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ORIGINAL WORK

The Role of Inhaled Estradiol and Myrtenol, Alone and in Combination, in Modulating Behavioral and Functional Outcomes Following Traumatic Experimental Brain Injury: Hemodynamic, Molecular, Histological and Behavioral Study

Mohammad Amin Rajizadeh[1†], Mohammad Khaksari[2,3†], Mohammad Abbas Bejeshk[4†], Ladan Amirkhosravi[3], Elham Jafari[5], Zahra Jamalpoor[6] and Akram Nezhadi[1*]

Abstract

Background: Traumatic brain injury (TBI) is an important and growing cause of disability worldwide, and its cognitive consequences may be particularly significant. This study assessed the neuroprotective impacts of estradiol (E2), myrtenol (Myr), and the combination of the two on the neurological outcome, hemodynamic parameters, learning and memory, brain-derived neurotrophic factor (BDNF) level, phosphoinositide 3-kinases (PI3K/AKT) signaling, and inflammatory and oxidative factors in the hippocampus after TBI.

Methods: Eighty-four adult male Wistar rats were randomly divided into 12 groups with seven rats in each (six groups to measure intracranial pressure, cerebral perfusion pressure, brain water content, and veterinary coma scale, and six groups for behavioral and molecular studies): sham, TBI, TBI/vehicle, TBI/Myr, TBI/E2, and TBI/Myr + E2 (Myr 50 mg/kg and E2 33.3 µg/kg via inhalation for 30 min after TBI induction). Brain injury was induced by using Marmarou's method. Briefly, a 300-g weight was dropped down from a 2-m height through a free-falling tube onto the head of the anesthetized animals.

Results: Veterinary coma scale, learning and memory, brain water content, intracranial pressure, and cerebral perfusion pressure were impaired following TBI, and inflammation and oxidative stress were raised in the hippocampus after TBI. The BDNF level and PI3K/AKT signaling were impaired due to TBI. Inhalation of Myr and E2 had protective effects against all negative consequences of TBI by decreasing brain edema and the hippocampal content of inflammatory and oxidant factors and also by improving BDNF and PI3K/AKT in the hippocampus. Based on these data, there were no differences between alone and combination administrations.

Conclusions: Our results propose that Myr and E2 have neuroprotective effects on cognition impairments due to TBI.

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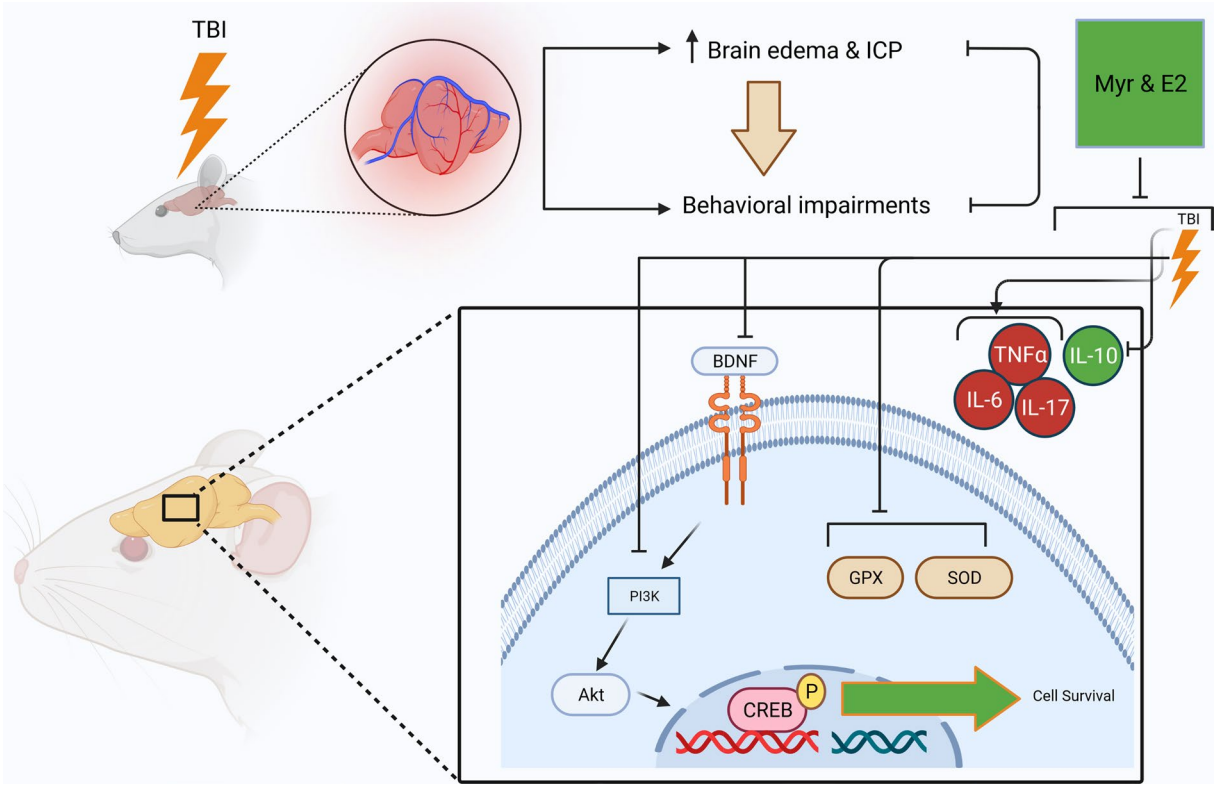
Graphical Abstract:



Keywords: TBI, Estradiol (E2), Myrtenol, Cognition, Inflammation, Oxidative stress, Inhalation therapy

Introduction

Traumatic brain injury (TBI) is a cause of mortality and disability worldwide [1]. The prevalence of TBI has been increasing since 1990. In 2016, the number of people with TBI was estimated at 55.5 million globally [2]. Improvements in emergency medicine and intensive care units have decreased TBI-related deaths, but those who survive suffer from neurological and neuropsychological problems [3]. TBI is a multifactorial neurological disorder caused by primary and secondary injury mechanisms. Primary injuries following TBI are those that are the direct result of the external mechanical forces causing brain tissue deformation and disrupting normal brain function. The types of mechanical forces involved in brain trauma include acceleration and deceleration linear forces, rotational forces, forces generated by blast winds associated with blast injury, blunt impact, and penetration by a projectile. These forces directly damage the neurons, axons, dendrites, glia, and blood vessels in a focal, multifocal, or diffuse pattern and initiate a dynamic series of complex cellular, inflammatory, mitochondrial, neurochemical, and metabolic alterations [4, 5]. The secondary neurologic damage produced by a cascade of secondary events after the primary injury has the potential to be reversible. Secondary brain injury occurs as a complication of the primary brain injury and includes ischemic and hypoxic damage, cerebral edema, raised intracranial pressure, hydrocephalus, infection, inflammation, blood–brain barrier damage, apoptosis, necrosis, varied energy metabolism, and free radical production [4–6]. Primary injury could be diffuse and localized damage that occurs during 100 ms pathologically [7]. The severity of secondary injuries depends on the severity and location of the primary injury [8]. Secondary injury processes are involved in many neurological outcomes after TBI, such as cognitive deficits [9]. The effectiveness of therapies including novel medications for TBI is investigated by evaluating neurobiological reflexes or motor function, as well as behavioral tests of cognitive function and memory. A proportion of survivors of severe TBI require long

rehabilitation after prolonged hospital care and may have long-term physical, cognitive, emotional, and behavioral disorders [10, 11]. Although most patients with TBI recover well from motor disorder, they still experience severe neuropsychological and behavioral problems months or years after the original head trauma, which is often called an invisible disability [10, 12, 13]. Cognitive disorders following TBI are the main complications of this lesion that involve the person, relatives, and community. Disturbances in attention, memory, and executive functions are frequent problems after TBI at any severity [14]. The hippocampus plays a major role in learning and memory processing in the brain and is a brain region highly sensitive to TBI.

Neurotrophic factors are a collection of structurally and functionally related proteins and play important roles in many aspects of neural development, survival, and plasticity [15]. The expression of neurotrophic factors following TBI is affected by the severity of the injury, genetic polymorphism, and different posttraumatic time points [15]. Neurotrophic factors play a major role in activating the repair mechanisms and stimulation of neurogenesis [16]. There are some neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor (BDNF), and neurotrophin that change and contribute to recovery after TBI [15]; still, BDNF may play a key role because it is the most abundant neurotrophin in the central nervous system [17] and the most widely studied, owing to its potential effects and wide distribution in the brain [18]. As a member of the family of neurotrophic proteins, BDNF is a secreted autocrine factor that promotes the development, maintenance, survival, differentiation, and regeneration of neurons [19]. It is also important for synaptic plasticity and memory processing [20]. BDNF has been implicated in reducing secondary brain injury, with elevations providing neuroprotection and restoring connectivity after TBI [21]. It has been shown that the level of BDNF declined in serum and hippocampus following TBI [15, 22–25]; in general, however, there are discrepancies among the results of BDNF expression at different post-injury times, central nervous system regions, genetic polymorphisms, and clinical outcomes [15, 23, 26]. The hippocampus is rich in receptors for BDNF [27]. The expression of hippocampal BDNF is affected by various factors, including sex, age, trauma intensity, neuronal activity, and glutamatergic activity [27]. The BDNF molecule can activate different signaling pathways and cascades after being connected to the receptor. One of these pathways is phosphoinositide 3-kinases (PI3K/AKT), activating which could finally inhibit neuronal apoptosis and increase neuronal survival [28].

Herbal remedies derived from plants and herbal extracts are traditionally applied to treat diseases. In recent decades, scientists have become interested in understanding the mechanism of these plants and identifying their main ingredients [29–31].

Myrtenol (Myr) is a bicyclic monoterpene alcohol found in some aromatic plants, such as *Myrtus communis* Linn, which could be obtained from the oxidation of α-pinene. Although this substance is used for its flavoring properties, some studies have suggested that Myr has antihypertensive, anti-inflammatory, and antioxidant effects [32]. Few studies have been conducted on the effects of *M. communis* on cognitive functions, but its analgesic and neuroprotective effects have been reported [33]. Because of the cognitive, anti-inflammatory, and antioxidant effects of myrtenol, this compound was selected for this study.

Estradiol (E2) is a hormone made naturally in the human body by the ovaries and is the most potent and abundant estrogen during the female reproductive years [34]. It can enhance neurogenesis and recover the nerves by reducing the activity of damaged neurons and glial cells in the brain. E2 acts as a powerful antioxidant against free radicals generated after TBI. It also suppresses and decreases the second wave of brain damage by reducing the concentration of proinflammatory cytokines following TBI [35, 36]. E2 is a potent, protective, restorative, and trophic factor after brain injury [37]. Although the results of E2 treatment after TBI in animal models are convincing, clinical studies indicate that E2 use is associated with detrimental effects, such as feminization and an increased risk of stroke and tumorigenesis, raising concerns about its overall safety [38]. Considering the side effects of E2, especially its carcinogenic effect, this compound was used in this study along with myrtenol to compare them and check the effectiveness of their combination.

