Neuroprotective Effect of Cinnamaldehyde on Secondary Brain Injury After Traumatic Brain Injury in a Rat Model

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■ OBJECTIVE: **The aim of this study was to investigate the possible neuroprotective effects of cinnamaldehyde (CA) on secondary brain injury after traumatic brain injury (TBI) in a rat model.**

■ METHODS: **Rats were randomly divided into 4 groups: control ($n = 9$), TBI ($n = 9$), vehicle (0.1% Tween 80; $n = 8$), and CA (100 mg/kg) ($n = 9$). TBI was induced by the weight-drop model. In brain tissues, myeloperoxidase activity and the levels of luminol-enhanced and lucigenin-enhanced chemiluminescence were measured. Interleukin 1β, interleukin 6, tumor necrosis factor α, tumor growth factor β, caspase-3, and cleaved caspase-3 were evaluated with an enzyme-linked immunosorbent assay method. Brain injury was histopathologically graded after hematoxylin-eosin staining. Y-maze and novel object recognition tests were performed before TBI and within 24 hours of TBI.**

■ RESULTS: **Higher myeloperoxidase activity levels in the TBI group ($P < 0.001$) were suppressed in the CA group ($P < 0.05$). Luminol-enhanced and lucigenin-enhanced chemiluminescence, which were increased in the TBI group ($P < 0.001$, for both), were decreased in the group that received CA treatment ($P < 0.001$ for both). Compared with the increased histologic damage scores in the cerebral cortex and dentate gyrus of the TBI group ($P < 0.001$), scores of the CA group were lower ($P < 0.001$). Decreased number of entries and spontaneous alternation percentage in the Y-maze test of the TBI group ($P < 0.05$ and $P < 0.01$, respectively) were not evident in the CA group.**

■ CONCLUSIONS: **CA has shown neuroprotective effects by limiting neutrophil recruitment, suppressing reactive oxygen species and reducing histologic damage and acute hippocampal dysfunction.**

INTRODUCTION

Traumatic brain injury (TBI), which occurs as a result of an external force, may severely alter brain function and result in several pathologic changes in the brain tissue.[1] Based on the clinical presentation of the neurologic signs and symptoms, the severity of TBI can be classified as mild, moderate, or severe.[2] Most patients with trauma (70%–90%) have mild TBI.[2-4] Patients with mild TBI mostly have somatic (e.

Key words
- Antiinflammatory
- Antioxidant
- Cinnamaldehyde
- Neuroprotection
- Rat
- Traumatic brain injury

Abbreviations and Acronyms
CA: Cinnamaldehyde
CA3: Cornu ammonis 3
CL: Chemiluminescence
DG: Dentate gyrus
DI: Discrimination index
IL-1β: Interleukin 1β
MPO: Myeloperoxidase
mRNA: Messenger RNA
RI: Recognition index
ROM: Reactive oxygen metabolites
TBI: Traumatic brain injury
TGF-β: Tumor growth factor β
TNF-α: Tumor necrosis factor α

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g., physical and/or sensory), cognitive, and affective (e.g., emotional) problems.[4] Neuropsychological evaluation after mild TBI is essential to follow up the recovery of these patients.[5]

Pathophysiologic mechanisms that initiate and progress TBI are divided into primary and secondary injury.[6] Primary injury is the mechanical damage that occurs directly in neurovascular structures and glial cells during insult. Secondary injury begins minutes after the insult and is associated with pathophysiologic mechanisms such as excitotoxicity, ionic imbalance caused by energy deficiency, inflammation, oxidative stress, and apoptosis.[7,8] All available treatment alternatives are aimed to prevent secondary injury. Despite many preclinical and clinical studies conducted to prevent secondary injury in TBI for decades, there are no treatment alternatives with proven efficacy.[9-16]

Cinnamaldehyde (CA) is the active ingredient in cinnamon, a natural spice, and has an important place in traditional Chinese medicine.[17] It is preferred because of its antioxidant, antiinflammatory, antithrombotic, antidiabetic, and anticancer effects.[17,18] CA shows its antioxidant activity by decreasing reactive oxygen metabolites (ROM) production and inhibiting nitric oxide production.[18-20] There is also a study reporting that it prevents neuronal damage by suppressing nuclear factor κB activation through activated microglia.[21] The efficacy of CA in many neurodegenerative disease animal models was examined. It was shown to be neuroprotective with its effects in regulating neuroinflammation, suppressing oxidative stress, improving synaptic connections, suppressing apoptosis, and preventing autophagy.[22-26] In the cerebral ischemic stroke model, it was shown that CA administration before the procedure reduces the damage by suppressing the release of inflammatory molecules.[27] In another study,[28] CA treatment was reported to be neuroprotective by showing antiinflammatory properties in the permanent cerebral ischemia model. In our previous study, it was reported that in the rabbit subarachnoid hemorrhage model, CA treatment reduced the hippocampal damage score by preventing cerebral vasospasm and showed neuroprotective activity.[29]

Based on these studies, CA treatment was hypothesized to show neuroprotective effects on TBI by reducing inflammation, oxidative stress, and apoptosis. Thus, possible neuroprotective effects of CA against secondary brain injury seen after TBI were examined by biochemical and histologic parameters. In addition, the neuroprotective effects of CA were further evaluated by the use of cognitive tests.

