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Original Article

Does Vitamin D Administration Increase the Neuroprotective Effect of Estrogen in Male Rats with Traumatic Brain Injury?

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Abstract

Background: In our previous studies, the effect of sex hormones on brain edema reduction after traumatic brain injury (TBI) was demonstrated. In the current study, alone and combined effects of 17-β estradiol (E2) and vitamin D (Vit D) on TBI in male rats were investigated.

Methods: Male rats were divided into six groups, including sham, TBI, vehicle, E2, Vit D, and E2+Vit D. In all groups except sham, moderate-intensity diffuse TBI was induced by the Marmarou's method. Vehicle, E2, Vit D and their combination were intramusculary injected one and 12 hours after the TBI. The brain water content, permeability of blood brain barrier (BBB) and histopathological outcome were assessed 24h after TBI. The neurological outcome score was determined using the veterinary coma scale (VCS).

Results: Significant reductions in brain water content ($P<0.001$, $P<0.05$ and $P<0.01$, respectively) and BBB permeability ($P<0.001$) appeared in the treated groups with E2, Vit D, and E2+Vit D compared to the vehicle group. Twenty-four hours after the injury, the neurological scores in the E2, Vit D, and E2+Vit D groups increased significantly compared to the vehicle group ($P<0.05$). Dramatic improvement in histopathological outcome was also observed in the treated groups compared to the vehicle group.

Conclusion: Alone and combined consumption of estrogen and vitamin D may similarly decrease the development of brain edema and improve the neurological and histopathological consequences of TBI. Therefore, consumption of vitamin D did not enhance the neuroprotective effect of estrogen in TBI.

Keywords: Traumatic brain injury, Vitamin D, Estrogen, Brain edema, Blood brain barrier

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Introduction

Traumatic brain injury (TBI) is one of the leading causes of disability and the third most common cause of death worldwide (1). Moreover, 2 100 000 TBI cases were admitted to the emergency departments from 2001 to 2010 in the United States, of whom 300 000 patients received emergency medical care while 53 000 patients expired (2). The incidence of TBI is approximately 56.3 per 100 individuals in Iran, 30% of which result in disability (3). In general, men are roughly twice as likely as women to be involved with TBI (4, 5).

TBI patients may experience primary and secondary brain injuries. The primary injury occurs at the moment of trauma and includes damage to neurons, axons, and blood vessels due to the tissues being stretched and torn, causing irreversible neuronal loss (6, 7). The secondary injury can be initiated by the activation of various biochemical and molecular pathways (8, 9). Secondary injury mechanisms are involved in the development of brain edema and increased intracranial pressure (10).

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Brain edema is considered to be the major cause of TBI deaths and neurologic deficits (11). Although the primary injury is a contributing factor for predicting the outcomes associated with these traumatic events, however, secondary injury triggered by physiological disorders may lead to further poor outcomes in TBI patients (12).

In spite of numerous research efforts (13-16), no successful treatment for TBI is yet available. The lack of an effective treatment can be due to the fact that the heterogeneous pathological processes of TBI have not fully been considered in these carried out studies. In order to target multiple pathological processes of TBI, a researcher should choose a number of treatments that affect multiple targets instead of a single one or rather use combination therapies (17).

Hormones, as homeostatic regulators, play a major role in maintaining the health and proper function of the body under changing environmental conditions. Moreover, Hormones maintain the nervous system homeostasis by regulating blood flow, access to nutrients, and the physiological activity of different cells in the nervous system. Thus, hormones play this protective functional role in the event of disease and injury too (18, 19).

Estradiol (E2), as a cholesterol-derived estrogen steroid hormone, is involved in the regulation of reproductive functions (20) and can act as a neuroprotective agent in some brain injuries (21). In addition, the anti-apoptotic (22), antioxidant (23), and anti-inflammatory (24) effects of E2 have been reported based on experimental TBI. It has been proposed that treatment with estradiol after ischemia can reduce injury to the cerebral cortex (25). Moreover, E2 can regulate brain edema and selective permeability of blood brain barrier (BBB) (26) in several models of trauma including systemic inflammation, neurotrauma, and ischemia (27).

