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Therapeutic benefits of quercetin in traumatic brain injury model exposed to cigarette smoke

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ABSTRACT

Scientific evidences reported the deleterious effect of cigarette smoking or passive smoking on brain health particularly cognitive functions, blood–brain barrier (BBB) permeability, up-regulation of inflammatory cascades, and depletion of the antioxidant system. These combined effects become more progressive in the events of stroke, traumatic brain injury (TBI), and many other neurodegenerative diseases. In the current study, we investigated the long-term administered therapeutic potential of quercetin in ameliorating the deleterious neurobiological consequences of chronic tobacco smoke exposure in TBI mice. After exposure to 21 days of cigarette smoke and treatment with 50 mg/kg of quercetin, C57BL/6 mice were challenged for the induction of TBI by the weight drop method. Subsequently, a battery of behavioral tests and immunohistochemical analyses revealed the beneficial effect of quercetin on the locomotive and cognitive function of TBI + smoked group mice ($p < 0.05$ vs control sham). Immunohistochemistry analysis (Nrf2, HO-1, NFkB, caspase 3) demonstrated a marked protection after 21 days of quercetin treatment in the chronic tobacco smoking group possibly by up-regulation of antioxidant pathways, and decreased apoptosis. In conclusion, our findings support the therapeutic effectiveness of quercetin in partly protecting the central neurological functions that become aberrantly impaired in combined habitual cigarette-smoking individuals impacted with TBI.

1. Introduction

Traumatic brain injury (TBI) is a brain insult induced by a direct or indirect external mechanical force (Qin et al., 2021; Sivandzade et al., 2020a).TBI is one of the leading causes of death and disability in children and adolescents, and has received much attention in clinical practice (Du et al., 2016). It is estimated that 69 million individuals suffer a TBI each year (Dewan et al., 2018). Due to a lack of research on relationship between smoking and TBI, we aimed to study the effect of smoking on the injury induced by TBI and find a treatment that mitigates the TBI-related injury that is exacerbated by prior cigarette smoke exposure.

TBI pathophysiology can be classified into primary and secondary injury pathways. Primary injury is caused by physical harm and may result in cranial hemorrhage as a result of damage to blood vessels and the blood–brain barrier (BBB). Secondary injury occurs after the initial damage and is caused by oxidative stress, inflammation, calcium homeostasis imbalance, excitotoxicity, apoptosis, increased vascular permeability, and BBB disruption (Thal and Neuhaus, 2014). Post-traumatic BBB disruption is one of the major factors influencing the progression of injury and the timing and amount of neuronal healing (Alves, 2014). A growing body of evidence suggests that oxidative stress by reactive oxygen species (ROS) has a negative impact on BBB failure (Chrissobolis et al., 2011).

Tobacco smoke (TS) is a complex mixture of approximately 4,700 toxic compounds, including carcinogens, mutagens, free radicals, and (ROS) (Valavanidis et al., 2009). Each year, TS kills around 6 million people across the world. Chronic smoking is the habit of smoking

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tobacco products for an extended period, which can cause several diseases and shorten life expectancy. Smoking is one of the leading preventable causes of morbidity and mortality, affecting the function of almost every organ in the body (Jha, 2020). It has been linked to an increased risk of stroke and other neurological and cerebrovascular diseases such as Alzheimer's, multiple sclerosis, and vascular dementia via a variety of mechanisms (Kaisar et al., 2018).

TBI patients with premorbid TS exposure may have worse post-traumatic cerebrovascular inflammatory and neurovascular disorders than non-smokers (Durazzo et al., 2013). However, it is not surprising that continuous smoking is one of the most common pre-morbid factors likely to influence TBI severity and post-TBI recovery (Sivandzade et al., 2020b). Chronic smoking has been demonstrated to cause BBB dysfunction by triggering oxidative, inflammatory, and immunological responses, all of which contribute to the genesis and progression of cerebrovascular and neurodegenerative disorders, including TBI. Additionally, nicotine in cigarettes activates nicotine receptors, increasing BBB permeability and causing a loss of brain homeostasis (Mazzone et al., 2010).

Quercetin (3,5,7,3́,4́-pentahydroxyflavone) is a well-known natural flavonoid found in a variety of fruits and vegetables, including apples, berries, onions, and capers (Li et al., 2018). Quercetin has been described as having the highest flavanol content, and its dietary intake can account for 70 % of the total intake of flavanol (Xiao et al., 2018). Quercetin has anti-oxidative, anti-apoptotic, anti-inflammatory, anti-cancer, antithrombotic, anti-aggregatory, and vasodilating effects (Khan et al., 2018). The mechanisms underlying these effects are mostly unclear; however, they could be preceded by a variety of metabolic events. Quercetin has recently been shown to have neuroprotective properties in the treatment of neurological diseases, including brain injury (Yang et al., 2014), and, it can improve or protect the integrity of the BBB (Jin et al., 2019; Selvakumar et al., 2013).

In a TBI model, a study discovered that quercetin reduced oxidative injury to mitochondria by increasing the expression and activity of the antioxidant enzyme superoxide dismutase, and this effect could be linked to its ability to upregulate the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (Li et al., 2016b). Another study from the same group revealed that quercetin could potentially minimize brain injury in a TBI model by increasing mitochondrial biogenesis activities via the peroxisome proliferator-activated receptor γ coactivator-1(PGC-1a) pathway (Li et al., 2016a). Accordingly, quercetin was found to improve cognitive performance in TBI rats by inhibiting oxidative stress, resulting in a lower inflammatory response, and thereby lowering neuronal death. In mild TBI-induced mice, another study observed that quercetin administration reduced anxiety-like behaviors, dysregulated the hypothalamus–pituitary–adrenal axis, and lowered the levels of adrenocorticotropic hormone and corticosterone (Kosari-Nasab et al., 2019). Moreover, rats administered with quercetin showed reduced brain edema and enhanced motor function after a TBI. These effects might be mediated through the inhibition of phosphorylation of extracellular signal-regulated kinase (ERK1/2) and activation of Akt serine/threonine protein kinase, which could lead to a reduction in neuronal apoptosis (Du et al., 2018).

Although some studies have investigated the relationship between TBI and TS, the effect of antioxidants on reducing the exacerbation of TBI by TS has not been reported. Here, we examined the proposed neuroprotective effect of quercetin in mouse brains through behavioral assays and biochemical analyses. The importance of this research is that it is the first to assess the effect of an antioxidant compound in this model.

2. Materials and methods

2.1. Experimental model

The study was conducted in 64 healthy male C57BL/6J mice aged 6–10 weeks (weight range 16–26 g). Mice were obtained from the Animal Center, College of Pharmacy, King Saud University. The mice were kept in standard conditions; a 12-h L/D cycle, a 25 °C, and free access to water and standard food. All experiments were performed during the light cycle (9:00 a.m. to 5:00p.m.).

2.2. Materials

Quercetin was purchased from BOC Sciences (Shirley, NY, United States). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, United States). Primary antibodies (NF-B-p65, Nrf2, HO-1, cleaved caspase-3), secondary antibody biotinylated goat anti-mouse, and diaminobenzidine DAB were purchased from Abcam Co. (Waltham, MA, United States).