METHODS

Experimental Groups

All experimental procedures used in this investigation were reviewed and approved by the Marmara University Animal Care and Use Committee (12.2019.mar). Animal care and all experiments were conducted in concordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) on the protection of animals for experimental use. Thirty-five adult male Wistar albino rats weighing 250–400 g were used. Animals were housed in an air-conditioned room with 12-hour light and dark cycles, maintained at constant temperature (22°C ± 2°C) and relative humidity (65%–70%). Rats were fed a standard laboratory chow and had free access to water.

The rats were randomly assigned to 4 groups as follows:

1) Control group ($n = 9$): rats underwent only a skin incision under anesthesia and received a single intraperitoneal injection of saline (0.9% NaCl, 0.1 mL/100 g) immediately after surgery.
2) TBI group ($n = 9$): rats underwent TBI as described later and received a single intraperitoneal dose of saline (0.9% NaCl, 0.1 mL/100 g) immediately after TBI.
3) Vehicle group ($n = 8$): rats underwent TBI as described later and received a single intraperitoneal dose of vehicle (0.1% Tween 80, 0.1 mL/100 g) immediately after TBI.
4) CA group ($n = 9$): rats received a single intraperitoneal dose of CA (100 mg/kg [Shandong Sigmachemical Co. Ltd., Qingdao, Shandong, China]) immediately after TBI. The rationale for the selected dose of CA was based on the previous studies.[30]

Anesthesia and Induction of TBI

The animals were anesthetized by an intraperitoneal injection of chlorpromazine (0.5 mg/kg Largactil [Eczacıbaşı, Istanbul, Turkey]) and ketamine (50 mg/kg Ketalar [Parke Davis, Istanbul, Turkey]) combination and were allowed to breathe spontaneously. A mild TBI model, described by Marmarou et al.[31] and modified by Ucar et al.,[32] was applied for head trauma. Rats were placed in a prone position on the table and supported on a 10-cm foam bed, which provided a deceleration of the impact. A midline incision was made on the head, and the coronal and lambdoid sutures were identified. A metallic disc with a 10-mm diameter and 3-mm thickness was fixed in the midline to the cranium using bone wax between the 2 cranial sutures. A lead object weighing 300 g was allowed to fall freely from a height of 1 meter through a copper tube on to the metal disc over the skull of the rat at the midline point where the disc was placed. After the induction of injury, the metallic disc was removed, the surgical area was cleaned, and the skin was sutured.

Collection and Storage of Brain Tissue Samples

All the animals were decapitated at the 24th hour after trauma, and the brains were carefully removed. Identical anatomic regions of the brain were obtained from each animal for the analysis of each of the parameters. Brain sections that were used for biochemical analysis were stored at −80°C, and those used for histologic investigations were stored in a paraformaldehyde solution.

Biochemical Analyses

Measurement of Myeloperoxidase Activity in Brain Tissue. Myeloperoxidase (MPO), an enzyme that is mainly located in the azurophilic granules of polymorphonuclear leukocytes, is commonly used to show the accumulation of neutrophils in tissues.[33] Tissue MPO activity was evaluated by measuring the hydrogen peroxide–dependent oxidation of *O*-dianizidinedihydrochloride. Tissues (0.2–0.4 g) were homogenized in potassium phosphate buffer containing hexadecyl-trimethylammonium bromide and centrifuged at 12,000 rpm for 10 minutes at 4°C. The

supernatant was discarded, the pellet was rehomogenized with 50 mmol/L potassium phosphate containing 0.5% (w/v) hexadecyltrimethyl ammonium bromide and 10-mmol/l ethylenediaminetetraacetic acid (Sigma Aldrich, St. Louis, Missouri, USA). One unit alteration of enzyme activity measured at 460 nm and 37°C was determined to be the amount of the MPO present per gram of tissue.[34]

Chemiluminescence Measurements in Brain Tissue. When added to in vitro biological systems, the enhancer probes luminol and lucigenin produce high levels of excited products, and thereby chemiluminescence (CL) measurement shows the generation of ROM by a direct and noninvasive method. 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.[35] ROM were numerically measured after the addition of 0.2 mM of enhancers (Sigma Aldrich). The final concentrations of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (bis-N-methylacridinium nitrate) probes (Sigma Aldrich) were measured at room temperature using a luminometer (Junior LB 9509 luminometer [EG&G Berthold, Bad Wildbad, Baden Württemberg, Germany]).[36] Counts were obtained at 1-minute intervals for a 5-minute period, 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 interleukin 1β (IL-1β), IL-6, tumor necrosis factor α (TNF-α), tumor growth factor β (TGF-β), caspase-3, and cleaved caspase-3 in the brain tissue, commercial kits (Sunlong Biotech Co. Ltd., Yuhang District, Hangzhou, Zhejiang, China) were used according to the manufacturer's instructions. Supernatants of tissue homogenates were used for measuring the levels of cytokines.

Behavioral Tests

The behavioral tests were performed 2 times for each rat: one before TBI and the second 24 hours after TBI. The rats were tested as the sequence the TBI was performed so the 24-hour window was the same for each rat. The scorings were performed by investigators blinded to treatment groups.

Modified Bederson Neurologic Examination Score. A 20-point neurologic scoring scale was used to assess motor and behavioral deficits,[37,38] in which consciousness, climbing performance on a smooth platform, limb tone, walking and posture reflexes, circling, and response to nociceptive stimuli were evaluated. A low score (minimum 0) indicates that the animal is awake and active and has normal reflexes, whereas increased scores (maximum 20) suggest the presence of a neurologic disorder.