Vitamin D (Vit D), as yet another neuroprotective hormone, is a potent secosteroid hormone. Vit D metabolism is highly complex and well-regulated while its metabolites bind to its protein receptor (VDR) and circulate in the bloodstream (28). Vit D has represented neuroprotective properties in various models of brain injury, including traumatic, ischemic, cytotoxic, degeneration, and autoimmune (29). While the level of endogenous Vit D is directly affected in response to a brain injury, Vit D can improve neurological outcomes in TBI patients (30).

Due to (*i*) the necessity of TBI research and the focus on combination therapy and treatment with multiple effects (31, 32), (*ii*) evidence which shows the neuroprotective effects of Vit D (28) and estrogen (33) and also (*iii*) not fully evaluation of the combined neuroprotective effects of Vit D and estrogen on TBI patients, the present study attempts to investigate the effect of alone and combined administration of estrogen and Vit D on brain edema, BBB permeability, and the neurological and histopathological outcomes in male rats with diffuse TBI.

Materials and Methods

Animals

The experiments were done on 72 male Wistar rats weighing 200-250 g and bred in Kerman University of Medical Sciences. They were randomly divided into the groups of 6 and kept in a temperature-controlled room (23 ± 2°C) on a 12/12 hours light/dark cycle, with free access to water and standard food. Animal care was conducted according to the standard ethical guidelines and the investigation was approved by the Ethical Committee of Kerman University of Medical Sciences (IR.KMU.REC.1396.1658). All anesthesia procedures were performed by the injection of ketamine (60 mg/kg) plus xylazine (10 mg/kg).

Experimental protocols

Animals were randomly divided into the six groups: (*i*) Sham: rats received all procedures to induce diffuse TBI except falling weight on their head, (*ii*) TBI: rats received a diffuse trauma by falling weight after anesthesia, (*iii*) Vehicle (Veh): rats intramuscularly received vehicle in a volume of 1 mL/kg at 1 and 12 hours after TBI, (*iv*) Estrogen (E2): rats intramuscularly received estrogen in a dose of 1 mg/kg (34) and a volume of 1 ml/kg at 1 and 12 hours after TBI, (*v*) Vit D: animals intramuscularly received Vit D in a dose of 1 µg/kg (35) and a volume of 1 mL/kg at 1 and 12 hours after TBI, (*vi*) Estrogen and Vit D (E2 + Vit D): rats intramuscularly received estrogen in a dose of 1mg/kg and Vit D in a dose of 1 µg/kg with a volume of 1 mL/kg for each treatment at 1 and 12 hours after TBI.

Induction of diffuse TBI

After the scalp incision and retraction of the soft tissue, a stainless steel disc 10 mm in diameter and 3 mm in thickness was centrally fixed by polyacrylamide glue between bregma and lambda. The animals were placed on a foam bed, and in all groups except the sham, a 300 g weight dropped from a 2 m height onto the disc. Then the rats were immediately connected to a respiratory pump, and after returning of spontaneous breathing, they were disconnected from the pump and placed in cage following recovery from the surgery (24). The rate of post-TBI mortality was 20% in the current research.

Assessment of BBB permeability

BBB permeability was evaluated by measuring the amount of Evans blue outside the cerebral vessels. Twenty-four hours after the false or actual trauma, 2% Evans blue (20 mg/kg) was injected into the jugular vein in the anesthetized rat. One hour after the injection, the animal's thorax was opened under anesthesia, and the dye was washed by injecting isotonic saline into

the left ventricle for 20 minutes until the clear solution came out through the jugular vein (36). The brain was then removed, weighed, sliced, and placed in 14 ml of acetone solution + 6 ml of 1% sodium sulfate and located in a shaker for 24 hours. The top solution was then removed and centrifuged at 2000 rpm for 10 minutes, and the amount of Evans blue was obtained based on absorption in 620 nm (37). The dye amount was reported in micrograms per 1 g of tissue.

