Cerebrovascular and neurological impact of chronic smoking on post traumatic brain injury outcome and recovery: an in vivo study

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Research

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Abstract

Background: Traumatic Brain Injury (TBI) is among the most prevalent causes of cerebrovascular and neurological damage worldwide. To this end, tobacco smoking (TS) has been shown to promote vascular inflammation, neurovascular impairments and risk of cerebrovascular and neurological disorders through oxidative stress (OS) stimuli targeting the blood-brain barrier (BBB) endothelium among others. It has been recently suggested that premorbid conditions such as TS may exacerbate post-TBI brain damage and impact recovery. The present study aims to investigate and dissect out the pathophysiological mechanisms underlying the exacerbation of TBI in a weight-drop model following chronic TS exposure. Methods: C57BL/6J male mice, age range 6–8 weeks were chronically exposed to TS for three weeks. Test animals were then subjected to TBI by guided vertical head weight drop using a 30 g metal weight free felling from an 80 cm distance before reaching the target. Physical activity and body weight of the mice were analyzed before TBI and 1 h, 24 h and 3 days post-injury. Finally, mice were sacrificed to collect blood and brain samples for subsequent biochemical and molecular analysis. Western blotting was applied to assess the expression of Nrf2 (a key antioxidant transcription factor) as well as tight junction proteins associated with BBB integrity including, ZO-1, Occludin, Claudin-5 from brain tissues homogenates. Levels of NF-kB (a pro-inflammatory transcript factor which antagonizes Nrf2 activity) along with pro-inflammatory cytokines IL-6, IL-10 and TNF-α were measured by ELISA on blood samples. Results: TS promoted significantly increased inflammation and loss of BBB integrity in TBI when compared to TS-Free test mice. Additionally, mice chronically exposed to TS prior to TBI experienced a more significant weight loss, behavioral, and motor activity deficiency and slower post-TBI recovery when compared to TS-free TBI mice. Conclusion: TS promotes a significant exacerbation of post-TBI neurovascular and neurological impairments. Whereas BBB impairment and pro-inflammatory vascular responses induced by chronic TS exposure are likely responsible for the retardation of post-traumatic recovery observed in these animals.

Background

Traumatic brain injury (TBI), has long been among the most common type of trauma and the leading cause of death and disability in the young-aged population in the United States, thus becoming a serious public health concern in modern society (1, 2). According to the Centers for Disease Control and Prevention (CDC), about 2.5 million people in the U.S. seek emergency care for TBI secondary to motor vehicle accidents, falls, assaults, sports-related events, and other mechanisms every year while more than 5.3 million Americans are currently living with a lifelong disability due to TBI (3). The effects of TBI can cause emotional, physiological, cognitive, motor and behavioral damage ranging from mild to severe deficits and death (4-7). Mild traumatic brain injury (mTBI) accounts for over 80% of head injuries (3, 8). mTBI typically results in transient symptoms including sensitivity to light and sound, headache, vision impairment, difficulties with cognition and balance.

The pathophysiology of TBI can be divided into primary and secondary injury mechanisms. The primary mechanical injury is due to the physical injury and may result in intracranial or extracranial hemorrhage

following damage to the blood vessels, brain tissue, and the BBB (9). The secondary injury occurs within days, weeks, months or even years after the first injury and derived from oxidative stress, inflammation, imbalanced calcium homeostasis, excitotoxicity, apoptosis, increased vascular permeability and BBB disruption (2, 10-12). Although the primary brain injury is the main pathogenic factor, the secondary brain injury is generally more severe and complex than the primary one and encompasses anatomical, cellular, molecular and behavioral changes (12-15). A series of delayed secondary biochemical and metabolic changes at the cellular level is prodromal to other pathological processes including oxidative stress, inflammation, excitotoxicity, enhanced vascular permeability, and BBB impairment resulting in exacerbated post-traumatic brain damage and eventual neuronal dysfunction (2, 15, 16).

It has long been recognized that chronic cigarette smoking is one of the most prevalent premorbid conditions which may influence the severity of TBI and retardation of post-TBI recovery (3). In fact, TS, which is highly enriched by ROS constituents and other reactive compounds which have been clearly shown to promote dysfunction of the BBB through activation of oxidative, inflammatory and immune responses, thus impacting the pathogenesis and progression of cerebrovascular and neurodegenerative disorders including TBI (17-22). TBI patients with previous exposure to TS have shown aggravated post-traumatic cerebrovascular inflammatory and neurovascular conditions when compared to non-smokers.