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.[39]

During the habituation phase, the rats were initially placed in a box (65 × 45 × 65 cm) for 10 minutes. The next day, animals were put in the same box, but this time containing 2 identical objects (F + F), and the animals' behavior was observed and recorded with a video camera for 10 minutes. After this familiarization phase, rats were allowed to stay in their housing cages for 1 hour. 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 minutes. During all experiments, boxes and objects were cleaned with 70% alcohol solution before placing the next animal.[40]

Difference score (in centiseconds) in was the difference in exploration time for the familiar object (TF) and the novel object (TN): difference score = TN−TF. Discrimination index (DI; in seconds) was calculated by dividing the difference in exploration TF and TN with 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.[40] Recognition index (RI; in seconds) was calculated by the formula: DI = TN/(TN + TF) and an increased RI indicates amelioration of cognitive functions.

Y-Maze Test. The Y maze is separated by three 120° branches and is similar to the T maze. The rat starts at the end of 1 arm, then chooses between the other two. In this setup, the number of entries into the arms and spontaneous alternation are measured. Spontaneous alternation is measured to assess spatial working memory.[41] The Y maze is generally preferred to the T maze, because gradual turns in the Y maze shorten the learning time compared with the sharp turns of the T maze. It is also a smaller labyrinth, which allows less free movement and enables clear focusing of the animal on the task.

The branches of the Y maze are named A, B, and C. Before each rat is evaluated, the Y maze is cleaned with 70% alcohol. The rat is left with 1 arm facing the center and making sure that it does not see the observer. Video is recorded for 10 minutes. When all 4 paws of the rat cross the threshold of the central zone and the face of the rat is oriented toward the end of the arm, it is considered as an arm entry. A spontaneous alternation occurs when a rat enters a different arm of the maze in each of 3 consecutive arm entries. Spontaneous alternation (%) is then calculated by dividing the number of spontaneous alterations with the total number of entries minus 2, and multiplying that with 100.[41]

Histopathologic Examinations

The brain samples were fixed in the 4% paraformaldehyde (in phosphate buffer saline, pH 7.4) for 24 hours at 4°C. After processing for light microscopic investigations, tissues were embedded in paraffin and 5-μm-thick coronal sections were created using a rotary microtome. The sections were stained with hematoxylin-eosin stain. Sections were examined under a photomicroscope (Olympus BX51, Tokyo, Japan). The severity of neuronal damage in the cortex and hippocampal dentate gyrus (DG), and cornu ammonis 3 (CA3) regions were scored semiquantitatively as follows: 0, no damage; 1, mild damage; 2,

moderate damage; and 3, severe damage. Pyknotic nuclei and intense staining of the shrunken neuronal perikarya were considered in scoring the degree of neuronal degeneration.

Statistical Analysis

Data were analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, California, USA) and expressed as means ± standard error of the mean. Biochemical data were analyzed using 1-way analysis of variance and a Tukey post hoc test or Kruskal-Wallis test for group comparisons and a Student t test was applied for control and TBI group comparisons. In behavior testing, a Sidak multiple comparison test was applied. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Effects of TBI Induction and CA Treatment on IL-1β, IL-6, TNF-α, TGF-β, Caspase-3, and Cleaved Caspase-3 Levels

When measured at the 24th hour of TBI, the brain levels of the IL-1β, IL-6, TNF-α, and TGF-β were not significantly altered among groups (Table 1). Similarly, no statistically significant difference was found between the brain caspase-3 and cleaved caspase-3 activities of the TBI and vehicle groups with respect to the control group (Table 1).

MPO Activity, Luminol-Enhanced, and Lucigenin-Enhanced CL Levels in Brain Tissues

In both the TBI and vehicle groups, a statistically significant increase was observed in MPO values compared with the control group ($P < 0.001$ for both comparisons). On the other hand, MPO level in the CA group was significantly reduced with respect to both TBI and vehicle groups ($P < 0.05$ and $P < 0.01$, respectively) (Figure 1A), showing that increased neutrophil infiltration in the damaged brain as a result of TBI is abolished by CA treatment.

A statistically significant increase in luminal-enhanced CL values was observed in the TBI and vehicle groups compared with the control group ($P < 0.001$ for both comparisons). A statistically significant decrease in luminal-enhanced CL values was observed with CA treatment compared with the TBI and vehicle groups ($P < 0.001$ for both comparison; Figure 1B).

There was a statistically significant increase in lucigenin-enhanced CL values in the TBI and vehicle groups compared with the control group ($P < 0.001$ for both comparisons). A statistically significant decrease in lucigenin-enhanced CL values was observed in the TBI and vehicle groups compared with CA group ($P < 0.001$ for both comparisons) (Figure 1C).

Increasing luminol-enhanced and lucigenin-enhanced CL levels after TBI are an indicator of increased oxidative stress and inflammation. The decrease in these values with CA treatment indicates that CA has antioxidant and antiinflammatory activity in TBI.

Modified Bederson Neurologic Examination Score

A subject's score in the TBI group was scored as 1, a subject in the vehicle group was scored as 3, and a subject in the CA group was scored as 1. There was no statistically significant difference among the experimental groups.

Novel Object Recognition Test

In the novel object recognition test, although the scores of the TBI and vehicle groups were in negative values in the difference score, there was no statistically significant difference compared with the control group. On the other hand, there was a statistically significant increase in the CA group compared with the TBI group ($P < 0.05$) (Figure 2A). When the vehicle and CA groups were compared, there was no statistical significance.

