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ORIGINAL ARTICLE

Possible anti-inflammatory, antioxidant, and neuroprotective effects of apigenin in the setting of mild traumatic brain injury: an investigation*

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ABSTRACT

Objective: Apigenin is a plant flavone proven with biological properties such as anti-inflammatory, antioxidant, and antimicrobial effects. This study, it was aimed to examine the possible anti-inflammatory, antioxidant, and neuroprotective effects of apigenin in the setting of the mild traumatic brain injury (TBI) model.

Methods: Wistar albino male rats were randomly assigned to groups: control ($n = 9$), TBI ($n = 9$), TBI + vehicle ($n = 8$), and TBI + apigenin (20 and 40 mg/kg, immediately after trauma; $n = 6$ and $n = 7$). TBI was performed by dropping a 300 g weight from a height of 1 m onto the skull under anesthesia. Neurological examination and tail suspension tests were applied before and 24 h after trauma, as well as Y-maze and object recognition tests, after that rats were decapitated. In brain tissue, luminol- and lucigenin-enhanced chemiluminescence levels and cytokine ELISA levels were measured. Histological damage was scored. Data were analyzed with one-way ANOVA.

Results: After TBI, luminol ($p < .001$) and lucigenin ($p < .001$) levels increased, and luminol and lucigenin levels decreased with apigenin treatments ($p < .01$–$.001$). The tail suspension test score increased with trauma ($p < .01$). According to the pre-traumatic values, the number of entrances to the arms ($p < .01$) in the Y-maze decreased after trauma ($p < .01$). In the object recognition test, discrimination ($p < .05$) and recognition indexes ($p < .05$) decreased with trauma. There was no significant difference among trauma apigenin groups in behavioral tests. Interleukin (IL)-10 levels, one of the anti-inflammatory cytokines, decreased with trauma ($p < .05$), and increased with 20 and 40 mg apigenin treatment ($p < .001$ and $p < .01$, respectively). The histological damage score in the cortex was decreased in the apigenin 20 mg treatment group significantly ($p < .05$), but the decrease observed in the apigenin 40 mg group was not significant.

Conclusion: The results of this study revealed that apigenin 20 and 40 mg treatment may have neuroprotective effects in mild TBI via decreasing the level of luminol and lucigenin and increasing the IL-10 levels. Additionally, apigenin 20 mg treatment ameliorated the trauma-induced cortical tissue damage.

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KEYWORDS
Apigenin; inflammation; oxidative stress; traumatic brain injury; neuroprotection

Introduction

Traumatic brain injury (TBI) is defined by an alteration in brain function, or other evidence of brain pathology, caused by an external force [1]. Forty percent of the adults reported a lifetime prevalence of TBI [2]. TBI is categorized as mild, moderate and severe, and mostly mild form of TBI is encountered [3]. At the time of trauma, mechanical damage results in primary injury in neurovascular structures and glial cells and this is not treatable but preventable [4]. All current efforts are aimed to prevent secondary injury which includes ischemia, hypoxia, oxidative stress, neuroinflammation, neurotoxicity, and cell death [5,6]. Early pharmacological interventions give hope for preventing these catastrophic cascades [7–10]. Besides the biochemical changes, cognitive changes must be evaluated in patients with TBI [11].

Apigenin (40,5,7-trihydroxyflavone) is a plant flavone proven to have a number of biological properties such as anti-inflammatory, antioxidant, anti-apoptotic, and neuroprotective effects [12–14]. Its neuroprotective activity has been demonstrated in models of cerebral ischemia, hypoxic-ischemic brain injury, Alzheimer's disease, Parkinson's disease, and depression [15–20]. Apigenin crosses the blood–brain barrier and has low levels of toxicity [21]. Anti-inflammatory, antioxidant, anti-apoptotic, and blood–brain-barrier protecting

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This study was presented at the 7th Annual Meeting of Serbian Neurosurgical Society, 2021, and was awarded second place in Best Young Neurosurgeon Paper Competition.

activity of apigenin treatment in subarachnoid hemorrhage models have been reported [22,23]. Also, in a cerebral ischemia and reperfusion study, anti-oxidant and anti-apoptotic activities of apigenin produced excellent neuroprotection [20].

In this study, the possible neuroprotective effects of apigenin against mild TBI were investigated with various parameters. It is thought that the administration of apigenin after mild TBI will show neuroprotective activity by reducing inflammation, oxidative stress, and apoptosis, and will have a positive impact on corticospinal tract functions and learning.

Materials and methods

In this study, all experimental procedures were reviewed and approved by the Marmara University Animal Care and Use Committee (12 March 2019). Animal care and all experiments were conducted in concordance with the European Communities Council Directive, 22 September 2010 (2010/63/EU) for experimental use. The ARRIVE guidelines were adopted for this study. 40 adult male (10- to 12-weeks-old) Wistar albino rats weighing 250–400 g were used. Since weight is one of the major determinants of the severity of the TBI, the age, weight, and species of the rats also affects the clinical outcome. In this study, we randomly assigned the rats among groups and the rats were equally distributed among groups for their weight and age difference. Animals were housed in an air-conditioned room with 12-h light and dark cycles maintained at constant temperature ($22 \pm 2\,^{\circ}C$) and relative humidity (65–70%). Rats were fed standard laboratory chow and had free access to water.

The rats were randomly assigned to five groups as follows:

1. Control group ($n = 9$): Rats underwent only a skin incision under anesthesia and received a single intraperitoneal dose of saline (0.9% NaCl, 0.1 ml/100 g) 15 min after surgery.
2. Trauma group ($n = 9$): Rats underwent TBI as described below and received a single intraperitoneal dose of saline (0.9% NaCl, 0.1 ml/100 g) 15 min after TBI.
3. Vehicle group ($n = 8$): Rats underwent TBI as described below and received a single intraperitoneal dose of vehicle (0.1% Tween 80, 0.1 ml/100 gr) 15 min after TBI.
4. Apigenin-20 mg group ($n = 6$): Rats underwent TBI as described below and received a single intraperitoneal dose of 20 mg/kg of apigenin 15 min after TBI. This dose was selected according to previous studies [22,23]. One animal died following TBI induction and data from that animal was not included in the analysis.
5. Apigenin-40 mg group ($n = 7$): Rats underwent TBI as described below and received a single intraperitoneal dose of 40 mg/kg apigenin 15 min after TBI. This dose was selected according to a previous study [18].

