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# Role of melatonin receptors in the effect of estrogen on brain edema, intracranial pressure and expression of aquaporin 4 after traumatic brain injury

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ABSTRACT

***Objective(s)***: Traumatic brain injury (TBI) is one of the most common causes of death and disability in modern societies. The role of steroids and melatonin is recognized as a neuroprotective factor in traumatic injuries. This study examined the role of melatonin receptors in the neuroprotective effects of estrogen.

***Materials and Methods:*** Seventy female ovariectomized Wistar rats were divided into five groups and two subgroups. All animals underwent brain trauma. The groups were as follow: 1) trauma, 2) melatonin receptor antagonist vehicle + estrogen, 3) MT1 melatonin receptor antagonist + estrogen, 4) MT2 melatonin receptor antagonist+ estrogen, 5) MT3 melatonin receptor antagonist+ estrogen. Brain edema (24 hr), intracranial pressure (ICP) (-1, 0, 1, 4 and 24 hr) and blood–brain barrier (BBB) permeability (5 hr) and aquaporin (AQP4) expression (24 hr) were evaluated after TBI.

***Results:*** MT1, MT2 and MT3 melatonin receptors had anti-edema effects while MT1 and MT2 have a role in protecting BBB by estrogen. Furthermore, the activity of MT3 and MT2 melatonin receptors weakened the effect of estrogen on ICP. However, melatonin receptors had no role in the effect of estrogen on AQP4 protein.

***Conclusion:*** Based on the above results, it seems that melatonin receptors appear to influence the effect of estrogen in TBI without altering AQP4 expression. The role of the receptors is different in this interaction.

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## Introduction

Traumatic brain injury (TBI) is the most common cause of mortality and disability among people below 35 years of age. About 50 -70% of TBI cases are due to car and motorcycle accidents, which lead to the death of 52000 and hospitalization of 500000 people in developed countries every year (1). In Iran, road-traffic crashes cause TBI-induced disability to more than 300,000 persons each year (2).

TBI is a complicated process that includes initial and secondary injuries, and regeneration (3). Initial injuries mainly happen due to the increase of intracranial pressure (ICP) and blood–brain barrier (BBB) permeability, which leads to edema (3).
Secondary injuries occur due to complex physiological and biochemical processes such as oxidative stress, the invasion of immune cells, the release of cytokines, etc. (3, 4). On the other hand, homeostasis and adjustment of water permeability in microvascular between brain and blood is necessary for the normal activity of the neurons. It is reported that the abnormality of aquaporins (APQS) in multiple brain diseases such as trauma, infection, and metabolic abnormalities leads to encephalitis and quick vulnerability of the brain (5). BBB dissociation is the most efficient inducer of mRNA AQp4 expression in hypertrophic astrocytes (6).

Pathophysiological imbalance in TBI leads to a series of aggressive events called delayed non-mechanical secondary injuries, including cerebral ischemia, hypoxia, inflammatory factors, BBB destruction, excitotoxicity, edema, apoptosis, necrosis, change in energy metabolism, free radical production and blood-spinal barrier destruction (7-9). It has been reported that the inflammation is one of the most important aforementioned factors. Thus, successful inhibition of inflammation caused by TBI can reduce cell death and improve clinical symptoms (10).

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The effects of estrogen on the reduction of cerebral edema, ICP, BBB permeability, improvement of the neurological performances and modification of the amount of cytokines in TBI have been shown (11, 12). The mechanisms in which estrogen reduces cerebral edema and BBB permeability after TBI are unknown, but some probable mechanisms have been reported such as the inhibition of lipid peroxidation, the inhibition of free radical production caused by edema and modulation of nitric oxide (NO) formation (13).

The protective role of melatonin as an antioxidant with internal source in brain injury, and also the low concentration of melatonin observed in brain injury in comparison with its normal state have been reported (14). The reduction of melatonin in patients with TBI has been shown to be due to the disruption in the circadian regulation of melatonin synthesis in response to injury (15). The acute consumption of melatonin reduces cell injury in the cerebral cortex and oxidative injury to proteins and lipids (16). Evidences show that melatonin increases the survival of glial cells, protects neurons in ischemia/ reperfusion and reduces the formation of cerebral edema in cerebral ischemia (17, 18). In addition, a study showed that melatonin had a role in decreasing edema and ICP after TBI (14).