In the novel object recognition test, although the values of the TBI and the vehicle groups were in negative values for DI, there was no statistically significant difference compared with the control group. There was a statistically significant increase in the CA group compared with the TBI group ($P < 0.01$) (Figure 2B). When the vehicle and the CA groups were compared, no statistical significance was observed among groups.

In the novel object recognition test, there was a statistically significant increase in the CA group compared with the TBI group ($P < 0.01$) (Figure 2C). Although an increase was observed in the CA group compared with the vehicle group, a statistically significant difference was not found.

Table 1. Interleukin 1β (IL-1β), IL-6, IL-10, Tumor Growth Factor β, Tumor Necrosis Factor, Caspase-3, and Cleaved Caspase-3 Levels of Experimental Groups

Variable	Control	Traumatic Brain Injury	Vehicle	Cinnamaldehyde
IL-1β (ng/L)	188.87 ± 14.42	277.69 ± 78.87	163.53 ± 9.97	250.49 ± 23.64
IL-6 (ng/L)	0.63 ± 0.04	0.85 ± 0.11	0.68 ± 0.09	0.69 ± 0.05
IL-10 (ng/L)	8.73 ± 1.22	7.74 ± 1.25	3.64 ± 1.39	10.26 ± 1.41
Tumor growth factor β (ng/L)	32.61 ± 1.89	50.77 ± 12.91	28.16 ± 2.35	39.12 ± 2.96
Tumor necrosis factor (ng/L)	10.06 ± 0.68	17.63 ± 4.12	11.74 ± 0.78	11.78 ± 1.10
Caspase 3 (ng/mL)	0.17 ± 0.01	0.23 ± 0.04	0.15 ± 0.01	0.21 ± 0.01
Cleaved caspase 3 (ng/L)	77.89 ± 4.48	110.20 ± 24.39	74.89 ± 6.11	103.25 ± 3.45

IL, interleukin.

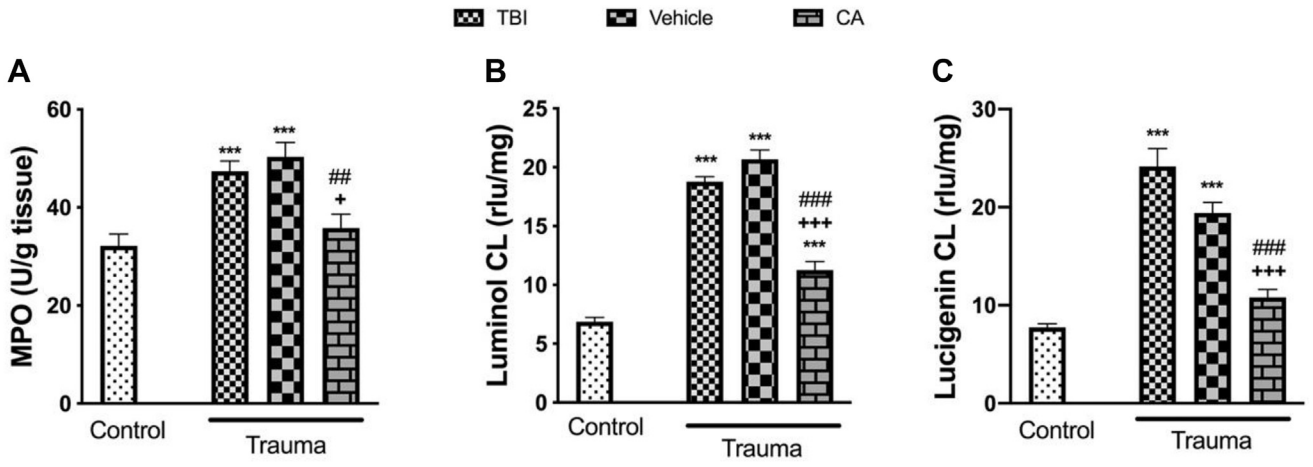

Figure 1. (**A**) Myeloperoxidase (MPO), (**B**) luminol-enhanced, and (**C**) lucigenin-enhanced chemiluminescence (CL) values of experimental groups (according to the control group, $***P < 0.001$; according to the traumatic brain injury [TBI] group, $+P < 0.05$, $+++P < 0.001$; according to the vehicle group, $\#\,\#P < 0.01$, $\#\,\#\,\#P < 0.001$). CA, cinnamaldehyde.

Y Maze Test

In the Y maze test, a statistically significant decrease was observed in the number of entries to the arms in the TBI group compared with the control group values before the procedure ($P < 0.05$; Figure 3A). Although a decrease was observed in the vehicle group compared with the control group values before the procedure, a statistically significant difference was not found. There was no statistically significant difference among other groups.

Although there was a decrease in the percentage of spontaneous alternation in the TBI group compared with the control group, no statistically significant difference was observed. A statistically significant decrease was observed in the vehicle group compared with pretreatment total values, pretreatment control, pretreatment TBI, and pretreatment vehicle groups ($P < 0.05$–0.01) (Figure 3B). Although there was an increase in the percentage of spontaneous alternation in the CA group, there was no statistical significance compared with the TBI and vehicle groups.