Anesthesia and induction of TBI

The animals were anesthetized with an intraperitoneal injection of 0.5 mg/kg chlorpromazine (Largactil, Eczacıbaşı, Türkiye) and 50 mg/kg ketamine (Ketalar, Parke Davis, Türkiye) combination and were allowed to breathe spontaneously. A mild TBI model, described by Marmarou et al. [24] and modified by Ucar et al. [25] was applied for head trauma. A lead object weighing 300-g was allowed to fall freely from a height of 1 m through a copper tube onto the metal disk over the skull of the rat. In order to reduce mortality thicker metal disk was used during experiments.

Collection and storage of brain tissue samples

All the animals were decapitated 24 h after trauma, and the brains were carefully removed immediately. For analysis of each parameter, the same parts of the brain were obtained from each animal. We used the left frontal part for chemiluminescence measurements, the right frontal part for myeloperoxidase (MPO) measurement, the left temporoparietal part for histopathological examinations, and the right temporoparietal part for ELISA measurements. Brain parts used for biochemical analysis were stored at $-80\,^{\circ}C$. For biochemical analysis, the brain samples thawed accordingly and for each study group, the results of each animal in their belonging group were included in the analysis. Brain parts that were used for histological investigations were stored in paraformaldehyde solution. All tissues were examined within first 6 months after decapitation.

Biochemical analyses

Chemiluminescence measurements in brain tissue

Chemiluminescence (CL) is a direct, noninvasive method for the measurement of reactive oxygen radicals that utilizes luminol and lucigenin as enhancer probes. When added to *in vitro* biological systems, luminol and lucigenin produce high levels of excited products. Excited electrons from these compounds generate radiating light energy or CL that can be detected by a luminometer. Luminol detects radicals such as hydroxyl ions, hydrogen peroxide, and hydrochloric acid, whereas lucigenin is selective to superoxide anions [26]. Reactive oxygen species (ROS) were numerically measured after the addition of 0.2 mM of enhancers luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (bis-N-methylacridinium nitrate) (Sigma Aldrich, USA). A purified luminol-hydrogen peroxide system was used for NO measurement. For this purpose, potassium carbonate (K_2CO_3) (0.4 mM), desferal (60 μM), H2O2 (4 mM), and purified luminol (3.6 M) were added to the tissues in 2 ml of phosphate-buffered saline (PBS) + HEPES buffer [27,28]. Counts were measured at room temperature using a luminometer (Junior LB 9509 luminometer; EG&G Berthold, Germany), and obtained at 1-min intervals for 5-min. The area under the curve was determined, and data were expressed as relative light units after counts were normalized to the weight of the brain tissue sample. Results were expressed as relative light units/mg tissue (rlu/mg).

Enzyme-linked immunosorbent assay measurements

To determine the levels of IL-1β, IL-6, TNF, IL-10, transforming growth factor-beta (TGF-β), caspase 3, and cleaved caspase 3 in the brain tissue, commercial enzyme-linked immunosorbent assay kits (Sunlong Biotech Co., Ltd., China)

was used according to the manufacturer's instructions. Supernatants of tissue homogenates were used for measuring the levels of cytokines.

Western blot for Nuclear Factor kappa B

The frozen tissues were weighed and homogenized in ice-cold 10 mM Tris–HCl buffer (pH = 7.2) containing 1 mM EDTA and protease inhibitors (0.2 mM PMSF, 1 μg/ml leupeptin, 1 μM pepstatin, 10 μg/ml soybean trypsin inhibitors) with Ultraturrax homogenizer. Whole homogenates were used in Western blots. The protein content of the whole homogenate was determined with the Lowry method [29]. Mixed with loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue) samples containing 50 mcg protein were denatured at 100 °C for 3–5 min and electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell, 0.45 μm, Germany) for 120 min at 80 V. The membranes were blocked at room temperature for 60 min with tris-buffered saline containing 1% bovine serum albumin (Sigma Aldrich, USA) and 0.05% Tween-20 (Sigma Aldrich, USA) and incubated overnight at 4 °C with antibody against Nuclear Factor kappa B (NF-κB) (1:500; NOVUS, CO, USA) and β-actin (1:1000). β-actin (Sigma Aldrich, USA) was used as an internal control.

The secondary antibodies were purchased from Sigma (St Louis, MO, USA). All chemicals were obtained from Sigma unless stated otherwise. The blots were washed three times with TBS containing 0.05% Tween-20 (TBS-T) and incubated with alkaline phosphatase-conjugated secondary antibodies for 1 h at room temperature (20 °C). The antibody-antigen complex was detected with NBT-BCIP(5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium method). The apparent molecular weights of NF-κB and β-actin are 65 kDa, and 42 kDa, respectively. The densitometric analyses were carried out with Image Studio Lite ver5.2 software (Lincoln, Nebraska, USA).

Histopathological examinations

Light microscopy

The brain samples were fixed in the 4% paraformaldehyde in phosphate buffer (pH 7.4) for 24 h at 4 °C. Tissues were then embedded in paraffin and 5-μm-thick coronal sections were created using a rotary microtome. The sections were stained with hematoxylin and eosin stains. Finally, sections were examined under a photomicroscope (Olympus BX51, Japan). The severity of neuronal damage in the cortex was scored semiquantitatively as follows: 0 = no damage, 1 = mild damage, 2 = moderate damage, and 3 = severe damage [5,30,31]. Pyknotic nuclei and intense staining of the shrunken neuronal perikarya were considered in scoring the degree of neuronal degeneration. The histopathological scorings were performed by investigators blinded to treatment groups to reduce the potential for bias.

Behavioral tests

The behavioral tests were performed twice for each rat, once before TBI and once again 24 h after TBI. The scorings were performed by the same investigators blinded to treatment groups.

Novel object recognition test

The novel object recognition test, which is efficient in evaluating short-term memory, has become a widely used model for the investigation of memory alterations. Alterations in the test results are accepted to be indicative of both hippocampal and cortical lesions [32]. During the 'habituation phase,' the rats were initially placed in a box ($65 \times 45 \times 65\,cm^3$) for 10 min. The next day, animals were put in the same box, but this time containing two identical objects (F + F), and their behavior was observed and recorded with a video camera for 10 min. After this 'familiarization phase,' rats were allowed to stay in their housing cages for 1 h. During the 'test phase,' the animals were put in the box with the familiar object (F) and a novel object (N), which were different both in color and shape. Similarly, the test phase was also videotaped for 5 min. During all experiments, boxes and objects were cleaned with 70% alcohol solution before placing the next animal [33].