Assessment of brain edema

The brain edema was assessed based on brain water content 24 hours after the TBI. Briefly, the rats were anesthetized, their brains were extracted, and the hemisphere weighed (wet weight). Then, the tissues were placed in an incubator at 60°C for 72 hours, and afterward re-weighed (dry weight). The percentage of brain water content was calculated as follows: ([(wet weight–dry weight)/wet weight] × 100) (38).

Evaluation of neurologic outcome

Neurologic outcome was assessed by the veterinary coma scale (VCS). VCS is a score of 3-15 as the total of three motor (1-8), ocular (1-4) and respiratory scores (1-3). The higher and lower scores reflect better and serious neurological outcomes, respectively. In the present study, this score was determined one hour before the trauma, and 1, 4 and 24 hours after the trauma (39, 40).

Evaluation of histopathological outcome

The histopathological outcome was assessed in the remaining hemisphere to show the edema, inflammation, vascular congestion, and neuronal damage 24 hours after the TBI. Briefly, the brain samples were washed with 0.9% cold saline and fixed in 10% formalin. Then, tissues were embedded in paraffin, coronal sections in 4μm thickness were obtained and stained with hematoxylin and eosin (H&E). Brain tissues were microscopically evaluated by a blind pathologist to the groups. The changes were made semi-quantitatively and graded as follows: (0) nil = unchanged (if no change is observed), (1) mild = slight change (1%-29% changes), (2) moderate = obvious change (30%-59% changes), (3) severe = clear change (60%-100% changes) (41).

Statistical analysis

Data were presented as mean ± SEM (standard error of mean). The data normality was checked by Shapiro-Wilk test using the SPSS software package version 20 (SPSS Inc, Chicago, IL, USA). The comparison of the data was done using one-way analysis of variance (ANOVA) and Tukey post hoc test. Two-way repeated measures ANOVA test with a Bonferroni post hoc test was performed to compare variables between groups in different times. The level of significance was considered as *P* values less than 0.05.

Results

The effect of alone and combined administration of estrogen and vitamin D on brain water content

The content of brain water 24 hours post-TBI is shown in Figure 1. Vehicle group showed an increase in brain water content in comparison with the sham group ($P < 0.001$). This increase was removed in E2, Vit D, and E2 + Vit D groups compared to the Veh group ($P < 0.00$, $P < 0.05$, and $P < 0.01$ respectively). Also, there were no significant differences among sham, E2, Vit D, and E2 + Vit D groups.

The effect of alone and combined administration of estrogen and vitamin D on brain Evans blue content

In Figure. 2, the content of brain Evans blue 24 hours post-TBI is shown in the study groups. Evans blue content increased in the Veh group as compared to the sham group ($P < 0.001$). The administration of E2, Vit D, or E2 + Vit D decreased Evans blue content as compared to the Veh group ($P < 0.001$). Also, there were no significant differences among sham, E2, Vit D, and E2 + Vit D groups.

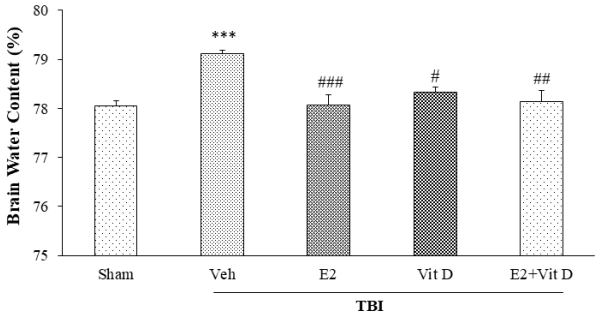

Figure 1. The comparison of brain water content (%) 24 h after the TBI in the different groups ($n = 6$ in each group). The data have been represented as mean ± SEM.*** $P < 0.001$ vs. Sham; ### $P < 0.001$ vs. Veh; ## $P < 0.01$ vs. Veh; # $P < 0.05$ vs. Veh. Veh: Vehicle; E2: Estrogen; Vit D: Vitamin D