Given the high prevalence of TBI and its numerous side effects, including cognitive disorders, finding an effective therapy to minimize the complications of this disorder is an urgent need. Many therapies have been reported to alleviate the side effects of TBI. Because the effects of Myr on TBI had not been reported before, in this research, the effects of E2 and Myr were studied. In the case of E2, despite the studies on TBI, there were no reports on BDNF and the hippocampus following TBI. In this regard, this research is a novel study. Another new point addressed herein is the inhalation of these compounds, which is similar to drug administration models in humans.

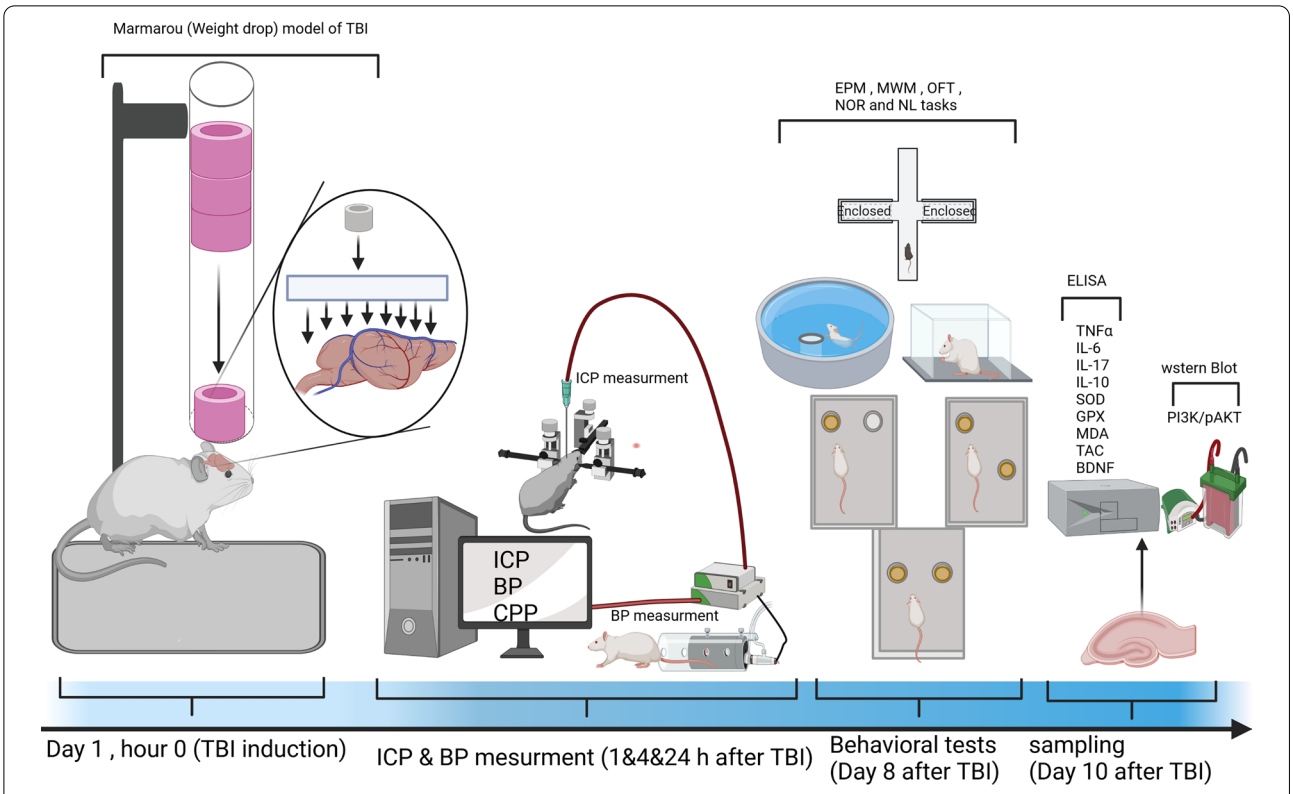


Fig. 1 Time-line diagram showing the experimental protocol used for TBI induction and treatment in study groups. BDNF, brain-derived neurotrophic factor, BP, blood pressure, CPP, cerebral perfusion pressure, ELISA, enzyme-linked immunosorbent assay, EPM, elevated plus maze, GPX, glutathione peroxidase, ICP, intracranial pressure, IL, interleukin, MDA, malonyldialdeide, MWM, Morris water maze, NL, novel location, NOR, novel object recognition, OFT, open field test, SOD, superoxide dismutase, TAC, total antioxidant capacity, TBI, traumatic brain injury

Methods

Animals

Eighty-four adult male Wistar rats (200–250 g, 2 months of age) were prepared for the current research. The animal house temperature was maintained at 22–25 °C with a 12:12-h light/dark cycle and access to food and water. The design of this study was approved by the Ethics Committee (Ethics and Animal Care Committee, No. IR.AJAUMS.REC.1401.027) at the AJA University of Medical Sciences (Iran).

Experimental Protocol

The male rats were randomly divided into 12 experimental groups ($n=7$ per group). Six groups were assigned to study the behavioral function of the animals (all behavioral tests were performed from 8 a.m. to 15 p.m). All the behavioral tests were performed in these six groups. All the tests were conducted in the same order for all groups. To prevent the tests from interfering with each other, first, the tests related to anxiety (open field and elevated plus maze, respectively) and then the tests related to learning and memory (novel recognition tests and Morris water maze test, respectively) were conducted. The behavioral tests were performed in 3 days (days 8–10 after TBI). On the 8th day, open field, elevated plus maze, and novel recognition tests were respectively performed; on the 9th day, the learning part of the Morris water maze was conducted; and on the 10th day, the memory part of this test was conducted. In addition, molecular studies were conducted in the hippocampus of these animals. In the other six groups, intracranial pressure (ICP), cerebral perfusion pressure (CPP), and brain edema were evaluated. The study groups included: a sham group in which the rats underwent an incision of skull skin after anesthesia, but brain trauma was not induced for them; TBI group, in which the rats underwent brain trauma; TBI + Vehicle group, in which the rats inhaled the vehicle (0.5% dimethyl sulfoxide) 30 min after TBI for 30 min; TBI + Myr group, in which the rats inhaled Myr (50 mg/kg) 30 min after TBI for 30 min [29]; TBI + E2 group in which the rats inhaled E2 (33.3 μg/kg) 30 min after TBI for 30 min [39]; and TBI + Myr/E2 group, in which the rats inhaled a combination of Myr and E2 30 min after TBI for 30 min. The experimenters were blinded to the groups in all stages of the experiments (Experimental protocols illustrated in Fig. 1).

Diffuse Moderate TBI Model

Intubation was performed in anesthetized rats (ketamine-xylazine mixture, 50/5 mg/kg, intraperitoneal (i.p.) before TBI. Diffuse brain trauma was induced by using Marmarou's method [40]. A steel disk (diameter 10 mm and thickness 3 mm) was firmly attached to the animal's skull, centrally between the lambda and bregma, and then, a 300-g weight (moderate model of TBI) was dropped from a 2-m height onto the disk. This model induces diffuse cellular and axonal injury in forebrain structures such as the sensorimotor cortex and the hippocampus but limited brain stem and cerebellum damage [41]. The percentage of mortality was 20–30%, which was consistent with other studies [42, 43].

Determination of Brain Edema

Twenty-four hours after the induction of TBI, brain edema was assessed by measuring the water content of the brain. The anesthetized animals' brains were removed and weighed to calculate the tissue's wet weight. The tissue was then incubated at 60 °C for 24 h in an incubator (Memmert, Germany) and reweighed to calculate the dry tissue weight. The percentage of BWC as an index of brain edema was calculated by using the following formula: brain water content (BWC) (%) = ([dry tissue weight − wet tissue weight]/wet tissue weight) × 100 [44].

Table 1 Veterinary comma scale (VCS) scores

Variable	Score
Motor	
Normal movement	8
Mildly drowsy with spontaneous, purposeful movements	7
Lethargic, unable to stand, but maintains sternal recumbency	6
Lethargic, withdraws to pinch, and lifts head with attention to visual stimuli; no sternal recumbency	5
Withdraws or pedals to pinch	4
Spontaneous pedaling	3
Extensor posturing (spontaneous or to stimuli)	2
Flaccid to stimuli	1
Eye	
Open	4
Open on stimulation	3
Normal eyelid reflexes	2
No eyelid response to stimuli	1
Respiration	
Normal	3
Ataxic	2
Apneic	1

ICP Measurement

The animals were anesthetized with ketamin and xylazine and were fixed in stereotaxic instruments such that the head was placed in the middle of the sagittal plane and the anterior–posterior point was located midway between the occipital, crest, and the lambda suture. After identifying the cisterna magna area, a 20-gauge needle connected to a transducer pressure via a short polyethylene tube of recording system (AD Instruments, Australia) was entered into the 5-mm depth of the cisterna magna area [45]. ICP was recorded before the induction of the trauma and 1 h, 4 h, and 24 h after the TBI.

Determination of CPP

Systolic and diastolic blood pressure was recorded by the tail-cuff technique using an NIBP ML125 system (AD Instruments, Australia). CPP was calculated based on the difference between the mean arterial pressure and ICP [44, 46]. The perfusion pressure was recorded before and 1 h, 4 h, and 24 h after the induction of the trauma.

Motor Function Evaluation

The motor performance was reported according to a motor score of the veterinary coma scale (VCS) (Table 1). The score range was from 1 to 8 as (1) flaccid to stimuli; (2) extensor posturing (spontaneous or to stimuli); (3) spontaneous pedaling; (4) withdraws or pedals to pinch; (5) lethargic, withdraws to pinch, and lifts head with attention to visual stimuli; no sternal recumbence; (6) lethargic, unable to stand, but maintains sternal recumbence; (7) mildly drowsy with spontaneous, purposeful movements; and (8) normal movement. Motor function was assessed at − 1, 1, 4, and 24 h post TBI [47] (Table 1).

Morris Water Maze

The Morris water maze (MWM) consisted of a black circular pool (160 cm in diameter and 80 cm in height) filled with water maintained at room temperature to a depth of 40 cm. The pool was geographically divided into four quadrants of equal sizes, and starting points were designated at each quadrant as N, S, E, and W. A square platform (10 cm in diameter) was hidden right below (1.5 cm) the surface of the water in the center of the northeast quadrant. The experiments were coducted in a dimly lit room with various and fixed extra maze geometric images (e.g., circles, squares, or triangles) attached to different points on the walls around the maze. Performances were recorded by a smart video tracing system (Noldus Ethovision system, version 7, the Netherlands), and the animals could be traced on a computer screen. The water temperature was fixed at 25 ± 2 °C.