The difference score (DS; in centisecond) was the difference in exploration time for the familiar object (TF) and the novel object (TN): DS = TN − TF. The discrimination index (DI; in seconds) was calculated by dividing the difference in exploration TF and TN by the total amount of exploration time for the novel and familiar objects: DI = (TN − TF)/(TN + TF). DI can vary between +1 and −1, where a positive score indicates that more time was spent with the novel object, a negative score indicates that more time was spent with the familiar object, and a zero score indicates a null preference. Recognition index (RI; in seconds) was calculated by the formula: DI = TN/(TN + TF) and an increased RI indicated amelioration of cognitive functions.

Y-maze test

The Y-maze is characterized by three branches that intersect at a single point at 120° angles. The rat starts at the end of one arm, then chooses between the other two. In this setup, the number of entries into the arms and spontaneous alternation is measured. Spontaneous alternation is measured to assess spatial working memory [34]. The number of entries is counted and the spontaneous alternation percentage is then calculated with the following formula [34]:

$$\text{Spontaneous alternation percentage} = \frac{\text{Number of spontaneous alternations}}{\text{Total number of arm entries} - 2} \times 100$$

Assessment of corticospinal function with limb clasping

Limb clasping is a functional motor test that measures the loss of corticospinal functions [34]. After 5–10 s of drowning, the subject is held by the tail and videotaped. Each animal is

evaluated by two independent observers. Possible scores range from 0 to 4 points from normal to immobile. Subjects with a difference of more than 1 point are scored again and the different scores are averaged.

Modified Bederson neurological examination score

A 20-point neuro-score was used to assess motor and behavioral deficits [6,35]. A low score (minimum 0) indicates that the animal is awake, active, and has normal reflexes. Increasing scores (maximum 20) indicate the occurrence of a neurological disorder. Consciousness, climbing performance on a smooth platform, limb tone, walking, posture reflexes, circling, and response to nociceptive stimuli were evaluated.

Statistical analysis

Data were analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) and expressed as means ± SEM. The effect size was calculated as 0.8, the standard statistical power ($1 - \beta$) 0.95, and the significance level (α) 0.05 with the G*Power program. Analysis of biochemical and histological data was performed using a one-way analysis of variance, followed by Tukey's posthoc test. Values of $p < .05$ were considered to be statistically significant.

Results

Biochemical results

Luminol and lucigenin enhanced chemiluminescence levels in brain tissues

Luminol-enhanced chemiluminescence values were significantly increased in the trauma and vehicle groups when compared to the control group ($p < .001$; for both comparisons). In apigenin 20 and 40 mg groups significant decrease was observed in luminol-enhanced chemiluminescence values when compared to the trauma ($p = .0008$, and $p = .006$, respectively) and vehicle groups ($p < .001$, for both groups, Figure 1(A)).

There was a statistically significant increase in lucigenin-enhanced chemiluminescence values in the trauma and vehicle groups compared to the control group ($p < .001$; for both comparisons). A statistically significant decrease in lucigenin-enhanced chemiluminescence values was observed in the apigenin 20 and 40 mg groups compared to trauma and vehicle groups ($p = .003$ and .0009; for both comparisons) (Figure 1(B)).

Increased luminol and lucigenin-enhanced chemiluminescence levels after TBI are an indicator of increased oxidative stress and inflammation. The decrease in these values with apigenin treatment indicates that apigenin has antioxidant and anti-inflammatory activity in TBI.

Effects of TBI induction and apigenin treatment on IL-1 β, IL-6, IL-10, TNF-α, TGF-β, caspase-3, and cleaved caspase-3 levels

At the 24th hour of TBI, levels of the IL-1β, IL-6, TNF-α, and TGF-β in the brain tissue were not significantly altered among groups (Figure 2(A–E)). However, when we evaluated the inflammatory cytokines, an increasing trend was observed in the trauma groups but not in the vehicle group. The apigenin 20 and 40 mg groups had similar values to the control group.

There was a decrease in IL-10 levels in the trauma group and a significant decrease in the vehicle group when compared to controls ($p = .017$). A significant increase in IL-10 level was observed in the apigenin 20 and 40 mg groups compared to the trauma ($p = .012$, for apigenin 20 mg), and vehicle groups ($p = .0001$, $p = .0035$, respectively).

When we evaluated the caspases, an increase was observed in the trauma group but not in the vehicle group. We found no statistically significant difference among control, trauma, and apigenin 20 and 40 mg groups in terms of the brain caspase-3 and cleaved caspase-3 activities (Figure 3(A–B)).

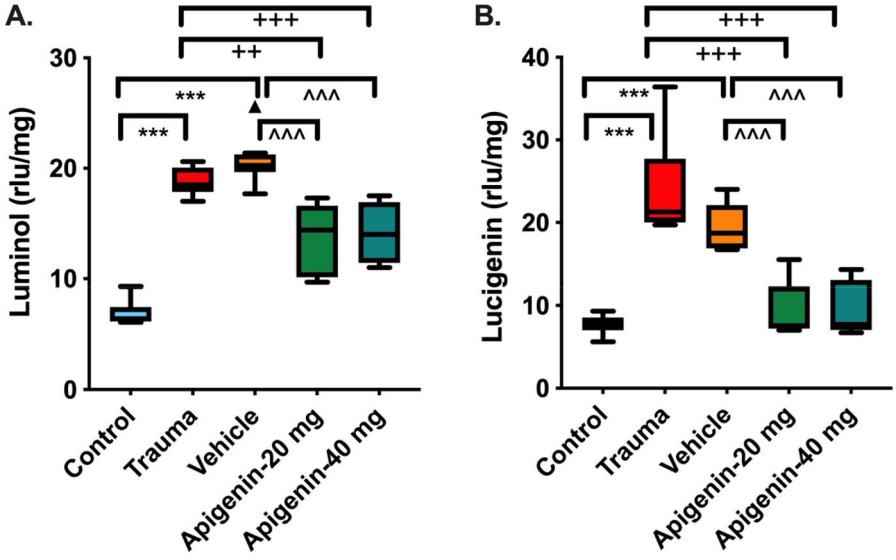

Figure 1. (A) Luminol- and (B) lucigenin-enhanced chemiluminescence (rlu/mg) values of experimental groups (according to the control group $^{***}p < .001$, according to the TBI group $+p < .05$, $+++p < .001$, according to the vehicle group $^{\wedge\wedge\wedge}p < .001$. Statistical test: One-way ANOVA followed by Tukey's post-hoc test).