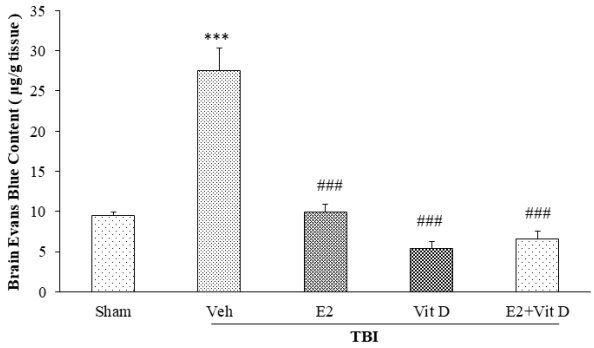

Figure 2. The comparison of brain Evans blue content (μg/g tissue) 24 h after TBI in study groups (n = 6 in each group). The data have been represented as mean ± SEM.*** $p < 0.001$ vs. Sham; ### $p < 0.001$ vs. Veh; ## $p < 0.01$ vs. Veh: Vehicle; E2: Estrogen; Vit D: Vitamin D

The effect of alone and combined administration of estrogen and vitamin D on VCS

The comparison of VCS in the study groups in different times is shown in Figure 3. Before TBI, there was no difference among the groups in VCS. The VCS decreased in all groups 1 hour after TBI compared to sham ($P<0.001$). In the fourth post-TBI hour, an increase in VCS appeared in Veh and E2 groups compared to the sham group ($P<0.001$ and $P<0.01$ respectively). The VCS in E2, Vit D, and E2 + Vit D groups were more than that in the Veh group ($P<0.001$). The VCS in the Veh group decreased compared to the sham group ($P<0.01$) 24 hours after TBI. Besides, VCS increased in E, Vit D, and E2 + Vit D groups compared to the Veh group 24 hours after TBI ($P<0.05$). No significant difference was seen among sham, E2, Vit D, and E2 + Vit D groups 4 hours and 24 hours after the TBI.

The effect of alone and combined administration of estrogen and vitamin D on histopathological outcome

Edema, inflammation, vascular congestion, and neuronal degeneration of brain tissue in the study groups are shown in Figure 4. The scores of brain edema in the Veh, Vit D, and E2 + Vit D groups were higher compared to the sham group ($P<0.001$, $P<0.05$, and $P<0.05$ respectively). As it is seen in Figure 4A, the administration of E2, Vit D, or E2 + Vit D decreased edema score as compared to the Veh group ($P<0.001$, $P<0.01$, and $P<0.01$ respectively). The scores of brain inflammation in the Veh, Vit D, and E2 + Vit D groups were higher than that in the sham group ($P<0.001$, $P<0.05$, and $P<0.05$ respectively). It also decreased in E2, Vit D, and E2 + Vit D groups compared to the Veh group ($P<0.001$, $P<0.01$, and $P<0.01$ respectively; Figure 4B). The scores of vascular congestion in the Veh, Vit D, and E2 + Vit D groups were higher than that in the sham group ($P<0.001$). It can be seen in Figure 4C that the administration of E2, Vit D, or E2 + Vit D decreased vascular congestion score as compared to the Veh group ($P<0.001$, $P<0.01$, and $P<0.01$ respectively). The score of neuronal degeneration increased in the Veh group compared to the sham group ($P<0.001$). It also decreased in E2, Vit D, and E2 + Vit D groups compared to the Veh group ($P<0.001$, Figure 4D). There was no significant difference among E2, Vit D, and E2 + Vit D groups in histopathological evaluation (Figure 4).