2.3. Experimental design

We designed the study in three stages: pre-TBI (3 weeks), TBI day, and behavioral assays (3 days). These three stages took around 1 month, followed by sacrificing mice and collecting blood and brains for the last stage, the biochemical assays (Fig. 1). Mice were divided into four groups of 16 mice each, as follows: control (non-smoke + vehicle + sham), TBI group (non-smoke + vehicle + TBI), TBI + smoke group (smoke + vehicle + TBI), and treated group (smoke + 50 mg/kg quercetin + TBI). We tested quercetin's solubility in water, saline, and DMSO, and we found that DMSO 10 % had the best solubility. Stock solution (12.5 mg/ml quercetin in DMSO10%) was prepared as needed. Each day, the required volume (100 μl/mouse/day) was injected into mice. All mice were injected once daily intraperitoneally with quercetin for the treated group, or isometric vehicle DMSO 10 % for the other groups as described previously [15]. During the same period, TBI + smoke and treated groups were chronically exposed to TS (through direct inhalation of 2 cigarettes/12 min (=1 cycle), 12 min /h, 6 times/day, 7 days/week, for 3 weeks) from 3R4F standardized research cigarettes (University of Kentucky., Lexington, KY, USA) as side stream smoke generated using a single cigarette smoking machine (CSM-STEP, CH Technologies Inc., Westwood, NJ, USA) as previously described (Prasad et al., 2017), following the International Organization for Standardization standard smoking protocol. Control and TBI groups were instead exposed to air. Throughout the study, the weights of the mice were measured every 5 days.

2.4. TBI induction

At 3 weeks from the start of the study, we performed TBI using the weight-drop method for the TBI, TBI + smoke, and treated groups as previously reported (Flierl et al., 2009). Briefly, mice were anesthetized for 1 min by inhaling isoflurane vapor before being placed on a spongy platform below the weight-drop apparatus. Then, a metal weight of 30 g was dropped from a height of 80 cm in free fall to induce brain trauma in the middle of the head between the ears. Then, we recorded the recovery time after induction. The control group was sham and followed the same steps in anesthesia but without hitting the brain.

2.5. Locomotion test

This test was carried out at 1 h and 24 h after TBI induction, exactly as instructed previously (Alqahtani et al., 2020). Using the locomotion apparatus (Muromachi Kikai Co, Tokyo, Japan) each mouse was individually housed in a 16″×16″ unobstructed glass cage with infrared sensors above the cage. Rodents have the curiosity to explore novel places; hence, this inbuilt characteristic is widely used by researchers to explore the impact of test treatments on an animal's locomotive function. In brief, the mice were initially acclimatized for 20 min to the arena of an open field with a subsequent testing phase of 10 min duration. The mice's behavior in the testing phase was recorded as their total distance

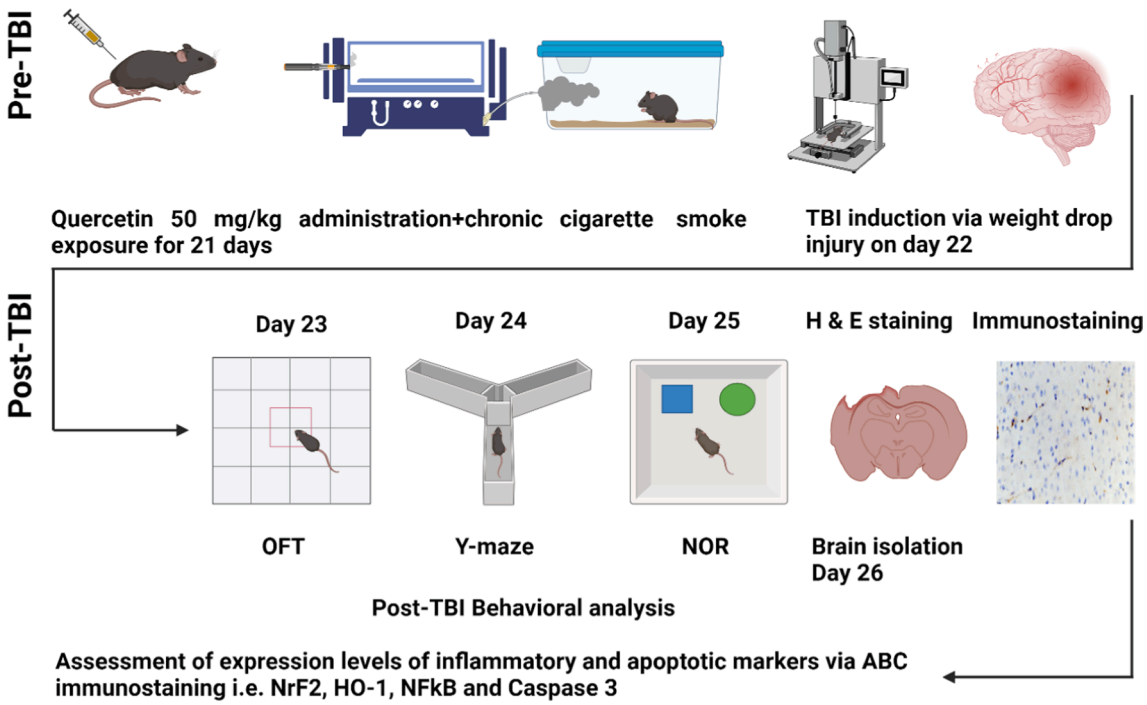

Fig. 1. Infographic abstract representing the experimental timeline.

traveled to evaluate their locomotive activity. A greater distance traveled was taken as a sign of increased locomotive activity in animals and vice versa.

2.6. Y-maze test

This test was performed to examine the impact of quercetin on the mice's short-term memory after TBI, precisely as described previously (Rehman et al., 2022). In summary, the test apparatus consisted of three closed arms (35 cm, 5 cm, and 10 cm) that were designed to be closely spaced at 120°. The mice were placed individually in the maze's center, facing one of the three arms (A, B, or C). Each individual mouse was permitted to explore the maze for 5 min, and the sequence of arm visits (ABC, BAC, CAB, etc.) with all four paws was recorded to calculate the number of alternations and number of entries. Rodents with appropriate cognition have the capacity to recall a previously visited arm and tend to explore the novel one; thus their alternation behavior will be increased, which was taken as an indication of improved cognition and memory.

2.7. Novel object recognition test

This test was carried out in two days as follows: On the first day, we placed the mice in an open field (a transparent acrylic glass box measuring 16″ by 16″) for 5 min to explore the area for acclimation. Following that, in the training session, two geometrically identical items were placed in the arena of the open field box, and each mouse was individually allowed to explore them for 10 min. On the second day, one of these two objects was replaced by a novel object of a different shape, and mice were allowed to explore it for 5 min (Javaid et al., 2023). The mice's time spent with older and novel objects was monitored to calculate the discrimination index, novelty preference, and absolute discrimination measure. Rodents with efficient memory can remember older objects and tend to show more inclination towards novel ones due to the innate curiosity seen in rodents. An increased discrimination index is a symbol of better cognition and memory in animals. The following formulas were used to calculate each parameter for each mouse: discrimination index = [(time spent with novel object - time spent with older object) (time spent with novel object + time spent with older object)], novelty preference = [(time spent with novel object - total exploring time) × 100], absolute discrimination measure = [time spent with novel object - time spent with older object].

2.8. Histopathological analysis

The cerebral cortex of mouse's brains was collected at 96 h after TBI and fixed in 10 % formalin, dehydrated, blocked in paraffin wax, and sectioned at 6 μm. Sections were stained with hematoxylin and eosin (H&E), stain and imaged using a light microscope (Nikon-Japan).