Given the well-known association between smoking and vascular endothelial dysfunction as well as the increased risk of onset and/or progression of neurological disorders (such as stroke, vascular dementia, small vessel ischemic disease, multiple sclerosis, Alzheimer's etc.), the goal of the present study was to characterize the pathogenic impact of chronic smoking on TBI and assess the post-traumatic exacerbation of TBI including post-TBI recovery using a well-established weight-drop mice model.

Methods

2.1 Materials and reagents

Reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Bio-Rad Laboratories (Hercules, CA, USA). All Quantikine ELISA kits were purchased from R & D systems; The antibodies used in this study were purchased from the various sources: rabbit anti-ZO-1 (#402200), mouse anti-Occludin (#331500) and anti-Claudin-5 (#4C3C2) from Life Technologies; mouse anti-PECAM-1 (#sc-376764), mouse anti-VCAM-1 (#sc-13160), rabbit anti-Nrf2 (#sc-722), mouse anti-NQO-1 (#sc-376023), mouse anti-HO1 (#sc-390991), mouse anti-NFκB-p65 (#sc-(F-6)-8008) from Santa Cruz Biotechnology. Donkey anti-rabbit (#NA934) and sheep anti-mouse (#NA931) HRP-linked secondary antibodies were obtained from GE Healthcare (Piscataway, NJ, USA).

2.2. In Vivo Experimental Design

The experiment protocol in this study was abided by the Institutional Animal Care and Use Committee, TTUHSC, Lubbock, Texas (23). Sixteen male C57BL/6J mice, ranging between 6–8 weeks old and a body weight comprised between 20–22 g) were purchased from Jackson Laboratory. Mice were given 3 days for acclimatization post arrival in the new location to recovery from the transport. All mice were given unlimited access to standard mouse chow and water. Test mice were chronically exposed (via direct inhalation) to side stream TS derived from 3R4F research cigarettes (9.4 mg tar and 0.726 mg nicotine/cigarette) 6 times a day, 2 cigarettes/hour/8 mice every day for three weeks (18). TS was generated using a Single Cigarette Smoking Machines (SCSM, CH Technologies Inc., Westwood, NJ, USA) following previously published methods (23, 24) (see also **Fig.1**). Cigarette exposure was performed according to the standard smoking protocol set by the International Organization for Standardization/ Federal Trade Commission (ISO/FTC). This consists of 35 ml puff volume, 2 s puff duration, 58 s intervals, 8 puffs per cigarette (25).

2.3. Induction of Head Injury in Mice

We used a weight-drop model of TBI (2, 4, 26). In brief, mice were anesthetized by inhaling Isoflurane vapor for several minutes and then placed on a spongy platform positioned under the weight-drop device. Head movements were allowed parallel to the injury plane at the time of the induction to mimic a head injury that occurs during a car accident. During the induction phase, mice were fixed in such a way as to direct the injury from the left anterior frontal area, at the same distance between the eye and the right ear. A hollow tube (with an internal diameter of 13 mm) was used a guiding system placed vertically above the head of the mouse whereas a metal weight (30 g) inserted into the hollow tube, which released in a free fall of 80 cm from the dropping point to the target (**Fig.1**). The sham-injured mice underwent the same procedures with the exclusion of being subjected to head impact by weight drop.

2.4. Open Field Test

Open field test as a common measure of exploratory behavior and general activity in rodents (27). Briefly, mice were housed into 16" × 16" unobstructed glass chamber containing infrared sensors along the perimeter and then the mice were monitored and recorded for 1 h and the first 30 min of 1 h were excluded as the acclimatization period (**Fig.1**). Automatic calculation of the activity of the animals (total distance traveled) was conducted by Versamax software (Accuscan Instruments., Columbus, OH). All behavioral tests were performed between 9 Am and 1 Pm.