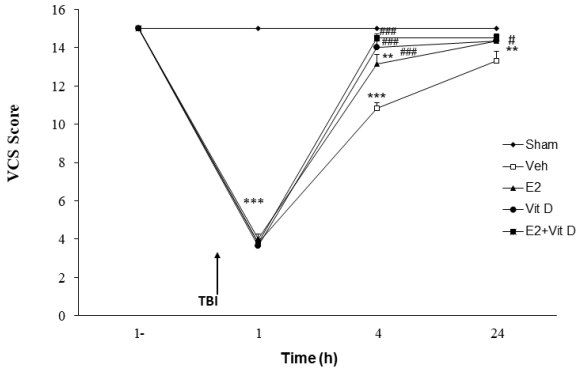

Figure 3. The effect of estrogen and Vit D administration alone and in combination on the veterinary coma scale (VCS) in the different groups at various times after TBI (n = 6 in each group). The data have been represented as mean ± SEM.*** $P<0.001$ vs. Sham 1 and 4 hours after TBI; ** $P<0.001$ vs Sham 4 hours and 24 hours after TBI; ### $P<0.001$ vs. Veh 4 hours after TBI; # $P<0.05$ vs. Veh 24 hours after TBI. Veh: Vehicle; E2: Estrogen; Vit D: Vitamin D

Discussion

The combined effects of estrogen and Vit D on diffuse TBI have been investigated for the first time in this study. The findings indicated that alone and combined administration of these hormones decreases the development of brain edema and BBB permeability while mitigating neurological disorders and histopathological injury after TBI. Furthermore, it was found that there was no significant difference in the effects of Vit D and estrogen when used alone or in combination.

BBB and neuronal deterioration and the formation of brain edema are only a number of delayed responses after the occurrence of TBI that are heavily involved in secondary injury (8, 42, 43). Numerous studies have indicated that maximum severity of brain edema is typically reached at 24 hours after TBI (34), thus, early management of brain edema plays a significant role in improving the outcomes of TBI patients. In addition, preventing major factors contributing to the onset of secondary brain injury may reduce neurological deterioration and mortality rate.

In this study, brain water content, as an indicator of brain edema, increased in the injured group compared to the sham group 24 hours after TBI. However, brain edema decreased due to the administration of estrogen in the injured group compared to the vehicle group, indicating the neuroprotective properties of estrogen after the occurrence of TBI. This result was consistent with the study carried out by Hajmohammadi et al, in which estrogen was alone administered in TBI (31). Moreover, Shin et al, also reported a decrease in brain water content following the administration of estrogen in TBI (44). In a study conducted by Naderi et al, in 2015, there was a significant decrease in the amount of brain water content following the administration of estrogen in TBI (26). Thus, previous studies confirm the findings of this study. Although this study has shown the neuroprotective properties of administering estrogen following TBI, this effect has not been stated in a number of other studies (45, 46). This discrepancy may be due to the innate differences in severity and mechanism of injury, drug dose, the type of estrogen administered, and animal species used in the study.