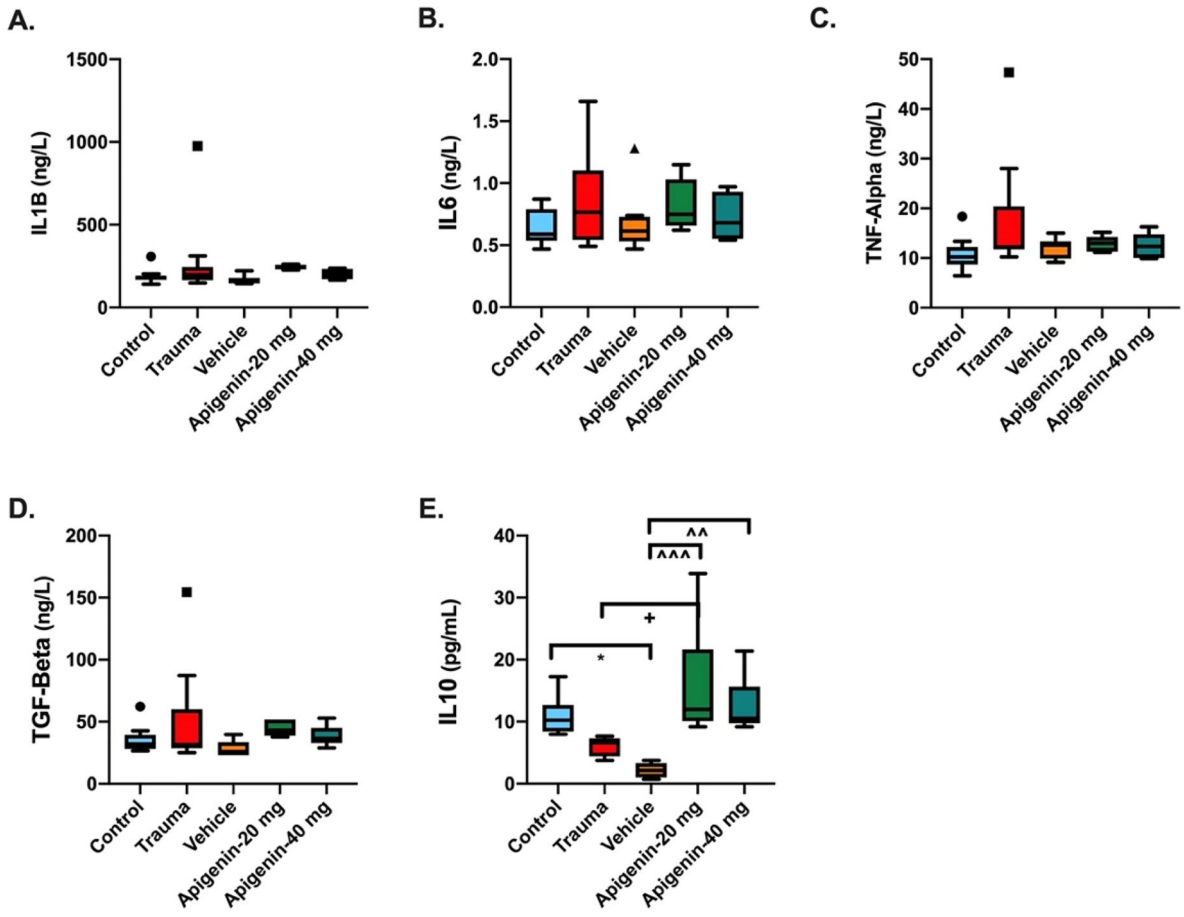

Figure 2. (A) IL-1β, (B) IL-6, (C) TNF, (D) TGF-β, (E) IL-10 levels of experimental groups (according to the control group $*p < .05$, according to the TBI group $++p < .01$, $+++p < .001$, according to the vehicle group $\text{^^}p < .01$, $\text{^^^}p < .001$. Statistical test: One-way ANOVA followed by Tukey's posthoc test).

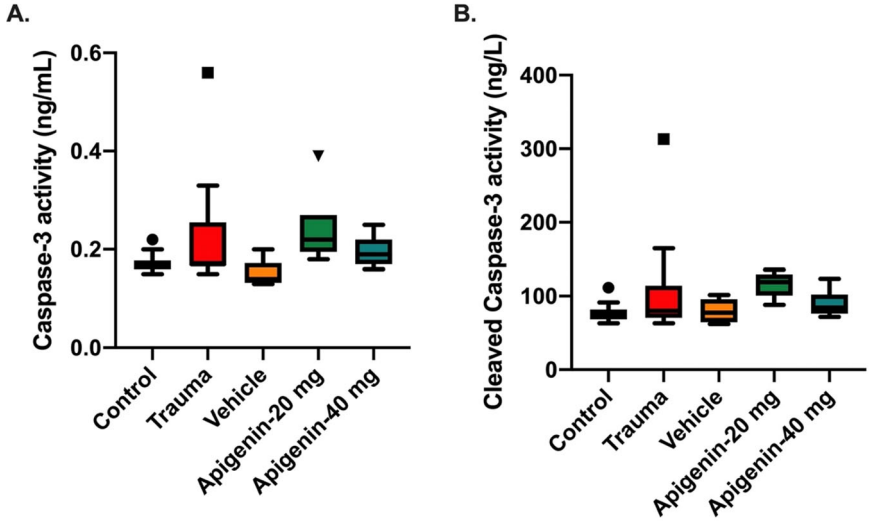

Figure 3. (A) Caspase-3, and B) cleaved caspase-3 levels of experimental groups (statistical test: One-way ANOVA followed by Tukey's posthoc test).

Measurements of NF-κB protein levels in brain tissue by Western blot method

There was a slight increase in NF-κB/β actin protein levels in the vehicle group but not in the trauma group when compared to the control group ($p = .1933$, and $p = .9434$, respectively). There was a significant decrease in the level of NF-κB/β actin in the apigenin-20 mg group compared to the trauma group ($p = .017$, Figure 4), and there was a significant difference between trauma and the apigenin-20 mg group ($p < .001$).