Light Microscopic Investigation

Cortex, DG, and CA3 regions in the hippocampus were evaluated with a semiquantitative method in terms of neuronal cell damage with hemotoxylin-eosin staining. In the cortex, DG, and CA3

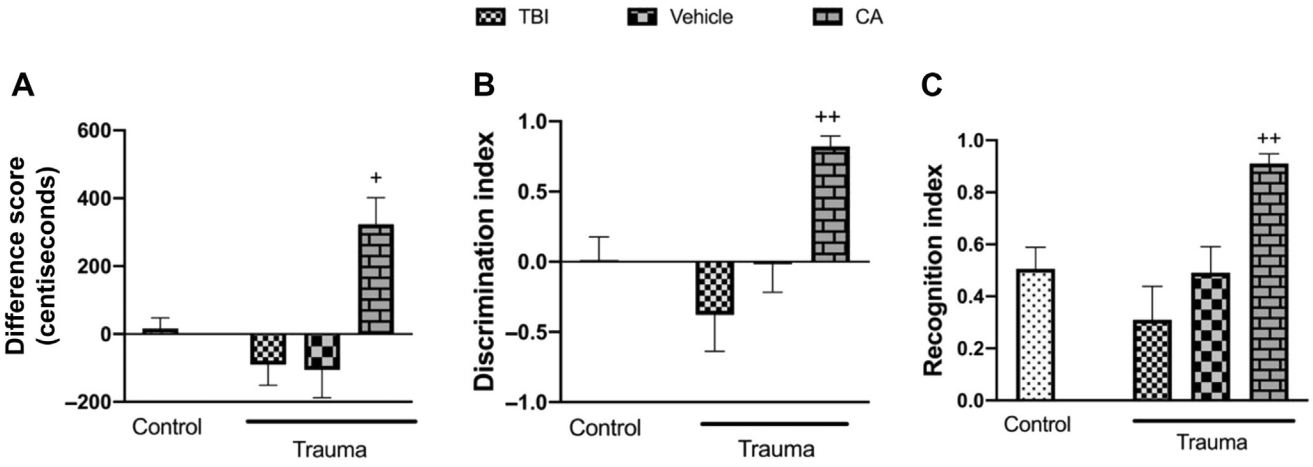

Figure 2. (**A**) Difference score, (**B**) discrimination index, and (**C**) recognition index among experimental groups (according to the traumatic brain injury [TBI] group, $+P < 0.05$, $++P < 0.01$). CA, cinnamaldehyde.

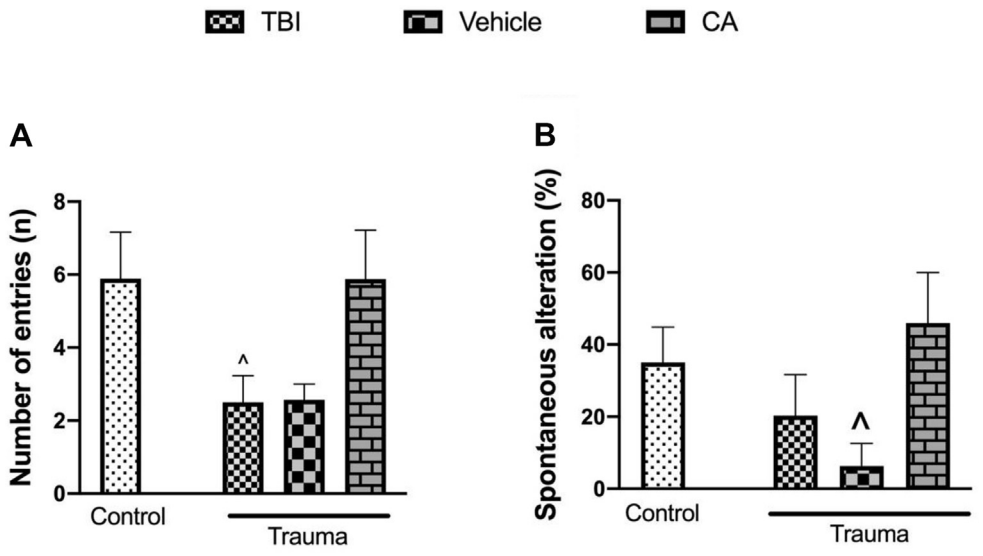

Figure 3. (**A**) The change in the number of entries into the arms. (**B**) The percentage of spontaneous alternation among the experimental groups (^$P < 0.05$ in the number of entries compared with the control group; in the spontaneous alternation test, (^) in the vehicle group there was a significant decrease compared with the total values before the procedure, pretreatment of control, pretreatment of vehicle, and pretreatment of the traumatic brain injury (TBI) groups ($P < 0.05$–0.01). CA, cinnamaldehyde.

regions of the rats in the control group, there were regular neuropil structure, neurons with uniform morphology and large nuclei, and prominent nucleolus structures were observed (Figure 4A–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 TBI and vehicle groups compared with the control group (Figure 4D–I). The damage to the cellular structure caused by TBI was less in the CA group (Figure 4J–L).

In the cortex, it was found that the histologic damage score increased significantly in the TBI and vehicle groups compared with the control group ($P < 0.001$ for both comparisons). A statistically significant difference for the histologic damage score was found between the TBI and vehicle groups ($P < 0.001$). In the CA group, the histologic damage score showed a statistically significant decrease compared with the TBI and vehicle groups ($P < 0.001$ for both comparisons) (Figure 5A).