The MWM task was conducted to assess spatial learning and memory. In a single training protocol, each rat

accomplished three blocks separated by a 30-min resting period. Each block consisted of four successive trials with a 60-s duration and about 60-s intertrial intervals. All the trials of all blocks were performed in one day (day 8 after TBI). In each trial, the rats were randomly released into the water from one of the four quadrants of the maze facing the wall of the quadrant where it was released. Each rat had four different releasing points. During acquisition, the location of the platform remained constant, and the rats were allowed to swim to the hidden escape platform. After the animal found the platform, it was allowed to remain there for 20–30 s and was then placed in an animal cage to wait for 20–30 s before the start of the next trial.

However, if a rat failed to find the platform in 60 s, the experimenter would guide it toward the platform; after 20–30 s of staying on the platform, the rat would be placed in an animal cage to wait for 20–30 s before the start of the next trial. The time and distance to find the hidden platform were collected and analyzed later. A single-probe trial was performed 24 h after the last training trial to test the spatial memory in the water maze. In this trial, the platform was removed, and the rat was allowed to swim for 60 s. The time and distance spent in the target quadrant (quadrant 4) were analyzed as a measure of spatial memory retention. The experimenter was blinded to the group membership of the rats [48–51].

Open Field Test

The open field test (OFT) yields some information about locomotion and anxiety-like behaviors in rodents. The apparatus consisted of an arena made of opaque Plexiglas ($90 \times 90 \times 45$ [H] cm). The arena was divided into 16 small squares so that the rats spent time in either central or peripheral squares. The total distance moved and velocity are indices for evaluating motor performance. The fecal pellets (fecal pellets excreted from the animal or the amount of defecation), grooming (licking its paws and using them to wash its face and ears, then nibbling and licking the fur on its shoulders, flanks, and hind quarters), and rearing (a variant of the search phase of the exploratory behavior, when the animal is moving around the environment attempting to contact relevant stimuli) numbers are indices to evaluate anxiety-like behaviors. The rats' behaviors were recorded via a video tracking system in a 5-min interval [52, 53].

Elevated Plus Maze

The elevated plus maze (EPM) test is one of the most important tasks for assessing rodents' anxiety. The time spent in the open arm and head dips (the number of times that a rodent either dips its head/the top half of its body below the open arm to see what is going on under the maze) numbers are indices for low anxiety. The two open and two closed arms of this task were made of wood ($50 \times 50 \times 50$ cm). The animals' behaviors in this task were recorded by a video camera for 5 min [54].

Novel Object Recognition and Novel Location Recognition Tasks

Both novel object recognition (NOR) and novel location recognition (NLR) tests exploit the inherent preference of rodents for novelty to reveal memory for previously encountered objects. However, NLR primarily evaluates spatial learning which relies heavily on hippocampal activity, whereas NOR evaluates nonspatial learning of object identity which relies on multiple brain regions [55]. Because rodents have an innate preference for novelty, a rodent that remembers familiar objects and locations will spend more time exploring the novel object and location [56, 57].

In the NOR task, the animals are permitted to detect familiar and novel objects in a box made of wood ($60 \times 60 \times 40$ cm) during two phases. The familiar and novel objects were similar in terms of material and size but differed in appearance or shape [55]. The first phase lasts 5 min (training phase), during which the objects are the same and placed in the same location. The second phase for assessing memory retention is accomplished 45 min after the training phase and lasts 3 min. In this phase, the objects are placed in locations similar to the training phase but one of the previous objects is substituted by a novel object. The time of sniffing or touching the objects with the nose is considered as the exploration time and recorded by the camera. Finally, the time that the rats paid attention to each object divided by the total attention time to both the objects multiplied by 100 in the training and test phases is the discrimination ratio (as the recognition index) [58, 59].

In the NLR task, as a hippocampus-dependent task, the rats are permitted to detect the objects in the novel location in a box made of wood ($60 \times 60 \times 40$ cm) during two phases. The first phase lasts 5 min (training phase), during which the objects are the same and placed in the same location. The second phase for assessing memory retention is accomplished 45 min after the training phase and lasts 3 min. In this phase, the objects are similar to the training phase but only one of the identical objects is moved to a novel location. The time of attention to the objects with the nose and hands is the exploration time and is recorded by the camera. Finally, the discrimination ratio (as the recognition index) is calculated similarly to the novel object test [59, 60]. Both NLR and NOR tasks were performed on the same day (day 8 after TBI). The habituation phase was conducted one day before the

main test (day 7 after the TBI), and the animals moved freely for 5 min in the empty open box (without any objects).

Biochemical Measurements in the Hippocampus Tissue

The rats were killed via anesthetic overdose (80 mg/kg ketamine and 50 mg/kg xylazine). After euthanization, the head was separated from the rest of the body. The skin covering the head was cut, and then, the bones were removed from the skull starting from the occipital part. Finally, the brain was removed from the skull. Hippocampal dissection was performed according to Jaszczyk's study [61]. Total proteins were measured by using Bradford's method. Hippocampal glutathione peroxidase (GPX), superoxide dismutase (SOD), malonyldialdeide (MDA), and total antioxidant capacity (TAC) were determined by using their assay kits, according to the manufacturers' protocols. The levels of BDNF and cytokines tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), IL-17, and IL-10) in the tissue were measured using enzyme-linked immunosorbent assay kits. The samples were analyzed on an automated enzyme-linked immunosorbent assay plate reader (Model No. ELX-80MS, Biotech, USA) [29, 62],

Western Blot

Western blotting was used to measure the amount of PI3K and phosphorylated AKT. In our study, the number of samples in each group was seven ($n=7$). The total protein concentration in the hippocampal samples was measured by the Lowry method, whereas bovine serum albumin was used as the standard. After matching the concentrations, in each Western blot, we loaded all groups from one sample. We did one Western from each sample with all groups and a total of seven Westerns with three repetitions. To this end, 40 µg of protein from each sample was mixed with a buffer sample. Then, electrophoresis was performed for 75 min using 11% SDS-PAGE gel. After that, the proteins separated in the gel were transferred to polyvinylidene difluoride (PVDF) paper. After the bands appeared, we placed the PVDF paper in the striping buffer for 30 min. The membrane was then incubated in a 2% block solution overnight (at 4 °C). In the next step, the membrane was quenched four times each washed with tris-buffered saline with 0.1% tween 20 (TBST) solution for 5 min and incubated for 3 h with the initial antibody (concentration 1.200) for each of the mentioned proteins. Then, the membrane was exposed to a secondary antibody (concentration 1.1000) for 1 h. In the next step, immune detection was recorded using a Chemi Doc XRS + imaging system (Bio-Rad Company, USA) and analyzed by ImageJ software [63–65]. B-actin was used as a control.

Histological Study

The neuronal damage score was assayed in the hippocampus. The rats were killed under deep anesthesia. Hippocampal sections were stained with hematoxylin and eosin, and the percentage of damaged neurons (apoptotic and necrotic neurons) in the CA1 area of the hippocampus was examined by a pathologist who was blinded to the treatment of each group [66, 67].

Statistical Analysis

A repeated-measures two-way analysis of variance (ANOVA) followed by Tukey's test (for VCS, ICP, CPP, and learning phase of MWM) and one-way ANOVA (for BWC, OFT, EPM, NOR, NLR, memory part of MWM, histological, biochemical, and molecular data) were used for data analysis. All the values were reported as means ± standard error of the mean (SEM), and *P*-values < 0.05 were considered significant. At all levels of the experiment (behavioral and biochemical tests), the experimenters were blinded to the study groups (Fig. 1).

Results

The Effects of Myr, E2, and Their Combination on BWC Following TBI

To measure the BWC, wet tissue weight (24 h after injury and immediately after brain removal) and dry tissue weight (24 h after brain removal) were measured. As depicted in Fig. 2, BWC was elevated in the TBI and TBI/vehicle groups compared with the sham group (Fig. 2, $P<0.\ 01$). BWC was reduced in the TBI/Myr ($P<0.01$), TBI/E2 ($P<0.01$), and TBI/Myr + E2 ($P<0.001$) groups compared with the TBI/vehicle group (one-way ANOVA; Fig. 2).

The Effects of Myr, E2, and Their Combination on ICP Following TBI

TBI increased the ICP level at 1, 4, and 24 h after trauma compared with the sham group ($P<0.001$ for TBI group). Myr, E2, and their combination prevented the elevation of ICP at 4 ($P<0.05$ for TBI/Myr, TBI/E2, and TBI/Myr + E2 groups) and 24 h ($P<0.01$ for TBI/Myr and TBI/E2 groups, and $P<0.001$ for TBI/Myr + E2 group) compared with the TBI/vehicle group (repeated-measure, two-way ANOVA; Fig. 3).

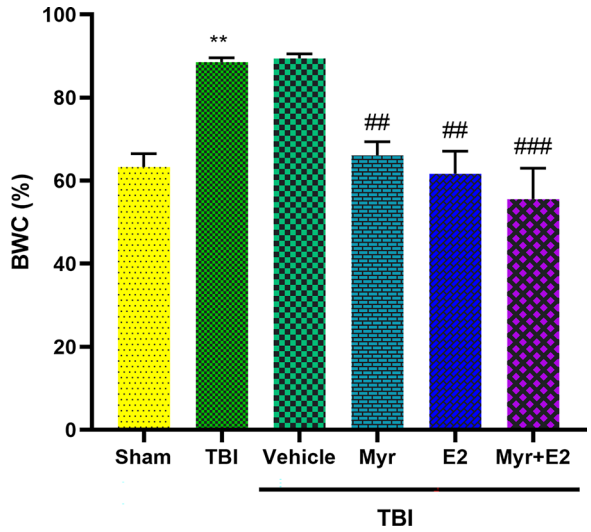


Fig. 2 The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on brain water content (BWC) in experimental traumatic brain injury (TBI) in male rats ($n=7$). Data are expressed as mean ± SEM. $^{**}P<0.01$ versus sham group; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ versus TBI/vehicle group. SEM, standard error of the mean

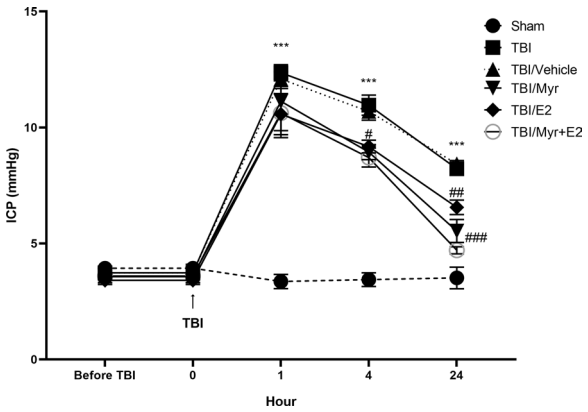


Fig. 3 The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on intracranial pressure (ICP) in experimental traumatic brain injury (TBI) in male rats in different times. Data are expressed as mean ± SEM ($n=7$). $^{***}P<0.001$ at 1, 4, 24 h (TBI vs. sham); $^{\#}P<0.05$ (4 h, all treatment groups vs. TBI/vehicle), $^{\#\#}P<0.01$ (24 h, TBI/Myr and TBI/E2 vs. TBI/vehicle) and $^{\#\#\#}P<0.001$ (24 h, TBI/Myr + E2 vs. TBI/vehicle). SEM, standard error of the mean

The Effects of Myr, E2, and Their Combination on CPP Following TBI

As shown in Fig. 4, the level of CPP decreased in the TBI and TBI/vehicle groups compared with the sham group at 1 h, 4 h, and 24 h after the trauma ($P<0.001$ for TBI group). Figure 4 shows the prevention of the decrement of CPP in the TBI/Myr, TBI/E2, and TBI/Myr + E2 groups compared with the TBI/vehicle group at 4 h ($P<0.05$ for TBI/E2 and TBI/Myr and $P<0.01$ for TBI/Myr + E2) and 24 h ($P<0.001$ for all treatment groups) (repeated-measure, two-way ANOVA; Fig. 4).