Light microscopic investigation

Cortex, DG, and CA3 regions in the hippocampus were evaluated for neuronal cell damage semi-quantitatively with

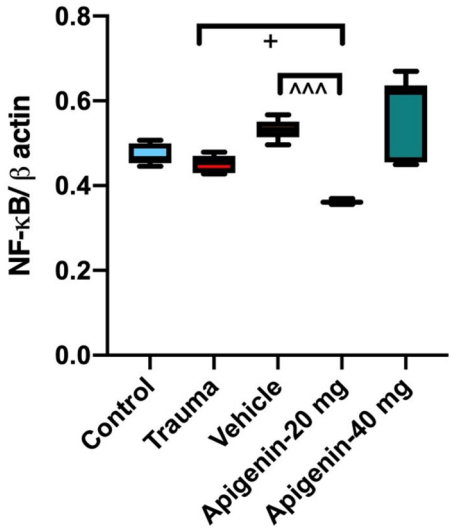

Figure 4. NF-κB/β actin levels of experimental groups (according to the TBI group $+p < .05$, according to the vehicle group $^{\wedge\wedge\wedge}p < .001$). Statistical test: One-way ANOVA followed by Tukey's posthoc test).

hematoxylin and eosin staining. In the cortex, DG, and CA3 regions of the rats in the control group, there were regular neuropil structures, neurons with uniform morphology and large nuclei, and prominent nucleolus structures were observed (Figure 5(A–C)). Neuronal damage, pycnotic cell nuclei, irregularity of cell structures, and cytoplasmic deterioration were observed in the cortex, DG, and CA3 regions of the rats in the trauma and vehicle groups compared to the control group (Figure 5(D–I)). This damage to the cellular structure caused by TBI was less in the apigenin 20 and 40 mg groups (Figure 5(J–L)).

In the cortex, it was found that the histological damage score increased significantly in the trauma and vehicle groups when compared to the control group ($p < .001$). In the apigenin 20 mg group, the histological damage score showed a statistically significant decrease when compared to the trauma group ($p = .0323$) (Figure 6(A)) but this difference was not significant for the apigenin 40 mg group.

In the hippocampal DG, the histological damage score was found to be increased in the trauma and vehicle groups compared to the control group ($p < .001$, for both comparisons). The histological damage score was shown to be decreased in the apigenin 20 and 40 mg groups compared to the trauma groups (Figure 6(B)).

In the hippocampal CA3 region, the histological damage score in the trauma and vehicle group increased significantly when compared to the control group ($p < .01$–$.001$). In the apigenin 20 and 40 mg groups, a decrease was observed in the histological damage score compared to the trauma and vehicle group (not significant) (Figure 6(C)).

Behavioral tests

Novel object recognition test

In the novel object recognition test, the scores of the trauma and vehicle groups were negative on the discrimination index, and there was a statistically significant difference in the trauma group when compared to the controls ($p = .0263$). Apigenin 20 mg group did not show a significant difference in scores compared to the trauma and control groups. There was an increase in the scores of the apigenin 40 mg group when compared to the trauma and vehicle groups (Figure 7(A)).

In the novel object recognition test, there was a statistically significant decrease in the trauma group compared to the control group for recognition index ($p = .0263$). There was no difference between the vehicle group when compared to the control group. Although there was an increase in the recognition index for the apigenin 20 and 40 mg groups when compared to the trauma group, a statistically significant difference was not found but the scores were close to control values (Figure 7(B)).

In the novel object recognition test, a decreased tendency in the discrimination and recognition indices was observed in the trauma group when compared to the control group. In most of the apigenin groups, values were close to the control values (not significant).

Y-maze test

In the Y-maze test, a statistically significant decrease was observed in the number of entries to the arms in the trauma and vehicle groups when compared to the pre-control group values ($p = .0137$, and $.0255$, respectively, Figure 8(A)). There was no significant difference between the apigenin 20 and 40 mg groups when compared to the trauma group, however, the apigenin 40 mg group had close values to the control groups.

When spontaneous alternation was analyzed, there was a statistically significant decrease in the vehicle group when compared to the pre-control group ($p = .0031$) (Figure 8(B)), but this decrease was not seen in the trauma group. Although there was an increase in the percentage of spontaneous alternation in the apigenin 20 and 40 mg groups, there was no statistical significance when compared to the trauma and vehicle groups.

In the Y-maze test, the number of entries was decreased in the trauma groups when compared to control values, and this response was found to be compatible with the freezing responses observed during the experiment. The spontaneous alternation percentages were lower in the vehicle group when compared to the control values. Additionally, an increase was noted in apigenin groups.

Tail suspension test

A statistically significant increase was observed in the tail suspension test score in the trauma group when compared to the control group ($p = .0043$), the increase in the vehicle group was not significant. There was a decrease in the test scores of the apigenin 20 and 40 mg groups, but no significance compared to the trauma group (Figure 9).

Modified Bederson neurological examination score

In the trauma group, a subject's score was 1, in the vehicle group a subject's score was 3; in the control and apigenin 20