In the hippocampal DG, it was observed that the histologic damage score increased in the TBI and vehicle groups compared with the control group ($P < 0.001$, for both comparisons). A significant difference was found between the TBI and vehicle groups when the histologic damage score in the DG was compared ($P < 0.05$). The histologic damage score was observed to be statistically decreased in the CA group compared with the TBI and vehicle groups ($P < 0.05$ and 0.001, respectively) (Figure 5B).

In the hippocampal CA3 region, it was found that the histologic damage score in the TBI group increased significantly compared with the control group ($P < 0.001$). In the CA group, a statistically significant decrease was observed in the histologic damage score compared with the TBI group ($P < 0.05$). When the CA and vehicle groups were compared, no statistically significant difference was found (Figure 5C).

DISCUSSION

Neuroinflammation is at a critical point after TBI.[21] It begins as a defense mechanism against neuronal damage, but the ongoing neuroinflammatory process itself causes neuronal damage.[21] Antioxidant, antiinflammatory, and neuroprotective efficacy of CA has been reported in several neuroinflammatory animal models.[21,24,29,42-44] MPO is an indicator of neutrophil migration to injured tissue and it was shown that MPO activity was increased after mild TBI.[10] On the other hand, it was reported that increased MPO activity as a result of neuronal injury induced by other experimental models was decreased with CA treatment.[28,45] In a model of persistent cerebral ischemia, it has been reported that CA treatment reduced MPO activity, showing that leukocyte infiltration into the ischemic area was reduced by CA.[28] Similarly, in the current study, we also showed that CA treatment applied immediately after TBI induction suppressed MPO activity, verifying the inhibitory effect of CA on the infiltration of the neutrophils to the injured brain tissue.

Previous studies have shown that the levels of luminol-enhanced and lucigenin-enhanced CL, which are the markers of oxidative stress and inflammation, were increased after TBI,[10] whereas glutathione peroxidase and superoxide dismutase levels were decreased and malondialdehyde levels were increased at the 24th hour after TBI.[13,16] Thus, there is an increase in oxidative stress and antioxidant enzymes are