2.9. Immunohistochemical analysis (IHC)

Brain sections were deparaffinized, rehydrated and then retrieved with citrate buffer in a microwave for 5 min. Sections were blocked with hydrogen peroxide for 10 min and then incubated with primary antibodies (anti-caspase-3, anti-HO-1, anti-NF-kB, anti-Nrf2) in the dark for 1 h. Sections were incubated with the secondary biotinylated antibody for 30 min, then immersed in (DAB) for 10 min, followed by 3 min in hematoxylin and finally dehydrated in 95 % alcohol and xylene and then mounted with a mixture of distyrene, a plasticizer, and xylene. Sections were imaged and images were subjected to analysis using ImageJ and Fiji software. The optical density (OD) of images and percentage (%) of immune reaction incidence were measured. The score was calculated as (S = OD x %), and score grades were presented as (weak $\leq$ 3, 3 $<$ moderate $<$ 8, or intense $\geq$ 8).

2.10. Statistical analysis

All data were expressed as mean ± SEM and were analyzed by one-way ANOVA (except for body weight, which was analyzed by two-way ANOVA) and evaluated using GraphPad Prism 6 Software Inc. (La Jolla, CA, USA). Post-multiple comparison tests were performed with Tukey's test. P-values $<$ 0.05 were considered statistically significant.

3. Results

3.1. Recovery time after TBI induction

Recovery time after anesthesia induction with isoflurane was calculated to assess associations between the post-TBI outcomes (i.e.; functional impairment and therapy response at baseline) (Fig. 2). One-way ANOVA followed by Tukey's post-hoc test revealed marked inter-group differences [$F(3, 60) = 55.43$, $p < 0.0001$]. Experimental TBI induction in mice significantly impaired the motor recovery and alertness in both TBI and TBI + smoke mice compared to the control ($p < 0.0001$). Contralaterally chronic therapy with quercetin promoted robust post-TBI recuperation from the anesthetized state in comparison to TBI and TBI + smoke mice ($p = 0.0026$ and $p = 0.0008$, respectively). However, there was no significant difference in recovery time between TBI and TBI + smoke mice.

3.2. Quercetin administration reduced the negative influence of TS exposure and trauma on weight loss and related feeding intolerance

Weight loss following a concussion often results from altered neural signals crucial for maintaining feeding rhythmicity. Moreover, dysphagia and increased metabolism are other causes of significant weight loss after brain injury. Weight analysis was executed throughout the study at different time points (Day 0, Day 5, Day 10, Day 15, Day 21, Day 26, and Day 30) to determine whether smoking or TBI alone had any detrimental impact on body weight and feeding behavior in rodents.

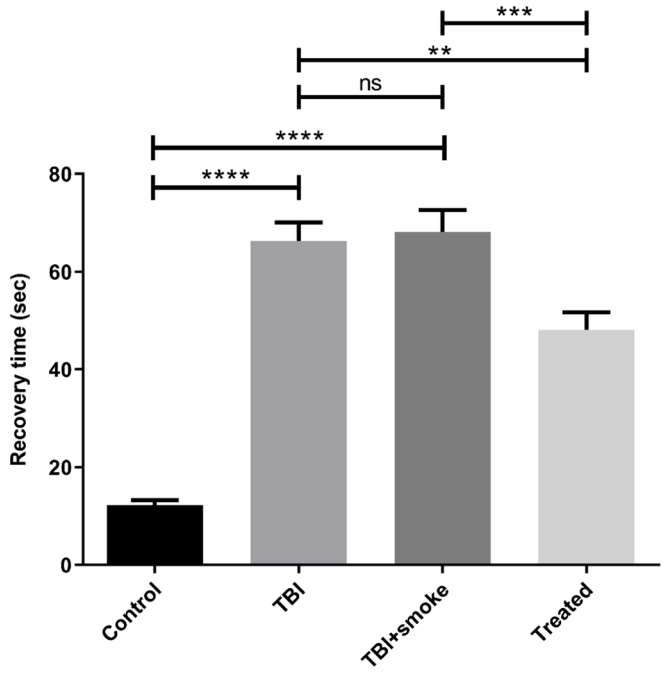

Fig. 2. Impact of quercetin treatment on post-TBI recovery time from the anesthetized state. The treated group was injected intraperitoneally with 50 mg/kg quercetin once daily for 21 days, and other groups were injected in the same manner with vehicle (DMSO 10 %). Smoking groups were exposed to TS (through direct inhalation of 2 cigarettes/12 min (cycle), 12 min/h, 6 times/day, 7 days/week, for 21 days) from 3R4F cigarettes. Control and TBI groups were instead exposed to air. TBI was performed on TBI, TBI + smoke, and treated groups. Sham was performed on the control only. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison tests: ****$p < 0.0001$ shows the comparison between control and TBI or TBI + smoke, **$p < 0.01$ the comparison between TBI and treated, ***$p < 0.001$ the comparison between TBI + smoke and treated, and ns the comparison between TBI and TBI + smoke mice. The results are expressed as mean ± SEM ($n = 16$ mice/group).

Mice were randomly divided into four test groups with $n = 16$ at Day 0. First, we noted and compared the baseline body weights in all groups at one day before starting the study (Day 0) and found that the mean animal weights in all groups fell within the same range (Fig. 3A). Second, we compared the animals' weights in all groups on TBI induction day (Day 21) (Fig. 3B). Last, we assessed for post-injury anorexia and the potential of long-term quercetin treatment to ameliorate TBI or TS-induced periodic loss of body weight in mice at Day 26. Two-way ANOVA demonstrated a statistically significant interaction between weight loss and chronic TS exposure over time [$F(3,344) = 5.596$, $p < 0.0001$)]. As shown in Fig. 3B on TBI induction day, the mean weight for control and TBI mice was significantly higher than that of TBI + smoke ($p < 0.0001$) and treated ($p < 0.0001$) mice, and there were no significant differences between TBI + smoke and treated mice or control and TBI mice. Next, we compared the body weights in all groups on sacrifice day (Day 26). We found that the mean weight of control mice was significantly higher TBI + smoke ($p < 0.001$) and treated ($p < 0.05$) mice, and the mean weight for TBI mice was significantly higher than TBI + smoke mice ($p < 0.05$). There were no significant differences between TBI + smoke and treated mice or control and TBI mice (Fig. 3C). Finally, we compared the body weights in all groups within 1 month of the experiment (Fig. 3D). We found that the mean weights for all groups were similar on Day 0. The mean weight for control and TBI mice increased over time, whereas that of TBI + smoke and treated mice decreased until Day 21 and then started to increase. On the last day, the mean weight for TBI + smoke and treated mice was lower than that for control and TBI mice. We also compared the mean weights beyond 30 days using two-way ANOVA and found a significant ($p < 0.005$) increase in weight in control and TBI mice compared to TBI + smoke and treated mice. This difference was related to the TS effect on the smoking group.