The Effects of Myr, E2, and Their Combination on VCS Following TBI

A significant reduction was observed in the VCS score in the TBI and TBI/vehicle groups in comparison with the sham group in 1, 4, and 24 h after TBI ($P<0.001$ for TBI group). The VCS in the treatment groups significantly increased compared with the TBI/Vehicle group in 24 h ($P<0.001$ for TBI/Myr + E2) after TBI. (repeated-measure, two-way ANOVA; Fig. 5).

The Effects of Myr, E2, and Their Combination on Anxiety-Like Behaviors and Locomotor Activity Following TBI in the OPT

The total distance moved (as an index of motor performance) decreased in the TBI group compared with the sham group on day 8 ($P<0.05$) and increased in the Myr ($P<0.05$), E2 ($P<0.01$), and their combination groups ($P<0.01$) compared with the TBI/vehicle group on day 8 after the trauma. Figure 6b presents a comparison of the travel velocity (as an index of motor performance) in the study groups. This variable was lower in the TBI and TBI/vehicle groups than in the sham group ($P<0.05$). Velocity increased in the treatment groups compared with the TBI/vehicle group ($P<0.05$ for TBI/Myr, $P<0.01$ for TBI/E2, and $P<0.001$ for TBI/Myr + E2 groups). According to these findings, TBI caused movement disorders in the rats, but treatments had protective effects. Our findings disclosed that TBI increased anxiety-like behaviors

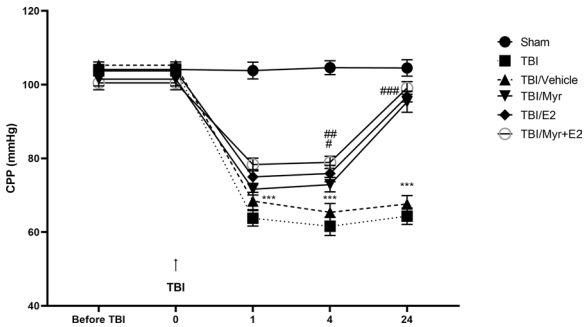


Fig. 4 The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on cerebral perfusion pressure (CPP) in experimental traumatic brain injury (TBI) in male rats in different times. Data are expressed as mean ± SEM ($n=7$). $^{***}P<0.001$ at 1, 4, 24 h (TBI vs. sham); $^{\#}P<0.05$ (4 h, TBI/Myr and TBI/E2 vs. TBI/vehicle), $^{\#\#}P<0.01$ (4 h, TBI/Myr + E2 vs. TBI/vehicle) and $^{\#\#\#}P<0.001$ (24 h, all treatment groups vs. TBI/vehicle). SEM, standard error of the mean

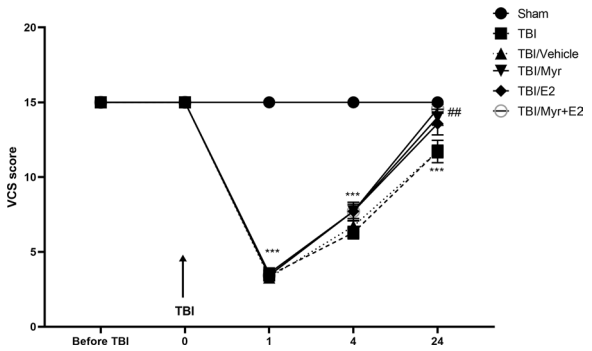


Fig. 5 The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on veterinary coma scale (VCS) in experimental traumatic brain injury (TBI) in male rats in different times. Data are expressed as mean ± SEM. ($n = 7$). ***$P < 0.001$ at 1, 4, 24 h (TBI vs. sham); ##$P < 0.01$ (24 h, TBI/Myr + E2 vs. TBI/vehicle). SEM, standard error of the mean

through elevation of grooming ($P < 0.001$) and fecal pellet ($P < 0.001$) numbers and reducing the rearing ($P < 0.001$) number. The inhalation of Myr ($P < 0.001$ for all three variables), E2 ($P < 0.001$ for all three variables), and their combination ($P < 0.001$ for all three variables) recovered anxiety-like behaviors by improving the mentioned indices (Fig. 6). These data showed that the level of anxiety increases after TBI, but the treatments demonstrated effective antianxiety impacts (one-way ANOVA; Fig. 6).

The Effects of Myr, E2, and Their Combination on Anxiety-Like Behaviors Following TBI in the EPM

The TBI rats revealed a significant reduction in the time spent in open arms in comparison with the sham group ($P < 0.001$). There was no significant difference in the time spent in closed arms among all the groups. Conversely, Myr ($P < 0.01$), E2 ($P < 0.01$), and their combination ($P < 0.001$) showed a significant elevation in the time spent in the open arms. Furthermore, the number of head dips significantly declined in the TBI group in comparison with the sham group ($P < 0.001$), and the inhalation of Myr ($P < 0.001$), E2 ($P < 0.001$), and their combination ($P < 0.001$) significantly raised head dips in the treatment group (Fig. 7). According to the reduction of the time spent in the open arm and the number of head dips after TBI, the increase in anxiety caused by TBI is evident. The

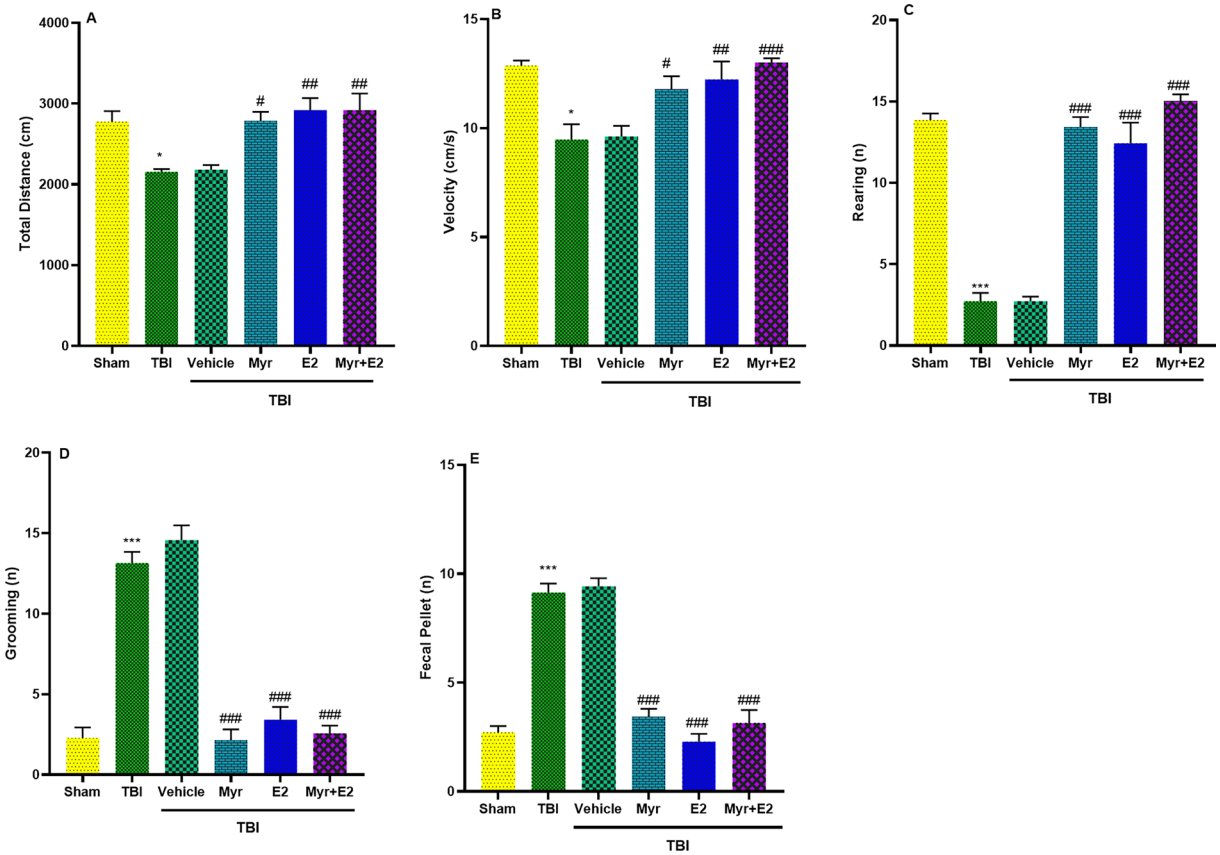


Fig. 6 The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on total distance moved (**a**), velocity (**b**), rearing (**c**), grooming (**d**), and fecal pellet (**e**) in open field task in experimental traumatic brain injury (TBI) in male rats. Data are expressed as mean ± SEM. ($n = 7$). *$P < 0.05$ and ***$P < 0.001$ versus sham; #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ versus TBI/vehicle group. SEM, standard error of the mean

antianxiety effects of treatments can also be observed here (one-way ANOVA; Fig. 7).

The Effects of Myr, E2, and Their Combination on Recognition Memories Following TBI in the NOR & NLR

The preference for a novel object means that the representation of the familiar object exists in the animals' memory. There was no significant difference among all the groups in terms of the time spent around similar objects in the first trial (Fig. 8a). In the test phase, the object recognition memory was impaired following TBI via spending less attention time exploring the novel object ($P<0.001$). Inhalation of Myr ($P<0.001$), E2, and their combination ($P<0.001$) improved the TBI-elicited disruption of the novel objective recognition test (Fig. 8a).