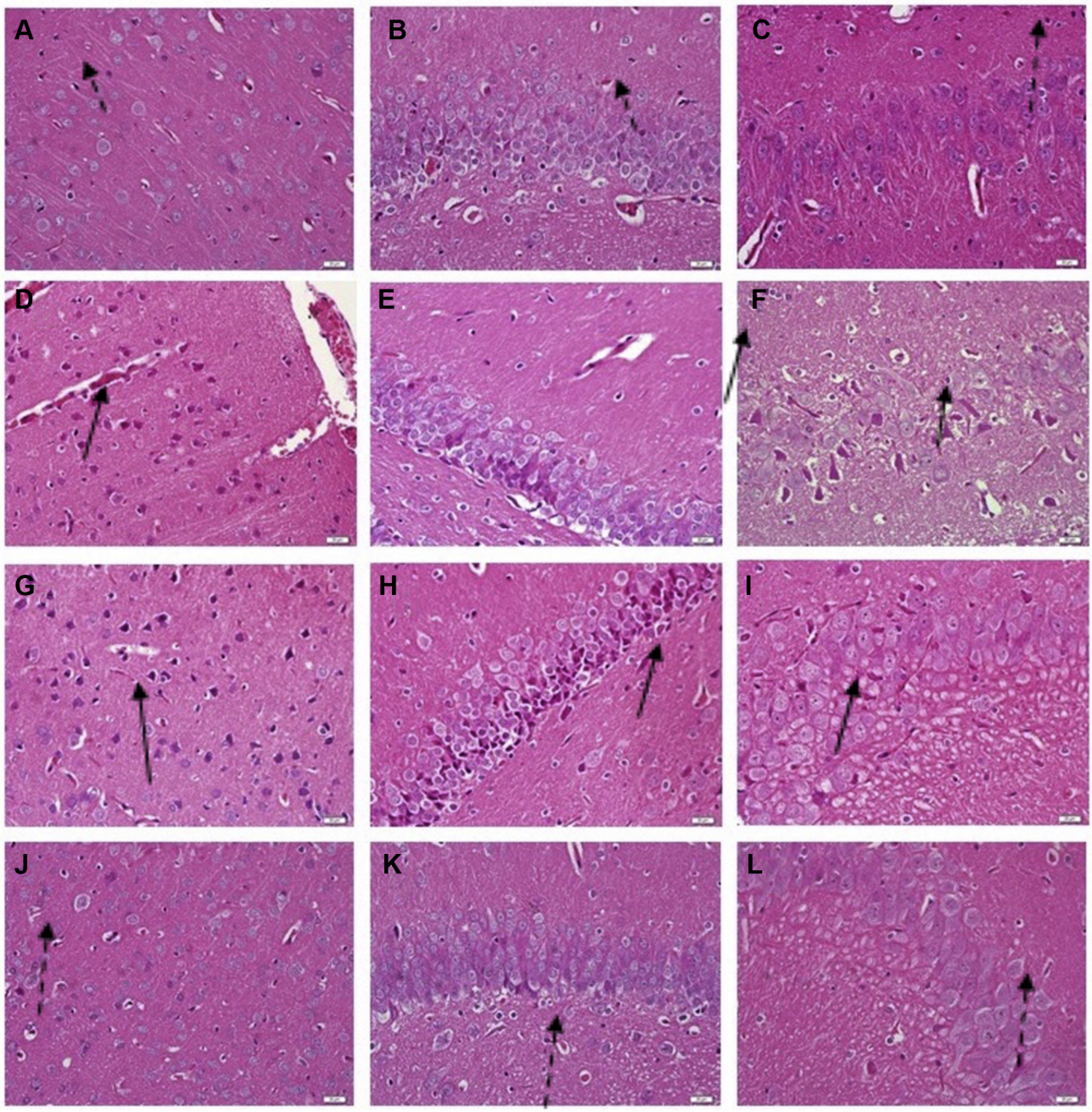

Figure 4. The histologic examinations performed with hematoxylin-eosin staining. (**A**–**C**) Representative sections of the cortex, hippocampal dentate gyrus (DG), and cornu ammonis 3 (CA3) regions, respectively, in the control group. (**D**–**F**) In the traumatic brain injury group, representative sections of the cortex, hippocampal DG, and CA3 regions are shown, respectively. (**G**–**I**) In the vehicle group, representative sections of the cortex, hippocampal DG, and CA3 regions are shown, respectively. (**J**–**L**) In the cinnamaldehyde group, representative sections of the cortex, hippocampal DG, and CA3 regions are shown, respectively. The *dashed black arrow* indicates normal neuron morphology, the *continuous black arrow* indicates the damaged neuron containing a pycnotic cell nucleus. Hematoxylin-eosin stain. Scale bar, 50 μm.

depleted in TBI.[10] In our study, we found that the luminol-enhanced and lucigenin-enhanced CL levels increased statistically significantly in TBI and vehicle groups. After CA treatment, a statistically significant decrease in the CA group was shown compared with the TBI and vehicle groups. Accordingly, it was concluded that CA treatment applied immediately after TBI suppressed oxidative stress and inflammation in the acute period.

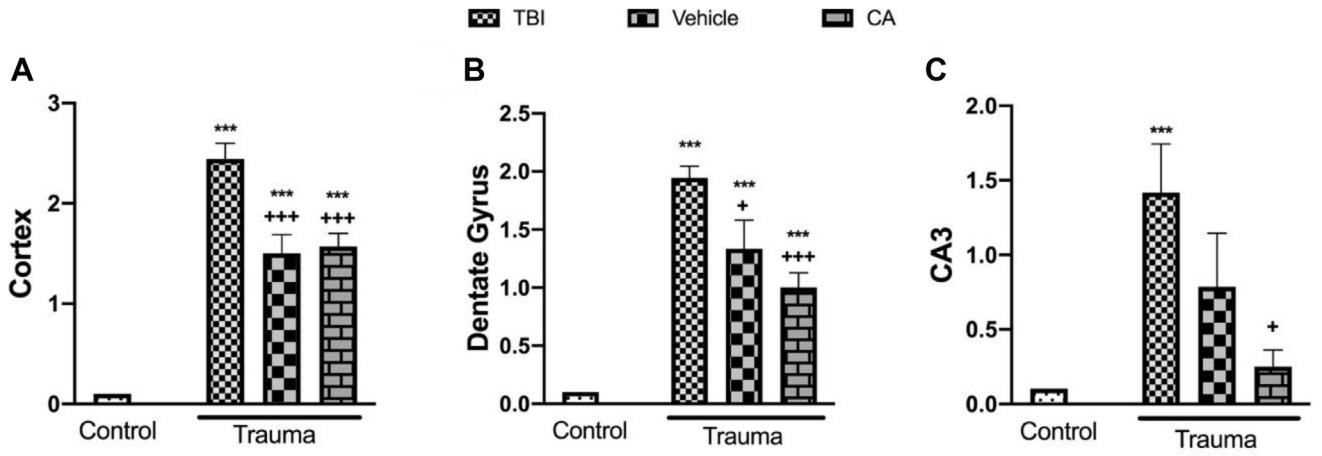

Figure 5. (**A**) Cortex, (**B**) hippocampal dentate gyrus, (**C**) hippocampal cornu ammonis 3 (CA3) region histologic damage scores among experimental groups (according to the control group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; according to the traumatic brain injury [TBI] group, + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$). CA, cinnamaldehyde.

Proinflammatory cytokines are released from damage-activated microglia.[21] After TBI, the amount of inflammatory cytokines increases in the acute period as a neuroinflammatory response.[46,47] It was suggested that increased levels of proinflammatory cytokines was associated with neuronal damage and death.[21] IL-1β messenger RNA (mRNA) levels, which is a proinflammatory cytokine, were increased at the first hour after TBI, whereas mRNA and protein expressions of IL-1β peak between 12 and 24 hours after TBI.[48-53] Moreover, the magnitude of its expression was positively correlated with the severity of the trauma.[50] Similarly, TNF-α level increased in the first hour after TBI and the expression of mRNA and protein reached a peak between 4 and 8 hours.[54-58] However, there is also a study showing that TNF-α levels increased after severe TBI, but did not increase after a mild TBI.[56] There are studies reporting that IL-6 expression rapidly increased and reached a peak between 2 and 8 hours after TBI.[57-63] TGF-β, an antiinflammatory cytokine that is activated by the effect of inflammatory cytokines after TBI,[64] was reported to show a peak at the 24th hour of TBI.[65,66] In a study investigating the effect of CA treatment on cerebral ischemia,[28] it has been reported that CA has suppressed inflammation, reduced brain edema, infarct volume, and neurologic deficit score, and decreases IL-1β and TNF mRNA levels, which were increased with ischemia. CA treatment has also been reported to suppress IL-1β-mediated cyclooxygenase-2 activity[67] and reduce IL-1β, IL-6, and TNF-α mRNA expression levels in other studies associated with inflammation.[68,69] TGF-β levels have also been reported to decrease with CA treatment.[69] In the current study, although a tendency to increase in proinflammatory cytokine levels (IL-1β, IL-6, and TNF-α) was observed at the 24th hour of the TBI, no statistically significant difference was found between the study groups. This finding may be because of the normalization of excessive inflammatory mediator release in the inflammatory cytokine levels at 24 hours in the mild TBI model. Another reason may be that the increased cytokine levels observed in severe TBI models could not be evident in mild TBI models.[56] Moreover, this lack of difference may be caused by the method of its measurement, because cytokine levels in our study were evaluated by the enzyme-linked immunosorbent assay method, but their mRNA levels were not determined.