3.3. Neuroprotective role of quercetin administration on exploratory profiles and locomotive activity in TBI mice model with chronic TS exposure

The effects of quercetin on neurobehavioral performance and locomotive activity were assessed at 1 h and 24 h after TBI induction. There was a marked tweaking in the exploratory profiles of mice challenged with TBI. The total distance traveled by each group in the test apparatus was evaluated as a measure of activity (Fig. 4A). One-way ANOVA revealed a significant interaction [$F(3, 60) = 6.439$, $p = 0.0007$)] and [$F(3, 60) = 9.129$, $p < 0.0001$)] between all tested groups at 1 h and 24 h post-TBI, respectively. We found that the total distance traveled decreased significantly in smoking groups compared to the control, immediately after TBI induction ($p = 0.0014$). Reciprocally, quercetin treatment prevented this aberrant behavior after TBI ($p = 0.0217$). Furthermore, a sharp decline in locomotive activity was observed at 24 h post-TBI in TBI + smoke mice, because injured smoker mice traveled only 1520.81 ± 127.89 m in comparison to the control distance of 2254.87 ± 101.402 m ($p = 0.0002$). As shown in Fig. 4b, quercetin-treated mice showed improvement in spontaneous movements because all mice actively explored the test arena with a mean of 2134.625 ± 91.10 m compared to TBI + smoke mice ($p = 0.0358$).

3.4. Ameliorative effect of quercetin on cognitive dysfunction induced by TS exposure and TBI

The Y-maze test was used to evaluate the potential of quercetin to prevent the loss of short-term working memory in TBI mice with premorbid TS exposure. ANOVA showed a significant inter-group difference for the number of spontaneous alternations [$F(3, 44) = 12.94$, $p < 0.0001$)]. The mice chronically exposed to TS did not remember the previously visited arms of the Y-maze and showed a greater inclination to traverse the pre-visited arms intermittently, leading to reduced spontaneous alternation ($p < 0.0001$) compared to the control. In comparison to TBI + smoke mice, the mice administered with 50 mg/kg

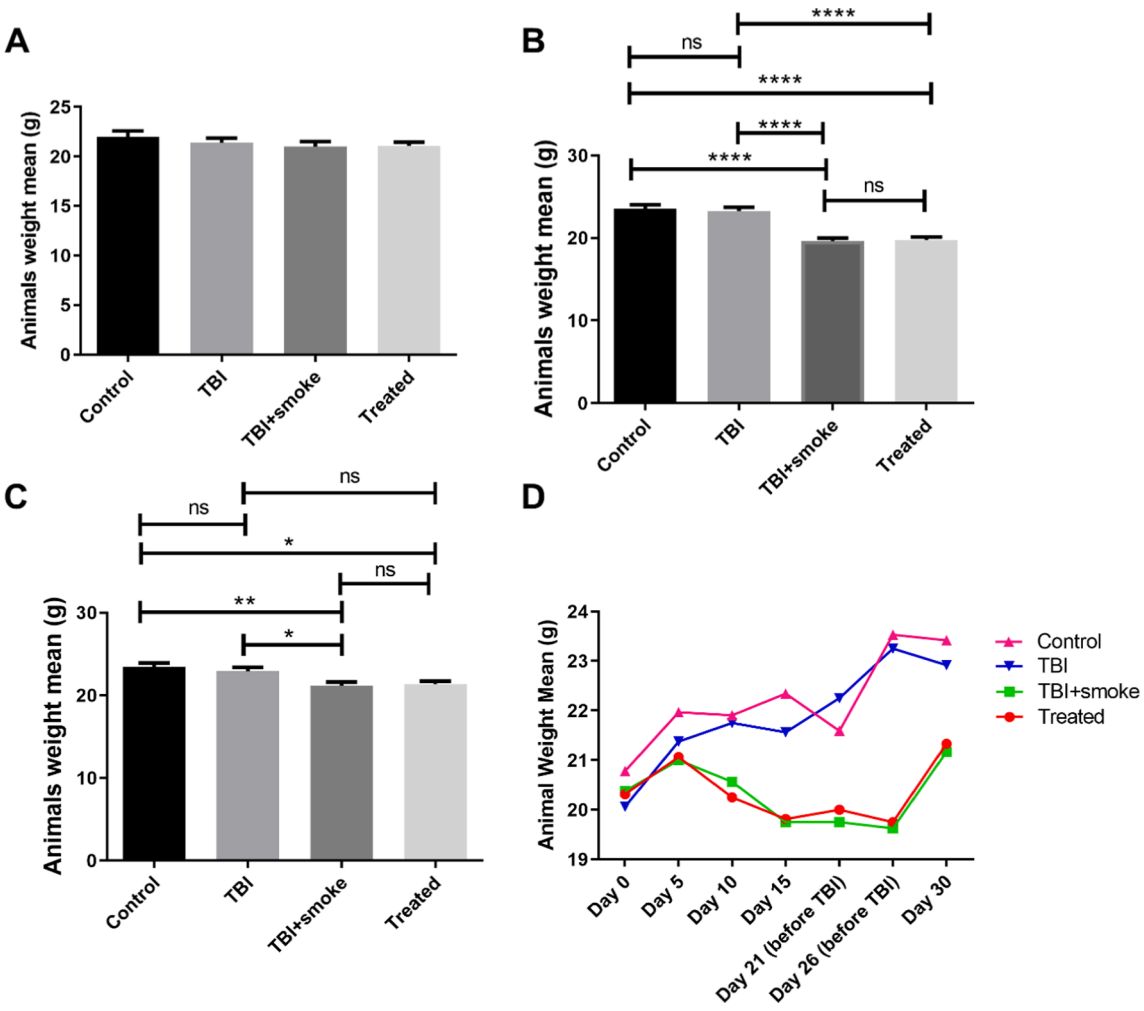

Fig. 3. Influence of premorbid TS exposure and TBI on the differential association between feeding behavior and mouse body growth rate over time. **(A)** Baseline body weights on Day 0 did not show any marked deviations between groups. **(B)** Chronic smoke inhalation led to a prominent reduction in body weight compared to the control on TBI induction day. **(C)** Assessment of body weight at 96 h post-TBI revealed trauma-induced aberrant feeding behavior, loss of appetite, and curtailing in body weight, whereas quercetin administration did not affect body weight alterations compared to the TBI + smoke group. **(D)** Mean animal weight over 4 weeks of study. Statistical analysis was carried out using two-way ANOVA followed by Tukey's multiple comparison tests: ****$p < 0.0001$ shows the comparison between TBI and TBI + smoke or control, *$p < 0.05$ the comparison between TBI and treated, and **$p < 0.01$ the comparison between TBI + smoke and treated mice. The results are expressed as mean ± SEM (n = 16 mice/group).

quercetin for 21 days had a notable increase in spontaneous alternation ($p = 0.0014$). Furthermore, TBI mice also had altered short-term recognition memory compared to control and treated mice ($p = 0.0004$ and $p = 0.0293$, respectively) (Fig. 5).