All the above-mentioned studies indicate the neuroprotective effect of melatonin on brain injury. Melatonin applies its effect through different receptors like MT1, MT2 and MT3 quinone reductase 2 (QR2) (19, 20). Several studies have shown that melatonin has an inhibitory impact on the activity of nitric oxide synthase (NOS) in cerebellum and hypothalamus (21), and reduces neutrophil infiltration through MT2 and MT3 (22). In addition, melatonin inhibits neuronal discharge and vasoconstriction through MT1 and also inhibits the release of dopamine, displacement of circadian and vasodilatation through MT2 (20).

Some studies have referred to the functional relationship between estrogen and melatonin receptors. Steroid receptors were recognized on the surface of pineal gland, and thus the pineal gland is one of the target tissues of steroid hormones (23, 24). High concentrations of estrogen stimulate the functional response of MT2 receptor. Estrogen increases vasodilation with MT2 mediation through the activity of classic steroid receptors (nuclear receptor) (25). Furthermore, it has been shown that estrogen reduces the expression of MT1 melatonin receptor in the smooth muscle. Another study on the arteries with removed endothelium reported that estradiol increases MT2 receptors in the smooth muscle (25).

Melatonin reduces the plasma level of 17-beta estradiol and adjusts estrogen and progesterone receptors differently in the reproductive system (16). The anti-cancer actions of melatonin (in breast cancer) are mediated through the response routes of estrogen. Melatonin reduces estrogen α-receptor mRNA in cancer cells in a dose-dependent manner (26). It seems that melatonin inhibits the connection of complex estrogen-estrogen receptor to DNA (27).

This effect of melatonin is mediated by MT1 membrane receptor and increased expression of this receptor (28). The ability of melatonin in the increase of vasoconstriction (by MT1) has a reverse relationship with the amount of estrogen (29). Melatonin inhibits the effects of estrogen on the increased number of mast cells (30).

Our previous studies showed that estrogen and melatonin had neuroprotective impacts on TBI (12, 14, 31). On the other hand, the interaction between estrogen and melatonin was reported in reproductive and non-reproductive tissues mentioned above. Thus, in the present study, the role of melatonin receptors in the neuroprotective effect of estrogen in TBI as a probable mechanism was studied.

## Materials and Methods

This experimental study was performed on female Wister rats weighting between 200 and 250 gr. The animals were kept at the temperature of 20-22˚C and a 12 hr photoperiodic cycle at the animal house of Kerman University of Medical Sciences with free access to adequate food and water. This study was performed under the ethics licenses No. 86.65 from the Lab. animals Ethics Committee of Kerman University of Medical Sciences.

### *Drugs and chemical materials*

17-β estradiol obtained from Aburaihan pharmaceutical company (Tehran, Iran). MT1 (Luzindole), MT2 (4-phenyl-2-propionamidotetraline) and MT3 (Prazosin) receptor antagonists were from Tocris or Sigma companies (USA). Rabbit polyclonal anti-AQP4 Sigma, HRP linked goat anti-rabbit IgG and anti-β-actin of Sigma Company (USA; cat. # A5971, #A9169 and # A2668, respectively) were used in this study. ECL kit was from Roche Company (Germany).

### *The studied groups*

After ovariectomy, 70 animals were divided into five groups and two subgroups. All groups underwent TBI and each group included three subgroups with seven in each. The groups were: 1) Trauma group: the animals that had TBI two weeks after the ovariectomy. 2) Melatonin receptor antagonist vehicle + estrogen (Veh+ E2): These animals received melatonin receptor antagonist vehicle (5% ethanol saline) (32) immediately after TBI and estrogen (1 mg/kg) 30 min later via intraperitoneal injection (IP) (12). 3) MT1 melatonin receptor antagonist + estrogen (Luz +E2): The animals received 100 μM of Luzindole (33) immediately after TBI and estrogen 30 min later via

IP (12). 4) MT2 melatonin receptor antagonist + estrogen (4PPDOP+ E2): The animals received 10 μM of melatonin MT2 receptor antagonist, 4PPDOP (4-phenyl-2-propionamidotetraline) (33) immediately after TBI and estrogen 30 min later via IP. 5) MT3 melatonin receptor antagonist+ estrogen (Prazo +E2): The animals received 0.5 mg/kg of MT3 melatonin receptor antagonist (Prazosin) (34) immediately after TBI and estrogen 30 min later through IP.

