are presented as mean±SEM (n=7 in each group). [a]Versus Veh (P<0.01), [b]Versus Oil + Veh (P<0.01). Veh: Vehicle of candesartan, LC: Low dose of candesartan, HC: High dose of candesartan, Oil + Veh: Vehicle of estrogen + vehicle of candesartan, E2 + LC: Estrogen+low dose of candesartan, E2 + HC: Estrogen + high dose of candesartan, SEM: Standard error of the mean

BWC (%) = ([weight of dry tissue – wet tissue weight]/wet tissue weight)× 100

Determining blood–brain barrier disruption

By measuring the extravascular Evans blue content using a spectrophotometry, the permeability of the BBB was determined. Four hours after the trauma, 20 mg/kg of Evans blue color was injected via the jugular vein. One hour after the injection (5 h after the trauma), to exit the blue color from blood, 200–300 mL of heparin isotonic saline solution was diffused into the left ventricle after opening the chest and cutting the jugular vein of the two sides. Then, the brain was quickly taken out of skull and weighted. After homogenization, it was put in 20 mL of solution (14 mL acetone + 6 mL sodium sulfate). After 24 h of shaking, 1 mL of the solution was mixed with trichloroacetic acid, and after 2–3 min exposure to cold temperature, it was centrifuged at 2000 g for 10 min.[17]

Finally, the optical density of 1 mL of supernatant was estimated using a spectrophotometer at 620-nm wavelength.

The color (μg/g of tissue) was calculated using the following formula:

Evans blue = 13.24 × 20 × optical density/weight tissue.

Investigating neurological outcome

According to the veterinary coma scale (VCS), the neurological consequences were measured in the form of neurological scores.[3-15] There are three type of scores: motor performance (score range: 1–8), eye performance (score range: 1–4), and respiratory performance (score range: 1–3),[22] which were estimated 1 h before the TBI, immediately after TBI, and 1, 4, and 24 h after the TBI. Based on the VCS index, the higher scores have a better representation of better neurologic outcomes, whereas the lower scores have worse representation of neurological outcomes.

Statistical analysis

The results were estimated based on mean ± standard error of the mean. To check for normal distribution of the data, the Shapiro–Wilk test was used. The data were not normally distributed; nonparametric Kruskal–Wallis and Friedman tests were used for analysis. $P < 0.05$ was considered as the significance level.

Results

Table 1 shows the BWC changes in different groups. The BWC in the TBI (78.83 ± 0.12) and oil groups (78.69 ± 0.13) was higher than that of sham + OVX (77.87 ± 0.11) group ($P < 0.001$). However, there was no significant difference in the BWC between the TBI and oil groups. The BWC in the E2 group (78.04 ± 0.14) was lower than that of the oil group ($P < 0.01$). Furthermore, Table 2 shows the effect of different doses of candesartan and combination of estrogen and candesartan on BWC. The BWC in the HC group (78.24 ± 0.15) was significantly reduced compared to the Veh group (78.83 ± 0.18) ($P < 0.05$). The BWC in LC and HC groups was not significant; it decreased significantly in E2 + LC and E2 + HC groups compared to the oil + Veh group ($P < 0.01$). The BWC was not different among E2 + LC, E2 + HC, and E2 groups [Figure 1a]. Figure 1b shows that BWC in the E2 + LC group significantly reduced compared to the LC group ($P < 0.05$). However, no significant difference in the BWC was observed between the HC and E2 + HC groups.

Figure 2 shows the changes in the content of Evans blue in different groups. This index was high in the TBI (38.3 ± 1.33) and oil (27.14 ± 0.78) groups compared to the sham + OVX group (7.77 ± 1.17) ($P < 0.001$), and it was not different between the TBI and oil groups [Figure 2a]. Figure 2b shows a significant reduction of the Evans blue content in the E2 group (12.87 ± 0.93) compared to the oil group ($P < 0.001$). The Evans blue content was less in the HC group (14.04 ± 1.5) compared to the LC (25.45 ± 1.08) and Veh (30.38 ± 1.09) groups ($P < 0.001$). This indicator was decreased in the LC group compared to the Veh group ($P < 0.01$) [Figure 2c]. Figure 2d