3.5. *Quercetin had on anti-amnesic activity and enhanced discrimination index in TBI mice with chronic smoke exposure*

The mice were further evaluated for their spatial memory by assessing their aptitude to discriminate a newly introduced unknown object from a previously acquainted one. ANOVA showed notable significant differences between all groups for discrimination index [$F(3, 44) = 12.04$, $p < 0.0001$)], novelty preference [$F(3, 44) = 11.85$, $p < 0.0001$)], and absolute discrimination index [$F(3, 44) = 6.624$, $p = 0.0009$)]. The mice treated with 50 mg/kg quercetin showed better remembrance of the known object, and they inspected the unknown/novel object more than TBI + smoke mice compared to the control ($p = 0.0010$). Moreover, in comparison to the injured mice with premorbid TS smoke exposure, treated mice had improved novelty preference ($p = 0.0120$), which is the time spent exploring novel objects throughout the duration of the test trial. However, TBI-impacted mice and those with prior chronic TS inhalation had frequent episodes of immobilization and did not show much interest in exploring newly introduced objects during the second phase of the trial ($p = 0.0001$ and $p < 0.0001$, respectively) compared to the control. Furthermore, the absolute discrimination index showed the difference in total time spent by mice in the exploration of novel objects versus the time spent in the exploration of old, familiar objects. As shown by Fig. 6C, TBI and TBI + smoke mice had a reduced absolute discrimination index in comparison with the control ($p = 0.0171$ and $p = 0.007$, respectively). However, treated mice had an enhanced absolute discrimination index compared to TBI + smoke mice ($p = 0.0139$).

3.6. *Histopathological analysis*

As shown in Fig. 7A, the cerebral cortex of control mice revealed the absence of characteristic neuropathological hallmarks. However, the cerebral cortex of TBI + smoke mice displayed marked changes in the cerebral vasculature, such as the dilatation of congested blood vessels, formation of cerebral infarction, and precipitation of hemosiderin granules (Fig. 7C). Furthermore, in comparison to TBI + smoke mice, the quercetin treatment prompted brain homeostasis and prevented long-

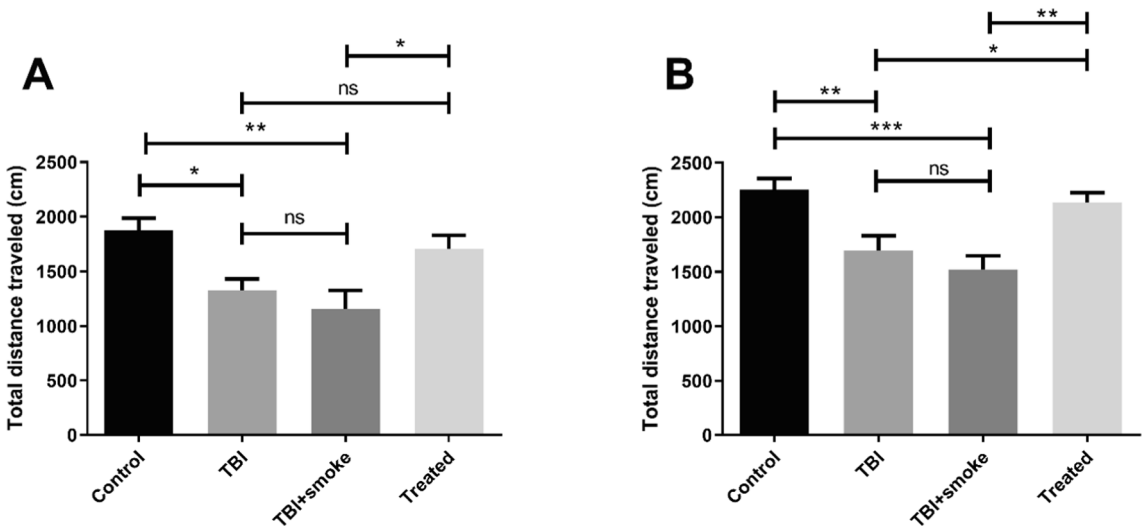

Fig. 4. Effect of quercetin administration on spontaneous locomotor activity. **(A)** Total distance traveled at 1 h post-TBI. **(B)** Total distance traveled at 24 h post-TBI in mice previously exposed to cigarette smoke. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison tests: *p < 0.05, **p < 0.01 show the comparison between control and TBI, **p < 0.01 and ***p < 0.001 the comparison between control and TBI + smoke, *p < 0.05 the comparison between TBI and treated, and *p < 0.05, **p < 0.01 the comparison between TBI + smoke and treated mice. The results are expressed as mean ± SEM (n = 16 mice).

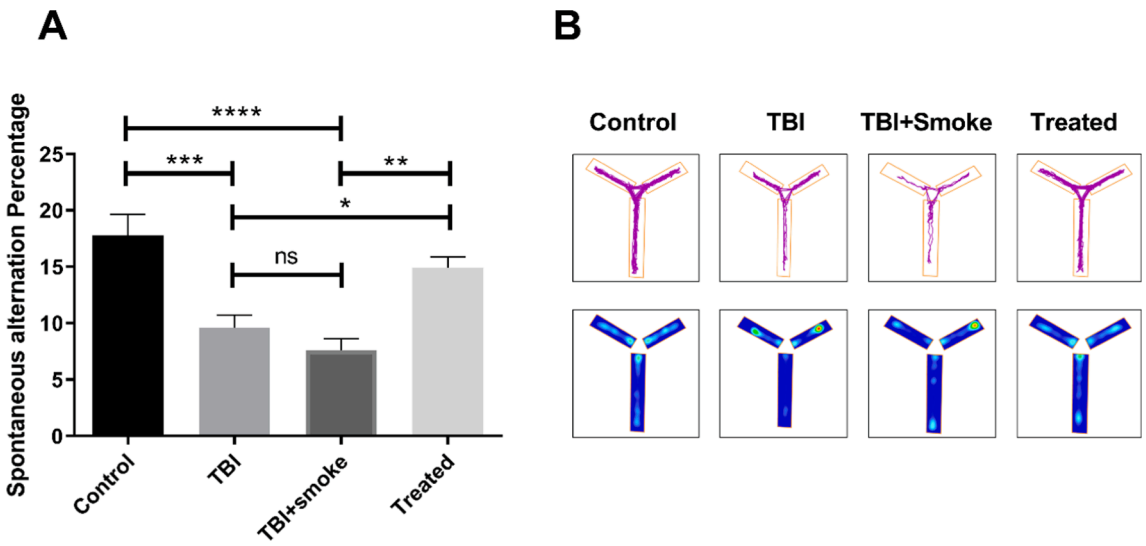

Fig. 5. Effects of quercetin on performance in the Y-maze test after induction of TBI in mice previously exposed to cigarette smoke. **(A)** Percentage spontaneous alternation. **(B)** Track plots with corresponding heat maps representing the mouse's pattern of exploration. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison tests: ***p < 0.001 shows the comparison between control and TBI, ****p < 0.0001 the comparison between control and TBI + smoke, *p < 0.05 the comparison between TBI and treated, and **p < 0.01 the comparison between TBI + smoke and treated mice. The results are expressed as mean ± SEM (n = 12).

term TS exposure-mediated modifications via regulation of inherent cerebral blood flow and, to some extent, attenuated vascular dysfunction. Moreover, the cerebral cortex of treated mice displayed marked neuroprotection and reduced damage to the neurovascular unit as evidenced by the minimal size of the cerebral infarction. Additionally, histopathological analysis hinted at the ability of quercetin to address the primary damage and related post-TBI secondary injury cascade in mice with chronic TS inhalation (Fig. 7D).