***Ovariectomy procedure***

First, the animals were anesthetized with 30 mg/kg thiopental through IP injection. Then, a horizontal incision with the length of 3-4 cm was created at the lower abdomen. After that, the skin, Fascia, and muscles of the abdomen were opened, and fat and intestines were sheared off until the uterus and its tubes were exposed. Then, the fallopian tubes and ovarian vascular pedicle were tied by catgut 4 at proximal area and then cut from distal area. This process was performed in both ovaries. At the end, 1-2 ml saline solvent was poured inside the abdomen and the muscles and skin were respectively sutured by catgut and 0-4 surgical silk suture through the continuous method. The wound area was sterilized by Betadine solution and the animals underwent intensive care for 2 hr. In order to prevent hormonal interference due to estrous cycle, the ovariectomy was performed at least 2 weeks before any other operation (35).

***Induction of TBI***

All animals were intubated before TBI. Moderate TBI of diffuse type was created by Marmarou method (36). The functioning of TBI induction machine (made by Kerman physiology group) was as follows: a 250 g weight was released from a 2 m height inside a pipe on the head of the anesthetized animal (40 mg/kg Ketamine and 10 mg/kg xylazine), while a steel plate was placed on the animal's skull in order to diffuse the trauma uniformly. After the induction of the TBI, the animal was connected to animals' respiratory pump (TSA animal respiratory compact, Germany). After the animal was able to breathe on its own, the animal was separated from the ventilation machine and kept in a cage and under care (5, 11). The mortality after TBI in animals was approximately 30%, which occurred following injury before 24 hr, and those who survived after 24 hr did not die due to TBI.

***Determination of brain edema***

To measure brain edema, brain water content was measured. The brain tissue of the animals was removed 24 hr after the induction of TBI and anesthesia. First, the weight of the wet tissue was measured, and then put in an incubator (Memmert, Germany) at 60 °C for 72 hr in order to evaporate its water and obtain a dry tissue. Then, its weight was re-measured and finally the water content of the brain tissue was calculated by using the following formula [(wet weight/dry weight- wet weight) x 100] in percentage (%) (37).

***Determination of BBB permeability***

The BBB permeability was obtained by measuring the amount of extravascular Evans blue and using spectrophotometer 5 hr after the trauma (13, 38). Thus, the animal was anesthetized 4 hr after the trauma (40 mg/kg ketamine and 10 mg/kg xylazine) and then 20 mg/kg Evans blue (1 ml/kg) was injected through the jugular vein by using needle No. 29. One hour after the injection (5 hr after the trauma), the thorax of the anesthetized animal was opened. After clipping the descending aorta, the isotonic saline solution (200-300 ml) was injected into the animal's blood circulation via its left ventricle in order to wash the dye inside the cerebrovascular veins. Also, the jugular vein at the both sides was cut, and washing continued until the light liquid was removed from the jugular vein (13). Then, the brain was immediately taken out and homogenized after weighting. After that, by adding 20 ml acetone solution (14 ml) + 1% sodium sulfate (6 ml), the brain was placed on the shaker for 24 hr. Then, 1 ml supernatant was mixed with 1 ml trichloroacetic acid and placed at a cool place (20 °C) for 2-3 min. After centrifugation of the solution at 2000 rpm for 10 min, the Evans blue absorption from 1 ml supernatant was measured at the 620 nm wavelength by using spectrophotometer (Biotech, Germany) and the color amount in terms of mg was calculated in 1 mg of the tissue. The more color in the brain tissue indicated more permeability of cerebral vessels and more destruction of the BBB (13).