The preference for a novel location means that the representation of the familiar location exists in the animals' memory. There was no significant difference among all the groups in terms of the time spent around objects in the first trial (Fig. 8b). In the test phase, the rats in sham, TBI/Myr ($P<0.001$), TBI/E2 ($P<0.001$), and TBI/Myr + E2 ($P<0.001$) groups spent significantly more time detecting the object in the novel place than the object in its original place compared with the TBI/vehicle group (in the training phase and test phase, only the comparison between the groups has been performed, and our aim was not to compare the two phases). On the contrary, the rats in TBI groups could not discriminate between the two locations in comparison with the sham group ($P<0.001$; one-way ANOVA; Fig. 8b).

The Effects of Myr, E2, and Their Combination on Spatial Learning and Memory Following TBI in the MWM Test

MWM examines hippocampal-dependent spatial learning and memory. The time spent (escape latency) and distance moved (path length) for finding the hidden platform are indices of spatial learning (Fig. 9a, b). Our

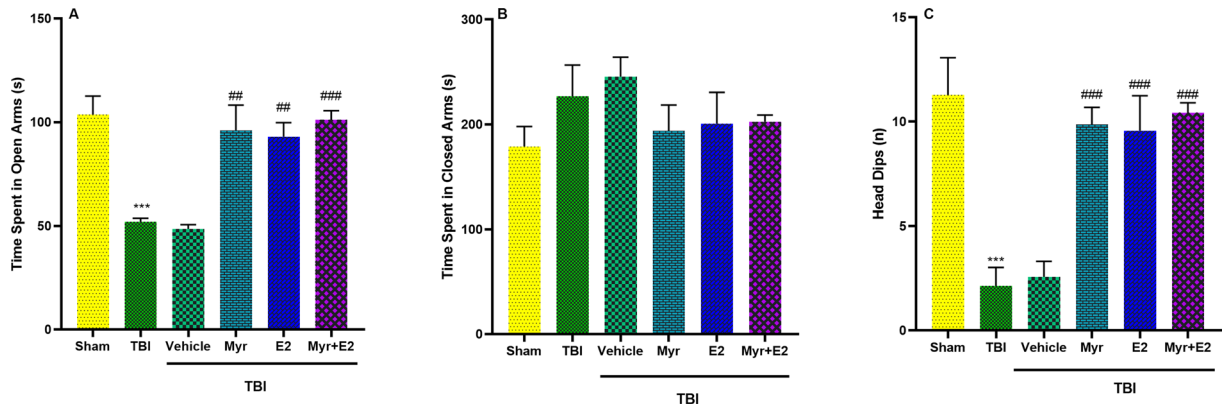


Fig. 7 The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on time spent in open (**a**) and closed (**b**) arms and head dips (**c**) in EPM task in experimental traumatic brain injury (TBI) in male rats. Data are expressed as mean ± SEM. ($n=7$). ***$P<0.001$ versus sham; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ versus TBI/vehicle group. EPM, elevated plus maze, SEM, standard error of the mean

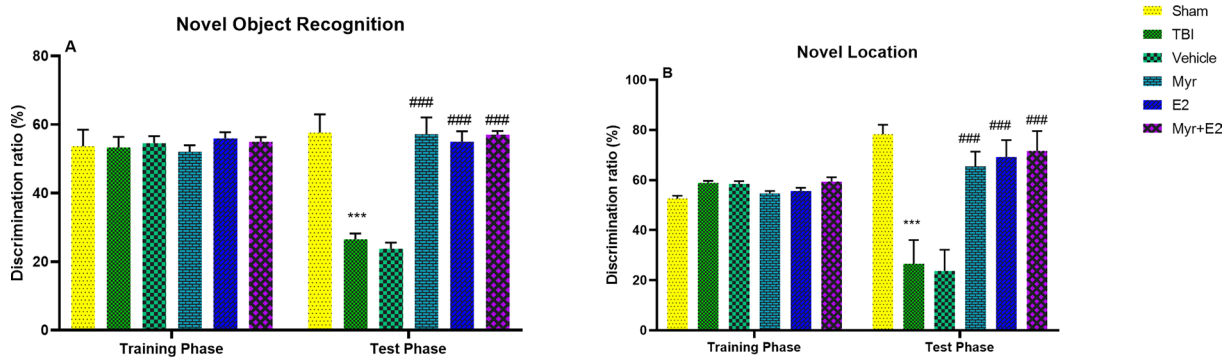


Fig. 8 The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on novel recognition (**a**) and novel location (**b**) tasks in experimental traumatic brain injury (TBI) in male rats. Data are expressed as mean ± SEM. ($n=7$). ***$P<0.001$ versus sham; $^{\#\#\#}P<0.001$ versus TBI/vehicle group. SEM, standard error of the mean

findings revealed spatial learning impairment due to TBI expressed via increased path length and escape latency in block 2 ($P<0.01$ for path length and $P<0.01$ for escape latency) and block 3 ($P<0.01$ for path length and $P<0.001$ for escape latency). Nevertheless, inhalation of Myr ($P<0.01$ and $P<0.001$ for path length in blocks 2 and 3, and $P<0.001$ for escape latency in blocks 2 and 3, respectively), E2 ($P<0.001$ for path length in blocks 2 and 3, and $P<0.001$ for escape latency in blocks 2 and 3, respectively), and their combination ($P<0.001$ for path length in blocks 2 and 3, and $P<0.001$ for escape latency in blocks 2 and 3, respectively) ameliorated this impairment. The mean percentage of the time spent and distance moved in the target quadrant are indices of spatial memory (Fig. 9c). Our results disclosed that TBI disrupted spatial memory ($P<0.001$ for both percentage distance and percentage time), whereas inhalation of Myr ($P<0.001$ for both percentage distance and percentage time), E2 ($P<0.001$ for both percentage distance and percentage time), and their combination ($P<0.001$ for both percentage distance and percentage time) reversed the negative impacts of TBI. (repeated-measure, two-way ANOVA for spatial learning, and one-way ANOVA for spatial memory).

The Effects of Myr, E2, and Their Combination on the Hippocampal BDNF Level Following TBI

TBI decreased the BDNF level in the hippocampus compared to the sham group ($P<0.001$), whereas the administration of Myr ($P<0.001$), E2 ($P<0.001$), and their combination ($P<0.001$) significantly increased the BDNF level in comparison with the TBI/Vehicle group (one-way ANOVA; Fig. 10).

The Effects of Myr, E2, and Their Combination on Hippocampal Cytokines Following TBI

The analysis of the hippocampus tissue sample suspensions showed that the levels of TNFα ($P<0.001$), IL-6 ($P<0.001$), and IL-17 ($P<0.001$) were significantly higher in the TBI group compared with the sham group. Furthermore, following the administration of Myr ($P<0.001$ for all cytokines), E2 ($P<0.001$ for all cytokines), and their combination ($P<0.001$ for all cytokines), the levels of TNFα, IL-6, and IL-17 significantly decreased compared with the TBI/vehicle group. In addition, the level of IL-10 as an anti-inflammatory cytokine was less in the TBI group compared to the sham group ($P<0.001$), and the administration of Myr ($P<0.001$), E2 ($P<0.001$), and their combination ($P<0.001$) increased IL-10 compared with the TBI/vehicle group (one-way ANOVA; Fig. 11).

The Effects of Myr, E2, and Their Combination on Hippocampal Oxidative Stress Following TBI

The level of MDA in the hippocampus was elevated in the TBI group in comparison with the sham group ($P<0.001$). The administration of Myr ($P<0.001$), E2 ($P<0.001$), and their combination ($P<0.001$) reduced MDA levels in comparison with the TBI/vehicle group. The levels of TAC in the hippocampus decreased in the TBI group in comparison with the sham group ($P<0.001$). Inhalation of Myr ($P<0.001$), E2 ($P<0.001$), and their combination ($P<0.001$) increased the levels of TAC in comparison with the TBI/vehicle group. The levels of SOD activity ($P<0.001$) and GPX activity ($P<0.001$) in the hippocampus tissue decreased in the TBI group compared to the sham group. Myr ($P<0.05$ for SOD and $P<0.001$ for GPX), E2 ($P<0.05$ for SOD and $P<0.001$ for GPX), and their combination ($P<0.01$ for SOD and $P<0.001$ for GPX) increased the levels of SOD activity and GPX activity compared to the TBI/vehicle group (one-way ANOVA; Fig. 12).

The Effects of Myr, E2, and Their Combination on the Hippocampal PI3K/AKT Signaling Pathway Following TBI

We loaded seven samples to evaluate the expression of each PI3K, AKT, and β-actin protein. We quantified

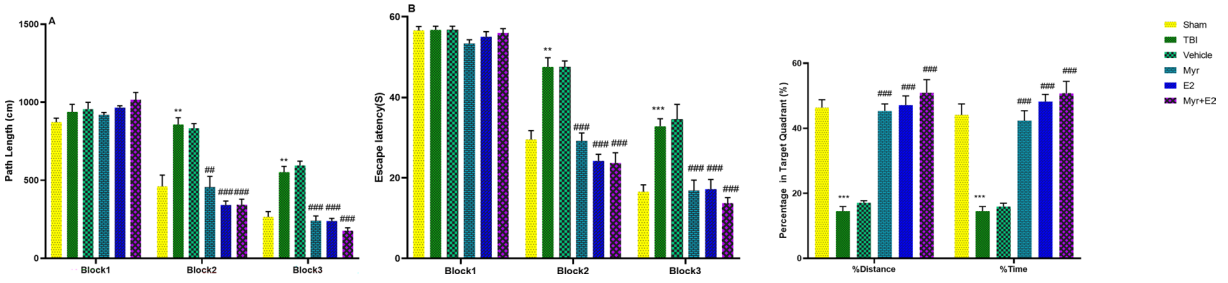


Fig. 9 The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on spatial learning and memory in MWM task in experimental traumatic brain injury (TBI) in male rats. Data are expressed as mean ± SEM. ($n=7$). **a** Path length. **b** Escape latency. **c** Percentage time and distance in target quadrant. $^{**}P<0.01$ and $^{***}P<0.001$ versus sham; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ versus TBI/vehicle group. MWM, Morris water maze, SEM, standard error of the mean