3.7. Quercetin therapy suppressed post-TBI inflammatory and apoptotic reactions in the cerebral cortex of mice exposed to chronic TS inhalation

To illustrate the therapeutic potential of quercetin on neuroinflammation in the injured brain, the expression level of inflammatory and apoptotic markers such as Nrf2, H0-1, NFkB, and caspase-3 were investigated through the avidin–biotin complex (ABC) method of IHC on Day 26 after TBI induction (Fig. 8). The cortex was stained immunohistochemically against anti-Nrf2 as an indicator of oxidative stress, which revealed a non-significant increase in immune staining optical density and a marked increase in Nrf2 positive cells in TBI + smoke mice compared to the control. However, in comparison to TBI + smoke, treated mice showed a notable immunoreactivity and a significant increase in the ratio of Nrf2 cell levels in the lesion area (Fig. 8A). One of the downstream detoxifying effector molecules of the Nrf2 signaling pathway is HO-1. In the IHC, TBI + smoke mice showed a significant increase in immune response and corresponding antibody concentration against anti-HO-1 compared to the control. However, treated mice demonstrated a moderate immune response and percentage compared to TBI + smoke mice. Furthermore, examination of the cerebral cortex stained immunohistochemically against anti-NF-kB, as

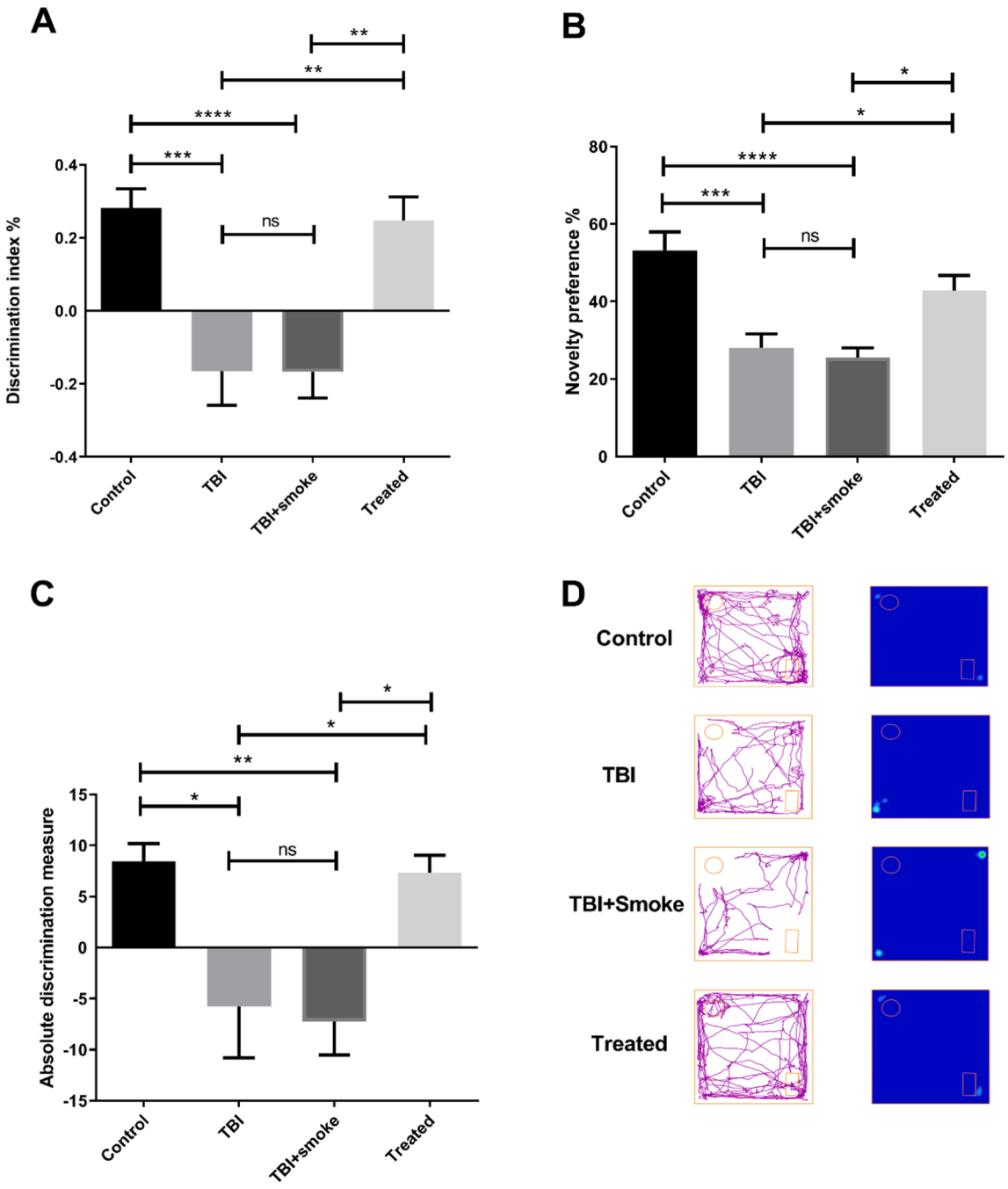

Fig. 6. Effect of quercetin on novel object recognition test after induction of TBI in mice previously exposed to cigarette smoke. **(A)** Discrimination index %. **(B)** Novelty preference. **(C)** Absolute discrimination measure. **(D)** Representative track plots and heat maps representing a mouse's exploratory profile in the NOR test. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison tests: ***$p < 0.001$, *$p < 0.05$ shows the comparison between control and TBI, ***$p < 0.001$, ****$p < 0.0001$, *$p < 0.05$ the comparison between control and TBI + smoke, **$p < 0.001$, *$p < 0.05$ the comparison between TBI and treated, and **$p < 0.001$, *$p < 0.05$ the comparison between TBI + smoke and treated mice. The results are expressed as mean ± SEM (n = 12).

an indicator for inflammation, revealed weak immune reactivity in control mice and moderate immune reactivity in TBI mice. Meanwhile, TBI + smoke mice showed a very intense immune response and a significantly high percentage of NF-kB-positive cells, which can be related to inflammation. Furthermore, the cortex of treated mice showed a moderate immune response and a notably lower percentage of NF-kB-positive cells compared to TBI + smoke mice. Additionally, the cerebral cortex tested immunohistochemically against anti-caspase-3 showed a weak immune response in control and TBI mice, with a low percentage of caspase-positive cells. However, TBI + smoke mice had an intense immune response with enhanced levels of caspase-3. Moreover, treated mice had a moderate immune response with a significantly lower percentage of caspase-3 ratio compared to TBI + smoke mice.

4. Discussion

TBI is a disorder that significantly contributes to global disability rates; it is estimated that 69 million individuals experience TBI annually (G/Michael et al., 2023). The likelihood of developing cerebrovascular and neurological problems is likely to be exacerbated by various comorbidities, such as smoking (Cho et al., 2021). The efficacy of antioxidants to reduce this multi-modal cascade of post-TBI secondary