***Evaluation of ICP***

First, a cut was made behind the animal on the skin middle line between the spinous processes of the 4$^{th}$ and 6$^{th}$ lumbar vertebra (L4-L6). Ilium was used as a guide to cut the skin because the surface above the ilium is similar to the top surface of L6 spinous processes. The spinous processes and L5 lamina were exposed carefully and the inferior half of L5 lamina (including both processes of the upper edge) was removed. After that, the yellow ligaments below the lamina were removed carefully in order to observe cauda equina. Then, a small cut was made on arachnoid membrane through dura mater by using needle No.25. Then, ICP monitoring system that was already prepared was used. The end of the poly-ethylene pipe no.10 (PE10) was entered slowly into the space below cauda equina for less than 10 mm, and after laminectomy, the dead space was filled with small pieces of gauze and then glue (Razi chemical Co.) was added to fix the gauzes. It helped

fixing PE pipe and fixing the lumbar vertebra. The end of main reservoir equalizing (MRE) pipe of ICP monitoring system embedded below the skin was fixed to the skin and then the wound was sutured. Then, the system was connected to a pressure transducer through stopcock, and the pressure was recorded by the computer (39). In all rats, ICP measurement started 1 hr before trauma induction and also performed immediately (0 min), 1, 4, and 24 hr after trauma.

### *Western blotting*

The AQP4 protein expression in the brain was measured by Western blot. The homogenized brain sample was centrifuged for 30 min at 12,000×g, 4 °C. Protein concentration of the supernatant was measured, and equal amounts of protein in each sample were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred to a nitrocellulose membrane. The membrane was blocked with 5% fat-free milk at room temperature for 2.5 hr and then incubated with rabbit polyclonal anti-AQP4 (1:500) at 4 °C overnight. The membrane was incubated with HRP-linked goat anti-rabbit IgG (1:1000) at room temperature for 2 hr, following three washes. ECL kit was used to visualize chemiluminescent signal bands. The membrane was exposed to the radiography film to reveal the AQP4 band (30 kDa). Image analysis software (Gel Pro Analyzer 4.0, Media Cybernetics, and USA) was used to analyze the densities. The β-actin band was used to normalize the AQP4 expression using anti-β-actin (Sigma, USA; cat. # A2668).

### *Statistical analysis*

One- way ANOVA was used to compare groups at any time evaluated post-TBI. In the case of significant differences, Tukey's test was used for the ANOVA *post hoc* analysis. All data were represented as mean±SEM; $P<0.05$ was considered statistically significant.

## Results

### *Brain edema*

The water content of brain tissue in different groups that used melatonin receptor antagonists + estrogen is shown in Figure 1. Brain water content in the trauma group (%78.84±0.27) was significantly higher compared to melatonin receptor antagonist vehicle + estrogen (%67.14±0.6) ($P<0.001$). Brain water content in the melatonin receptor antagonist vehicle+ estrogen group showed a significant decrease in comparison with 4-phenyl-2-propionamidotetralin+ estrogen (%70.2±0.16), prazosin+ estrogen (%70.9±0.38) and luzindole + estrogen (%69.7+0.35) ($P<0.01$). Brain tissue water in the group that received Luz+E2 was lesser than other three groups.

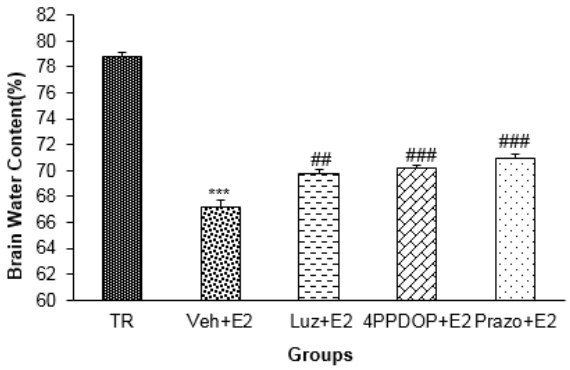


**Figure 1.** The effect of melatonin receptor antagonists + estrogen on the brain tissue water content after traumatic brain injury in ovariectomized rats (n=7 in each group). The results were shown as mean±SEM. ***: $P<0.001$ significant difference with TR. ###: $P<0.001$ significant difference with Veh +E2 group. ##: $P<0.01$ significant difference with Veh +E2 group. TR: trauma, E2: estrogen, Luz: luzindole, PPDOP4: 4-phenyl-2-propionamidotetralin, Proz: prazosin.