Nuclear estrogen receptors are responsible for the

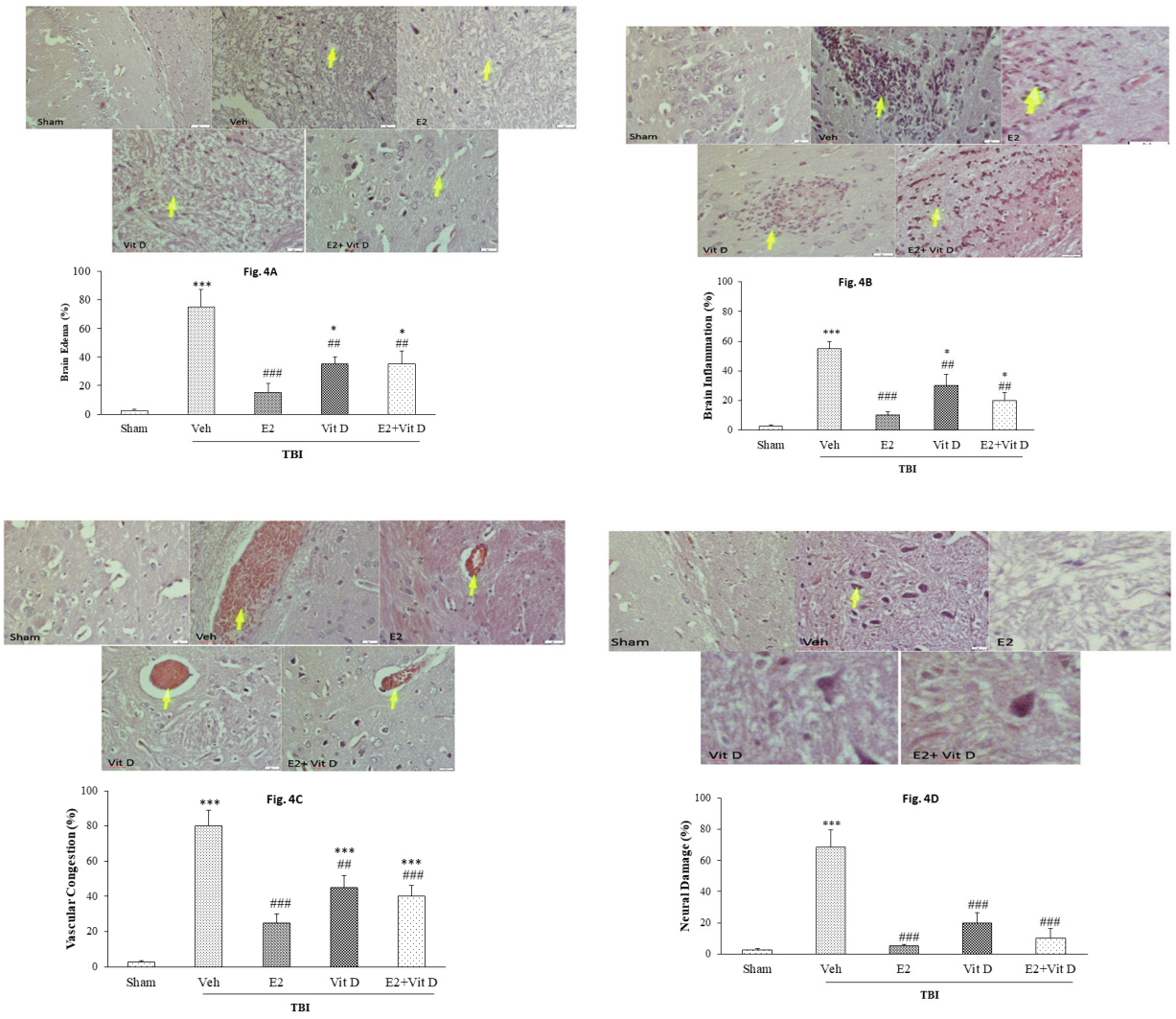

Figure 4 . Histopathological (with magnification of 400X) and quantitative (%) changes of edema, inflammation, vascular congestion and neuronal degeneration (A, B, C, D; respectively) in the experimental groups 24 h post-TBI. The yellow arrow shows edema, inflammation, vascular congestion and neuronal degeneration in the group. The data have been represented as mean ± SEM. *** $P<0.001$ vs. Sham; * $P<$ vs. Sham; ### $P<0.001$ vs. Veh; ## $P<0.01$ vs. Veh. Veh: Vehicle; E2: Estrogen; Vit D: Vitamin D

delayed desirable actions of estrogen, including an increase in the generation of anti-apoptotic proteins, a decrease in the generation of pro-inflammatory cytokines such as tumour necrosis factor α (TNF-α), and a reduction in cytokine receptors (26). On the other hand, mechanisms independent of classical estrogen receptors may mediate a number of rapidly-progressed estrogen actions.

There are several possible mechanisms by which estrogen can slow or inhibit the progression of brain edema and BBB deterioration, including trapping free radicals, modulating NO generation, regulating cerebral blood flow, inhibiting the expression of matrix metalloproteinase 9, and reducing the generation of pro-inflammatory cytokine and prostaglandins (PG) as a means of preventing inflammation, the inhibition of lipid peroxidation and generation of oxygen free radicals, prevention of cell death, and gene expression regulation of water channels, aquaporin 4 (34, 47, 48). It has been stated that the management of brain edema following TBI can improve neurological outcomes (49).