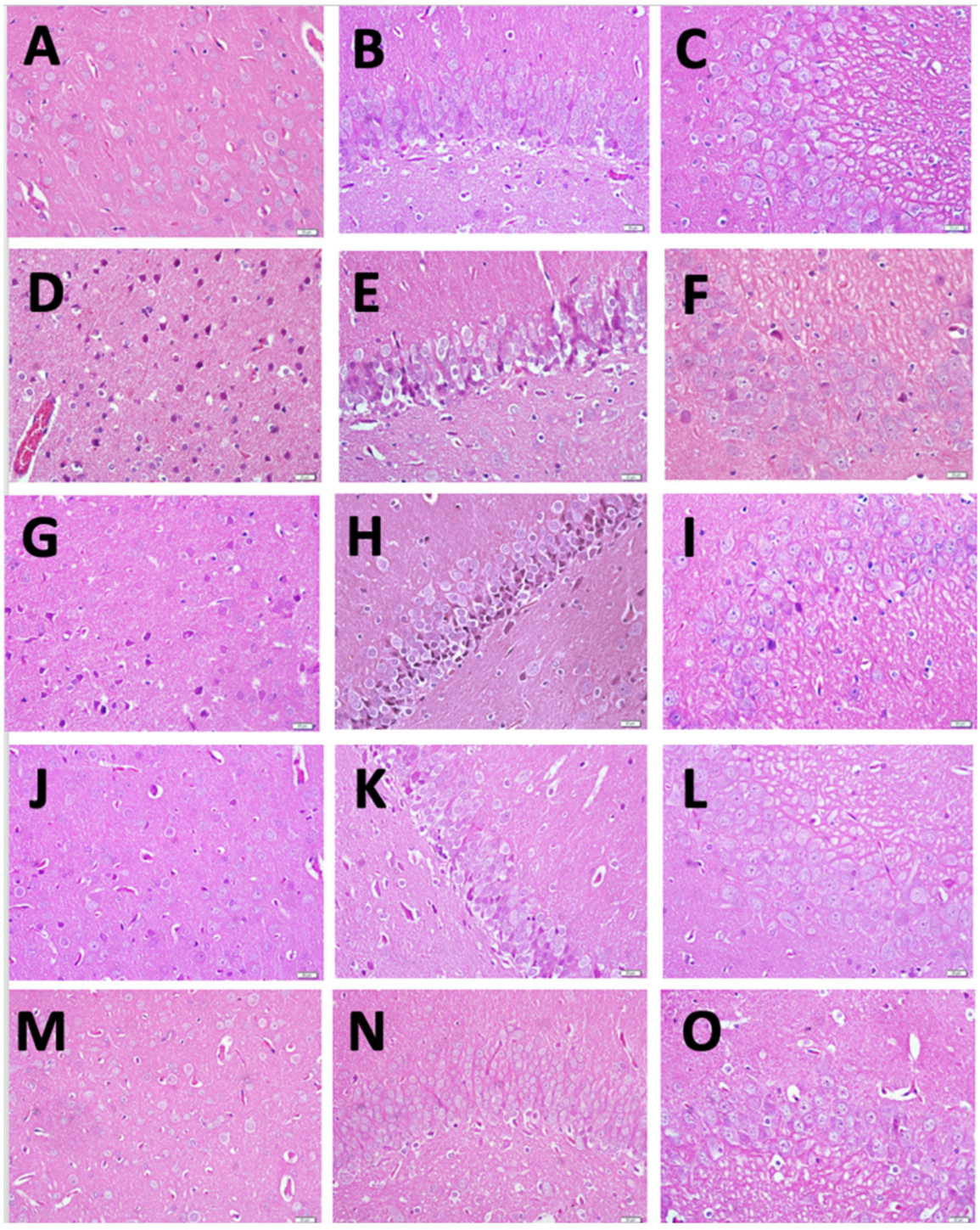

Figure 5. Histological examinations performed with hematoxylin and eosin (H&E) staining. (A–C) Representative sections of the cortex, hippocampal dentate gyrus (DG), and CA3 regions, in the control group. (D–F) Representative sections of the cortex, hippocampal DG, and CA3 regions in the trauma group. (G–I) Representative sections of the cortex, hippocampal DG, and CA3 regions in the vehicle group. (J–O) Representative sections of the cortex, hippocampal DG, and CA3 regions in the Apigenin groups. H&E stain. Scale bar: 50 μm.

and 40 mg groups, all subjects scored 0. There was no statistically significant difference among the experimental groups.

Discussion

Traumatic brain injury results from trauma to the brain from an external source, causing temporary or permanent functional impairments [4]. The most common form of TBI is the mild form, which mainly presents with cognitive problems. In the current study, we chose a mild TBI model to replicate the most frequently encountered form of TBI. In rats, weight drop TBI models have a high mortality rate [24]. Marmarou et al. [24] defined moderate (450–500 g from 1 m, 0% mortality rate), and severe (450–500 g from 2 m, 50% mortality rate) forms of the rat TBI model. Ucar et al. [25]

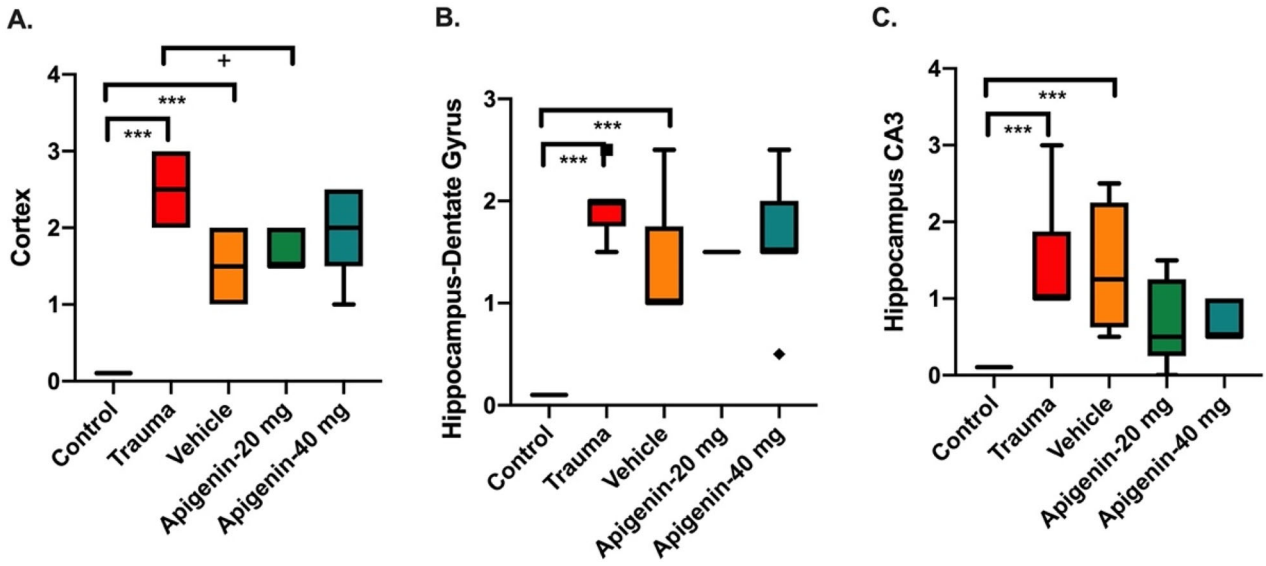

Figure 6. Hematoxylen-eosin (HE) staining histological damage scores among experimental groups. (A) cortex, (B) hippocampal dentate gyrus, (C) hippocampal CA3-region (according to the control group; $^{**}p < .01$, $^{***}p < .001$; according to the trauma group; $+p < .05$. Statistical test: One-way ANOVA followed by Tukey's posthoc test).

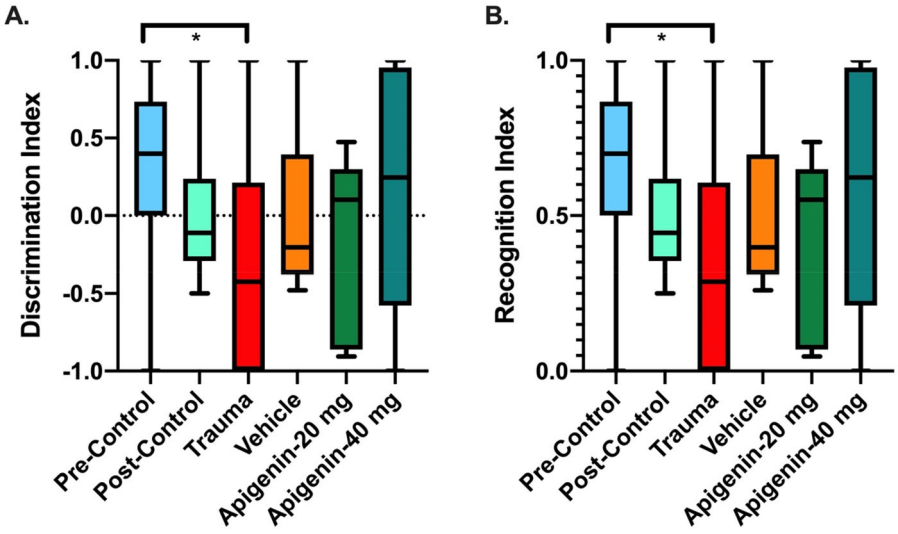

Figure 7. (A) Discrimination index, and (B) Recognition index among experimental groups, novel object recognition test indices (according to the pre-control group; $^{*}p < .05$. Statistical test: One-way ANOVA followed by Tukey's posthoc test).