### *BBB permeability*

The Evans blue content of brain tissue in different groups using melatonin receptor antagonists + estrogen is shown in Figure 2. The Evans blue content of brain tissue in the trauma group (68.48±0.47 µg/g tissue) was significantly higher than in melatonin receptor antagonist vehicle+ estrogen group (49.96±1.2 µg/g tissue) ($P<0.001$). This amount was higher in the groups of luzindole + estrogen (90.33±1.4 µg/g tissue) and 4-phenyl-2-+ estrogen (77.62±1.6 µg /g tissue) compared to the group of vehicle+ estrogen ($P<0.001$), while prazosin+ estrogen (23.59±0.87 µg/g tissue) showed a significant reduction in comparison with melatonin receptor antagonists vehicle+ estrogen ($P<0.001$).

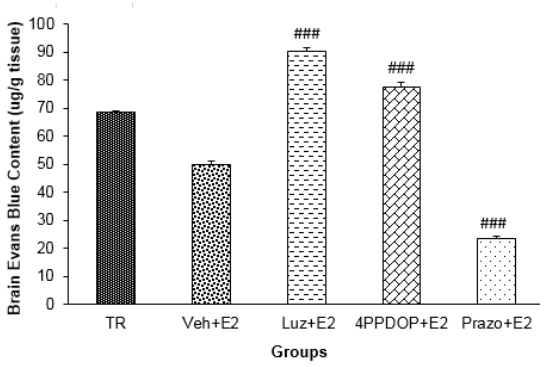


**Figure 2**. The effect of melatonin receptor antagonists + estrogen on brain Evans blue content (µg/g tissue) in the ovariectomized rats after traumatic brain injury (n=7 in each group). The results were shown as mean ± SEM. ###: ***P***<0.001 significant difference with Veh+E2. TR: trauma, E2: estrogen, Luz: luzindole, PPDOP4: 4-phenyl-2-propionamidotetralin, Proz: prazosin

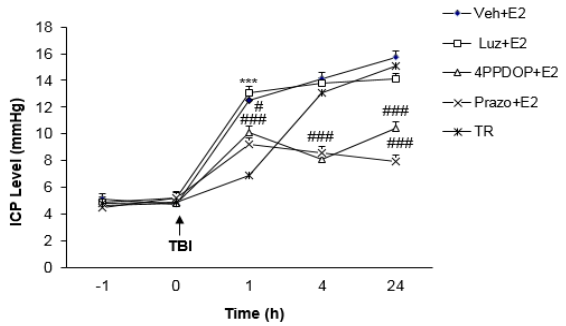


**Figure 3.** The effect of using estrogen + melatonin receptor antagonists on intracranial pressure (ICP) in the ovariectomized rats at different times after traumatic brain injury (TBI) (n=7 in each group). The results were shown as mean±SEM. ***: *P*<0.001 significant difference between TR and Veh + E2 1 hr after TBI. #: *P*<0.05 significant difference between 4PPDOP+ E2 and Veh + E2 1 hr after TBI. ###: *P*<0.001 significant difference between Prazo+E2 and Veh+E2 1 hr after TBI; significant difference 4PPDOP +E2 and Proz + E2 with Veh + E2 4 and 24 hr after TBI. TR: trauma, E2: estrogen, Luz: luzindole, PPDOP4: 4-phenyl-2-propionamidotetralin, Proz: prazosin