In this study, the Evans blue content, as an indicator of BBB permeability, increased following TBI in comparison to the sham group while a significant decrease was observed following the administration of estrogen. In a study carried out by Naderi et al, it was also observed that the administration of estrogen following TBI reduces BBB permeability (26). Zahedi Asl et al, also reported that estrogen administration following TBI reduces the Evans blue content (49).

Multiple neuroprotective actions of estrogen on the cerebrovascular system and BBB include the regulation of tight junction proteins, occludin and claudin-5, promoting vasodilation by upregulating the expression of endothelial nitric oxide synthase (eNOS), enhancing mitochondrial function, and inhibiting vascular

inflammation by reducing pro-inflammatory endothelial molecules such as cytokines, E-selectin, ICAM-1, and VCAM-1 (24, 50). It is worth mentioning that 17β-E2 upregulates the expression of eNOS and consequently stimulated the dilatation of vessels in the cerebrovascular system by both genomic and non-genomic signaling mechanisms (51, 52). Furthermore, estrogen resulted in adequate cerebral perfusion and reduced brain edema following TBI (33).

In this study, it was revealed that estrogen is able to improve neurological outcomes within 24 hours following TBI. Shahrokhi et al, also reported an improvement in the neurological outcomes within 4 hours following TBI due to estrogen administration and the effects of this intervention persisted 24 hours following injury compared to the vehicle group (33). The evaluation of symptoms using neurological scores (motor, eye, and respiratory functions) is correlated with intracranial pressure (ICP) and brain edema; thus, early management of brain edema in TBI can lead to improvements in the neurological outcomes; i. e., VCS. Based on ample evidence, the administration of estrogen can be effective in improving neurological outcomes in the initial hours following TBI; this may be due to the increased cerebral blood flow, decreased ICP, and reduced brain edema following the administration of estrogen (53, 54).

In this study, brain edema, inflammation, vascular congestion, and neurodegeneration increased following TBI. On the other hand, it was indicated that the administration of estrogen results in an improvement in the histological outcomes, including edema, inflammation, vascular congestion, and neurodegeneration. Moreover, decreased inflammation and improved histopathologic outcomes following estrogen administration have been reported in a study carried out by Khaksari et al, which is consistent with our findings (55).

The generation of pro-inflammatory cytokines following TBI has been proposed in animal and human models (56, 57). The formation of a complex network of cytokines around the injured area is observed in the primary and secondary injury stages. While cytokines such as IL-1β, IL-6, and TNF-α increase the inflammatory response following TBI (58), estrogen inhibits the release of inflammatory molecules including TNF-α, NO, ROS and prostaglandin E2 (PGE2) from microglia (59). The inhibitory effects of estrogen on neuroinflammation can be considered as a novel therapeutic approach to delay the onset or progression of neurodegenerative injuries by modulating the activity of microglia. In addition to modulating the expression of eNOS in the cerebrovascular system, estrogen downregulates the expression of iNOS; thus, reduces the generation of NO as a part of its innate inflammatory response (60).

In this study, the effects of Vit D administration on brain edema, BBB, and neurological and histopathological outcomes were investigated 24 hours following TBI. Vit D is mostly associated with the regulation of calcium homeostasis. The biologically active metabolite of this vitamin, calcitriol, exerts its endocrine effect via a nuclear receptor. The widespread distribution of Vit D receptors suggests the fact that Vit D may regulate various physiological pathways, including brain development, inflammation, neural function, cell cycle, and modulation of the immune system and apoptosis (28).

It was revealed that Vit D is able to reduce brain edema and the Evans blue content in TBI-induced rats, which is consistent with previous studies conducted on animal models. In a study carried out by Cui et al, it was stated that Vit D and its metabolites reduce brain water content following TBI (61). Dong et al, reached similar results using different animal models (62). The neuroprotective effects of Vit D are based on a number of concepts that calcitriol can indirectly inhibit the synthesis of NO, a free radical able to damage cells. Moreover, calcitriol can indirectly stimulate the synthesis of glutathione. Vit D may act as a nerve agent by stimulating the nerve growth factor, glial cell-derived neurotrophic factor and neurotrophin-3, thereby reducing brain edema and BBB permeability (63-65).