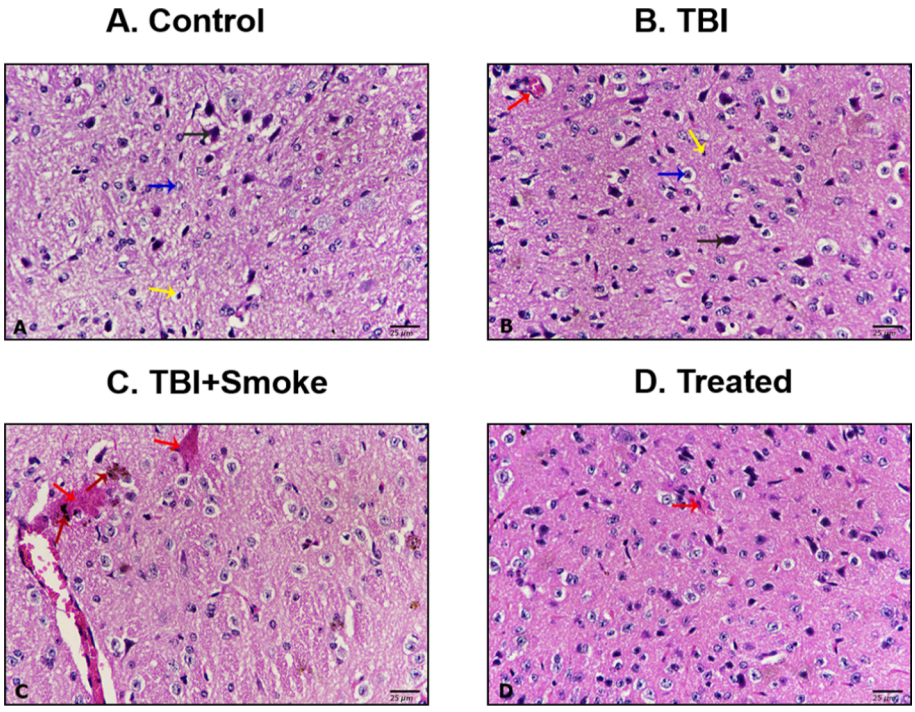

Fig. 7. Photomicrographs of cerebral cortex at 96 h post-TBI. **(A)** Control mice showed normal cerebral cortex pyramidal cells (black arrow), granular cells (blue arrow), and glial cells (yellow arrow). **(B)** TBI mice had a primary injury with local destruction of blood vessels in the area of concussion (red arrow). **(C)** TBI + smoke mice, injected with the vehicle for 21 days followed by head trauma, displayed infarction (red arrows) and hemosiderin granules (brown arrows). **(D)** Treated mice, exposed chronically to TS and injected with quercetin for 21 days and then followed by head trauma, demonstrated a smaller infarction (red arrows). n = 3. (H&E-400X).

injury has not been studied, although some studies have disentangled the relationship between TBI and TS. This study sought to show that quercetin can mitigate the negative detrimental effects of a TBI-like injury in mice that were chronically exposed to cigarette smoke. We have demonstrated that long-term quercetin administration attenuated multiple measures of TBI-induced cerebral damage in mice with pre-morbid TS exposure. Owing to its neuroprotective and antioxidant potential, our outcomes provide further evidence that this flavonoid might serve as a potential therapeutic regimen for patients with head trauma.

First, we assessed the body weight of mice every five days and found that the weight of mice in groups exposed to smoke was significantly decreased compared to control and TBI mice, which confirms that TS reduces weight over time. The consequences of chronic TS exposure are weight suppression, reduced appetite, and a sustained decrease in caloric intake (Perkins, 1992). This result is consistent with another study (Sivandzade et al., 2020a). Furthermore, they found that TBI alone caused a reduction in body weight, which is in line with our outcomes. On the other hand, quercetin ameliorated the weight-suppressive negative effect of long-term TS exposure and the subsequent impact of TBI induction on mouse body weight.

Second, we performed a set of behavioral tests to examine the general locomotive activity and memory in mice. The assessment of the mice's activity at 1 h and 24 h after TBI revealed a significant decrease in locomotion in TBI + smoke mice compared to control and TBI mice. Quercetin administration enhanced locomotive activity significantly compared to TBI + smoke mice, because treated mice actively wandered around the test arena. However, TBI + smoke mice showed a poor exploratory profile and locomotive activity.

Third, we performed the Y-maze test to assess spatial memory in the mice. The outcomes of TBI on memory consolidation have been reported in preclinical models (Xu et al., 2021). In line with the earlier published reports, we found that TBI impaired cognitive functions as evidenced by a reduced percentage of spontaneous alternations in the Y-maze test. Chronic TS inhalation caused a further decrease in the percentage. In comparison to TBI + smoke mice, the mice administered with 50 mg/kg quercetin for 21 days had a notable increase in spontaneous alternation ($p = 0.0014$), suggesting that long-term quercetin therapy results in improved cognition (Khan et al., 2018).

Fourth, we ran the novel object recognition test to assess the visual memory in mice. We found that TBI alone as well as TBI + smoke reduced the discrimination index, novelty preference, and absolute discrimination measure, which means the mice's memory was negatively affected by trauma to the brain. Quercetin restored recognition memory as indicated by increased exploration of novel articles compared to old, familiar ones. Another study also reported that quercetin enhances the discrimination index and ameliorates the cognitive impairments induced by doxorubicin treatment in rats (Ramalingayya et al., 2023). The loss of neurons following trauma often results in co-morbid neurocognitive alterations (Neuberger et al., 2017). Chiaretti *et al.* reported that quercetin upregulates doublecortin (DCX) expression, particularly in the frontal cortex and hippocampus (Balasubramanian et al., 2022; Chiaretti et al., 2008). Because DCX concentration predicts neurogenesis and related neuronal repair, we can presume that the enhanced memory and exploration in quercetin-treated mice may be linked to the ability of DCX to modulate neurogenesis markers. However, explicit neurogenesis markers were not quantified, which may be a limitation of the current study. Thus, these findings are indicative of the therapeutic capacity of quercetin in the amelioration of cognitive deficits.

To evaluate the histological changes further, we performed H&E staining. The cortex of TBI + smoke mice displayed marked changes, such as dilatation of congested blood vessels, formation of infarction, and precipitation of hemosiderin granules. We found that quercetin reduced the pathological changes induced by TBI + smoke. Quercetin also significantly improved the survival rate of cortical neurons in the mice model.

The transcription factor Nrf2 has become a research hot spot as a chief regulator of inflammation and oxidant resistance in a wide