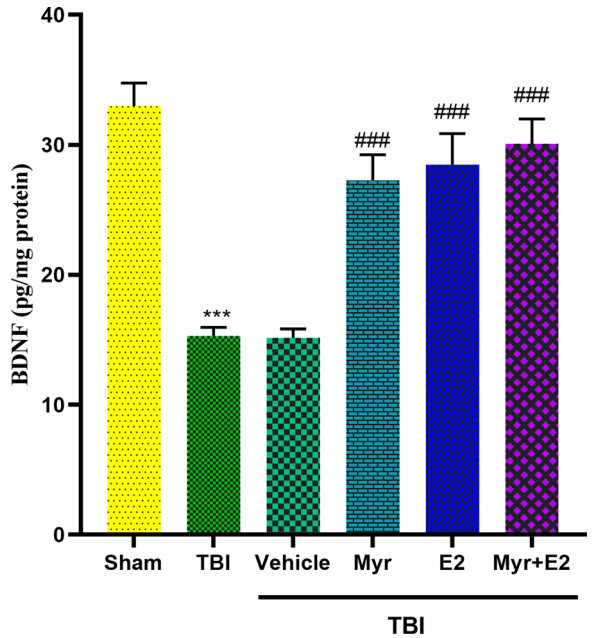


Fig. 10 The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on hippocampus BDNF in experimental traumatic brain injury (TBI) in male rats. Data are expressed as mean ± SEM. ($n = 7$). $^{***}P < 0.001$ versus sham; $^{###}P < 0.001$ versus TBI/vehicle group. BDNF, brain-derived neurotrophic factor, SEM, standard error of the mean

the Western blot results with the same light by Image J software. Then, the expression level of each protein was measured relatively to β-actin. A significant decrease in the hippocampal PI3K expression was observed in TBI and TBI /vehicle groups in comparison with the sham group ($P < 0.001$). The inhalation of Myr ($P < 0.001$), E2 ($P < 0.001$), and their combination ($P < 0.001$) increased PI3K expression in comparison with the TBI/vehicle group. AKT activity, which is determined by its phosphorylation form pAkt, decreased in the TBI and TBI/vehicle groups compared to the sham group ($P < 0.001$). As for the effect of the treatments on PI3K expression, the pAkt expression was significantly elevated compared to the TBI/vehicle group following treatments ($P < 0.001$ for all treatment groups) (one-way ANOVA; Fig. 13).

The Effects of Myr, E2, and Their Combination on Hippocampal Neuronal Damage Following TBI

The results of hematoxylin and eosin staining revealed that the percentage of neuronal damage in the TBI group was higher than in the sham group, but the inhalation of the compounds reduced this damage compared to the TBI/Vehicle group (one-way ANOVA; Fig. 14).

Discussion

In the current research, the impacts of inhaling Myr, E2, and their combination on posttraumatic behaviors, including spatial learning, memory, and anxiety-like behaviors were evaluated. We also investigated the effects of these compounds on the BDNF level, PI3K/AKT pathway, inflammation, and oxidative stress in the hippocampus of rats. The main findings showed that the post-TBI inhalation of Myr, E2, and their combination reduced brain edema and ICP and increased CPP. Furthermore, these compounds alleviated cognitive dysfunction and reduced anxiety-like behaviors. The other results of this study revealed that Myr, E2, and their co-administration increased the BDNF level and expression of PI3K/P-AKT proteins but reduced inflammation and oxidative stress in the hippocampus of rats after TBI.

We observed that BWC was elevated following TBI, and Myr, E2, and their combination reduced this index. In agreement with our results, studies have shown that estrogen decreases the BWC [68, 69]. To the best of our knowledge, no study has demonstrated the effects of Myr on brain edema after TBI, but its effect on other brain injuries such as cerebral infarction is reported, and Myr improved brain edema in this injury [70]. The probable mechanism(s) for this protective effect included scavenging free radicals [71], modulation of nitric oxide generation [72], lowering the production of inflammatory cytokines and prostaglandins, and reducing blood brain barrier (BBB) permeability [73] by E2. Moreover, anti-inflammatory and antioxidative mechanisms have been reported for Myr effects [70].

Other results of this research showed that both compounds and their combination reduced ICP elevation and increased the diminished CPP following TBI. An elevation in ICP in patients with cerebral hemorrhage and cerebral infarction is accompanied by edema; therefore, ICP modulation is advised in the care of patients with TBI [74]. Brain edema increases ICP and leads to neurologic disturbance [75]. In agreement with our findings, this effect of TBI was demonstrated in some investigations [43, 76]. Our results showed an increase in the ICP level in the first hour of TBI that continued for 24 h. Similar to our observations, it is reported that the elevation in ICP after TBI persisted for more than 24 h [74, 77, 78]. Possible causes of elevated ICP due to TBI are cerebral edema, ischemia, hyperperfusion, alterations in the expression of aquaporin-4, and impaired autoregulation [45, 79]. On the other hand, E2 reduced ICP. Consistent with our results, some studies showed ICP reduction due to E2 administration after TBI [45, 74]. It seems that this effect of E2 is also because of its positive effect on brain edema reduction [71]. Although Myr, like E2, decreased ICP after TBI in the present study, we did not

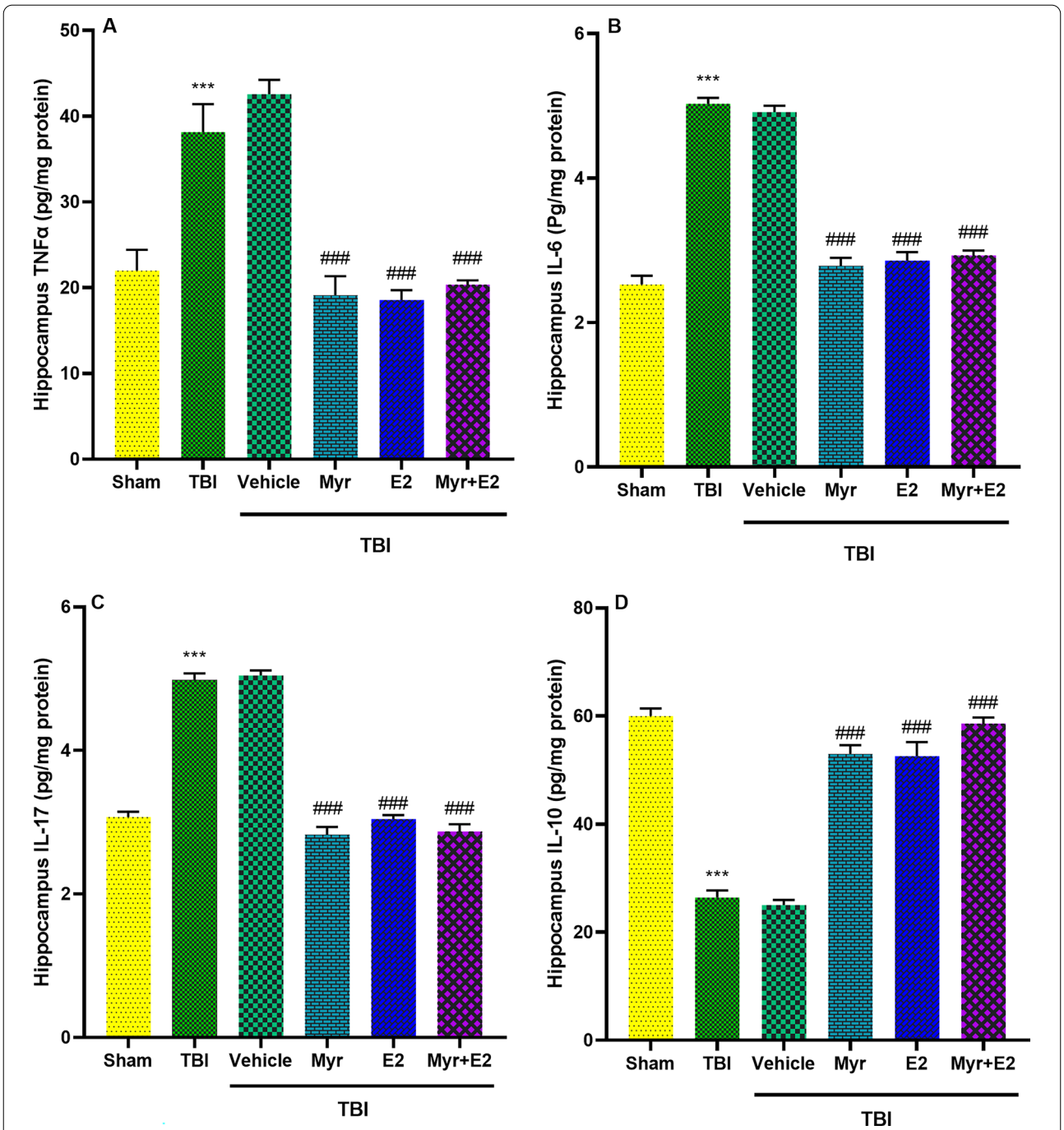


Fig. 11 The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on hippocampus inflammatory factors in experimental traumatic brain injury (TBI) in male rats. **a** Tumor necrosis factor alpha (TNFα). **b** Interleukin-6 (IL-6). **c** IL-17. **d** IL-10. Data are expressed as mean ± SEM. ($n = 7$). $^{***}P < 0.001$ versus sham; $^{###}P < 0.001$ versus TBI/vehicle group. IL, interleukin, SEM, standard error of the mean

find any similar investigation. The decreasing pattern of ICP changes in treatment groups can be attributed to the beneficial effects of the drugs (E2 and Myr). The decreasing pattern of ICP changes in the untreated groups (TBI and TBI/Vehicle) can be ascribed to several factors such as the intensity of the model (moderate model) and the compensatory mechanisms of the body to deal with the increased ICP such as Cushing's syndrome (as a physiological nervous system response to acute elevations of ICP, resulting in Cushing's triad of widened pulse