2.5. Blood Collection and Brain Isolation

Mice were sacrificed under terminal anesthesia 3 days after TBI to collect blood samples and brains for subsequent biochemical and molecular analysis. In order to collect blood samples, cardiac puncture blood collection method through an open approach was employed (28, 29). briefly, mice were posed on its back with its nose in a tube connected to the isoflurane device (, which is also more relaxing not to get worried about the mouse waking up. Then a V-cut was made through the skin and abdominal wall and internal organs were moved to the side. The needle was inserted through the diaphragm and into the heart and blood was collected by applying negative pressure on the syringe plunger. For brain isolation, briefly, a cut was made at the nape and extended along the midline from the dorsal cervical area to the tip of the nose and then the skin was pulled away from the skull laterally. The skull was cut and opened by placing the point of the scissors in the foramen magnum and cutting along the midline. After levering away of parietal bones from the brain and disrupting the nerve attachments at the brain stem and the optic chiasm, the brain was dropped from the skull into the sterile medium (17, 23).

2.6. Preparation of Protein Extracts and Western Blotting

To harvest the proteins homogenized brain tissues were lysed using RIPA lysis buffer so that total, nuclear, cytosolic and membrane fractions were collected by centrifugation at 14000g for 30 mins. Samples were then aliquoted and stored at −80 °C for the subsequent protein expression analysis by western blotting. Protein quantification was conducted using Pierce BCA Protein Assay Kit (Thermo Scientific, # 23225). Samples (60–90 µg for tissue lysates) were then prepared as described in our previous lab report (18, 23, 30). Briefly, denatured samples were run on SDS-PAGE (4–15% gradient gel) and transferred to polyvinylidene fluoride (PVDF) membranes or nitrocellulose membranes for further blotting. After the membrane was washed The membranes were washed with Tween-Tris-buffered Saline (TTBS) (10 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl containing 0.1% Tween-20), they were blocked for 1 h with Tween-TBS containing 5% non-fat dry milk, and incubated with primary antibodies prepared in TTBS containing 5% bovine serum albumin (BSA) for overnight at 4 °C. The following day, for immunodetection, cells were washed and then incubated with the secondary antibody prepared in Tween-TBS containing 5% BSA for 2h. The protein band densities were visualized using chemiluminescent reagents according to the manufacturer's instructions and quantified using Image Studio Lite Ver 3.1 and calculated as fold change/ percentage change over control protein expression. All protein quantifications were adjusted for the corresponding β-actin level, which was not consistently changed by the different treatment conditions.

2.7. Enzyme-Linked Immunoassay (ELISA)

Blood sample collected from mice were analyzed by Quantikine ELISA kits (R & D systems, Minneapolis, MN, USA) for the quantitative determination of Thrombomodulin and Cytokines

TNF-α, IL-6, and IL-10 according to the procedure in accordance with the manufacturer's protocol.

2.8. Statistical analysis

All collected data were expressed as mean standard ± deviation (SD). The sample size was chosen based on a previous work by us and others to generate 80% power and a type 1 error rate = 0.05. The blind analysis was performed by one-way ANOVA using GraphPad Prism 8 Software Inc. (La Jolla, CA, USA). Post multiple comparison tests were performed as with Tukey's or Dunnett's test as recommended by the software. P values < 0.05 were considered statistically significant.

Results

As shown in **Fig.1** TS generated by a CSM-SCSM cigarette smoking machine (CH Technologies, Westwood, NJ) was forced directly into two airtight smoking chambers with dimensions of 24 L X 12 W X 12 H. The smoking inlet is dually connected to a feeding tube and a ventilator system supplying O_2 (2 L/min) at atmospheric pressure (1 bar). Mice were housed in the smoking chambers (4 mice/chamber) receiving an uninterrupted supply of normal oxygenated air in between puffs. At the end of each smoking cycle, mice were transferred immediately back to their regular housings with standard food and water supply.

3.1. TBI and TS- Exposure Negatively Affect Body Weight

Weight analysis was regularly performed to assess whether smoke and TBI had any negative impact on body weight. Animals were divided into 4 groups at day 0 (**Fig. 2A**) representing the 4 main test categories: Control (no smoke exposure and no TBI); No smoke exposure TBI, Smoke exposure - no TBI and smoke exposure + TBI. As shown in **Fig.2B** we observed that chronic TS exposure alone was enough to reduce the growth rate of body weight over time. This is consistent with published data (including clinical) likely conducible to an appetite suppressant effect as well as moderate increase of metabolism promoted by TS. Following TBI, we also observed a post-traumatic reduction in body weight in animals that underwent TBI w and w/o TS exposure (**Fig. 2C**). Note also that post-TBI animals did not receive any further TS exposure. In summary, our data show that both TBI and chronic TS exposure negatively affected body weight whereas in the case of TBI this is likely due to a post-traumatic reduction of appetite.