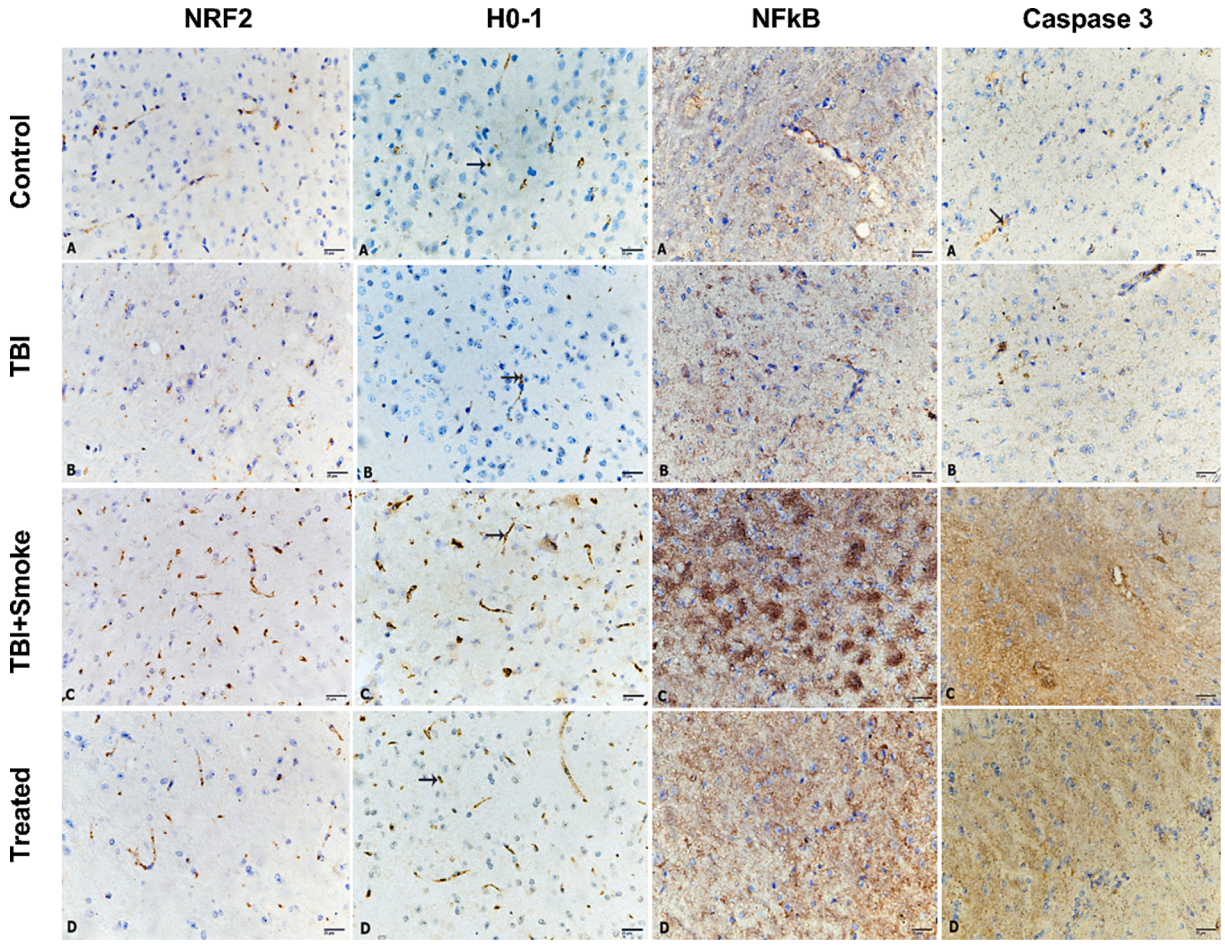

Fig. 8. Photomicrographs illustrating the avidin–biotin complex (ABC) immune staining of Nrf-2, HO-1, NF-kB, and caspase-3 in cortical brain tissue sections isolated from **(A)** control mice, **(B)** TBI mice, **(C)** TBI + smoke mice injected with the vehicle for 21 days followed by head trauma, and **(D)** treated mice exposed chronically to TS and injected with quercetin for 21 days followed by head trauma. n = 3. (ABC method-400X).

spectrum of chronic diseases associated with altered redox balance. As a result of Nrf2 translocation from the cytoplasm to the nucleus in response to oxidative or xenobiotic stress, cells exhibit a cytoprotective response that is characterized by the overexpression of a group of antioxidant enzymes and a lower sensitivity to oxidative damage. In this study, using IHC, we found that the level of Nrf2 in TBI + smoke mice increased from 24 h after TBI induction and remained elevated until 96 h post-TBI. However, this result is not consistent with other studies that found that the level of Nrf2 decreased in the TBI + smoke group in *in vivo* and *in vitro* models (Sivandzade et al., 2021). We suggest that this decreased level of Nrf2 could be due to the resolution of the acute phase of inflammation or the activation of other defense mechanisms in the injured brain. Further studies are needed to fully understand the effect of quercetin on the dynamics of Nrf2 expression in this model.

One of the Nrf2 detoxifying effector molecules is HO-1. A study found that HO-1 expression was not significantly altered after 2 weeks of TS exposure but was substantially upregulated at 4 weeks of TS exposure ($p < 0.001$) (Prasad et al., 2017). The IHC results showed there was a significant increase in immune response and percentage against anti-HO-1 at 96 h after TBI in TBI + smoke mice compared to the control, whereas quercetin treatment increased the levels of HO-1 positive cells in IHC. Thus, quercetin attenuated the TBI-mediated neuroinflammation and increased the oxidative stress in the cortex of mice pre-exposed to TS via activation of the Nrf2/HO-1 signaling pathway. Furthermore, activation of caspase-dependent pathways after TBI leads to an imbalance between proapoptotic Bax and Bcl-2 associated agonist of cell death (Bad) and between anti-apoptotic B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra-large (Bcl-xL) molecules, which increases cell death (Singh et al., 2019). We examined the cerebral cortex using IHC against anti-caspase-3 and found an intense immune response with a high percentage of caspase-3 in TBI + smoke mice. This result is not consistent with a study that found that the expression of cleaved caspase-3 was downregulated in TBI rats with TS exposure at 24 h after TBI (Lee et al., 2012). However, the percentage of caspase-3 incidence was significantly decreased by treatment with quercetin compared to TBI + smoke mice.

NF-kB is a protein complex that regulates the production of pro-inflammatory cytokines in most cell types, including neurons, astrocytes, microglia, oligodendrocytes, and endothelial cells of neurovascular and cerebrovascular units (Dresselhaus and Meffert, 2019). It is widely known that glial cells and neurons in regions of the brain that are atrophying after TBI activate NF-kB, which is linked to inflammatory processes (Sivandzade et al., 2019). Using IHC, the cerebral cortex was examined at 96 h after TBI and revealed very intense immunostaining against anti-NF-kB and a significantly high percentage of NF-kB incidence in TBI + smoke mice. These results are consistent with a study that showed using a western blot method that NF-kB was increased by prior smoke exposure in a TBI mouse model (Sivandzade et al., 2020b). Furthermore, in IHC, the cortex of treated mice showed moderate immunostaining and a significantly lower percentage compared to TBI + smoke mice.

Thus, quercetin, due to its free-radical scavenging properties, can effectively prevent behavioral and histological perturbations induced by elevated oxidative stress in TBI + smoke mice. Therefore, these results may provide a mechanistic understanding of the neuroprotective

potential of quercetin and emphasize the benefits of using this antioxidant as adjuvant therapy in habitual cigarette-smoking individuals impacted with TBI.

5. Conclusion

This study revealed the multifaceted therapeutic effects of quercetin on functional recovery in TBI + smoke mouse model-mediated neuroinflammation and the associated pathological, behavioral, and signaling alterations. Quercetin (50 mg/kg) exhibited antioxidant properties and downregulated the deleterious neurodegenerative and apoptotic events associated with TBI. These effects were confirmed by biochemical assays using ABC IHC. IHC data showed that quercetin mitigated the TS-induced neurovascular dysfunction, brain edema, and cortical cell death. Future studies are required to elucidate the neuroprotective mechanisms and precise ameliorative targets of quercetin after TBI + smoke-induced neural damage.

6. Institutional review board statement

The project was approved by the Local Research Committee at king Saud University (No: KSU-SE-21–70).

CRediT authorship contribution statement

Faleh Alqahtani: Conceptualization, Writing – original draft, Supervision. **Yousif S. Mohamed Ali: . Mohammed M. Almutairi: . Abdullah F. Alotaibi:** Writing – review & editing. **Imran Imran:** Conceptualization, Writing – original draft. **Musaad A Alshammari:** Writing – review & editing, Conceptualization. **Abdullah K. Alshememry:** Writing – review & editing. **Shakir D. AlSharari:** Conceptualization, Writing – review & editing. **Thamer H. Albekairi:** Conceptualization, Writing – original draft, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2023.101895.

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