modified the original weight-drop model in order to decrease mortality and suggested dropping 300-g weight from 1 m height as a mild TBI model (7.1% mortality). Severe models have over %50 mortality making it difficult to follow up on those rats for hours. This study replicated clinical situations where patients suffer from mild TBI and their cognitive deteriorations are overlooked because there are no major symptoms such as unconsciousness and seizures. It is thought that because the hippocampus is a delicate structure and has thin caliber vascular feeders, they are more prone to hypoxia observed after TBI. We reported a mortality rate of 2.5%, which is close to the results of Uçar et al. [25]. TBI-related neuropathological changes mainly take two forms: primary injury, preventable but irreversible; and secondary injury, reversible, with most treatment methods aiming to prevent inflammation-response cascades [30,31].

Apigenin is a potent plant flavonoid and is well-known for its anti-oxidant, anti-inflammatory, anti-apoptotic, and neuroprotective activities [19]. Recently, it has been shown that apigenin treatment reduces infarct volume, decreases cerebral edema, ameliorates inflammatory response, inhibits apoptotic cascades, and improves prognosis in a hypoxic-ischemic brain injury model [19]. Also, in a chronic neuroinflammation model, long-term apigenin supplementation ameliorated microglial activation [36]. Apigenin has a diverse mode of action via its potential therapeutic effects with regulating cell cycle arrest, turning on apoptosis, and anti-inflammatory, and antioxidant functions [37]. Apigenin regulated intrinsic and extrinsic apoptotic pathways and activates apoptosis [37]. It promotes different anti-inflammatory pathways (i.e. p38/MAPK and PI3K/Akt). Also, it prevents nuclear translocation of the NF-κB and the IKB degradation.

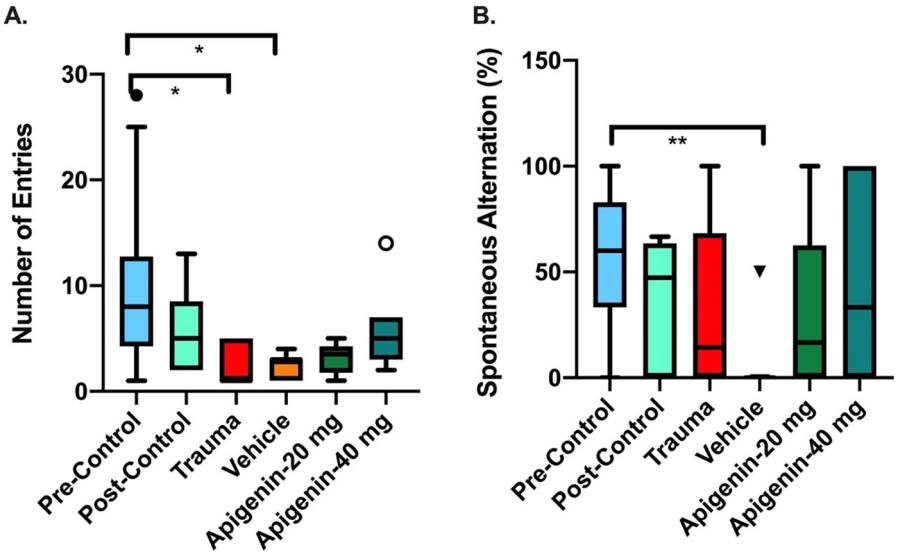

Figure 8. (A) The change in the number of entries into the arms. (B) The percentage of spontaneous alternation among the experimental groups (according to the pre-control group; $^{*}p < .05$, $^{**}p < .01$. Statistical test: One-way ANOVA followed by Tukey's posthoc test).

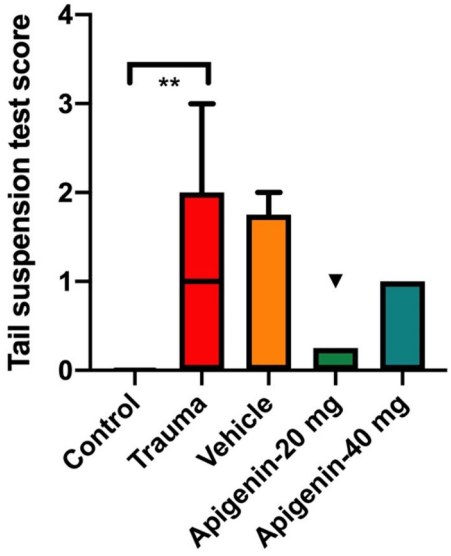

Figure 9. Tail suspension test scores among experimental groups (according to control group $^{**}p < .01$. Statistical test: One-way ANOVA followed by Tukey's posthoc test).

The anti-inflammatory properties of apigenin inhibit inducible nitric oxide (NO) synthase and cyclooxygenase-2 and suppress NO and prostaglandin [38]. Apigenin treatment also inhibits interferon-gamma-induced phosphorylation of signal transducer and activator of transcription 1 and suppresses levels of interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) [39]. Apigenin enhances the expression of antioxidant enzymes (i.e. catalase, glutathione-synthase, and superoxide dismutase), and counteracts cellular oxidative and electrophilic stress [37].

One of the main findings of this study is the TBI-related increase in luminol and lucigenin-enhanced chemiluminescence levels and their reversal with apigenin treatment. In our previous mild TBI studies, luminol- and lucigenin-enhanced chemiluminescence levels were preferred as reliable biomarkers of oxidative stress [5,30,31]. Potent antioxidant activity of apigenin treatment has also been observed in a subarachnoid hemorrhage model [22]. The TBI model we preferred in this study is enough for showing the initiation of observable oxidation. Apigenin treatment decreased these luminol- and lucigenin-enhanced chemiluminescence levels, which supports its anti-oxidant and anti-inflammatory activities.