3.2. TS-Exposure and TBI Promote Vascular Inflammatory Responses and Potentially Impact Blood Hemostasis

Our basic hypothesis refers to the alteration in pro-inflammatory markers as one of the mechanisms that underlie the damage in the brains of TBI- induced mice. Hence, we evaluated the expression levels of inflammatory marker NF-kB, inflammatory adhesion molecules VCAM-1 and PECAM using western blotting and ELISA. As shown in **Fig.3** western blotting analysis revealed a significant increase in the expression level of NF-kB (Fig. 3A) as well as vascular adhesion molecules VCAM-1 (**Fig 3B**), and PECAM-1 (**Fig. 3C**) in mice exposed to TS w or w/o TBI as well as mice undergoing TBI but not TS exposure. As evident from the results both TS and TBI can elicit inflammation as standalone stimuli. However, the effect was significantly increased where the two stimuli were combined, thus suggesting a synergistic effect additive effect. Furthermore, as shown in **Fig.4**, the inflammatory activity of TBI and TS were confirmed by analysis (via ELISA) of pro-inflammatory cytokines IL-6 (**Fig. 4A**), IL-10 (**Fig. 4B**) and TNF-α (**Fig. 4C**) in blood samples collected 24h and 3 days after TBI. As for NF-kB and vascular endothelial adhesion molecules, we observed a synergistic effect between TS and TBI.

Of relevant interest, TS exposure decreased the expression level of the anticoagulant factor thrombomodulin (**Fig. 5A&B**) similar to what we previously observed in vitro and in vivo (23). TBI had instead an opposite effect as a stand-alone factor, however, in animals previously exposed to TS, the levels of thrombomodulin resulted still downregulated despite TBI. This suggests that TS in TBI patients can increase the risk of blood coagulation thus adding to the risk of post-traumatic brain damage. The effect remained statistically significant even 3 days post-TBI **(Fig. 5B)**.

3.3. Downregulation of NRF2 and its Downstream Effector NQO-1 and HO-1 by TS-Exposure and TBI

The effect of TS-exposure and TBI on the expression of the antioxidative response Nuclear factor erythroid-2-related factor 2 (Nrf2) was also evaluated, as demonstrated by western blot analysis of whole brain homogenate in **Fig. 6**. Consistent with our previous finding, chronic TS exposure significantly downregulated Nrf2 expression (**Fig. 6A**) and its immediate downstream effector NAD(P)H dehydrogenase [quinone] 1 (NQO-1; **Fig. 6B**) and Heme oxygenase 1 (HO-1**; Fig. 6C**) indicating impairment of the antioxidative response system. By contrast, TBI, as a stand-alone stimulus had the opposite effect were Nrf2 was upregulated. The effect of TBI on Nrf2 expression was abrogated in mice chronically exposed to TS preventing the antioxidative response system to mount an effective response to injury by concomitant inflammation and ROS generation, thus further confirming the detrimental role of TS on post-traumatic brain injury.

3.4. Chronic TS Exposure Hamper BBB Integrity in TBI

Additional experiments were performed to assess the effect of TS-exposure and TBI on BBB integrity. Specifically, we assessed the expression level of the BBB accessory protein zonula occludens-1 (ZO-1)

and tight junction (TJ) protein expression encompassing the main regulator of BBB paracellular permeability Occludin and Claudin-5.

As demonstrated in **Fig.7**, chronic TS-exposure significantly downregulated the expression of ZO-1. Moreover, the expression level of the main TJ proteins, including Occludin and Claudin-5 were also significantly downregulated when compared to controls. Even though Occludin expression levels were also significantly downregulated by TBI (the impact on claudin-5 was marginal and no effect was observed for ZO-1), our data and previous work by our group suggest that the main effector of TJ downregulation and loss of BBB integrity is indeed TS exposure (23, 25, 30).

3.5. TS Promotes Increased Motor Activity in Mice but Aggravates Post-Traumatic Behavior in Mice Undergoing TBI

Exploratory behavior and general activity of the test mice were regularly recorded to evaluate the impact of smoke and TBI on their motor activity before and after induction of traumatic injury.