The proposed mechanism of Vit D neuroprotective properties is rather complex and multidimensional (66). The activation of Vit D receptor reduces the amount of intracellular $Ca^{++}$by its intracellular buffering properties and decreases voltage-sensitive Ca^{++}channels, L-type, which, in turn, causes a decline in the release of glutamate and its associated toxicity (67, 68). In particular, cell apoptosis can be stimulated by the release of neuroexcitotoxic glutamate and the extravasation of calcium following TBI (66, 69). In this study, it was also shown that Vit D can lead to a reduction in inflammation. In a study by Cekic et al, it was indicated that this vitamin can reduce the inflammatory response induced by TBI, which is consistent with our results (70). It has been proposed in previous studies that a decrease in neuroinflammation following TBI results in a reduction in brain injury and cell apoptosis and improved outcome (66, 71, 72). Cui et al, stated that the activation of Vit D receptor leads to the upregulation of free radical trapping and the inhibition of oxidative stress (61). Finally, the activation of this receptor can also enhance the stability of microtubule and neuro-cytoskeleton, which, in turn, promote axonal regeneration following the injury (69, 73).

In this study, Vit D improved the neurological outcome following TBI. It was also reported in a previously-conducted study that Vit D and its biologically active metabolites can mitigate the neurological deficits following TBI, which confirms the results of the present study. Yalbuzdag et al, and Cekic et al have reported similar results as well (74, 75).

It was also revealed in the current study that Vit

D improves histopathological outcomes, including inflammation and neuronal degeneration following TBI. In a study by Cekic et al, it was also observed that the administration of Vit D reduces the inflammatory response following TBI (70). Another research indicated that Vit D reduces neuronal degeneration and cell apoptosis following TBI, which leads to a decreased inflammatory response (61).

A recent study suggested that the TLR is an important factor in controlling the extent of brain injury. This is due to the fact that a cascade of inflammatory signaling events is stimulated, thereby activating NF-κB and subsequent generation of inflammatory mediators. Similar to estrogen, Vit D is a potent hormone that exerts its specific anti-inflammatory effects by regulating the negative feedback loop of TLR4 signaling in macrophages (32, 76). It also reduces the amounts of cyclooxygenase-2 and iNOS, enzymes involved in inflammation response (77).

According to the findings of this study, one can propose that the neuroprotective effects of alone and combined administration of estrogen and Vit D are similar following TBI; therefore, if the administration of estrogen is deemed as a risk factor for a TBI patient, Vit D can be suggested to be safely used as its alternative while providing the same benefits. In the current study, the combined administration of estrogen and Vit D decreased brain edema and BBB deterioration and improved neurological and histopathologic outcomes 24 hours following TBI. Moreover, the obtained results were almost identical to that of alone administration of estrogen and Vit D.

Since the heterogeneous nature of TBI and interventions characterized by their multiple and combined targets are rather critical to be taken into serious consideration in TBI research, the results of this study proposed that if their use is approved, Vit D and estrogen can be alternatively administrated in TBI patients while combination therapy is not required. That is to say, multiple therapeutic targets can be achieved by the alone administration of Vit D and estrogen following TBI.

Alone and combined administration of estrogen and Vit D may similarly decrease the development of brain edema and BBB permeability while improving the neurological and histopathologic outcomes of moderate diffuse TBI. Further research is required to confirm this hypothesis, however, based on our findings, it can be proposed that Vit D administration did not increase neuroprotective effect of estrogen on TBI outcome.

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Authors' Contribution
Investigation: AAL, SMJ, RR, MA, SA, NE and SD. Data Curation: ZS, NS. Writing—Original Draft Preparation: NS, HB. Writing—Review and Editing: ZS, NS. Supervision: ZS. Project Administration: ZS.

Conflict of Interests
The authors state no conflict of interest.

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