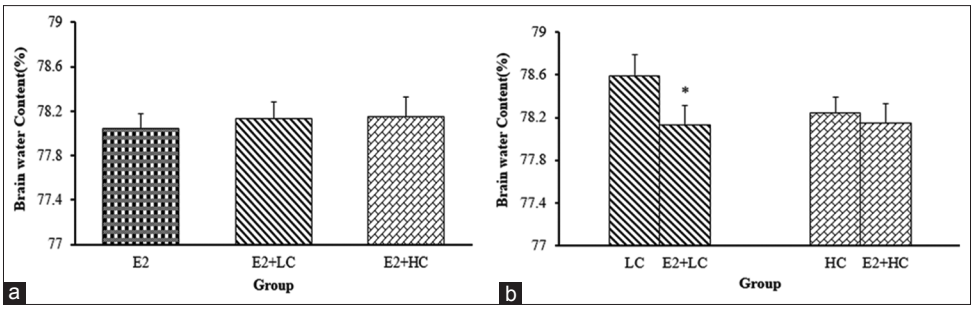

Figure 1: (a) The brain water content (%) in different groups treated with estrogen and combination of estrogen and candesartan (n = 7 in each group). The results are based on mean ± standard error of the mean. *: P <0.05 versus LC (b). E2: Estrogen, E2 + LC: Estrogen + low dose of candesartan, E2 + HC: Estrogen + high dose of candesartan, LC: Low dose of candesartan, HC: high dose of candesartan

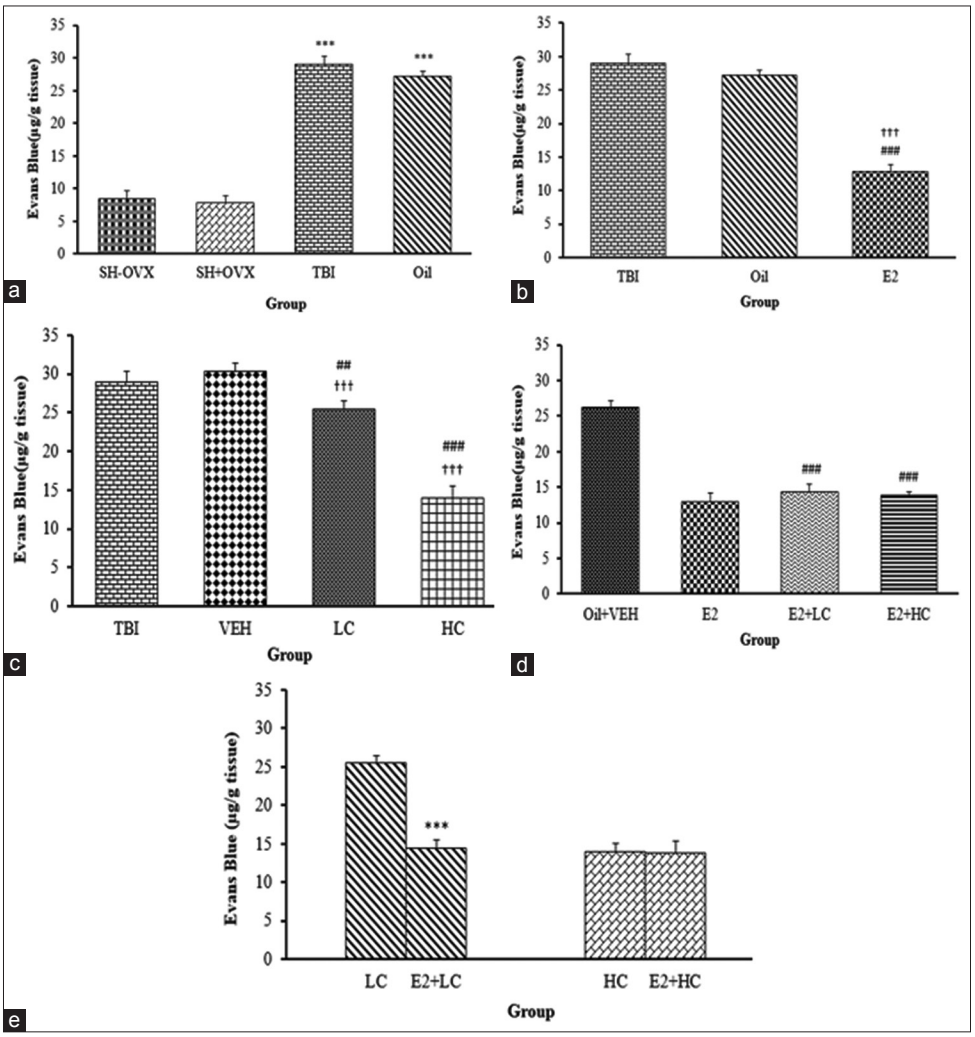

Figure 2: Comparison of the Evans blue color (µg/g tissue) in treated and nontreated groups (n = 7 in each group). The results are based on mean ± standard error of the mean. ***P < 0.001 versus Sham-OVX and Sham + OVX (a). †††P < 0.001 versus traumatic brain injury. ###P < 0.001 versus oil (b). †††P < 0.001 versus traumatic brain injury. ##P < 0.01 versus Veh. ###P < 0.001 versus Veh (c). ###P < 0.001 versus oil + Veh (d). ***P < 0.001 versus LC (e). TBI: Traumatic brain injury, oil: Estrogen vehicle, E2: Estrogen, LC: Low dose of candesartan, HC: High dose of candesartan, Veh: Candesartan vehicle, E2 + LC: Estrogen + low dose of candesartan, E2 + HC: Estrogen + high dose of candesartan, Oil + Veh: Estrogen vehicle + candesartan vehicle