As shown in **Fig.8** we observe that in compared to both groups of non-smoked mice there was an increase in totaled distance traveled by mice chronically exposed to TS. This is consistent with the metabolic effect of TS as well as craving for TS itself (**Fig. 8B1** to **8B3**). While it was expected that mice undergoing TBI would have a reduced motor activity when compared to controls (the effect of TBI became manifest as early as 1h post-TBI) and gradually recovered (partially) later on at 24h and 3d post TBI), we observed that the impact on motor behavior/activity was aggravated (further reduced) in mice that were also chronically exposed to TS. TS did not only affect the overall decrease in motor activity, but further impaired motor recovery as shown in **Fig. 8B1** to **8C**, thus confirming the negative impact of TS on TBI.

Discussion

TBI is a global health issue and one of the major leading cause of death and disability for individuals under the age of 50. According to the statistics, approximately half the global population will experience one or more TBI events over their lifetime (8). Oxidative stress caused by the redox imbalance promoted by highly reactive oxygen species (ROS; including free oxygen radicals and reactive anions) has been proven to play a major role in post-traumatic secondary brain damage (17, 31). Recent studies have shown that TS is highly enriched with ROS and that chronic TS exposure is associated with impairment of the antioxidative response system and dysfunction of normal endothelial physiology, thus promoting the onset of major cerebrovascular and neuroinflammatory/degenerative disorders (20, 24, 25, 32-35). However, there is still considerable controversy regarding the influence of cigarette smoking as a commonly premorbid factor on how it relates to TBI and its impact on post-traumatic secondary brain injury and post-TBI recovery (3).

In the present study, we evaluated the potential influence of chronic tobacco smoking on pathophysiological mechanisms underlying the exacerbation of TBI and retardation of post TBI recovery using a weight-drop mice model. In this model, a fixed weight is released for a free fall based on a defined path and height. The weight and the height from which it is imposed determines the severity of the injury, and it can range from a mild level of injury to severe brain injury. This model was chosen because of its ability to simulate traumatic head injuries comparable to those observed in road accidents or falls (4). Based on our results, both groups of smoked mice demonstrated a loss of body weight when compared to control, confirming the common metabolic stimulatory effect of TS. Longitudinal increase of body weight was also significantly dampened after TBI induction which is consistent with the well-observed reduced appetite after TBI. In line with these findings, the behavioral analysis confirmed similar changes in the state of consciousness and *awareness* immediately after TBI and during recovery. While TS was consistently associated with an increase in motor activity as a standalone stimulus, when it was combined with TBI (see Fig. 8) is further depressed motor activity when data were compared to TBI mice that were not exposed to TS. The Post-traumatic motor recovery was also significantly reduced when compared against the same group. These data are consistent with increased severity of post-traumatic brain injury promoted by TS and well correlated with the analysis of inflammatory biomarkers as well as BBB integrity.

Nrf2, a basic region-leucine zipper (bZip) redox-sensitive transcription Factor is the master regulator of multiple cytoprotective responses which controls the redox state of cells in harmful stresses (17, 36, 37). Under basal conditions, Nrf2 is localized in the cytoplasm by its inhibitor, Kelch-like ECH-associated protein 1 (Keap1). Nevertheless, under conditions of oxidative or xenobiotic stress, the cysteine residues of Keap1 become oxidized, Nrf2 dissociates from Keap1, translocate into the nucleus, binds to the antioxidant response element (ARE), and promotes the transcription of over 500 genes encompassing phase 1 and 2 enzymes, regulators of redox metabolism; production of ATP and antioxidative agents (including NADH and glutathione), and TJ expression at the BBB (16, 38, 39). Based on valid evidence, Nrf2 also promotes anti-inflammatory mediators, the activity of the proteasome and other transcription factors involved in mitochondrial biogenesis (40). According to recent studies, while suppression of Nrf2 activity and impairments of the Nrf2–ARE pathway exacerbate TBI-induced oxidative damage as well as post-traumatic neurological deficits, NRF2 played a significant neuroprotective role in TBI and neurodegenerative disorders (4, 11). Since the upregulation of Nrf2 activity ameliorates TBI-induced brain injury it could be suggested that positive modulation of Nrf2 could better TBI outcomes through reduction of oxidative stress, inflammation, and protection of BBB integrity (23, 24, 41-45).