As part of the neuroinflammatory response, pro-inflammatory cytokines are released from damage-activated microglia following TBI [40,41]. Increased proinflammatory cytokine response is related to neuronal damage and death. IL-1β mRNA expression increases after TBI and peaks around 12–24 h after TBI [42–47]. There is a positive correlation between the severity of TBI and the level of IL-1β levels [44]. IL-6 levels were also rapidly increased following TBI and reach their maximum level around 2–8 h after TBI [48–54]. The TNF-α level also increased starting from 1st hour of TBI and peaked around 4–8 h after TBI [49,50,55–57]. Although it is expected to see increased levels of TNF-α following severe TBI, it may not change following mild TBI [57]. TGF-β is triggered by inflammatory cytokines and peak around the 24th hour after TBI and act as an anti-inflammatory cytokine with IL-10 [58–60]. In this study, there is a tendency to increase IL-1β, IL-6 and TNF-α, and TGF-β cytokine levels at the 24th hour of the TBI. However, there was no statistically significant difference among the groups. There was a tendency for an increase in pro-inflammatory cytokine levels in the trauma group which is not observed in the vehicle group. Because there is no significant difference between trauma and vehicle groups we can not address any specific explanation for this observation.

There was a slight increase in NF-κB/β actin protein levels in the vehicle group when compared to the control group and in the apigenin 20 mg group, there was a significant decrease in NF-κB/β actin levels when compared to the trauma group. However, this decrease was not observed in the apigenin 40 mg group. We preferred the mild TBI model and this form of diffuse mild trauma may not increase brain IL-1β, IL-6, TNF-α, and TGF-β cytokine levels and NF-κB/β

actin protein levels significantly [57,61]. Either, these cytokine levels may return to normal values at the 24th hour of TBI [61].

There were decreased levels of IL-10 in the trauma group and a significant decrease in IL-10 levels in the vehicle group when compared to control groups and, the IL-10 levels were significantly increased above control levels in apigenin 20 and 40 mg groups. This may be evidence of the anti-inflammatory actions of apigenin treatment in mild TBI models in acute applications.

The caspase family is cysteine proteases that regulate many steps of programmed cell death.

Caspase-3 levels, which is a reliable marker of apoptotic activity increased at the 24th hour after TBI [5,8]. Cleaved caspase-3 is the active form of caspase-3. Apigenin has anti-apoptotic activity and it has previously been shown in a subarachnoid hemorrhage model that apigenin treatment decreased Bax and caspase-3 expressions [22]. In this study, caspase-3 and cleaved caspase-3 levels are seen to increase trend in the trauma group, however, there was no statistically significant difference among groups. Apigenin 20 and 40 mg groups also did not show any significant difference. It may be assumed that the caspase-related apoptotic cascades are not yet activated enough to make a significant difference among groups or they may return to their baseline values [30,31].

Neurological and cognitive problems that are seen following TBI has detrimental effects on the quality of life of patients [62]. Therefore, we preferred to include motor and cognitive tests besides biochemical and histological evaluations in this study. There were no statistically significant differences were found among groups in modified Bederson neurological examination scores. This result is compatible with previous findings of a pioneer study conducted by Uçar et al. [25], in which a mild TBI model was described. As expected, when the magnitude of the impact increases, the encountered deficits also worsen [24,25]. Apigenin treatment also was proven to be effective as a cognitive enhancer. Acute apigenin treatment was shown to improve long-term memory [63]. We applied a novel object recognition test to evaluate short-term recognition memory, learning, distinguishing novel, or remembering [64,65]. In the current study, the novel object recognition test revealed that the discrimination index and recognition index were decreased in the trauma group and the differences were statistically significant. This significance was not observed with vehicle groups. Especially the apigenin 40 mg group exerted an ameliorative effect on the discrimination index and recognition index, which can be interpreted as improved recognition memory caused by apigenin 40 mg treatment in the mild TBI model. This effect was lighter for the apigenin 20 mg group for behavioral tests. Spatial working memory was evaluated with spontaneous alternation in the Y-maze test [34]. In the current study, the number of entries to the arms and the percentage of spontaneous alternation in the Y-maze test was decreased in trauma and vehicle groups as compared to pre-procedure values, indicating the cognitive dysfunction within the first 24 h of TBI. In a TBI model, in the Morris water maze test the time to find the platform was prolonged in the trauma group [66]. The percentage of spontaneous alternation showed an increasing tendency in apigenin 20 and 40 mg groups; these can be interpreted as a reduction in memory dysfunction.

The histological changes in the brain after TBI included edema, presence of vascular congestion areas, hyperchromasia in the neuron nucleus, nuclear pycnosis, and cytoplasmic eosinophilic degeneration. They were signs of neuronal damage, focal neuronal loss, axonal edema, and formation of gliotic areas [8]. In the present study, the histological damage score of the cortex, DG, and CA3 increased with TBI and was seen to decrease trend in the apigenin groups. A significant decrease was observed in the cortex in the apigenin 20 mg group when compared to control values. The decrease in the apigenin 40 mg group was not significant. Damage findings in the trauma group were consistent with the histological damage that was reported in our previous studies [5,30,31]. The reduced TBI-related histological damage in the cortex and hippocampus by apigenin treatment supports the neuroprotective activity of apigenin.

The current study is not without limitations. In this study, we aimed to eliminate the effects of secondary injury mechanisms via immediate apigenin treatment after TBI induction and by reaching an effective blood concentration readily. For clinical applications in cases of acute injuries, this timing may not be feasible or appropriate. We investigated 20 and 40 mg treatments and concluded that given the overall results, apigenin 20 mg treatment is enough for the desired neuroprotective effect. In a recent study, a 20 mg/kg dose of apigenin was nontoxic and 40 mg/kg was found to be ineffective and escalated the inflammatory and oxidative response [18]. In future studies, 20 mg/kg of apigenin treatment may be enough for investigating efficacy. The neuroprotective efficacy of apigenin treatment at different treatment durations could be investigated more comprehensively within various TBI models. The cytokine levels, except IL-10, were not significantly altered among groups. The reason for this may be the timing and method of measurement. Otherwise, mild TBI may not alter cytokine levels as expected; or they may return to baseline values 24 h after TBI. Further research is needed in order to clarify the effectiveness of apigenin treatment after TBI.

Conclusion

For the first time in the literature, this study shows that apigenin treatment after mild TBI has an ameliorative effect on TBI-related oxidative stress, inflammation, and neuronal damage. Apigenin is a potent antioxidant, anti-inflammatory, and antiapoptotic compound, and examining the effects of apigenin on both pathophysiology and function in TBI would lead the way to future clinical studies.

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