Caspase-3 is an IL-converting enzyme and is a reliable marker of apoptotic activity.[70,71] Caspase-3 activity was increased at 24 hours after TBI.[10,13] It has been reported that CA prevents apoptosis by preventing mitochondrial membrane potential loss and reducing oxidative stress.[25] In our study, although there was an increasing trend in the levels of caspase-3 and cleaved caspase-3 in the TBI group, no statistically significant difference was found between the groups. It can be suggested that the caspase-related apoptotic processes or the caspase system is not yet sufficiently activated to make a measurable difference between the groups induced with a mild TBI.

A modified Bederson neurologic examination score was used in our study to evaluate motor deficits and no statistically significant differences were found between the experimental groups. Ucar et al..[32] have reported that the modified Bederson neurologic examination score of the rats subjected to mild TBI was lower compared with the animals in the groups with a more severe trauma. In the weight-drop model, it has been shown that the magnitude of the mechanical impact is directly correlated with the neurologic deficit and the survival of the animal.[31,32] Because recognition memory consists of familiarity and recall components,[72] the novel object recognition test evaluates short-term recognition memory, learning, distinguishing novel, or remembering.[73] In the current study, the novel object recognition test showed that the difference score, DI, and RI were decreased in the TBI group, but no statistical significance was found. Moreover, it was observed that the difference score, DI, and RI were significantly increased in the CA group, showing for the

first time that CA treatment improved the recognition memory. Spontaneous alternation in the Y maze is measured to assess spatial working memory.[41] In our study, the number of entries to the arms and the percentage of spontaneous alternation in the Y maze test were decreased compared with preprocedure values, showing the presence of cognitive dysfunction within the first 24 hours of TBI. Hoane et al.[74] have shown in the Morris water maze test that the time to find the platform in the TBI group was prolonged. Although the finding was not statistically significant, the percentage of spontaneous alternation was increased in the CA group, implicating a tendency of reduction in memory dysfunction.

In the diffuse TBI model with weight-drop method, the histologic changes seen in the brain are edema, presence of vascular congestion areas, hyperchromasia in the neuron nucleus, nuclear pycnosis, and cytoplasmic eosinophilic degeneration, which are signs of neuronal damage, focal neuronal loss, axonal edema, and formation of gliotic areas.[13] In this study, it was observed that the histologic damage score of cortex, DG, and CA3, which were increased with TBI, were decreased in the CA group. Damage findings in the TBI group are consistent with the histologic damage that was reported in our previous study.[10] The reduction of TBI-induced histologic damage by CA treatment strengthens the argument that this agent could have neuroprotective actions.

There are also limitations in this study as seen in any laboratory study. In the present study, the aim of applying CA treatment immediately after TBI was to reduce the effects of damage mechanisms by reaching an effective blood concentration immediately after the trauma. However, the effectiveness of the CA treatment needs to be further examined when applied after a certain period after TBI, which would more likely mimic the clinical scenario. The neuroprotective efficacy of CA treatment at different doses and different treatment durations can be studied more comprehensively with different TBI models. Especially in learning-memory tests, the number of subjects is insufficient to show possible differences among groups because the data do not show a normal distribution. The changes in behavioral test within different time points could also be evaluated.

CONCLUSIONS

Our findings show that CA treatment applied after TBI suppresses leukocyte infiltration and ROM generation, whereas neuronal damage in both the cortex and hippocampus were decreased with CA treatment, indicating that CA has a neuroprotective efficiency within the early hours of brain injury. Moreover, improvement in the short-term memory supports that CA treatment facilitates functional recovery along with the amelioration of the oxidative injury. Thus, our findings show for the first time that CA treatment through its antioxidant mechanisms exerts neuroprotective efficacy in the acute period of mild TBI. Further experimental and clinical studies are needed to clarify the neuroprotective efficacy of CA on the secondary brain injury seen after TBI.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Pınar Kuru Bektaşoğlu: Conceptualization, Methodology, Data curation, Writing — original draft, Writing — review & editing. **Türkan Koyuncuoğlu:** Data curation. **Dilan Demir:** Data curation. **Gizem Sucu:** Data curation. **Dilek Akakın:** Data curation, Writing — review & editing. **İrem Peker Eyüboğlu:** Data curation. **Meral Yüksel:** Data curation, Writing — review & editing. **Erhan Çelikoğlu:** Supervision. **Berrak Ç. Yeğen:** Visualization, Investigation, Supervision, Writing — review & editing. **Bora Gürer:** Visualization, Investigation, Supervision, Writing — review & editing.

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