### *ICP level*

The mean ICP in different groups that received melatonin receptor antagonists + estrogen is shown in Figure 3. ICP increased in melatonin receptor antagonist vehicle + estrogen in comparison with the trauma group 1 hr after TBI (*P*<0.001). Also, ICP reduced 1 hr after TBI in prazosin + estrogen (9.2±0.49 mmHg) and 4-phenyl-2-propionamidotetralin+ estrogen (10.8± 0.26 mmHg) in comparison with antagonist vehicle+ estrogen (12.48±0.51 mmHg) (respectively as *P*<0.001 and *P*<0.05). Furthermore, 4 and 24 hr after TBI, ICP in prazosin + estrogen (8.6±0.47 and 7.9±0.47 mmHg, respectively) and 4-phenyl-2-propionamido-tetralin + estrogen (8.08±0.33 and 10.4±0.27 mmHg, respectively) groups was lower than antagonist vehicle+ estrogen group (14.1±0.48 mmHg) (*P*<0.001).

### *Expression of AQP4 protein*

The concentration of AQP4 in different groups that used melatonin receptor antagonist+ estrogen is shown in Figure 4. The AQP4 increased in melatonin receptor antagonist vehicle+ estrogen in comparison with the trauma group (*P*<0.05). The concentration of AQP4 in melatonin antagonist vehicle+ estrogen (58.2±9.98) was not different from that of prazosin+estrogen (74.25±5.7), 4-phenyl-2-propionamido-tetralin+ estrogen (64.02±14.9), and luzindole+ estrogen (42.83±6.06) groups.

## Discussion

In this study, for the first time, the interference of estrogen and different melatonin receptors in TBI was studied. The three main findings of this study were: 1. Melatonin receptors affect reducing brain edema and protecting the BBB after TBI by estrogen. 2. The activity of melatonin receptors (MT2 and MT3) weakened the effect of estrogen on ICP. 3. Melatonin receptors played no role in the effect of estrogen on AQP4.

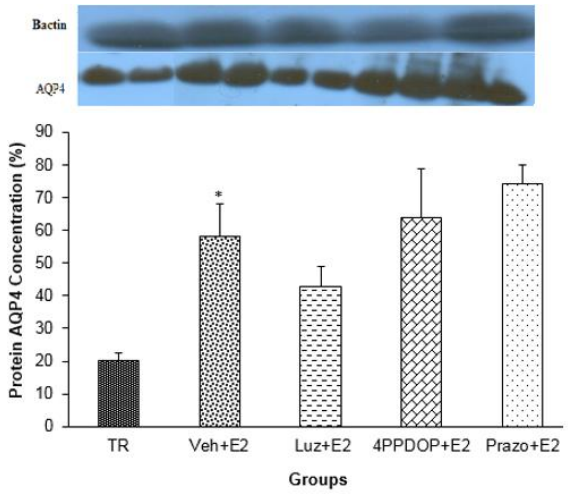


**Figure 4.** Western blot analysis for the concentration of AQP4 in brain tissue in different groups by using melatonin receptor antagonists + estrogen after traumatic brain injury in the ovariectomized rats (n=7 in each group). The results were shown as mean±SEM. *: *P*<0.05 significant difference with the TR group. TR: trauma, E2: estrogen, Luz: luzindole, PPDOP4: 4-phenyl-2-propionamidotetralin, Proz: prazosin.

In current study, TBI resulted in an enhancement in brain edema, BBB permeability and ICP and decrement in AQP4 expression compared to sham (data not shown).

The results of this study showed that the inhibition of different melatonin receptors changes the effect of estrogen on the amount of cerebral edema, BBB permeability and ICP. If the inhibition of different melatonin receptors occurs before using estrogen, the increase of BBB permeability after inducing TBI will occur, and on the other hand, the inhibition of MT3 receptor will reduce BBB permeability. Thus, estrogen will probably reduce the brain tissue water content through the activity of MT1, MT2 and MT3 receptors and will also reduce BBB permeability through the activity of MT1 and MT2.

MT1 and MT2 receptors are proteins coupled to G protein, which works through the stimulation of phospholipase C (PLC). MT3 is from the family of quinine reductase enzymes and probably works through the inhibition of adenylate cyclase enzyme (19, 40).