shows that the Evans blue content significantly decreased in the E2 + LC (14.39 ± 1.03) and E2 + HC (13.82 ± 0.59) groups compared to the oil + Veh group, and it was not different among E2, E2 + LC, and E2 + HC groups. Furthermore, this indicator was significantly lower in the E2 + LC group compared to the LC group (P < 0.001) [Figure 2e].

Neurological outcomes in the different groups and at different times of the study in terms of the neurological score are shown in Figure 3. Figure 3a shows that there was no significant difference in the VCS between the TBI and oil groups in any hour of the study. Immediately after the injury, this index was significantly less in the TBI (3.33 ± 0.21, $P < 0.001$) and oil groups (4 ± 0.36, $P < 0.001$) compared to the sham-OVX and sham + OVX (15 ± 0.0) groups. The indicator was significantly decreased in the oil and TBI groups compared to the sham + OVX group, 1, 4, and 24 h after TBI ($P < 0.001$). Figure 3b shows a significant increase in neurological scores of the E2 group (12.83 ± 0.16 and 14.33 ± 0.21) compared to the oil group, 4 and 24 h after TBI, respectively ($P < 0.01$). This index in the E2 group was lower compared to that in the TBI, 1, 4 and 24 h after TBI ($P < 0.01$). Figure 3c shows that there was no significant difference in the VCS between the LC and Veh groups in all hours. In addition, the neurological score had a significant decrease in the HC group compared to the Veh group, 4 and 24 h after TBI ($P < 0.01$).

The index was decreased in the E2 + LC group (9.66 ± 0.3) compared to the oil + Veh group, 1 h after TBI ($P < 0.05$). Furthermore, the neurological score was significantly high in the E2 + LC and E2 + HC groups compared to the oil + Veh group, 4 ($P < 0.05$) and 24 h ($P < 0.01$) after TBI. There was no significant difference in the VCS among the E2 + LC, E2 + HC, and E2 groups in any time after TBI [Figure 3d].

Discussion

The results of this study showed the reduction of cerebral edema, prevention of BBB destruction, and improvement of neurologic outcomes by estrogen and 0.3 mg/kg dose of candesartan. Furthermore, although 0.1 mg/kg dose of candesartan had no neuroprotective effect, its effects were similar to the consumption of a high dose of candesartan when combined with estrogen. The BWC and Evans blue content increased following TBI 1.23% and 270%, respectively, compared to the sham groups; similar changes were also caused by oil. The amount of the brain edema content was decreased following estrogen administration by 0.83%. Estrogen also decreased the Evans blue content (52.58%) compared to the oil group.