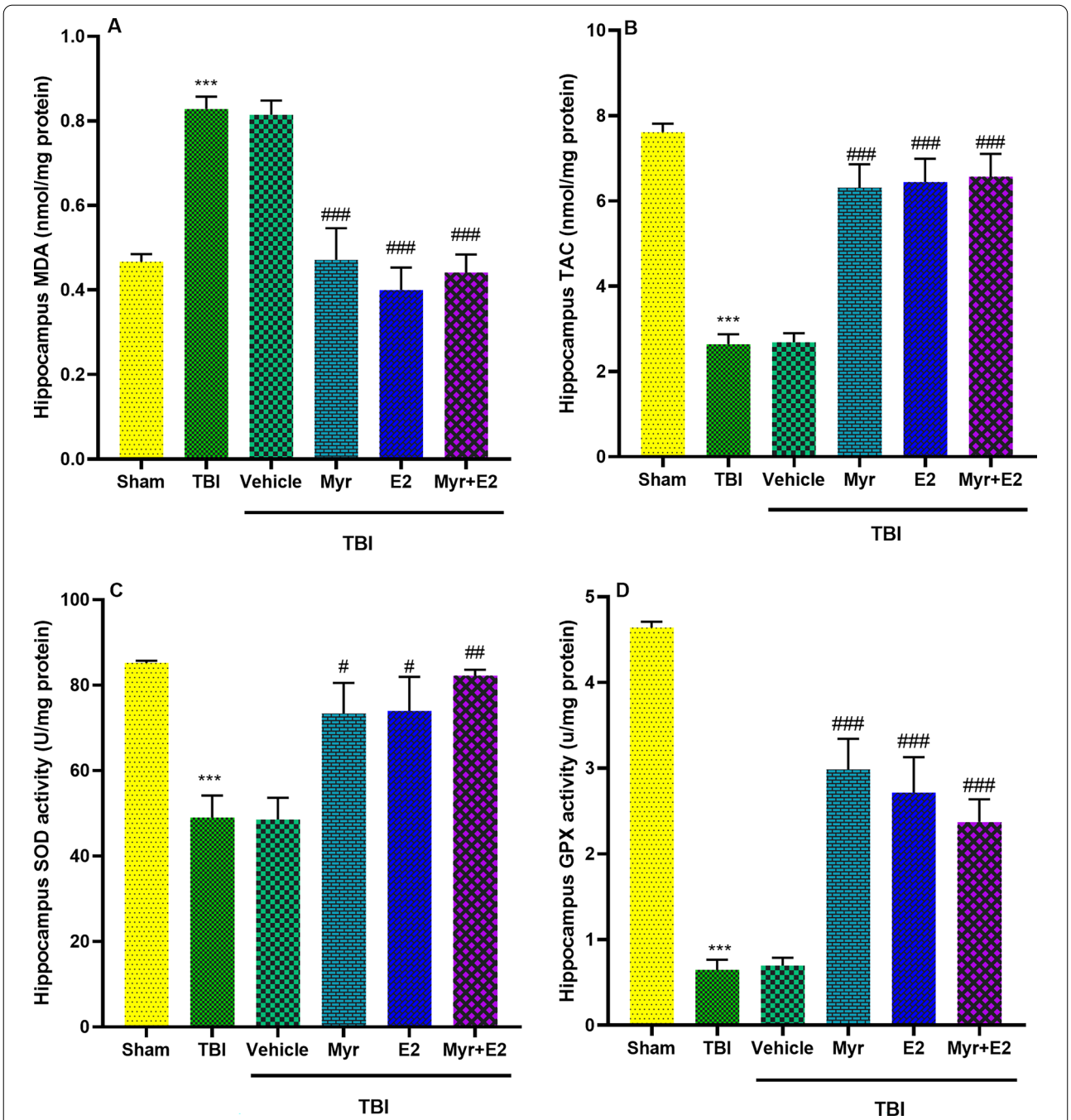


Fig. 12 The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on hippocampus oxidative stress factors in experimental traumatic brain injury (TBI) in male rats. **a** MDA. **b** TAC. **c** SOD. **d** GPX. Data are expressed as mean ± SEM. ($n = 7$). $^{***}P < 0.001$ versus sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus TBI/vehicle group. SEM, standard error of the mean, GPX, glutathione peroxidase, MDA, malonyldialdeide, SOD, superoxide dismutase, TAC, total antioxidant capacity

pressure (increasing systolic, decreasing diastolic), bradycardia, and irregular respirations), displacing or shifting cerebro spinal fluid (CSF), increasing the absorption of CSF or decreasing cerebral blood flow, immune system activity modulation through increased anti-inflammatory factors and decreasing proinflammatory factors, and blood flow autoregulation [80, 81]. However, the decreasing pattern of ICP changes in the untreated groups (TBI and TBI/vehicle) was milder than in the treatment groups.

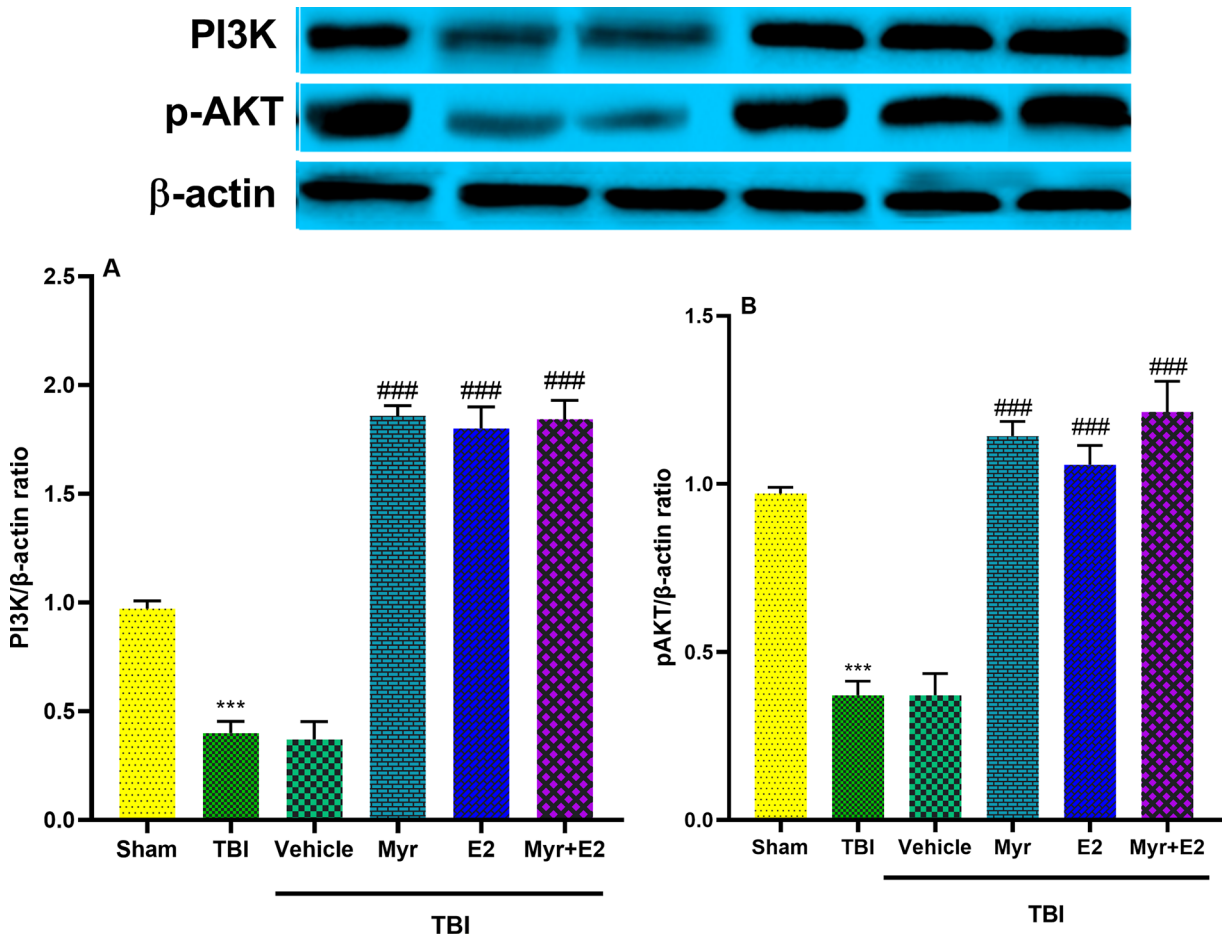


Fig. 13 **a**, **b** The effects of Myrtenol (Myr) and Estradiol (E2) inhalation on hippocampus PI3K/pAKT expression in experimental traumatic brain injury (TBI) in male rats. Data are expressed as mean ± SEM. ($n = 7$). ***$P < 0.001$ versus sham; $^{###}P < 0.001$ versus TBI/vehicle group. SEM, standard error of the mean

CPP is the net pressure gradient that causes blood flow to the brain, and its change can cause cerebral ischemia [45] and CPP reduction, resulting in more injury to the brain [82]. In another part of this study, we assessed the CPP. This index declined after brain trauma. The results of this research, in agreement with other investigations, revealed a decrease in the CPP after TBI [83, 84]. Furthermore, in agreement with our results, some investigations demonstrated an increase in CPP by the administration of E2 [45, 84]. Since it has been disclosed that brain edema can cause ICP elevation and CPP reduction [85], the main possible mechanism through which E2 inhibits the increase in ICP and decreases in CPP following TBI is the reduction of brain edema. Other mechanisms include elevations in the cerebral blood flow or maintenance of the cerebral blood flow, antioxidant effect, and nitric oxide synthesis [86]. In addition, the prevention of oxygen reduction after TBI is suggested as a mechanism [87]. Our results demonstrated that Myr could increase CPP after TBI. It seems that Myr can decrease ICP and increase CPP by reducing brain edema. We did not find any similar research about the effect of Myr on CPP and its mechanisms.

This study, like other studies [47, 88], disclosed that VCS (neurological outcomes) diminished after TBI. Our findings also showed that the E2 improves the VCS at 4 and 24 h after TBI. These results are confirmed by other studies [39, 83]. The mechanism for the increased VCS score by E2 may be due to the decrease in brain edema and lowering of ICP. Myr, similar to E2, can improve VCS after TBI. As mentioned before, the protective effect of Myr on VCS may be due to its impact on brain edema.

TBI is associated with cognitive symptoms such as executive function and memory impairments. The MWM is a test for assessing hippocampal-dependent learning and memory [89]. In the current research, the indices of cognitive behaviors were impaired, such that we observed dysfunction in the MWM task following