It has been reported that the stimulation of MT1 receptor inhibits neuronal discharge, cAMP production and arterial vasoconstriction. The stimulation of MT2 receptor inhibits leukotriene B4 (the adhesive factor of leukocytes), and the stimulation of MT3/QR2 receptors leads to strong detoxification mechanisms (19, 20). It has been shown that estrogen increases vascular vasodilatation by MT2 receptor that changes the expression of MT2 receptor in vascular smooth muscles and stimulates the practical response of MT2 receptor (25). The decrease of BBB destruction by melatonin

has been shown in different models of brain injury (14, 41). Also, the use of melatonin effectively reduces the induction of edema in animals with ischemia and also in injuries caused by brain trauma (18). Since the continuity of ischemia can enhance edema, and because vascular vasodilatation probably prevents the continuity of ischemia by increasing the blood flow, it can be stated that perhaps in the current study, melatonin and estrogen affected the reduction of edema through vascular vasodilatation by affecting melatonin receptors. Estrogen affects the amount of plasma melatonin through its activity in the pineal gland (29).

Since the reduced concentration of melatonin has been shown in patients with TBI (40), it has been suggested that melatonin and estrogen may exert synergic antioxidant and neuroprotective actions in the ischemia/reperfusion of female rats (25, 42). Synergic anti-inflammatory and antioxidant actions were reported in the simultaneous use of melatonin and estrogen after ischemia/reperfusion (43). Thus, it is possible that a small amount of melatonin in TBI after using estrogen has a positive role in the reduction of cerebral edema.

Thus, according to the available findings, more studies are needed to explain the role of each melatonin receptor and the intensity of the role of exogenous estrogen and endogenous melatonin in the reduction of edema formation and BBB stability after TBI.

The results of another part of this study showed that the simultaneous use of estrogen and MT2 receptor antagonist (4-phenyl-2-propionamidotetralin) or MT3 (prazosin) reduces ICP at all hours after TBI. Thus, it should be noted that estrogen increases ICP at the presence of MT2 and MT3 receptors. The inverse inhibition between melatonin and estrogen to adjust vascular tone and receptor expression was shown (44). In a study, it was shown that estrogen reduced the expression of MT2 receptor and adjusts these receptors in vascular smooth muscles (29). Furthermore, it was reported that melatonin reduces the action of estrogen in the cells with ERα by inhibiting Aromatase (45, 46). The inhibition of estrogen by melatonin was also reported through changing the concentration of intracellular calcium (43). The reduction of blood pressure was shown by melatonin (47), and perhaps the increase of ICP by its receptors is a compensatory mechanism to maintain cerebral perfusion pressure (CPP) and reduce the expansion of edema. Since the disruption of BBB in TBI increases ICP and inflammatory response (48), it seems that the activity of MT1 and MT2 receptors must be performed to prevent the high increase of ICP.

The last section of this study showed that the inhibition of melatonin receptors in the presence of estrogen did not change the amount of AQP4. Although, it was shown that estrogen affects the amount of plasma melatonin through its activity in the pineal gland (29) and that female steroid hormones have a role in the relationship between the action of melatonin and aggression, the use of melatonin in the mice that causes aggression through melatonin receptor-dependent/independent mechanisms does not cause any change in estrogen-dependent genes (49). Thus, estrogen changes the expression of AQP4 probably through melatonin receptor-dependent/ independent mechanisms. More studies are required to confirm the effect of estrogen on the amount of AQP4.

Thus, according to the current findings, more studies are needed to explain the role of each melatonin receptor in neuprotection after TBI in the presence of estrogen.

## Conclusion

It can be concluded that: 1. probably the effect of estrogen on the reduction of cerebral edema has a relationship with the activity of melatonin receptors (MT1, MT2 and MT3). 2. The activity of melatonin receptors (MT1 and MT2) is probably a factor in the reduction of BBB permeability by estrogen. 3. Furthermore, the interaction of melatonin receptors (MT2 and MT3) with estrogen on ICP can be expected. 4. Melatonin receptors play no role in the effect of estrogen on the expression of AQP4.

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