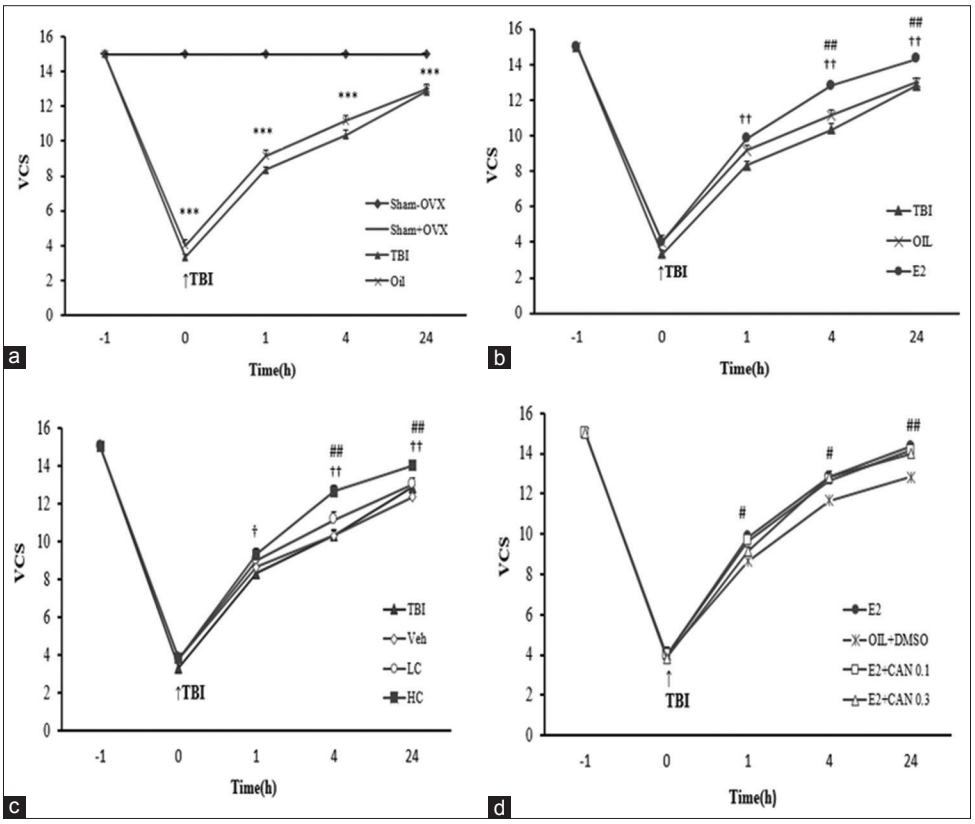

Figure 3: Changes in treated and non-treated groups at different hours ($n = 7$ in each group). The results are based on mean ± standard error of the mean. $^{***}P < 0.001$ in the traumatic brain injury and oil compared to Sham-OVX and Sham + OVX and for all hours after traumatic brain injury for all hours after traumatic brain injury (a). $^{\dagger\dagger}P < 0.01$ for E2 compared to traumatic brain injury, 1, 4, and 24 h after traumatic brain injury. $^{\#\#}P < 0.01$ for E2 compared to oil at 4 and 24 h after traumatic brain injury (b). $^{\dagger}P < 0.05$ for HC compared to traumatic brain injury in the 1st h after traumatic brain injury. $^{\dagger\dagger}P < 0.01$ for HC compared to traumatic brain injury 4 and 24 h after traumatic brain injury. $^{\#\#}P < 0.01$ for HC compared to Veh 4 and 24 h after traumatic brain injury (c). $^{\#}P < 0.05$ for E2 + HC in comparison with oil + Veh 1 h of traumatic brain injury. $^{\#}P < 0.05$ for E2 + LC in comparison oil + Veh 4 h after traumatic brain injury. $^{\#\#}P < 0.01$ for E2 + HC and E2 + LC compared to oil + Veh 24 h after traumatic brain injury (d). TBI: Traumatic brain injury, Oil: Estrogen vehicle, E2: Estrogen, LC: Low dose of candesartan, HC: High dose of candesartan, Veh: Candesartan vehicle, E2 + LC: Estrogen + low dose of candesartan, E2 + HC: Estrogen + high dose of candesartan, oil + Veh: Estrogen vehicle + candesartan vehicle

The loss of BBB integrity could be due to initial damage,[23] upregulation of the aquaporins (AQPs),[24] metalloproteinase-9 (MMP-9),[25] inflammation, and free radicals produced[26] after TBI as mechanisms involved in the development of cerebral edema. By stabilizing the BBB,[10] reducing AQP4,[27] decreasing oxidative stress,[8] reducing cytokines, driving inflammation, increasing anti-inflammatory cytokines,[28] maintaining cerebral blood flow,[29] inhibiting MMP9,[30] estrogen may maintain its antiapoptotic[12] and protective effect on keeping the integrity of BBB in turn brain edema reduction.[31]

Estrogen has been widely investigated in experimental studies of ischemia and hemorrhage brain injury. Almost without exception, these studies found that estrogen reduces tissue damage and improves performance following injury. The results of this study are in line with the results of our previous laboratory studies.[9,10,16] Furthermore, other studies have reported neuroprotective effects of estrogen on reducing cerebral edema, protecting the BBB, and improving neurological outcomes. In a study conducted by O'Connor *et al*., it was observed that administration of a single dose of estradiol to female rats ½ h after injury caused a significant decrease in BWC 24 h after injury in comparison with the control group.[17] The results of Naderi *et al*.'s study also showed that estrogen activates neuroprotection against brain edema and destruction of BBB through the both receivers.[10]

In spite of much evidence that introduces estrogen as a neuroprotective agent, some studies never observed any negative or positive impact derived from estrogen consumption. The results of the present study could be due to a consumed estrogen dose,[32,33] type of TBI, and severity of created TBI.[34] In another section of this study, in which the effect of different doses of candesartan was investigated, the findings show that only high doses of candesartan could decrease BWC for 75% and reduce neurological score in different hours after TBI. Although the inhibitory effect of candesartan on the Evans blue content was applied by both doses, the effects of a high dose were much more than a low dose (53.79% compared to 16.23%).