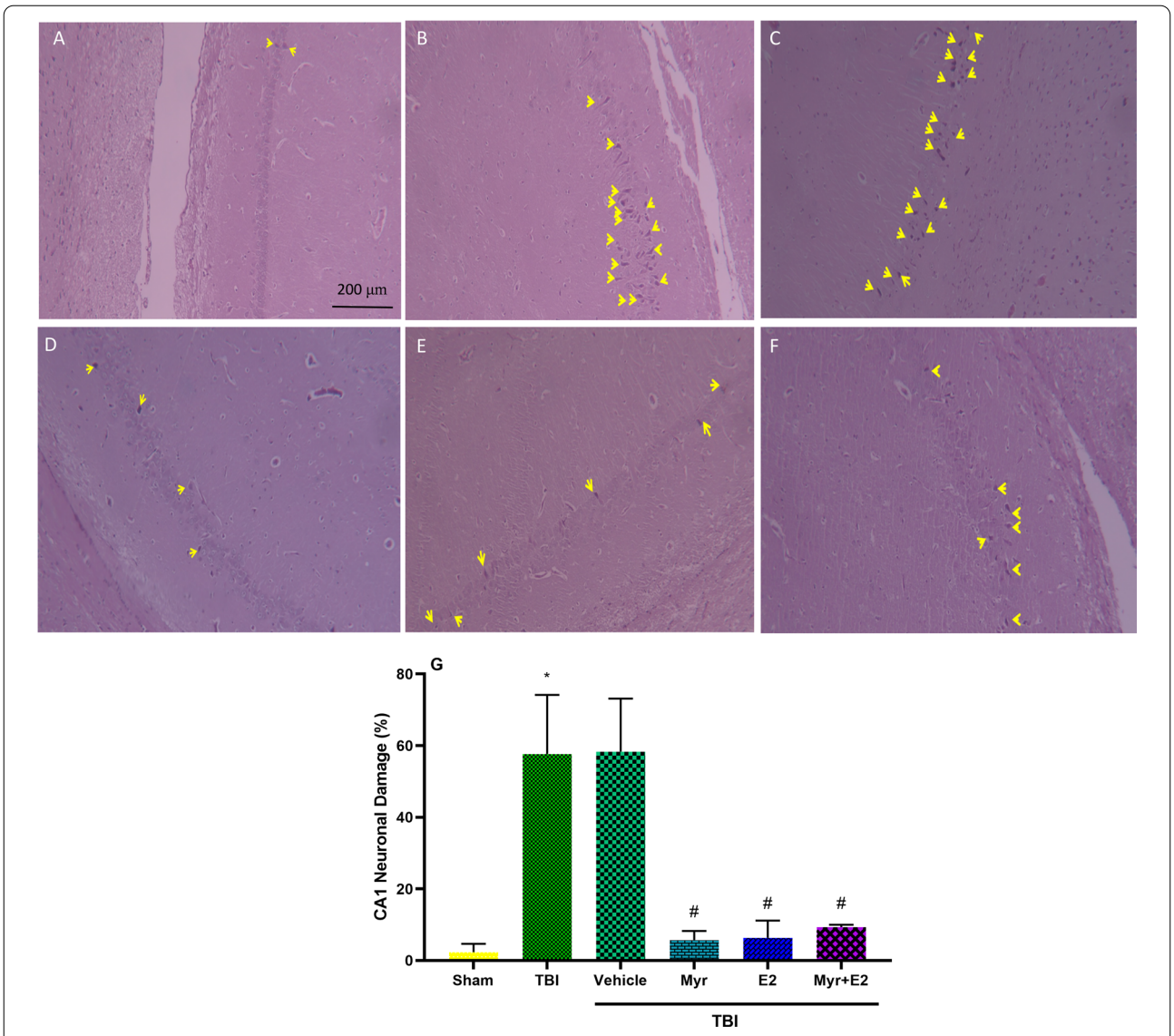


Fig. 14 Micrographs of the hippocampus stained with h & e showing neuronal damage in the CA1 among all the groups (arrows indicate apoptotic and damaged neurons). × 100 magnifications, scale bar 200 µm. **a** Sham. **b** TBI. **c** TBI/Vehicle. **d** TBI/Myrtenol. **e** TBI/Estradiol (E2), **f** TBI/Myr + E2. **g** The percentage of neuronal damage in the studied groups. Mean ± SEM, $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus TBI/vehicle. The data were analyzed using One-way ANOVA. ANOVA, analysis of variance, SEM, standard error of the mean

TBI. In line with our research, numerous studies have reported hippocampal-dependent spatial learning and memory impairments post-TBI [78, 90]. The cell loss in the hippocampus elicited by secondary injury is the major reason for learning and memory impairments, mood disorders, and hypothalamic–pituitary–adrenal axis activity disorder [91]. The hippocampus, which is one of the main damage areas, is a key structure of many functional brain circuits that regulate cognition, mood, and stress (hypothalamic–pituitary–adrenal axis function). On the other hand, our findings disclosed the positive impacts of E2 on spatial cognitive disturbances following TBI. It has been reported that E2 augments both long- and short-term memory [92] by affecting the hippocampal synaptic plasticity, as well as restructuring dendrites and synapses [93]. There are few studies on the effects of Myr on cognitive functions, e.g., its role in stress-induced spatial learning disorder [94].

A common complication of TBI is anxiety, which constitutes a noticeable part of the long-term symptoms and complications of TBI [95]. In another section of this study, we evaluated posttraumatic anxiety-like

behaviors by open field and EPM tasks. Our observations showed a decrease in the time spent in the open arms and the number of head dips in EPM after TBI. Furthermore, after TBI, grooming and fecal pellet number increased and the rearing number decreased in OFT. Many researchers have reported that experimental TBI increases anxiety-like behaviors in rats in both open field and EPM tests [83, 90]. Based on previous studies and clinical trials, TBI-induced anxiety is associated with progressive atrophy induced by injury in the posterolateral area of the prefrontal lobe and the left side of the basal nuclei [96]. Our findings demonstrated that both E2 and Myr increased the time spent in the open arms and the number of head dips in EPM. Besides, they decreased grooming and fecal pellet number and increased rearing number in OFT. Some studies like ours showed that E2 can mitigate anxiety following TBI. E2 can improve anxiety-like behaviors in TBI probably by decreasing brain edema, microglia proliferation, and neuronal damage [83]. The E2 receptors α and β mediated this improvement in anxiety-like behaviors [97]. The effects of Myr on anxiety and insomnia are also reported [98].

The results in another part of the present study showed that both NOR and NLR memories were impaired after TBI. Indeed, TBI can impair both spatial and nonspatial memories. Some researchers reported NOR [99, 100] and NLR [101] impairments following TBI. On the other hand, E2 improved NOR and NLR memories after TBI. NOR memory is sensitive to hormonal fluctuations, so it is enhanced in cycling female rats at the proestrus, as well as in ovariectomized (OVX) rats treated with exogenous E2 [102]. While many brain regions contribute to this task, this E2 effect is specifically due to hippocampal NMDARs [103]. NLR memory is a hippocampus-dependent test and the effects of E2 are probably mediated by the improvement of synaptic plasticity and dendritic spines in the hippocampus [104]. Myr neuroprotective effects have also been reported [33, 105]. Furthermore, its neuroprotective effect has been demonstrated in an Alzheimer's disease model [106] and diabetic postmenopausal rats [107].

BDNF is a key molecule involved in plastic changes related to learning and memory [108]. The level of BDNF was reduced in the hippocampus due to TBI in another part of this study. Many studies, in agreement with our results, have shown that the level of BDNF decreases after TBI in the hippocampus [109, 110]. In the TBI, the expression of neurotrophic factors began to decrease near the injury site [111]. Some studies have shown that the levels of BDNF are elevated several hours after TBI, and this increment of BDNF might be considered a mechanism promoting neuroprotection and neuronal repair after damage [25, 112]. Factors such as the time of BDNF measurement after TBI, the affected and studied area, the type of TBI induction model, and sex can explain the difference between these and our results. Our findings showed that E2 increased hippocampal BDNF levels after TBI. In this regard, some studies have reported that E2 can induce BDNF [113, 114]. We also found that Myr increased BDNF levels after TBI. One study reported that Myr had positive effects on BDNF in a stroke model [70].

Our results showed that PI3K and p-AKT expression decreased in the hippocampus after TBI. Consistent with our findings, some studies demonstrated that TBI inhibited the activation of the PI3K/AKT signaling pathway, and the expression of p-AKT declined after TBI [115, 116]. Activation of the PI3K/AKT pathway through BDNF/tropomyosin receptor kinase B (TrkB) interaction inhibits cell apoptosis and activates the mTOR pathway and, subsequently, protein synthesis [117]. AKT can activate cAMP response element-binding protein (CREB) and, thus induces the expression of multiple genes related to cell growth [118] and decreases apoptosis [110]. Furthermore, BDNF increases synaptic density through the TrkB/PI3K pathway [119]. Our results revealed that E2 and Myr increased both pAKT and PI3K expression in the hippocampus after TBI. Consistent with our findings, many studies have shown the stimulatory role of E2 on the PI3K/AKT pathway [120, 121].

Focal brain inflammation causes secondary brain injury following TBI by exacerbating brain edema and neuronal death [122]. Elevated serum and brain levels of inflammation-related cytokines in mild TBI are associated with cognitive performance [123, 124]. Our observations showed that the levels of TNFα, IL-6, and IL-17 increased, while IL-10 decreased in the hippocampus following TBI. Consistent with our results, some studies found an increase in TNFα [125], IL-6 [125], and IL-17[126] and a decrease in IL-10 [127, 128] levels after brain injury. In general, the level of inflammation and the levels of cytokines have a significant increase 24 h and seven days after the TBI [129, 130]. In another part of this study, the findings showed that E2 had neuroprotective effects by decreasing TNFα, IL-6, and IL-17 levels and increasing IL-10 levels in the hippocampus after TBI. In line with this study, it has been reported that E2 has anti-inflammatory effects in the hippocampus by changing the cytokines levels [131, 132]. Myr, similar to E2, decreased TNFα, IL-6, and IL-17 and increased IL-10 levels in the hippocampus. Although there are some studies indicating that Myr has anti-inflammatory effects on other organs such as the lungs and blood [29, 133], there are no reports about this anti-inflammatory effect after TBI.

Emotional and cognitive disorders following TBI are related to oxidative stress parameters [134], and the

improvement of oxidative stress can protect against cognitive impairments after TBI [135]. Excessive calcium influx into neuronal cells occurs following TBI, leading to the generation of oxidative stress and resulting in mitochondrial dysfunction, lipid peroxidation, and oxidation of proteins and DNA [136, 137]. Our data revealed that hippocampal levels of MDA increased, whereas the levels of TAC, SOD, and GPX decreased after brain injury. Other researchers reported that oxidative stress increased following TBI [138, 139]. Our results showed the protective effects of E2 against oxidative stress after TBI. E2 is a potent activator of antioxidant defense systems [140]. In line with our results, many studies have also shown that Myr has strong antioxidant effects [29, 62].

In conclusion, our results indicated that the inhalation of E2, Myr, and their combination had a neuroprotective effect on TBI-induced secondary injuries, including brain edema, CPP, ICP, inflammatory and oxidative status, BDNF level, and the PI3K/AKT signaling pathway in the hippocampus. In addition, post-TBI administration of E2, Myr, and their combination improved spatial learning and memory, recognition memory, and anxiety-like behaviors. There were no differences between the alone consumption and combination inhalation of these two compounds; therefore, to decrease the side effects of E2, we should not use Myr with E2. Considering the similar effects of Myr and E2, we recommend that Myr be administered instead of E2 for the elimination of E2 side effects following brain injury. Furthermore, studies on female animals are recommended. We only examined the hippocampal tissue and did not examine the nonhippocampal tissues, which was a limitation of our study. Therefore, nonhippocampal tissues should also be examined in future studies.

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MAR: acquisition, analysis, or interpretation of data. MK: acquisition, analysis, or interpretation of data. MAB: drafting the work. LA: drafting the work. EJ: acquisition, analysis, or interpretation of data. ZJ: Data analyzes. AN: acquisition, analysis, or interpretation of data, conception or design of the work. The final manuscript was approved by all authors.

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Declarations

Conflicts of interest
The authors have no relevant financial or nonfinancial interests to disclose.

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