This may indicate the dose-dependent effect of candesartan on brain edema and neurological outcome. Furthermore, unlike other studies, in this research, 0.1 mg/kg dose of candesartan might affect the Evans blue content because of blocking AT1R in vascular endothelial brain and the resulting effects on the BBB. On the other hand, it seems to have an impact on brain edema and neurological outcomes, yet there is a need for more concentration. Since it has been reported that there is an inverse relationship between brain edema and neurological outcome,[35] no effect on neurological outcomes in low concentrations may be due to lack of concentration impact on cerebral edema.

ARBs are known for their multiple functions in the brain such as reducing inflammation, providing protection against stroke, and having direct neuroprotective effect. Candesartan is reported to cause reduction of brain injury,[36] increase in coronary microcirculation,[37] and the expression of endothelial NOS in the brain vessels,[6] reduction of inflammation[38] and oxidative stress,[39] prevention of endothelial function defection,[6] reduction of TGF1β,[36] activation of peroxisome proliferator-activated receptor-gamma,[36] normalization of cerebral vascular self-regulation, and inhibition of inflammatory signals that reach to brain through the vagus.[40]

Since the AT1R expression in the cerebral cortex is little, it is likely that the candesartan neuroprotection has a more general effect in reduction of inflammation and oxidative stress through its function on other types of cells such as brain vessels.[41] In addition, it has been reported that treatment with candesartan reduces microglial activity in the injured cortex after TBI,[36] and this may count for the beneficial therapeutic effect of candesartan. Furthermore, a research conducted by Brdon *et al*. showed improved neurological outcome and reduced infarct size in systemic candesartan administration after ischemic damage.[42] Panahpour *et al*. showed that candesartan reduces the size of injury and cerebral edema, and it improved the outcome of ischemic injury in animal tests.[18]

As it has been stated, several functions in the brain are modulated by estrogen, and estrogen probably interferes with the AT1R signaling pathway. In another part of this study, the effect of different doses of estrogen combined with candesartan on various indices was investigated. Given the results, it was found that when estrogen is taken with low concentrations of candesartan, the effects on the Evans blue will be similar to the effects on BWC. In other words, there was no difference between estrogen and estrogen and candesartan regarding the content of brain water. This was also similar for the Evans blue content. In other words, although a low dose reduced the Evans blue content, when it was used with estrogen, the maximum inhibitory effect was similar to when estrogen was applied individually. As a matter of fact, this was true for both the groups. On the one hand, the combined effect was not greater than the effect of estrogen alone, and on the other hand, the impact of using a combination of estrogen and candesartan was similar to the effect of estrogen or candesartan alone. The impact of consuming a combination of these drugs is not greater than the effect of a single dose because maximum efficacy of these two drugs cannot go any further or they exerted their neuroprotective effects through a common signaling.

Although few studies have examined the effects of estrogen in the RAS, the results indicate modulation of RAR activities and thus reduction of the harmful effects caused by estrogen, insensitivity of AT1R, and increase of protection by AT1R blockers.

Possible mechanisms for strengthening candesartan effects by estrogen include weakening of AT1R response by estrogen,[43] increasing the expression of AT2R,[14] upregulation of ACE2,[44] increasing the MASS receptor by estrogen, increasing Ang I–VII, and strengthening its signaling pathway.[45]

The results of the present study showed that administration of an individual dose or ineffective dose of candesartan on

blood pressure (0.3 mg/kg) following diffuse TBI in the female rats led to the decrease in cerebral edema, prevention of BBB destruction, and improvement of neurological outcome. However, a low dose of candesartan (0.1 mg/kg) did not have such a neuroprotective effect, yet taking this dose in combination with estrogen resulted in the emergence of neuroprotective effects.

Moreover, there was no significant difference between the effects of estrogen and a high dose of candesartan. A combination of estrogen and a high dose of ARB did not increase the effects of any of the two drugs, which can suggest that probably each of them had different paths for exerting their effects or decreasing effect on brain edema cannot be more than this. In addition, this study also showed that estrogen and candesartan caused neurological improvement probably by reducing cerebral edema. However, since the effect of candesartan low dose emerged after the consumption of estrogen, it may suggest the interaction between estrogen and a low dose of candesartan. In the future, it is necessary to do more studies to determine the mechanisms and receptors of estrogen involved interaction between estrogen and ATR1.

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Conflicts of interest

There are no conflicts of interest.

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