In line with these findings, we assessed the impact of TS-exposure and TBI on Nrf2 expression levels, as well as its downstream effector molecules NQO-1 and HO-1, which are known for exerting acute detoxification and cytoprotective functions. Our in vivo data that the Nrf2-ARE system gets activated in response to TBI (**see Fig. 6**) This effect could be due to a direct modulatory activity toward Nrf2 expression and activation of the Nrf2–ARE pathway in response to trauma and are in line with the results obtained in a previous study by Li et al. indicating significantly enhanced Nrf2, NQO-1 and HO-1 protein expression following TBI (44). However, chronic TS exposure as a stand-alone stimulus has the opposite

effect (see **Fig. 6A**) which is also in line with previous in vitro and in vivo observations recently published by our group (22-24, 46). As a comorbid stimulus, when TS exposure is combined with TBI, it abrogates the post-traumatic activation/upregulation of Nrf2 that follows brain trauma, thus preventing this physiological recovery system from being activated. The overall effect is the impairment of the BBB (not observed in TBI animals not exposed to TS; see also **Fig. 7**) and an overall increase of post-traumatic inflammatory responses including overexpression of cytokines (see **Fig. 4**), the pro-inflammatory transcription factor NF-kB and vascular endothelial adhesion molecules (**see Fig. 5**). These findings are consistent with the analysis of post traumatic motor activity showing that animals chronically exposed to TS prior TBI fared significantly worse than those undergoing the same traumatic injury but were not exposed to TS (see **Fig. 8**). In this specific case, the overall cerebrovascular/BBB impairment promoted by TS could explain the phenomenon.

The BBB is a dynamic and complex interface between the blood and the central nervous system (CNS)which strictly maintains the brain homeostasis and controls the passage of substances in and out of the brain environment. Among the various control functions of the BBB, the inter-endothelial TJs rigidly control the paracellular pathways blocking the passage of polar molecules (including ions) from moving between adjacent endothelial cells (47). The most important TJ proteins modulating the extremely low BBB permeability to polar molecules are Occludin and Claudins (more specifically claudin-5) forming homotypic bonding with their corresponding counterparts on adjacent endothelial cells. ZO-1 plays the critical function of anchoring this TJ protein to the cell cytoskeleton, thus allowing the cell to direct the distribution of these TJ proteins around the membrane (48). Recent findings have demonstrated that BBB impairment is a key component of post-TBI secondary brain injury and can significantly affect the outcome (49).

Additional evidence also indicate that inflammation is an important contributor to the TBI pathophysiology and exacerbates neuronal damage during post-traumatic brain injury so that sustained and excessive inflammation through secretion of proinflammatory mediators can exacerbate subsequent neurological impairment (50-52). This process involves resident microglia and astrocytes, peripheral leukocytes penetrating through the leaking BBB and inflammatory mediators including cytokines that interfere with normal restorative processes of the brain, thus promoting neuronal cell death (31, 53, 54). Proinflammatory cytokines like IL6, IL-10, and TNF-α, are increased in posttraumatic brain tissue, followed by synthesis of chemokines, prostaglandins and expression of cell adhesion molecules like VCAM-1 and PECAM-1 on the surface of the cerebrovascular endothelium. This latter process can favor the extravasation of inflammatory cells from the blood into the brain (31, 50). There is also a growing consensus that all these processes are the key promoters of the secondary brain damage associated with TBI including dysfunction of astrocytes and microglia, as well as BBB impairment contributing to the increased paracellular permeability and the loss of neurons (50, 55, 56). In fact, proinflammatory molecules play a supplementary role in increased BBB permeability related to loss of Occludin/ZO-1 as well as other tight junction (57, 58). It is also well described that BBB integrity is deeply affected by oxidative stress, so that enhanced ROS production leads to redistribution and/or altered expression of tight-junction proteins, endothelium dysfunction and increased BBB permeability (59-61). Inflammation is

also linked to oxidative stress, whereas ROS (such as those released within TS) are considered among the most potent inflammatory mediators (62). In fact, oxidative stress caused by TS is increasingly recognized as a negative contributing factor for neurological outcomes following brain injury (41) whereas TS modulates a cascade of events leading to the activation of NF-κB and the expression of pro-inflammatory cytokines and vascular adhesion molecules (46). This has been observed in glial cells and neurons following TBI and is associated with long-term inflammatory processes (10). Mettang et al., using an experimental model of closed-head injury promoted neuronal cell death, demonstrated the repression of the NF-κB inhibitor system exacerbating the neurological outcome and increasing posttraumatic mortality rate (63). In the contest of Nrf2 - NF-κB interplay, a recent study confirms the cytoprotective mechanisms of action associated with Nrf2 which leads to the downregulation of proapoptotic mediators such as Bax, BAD, and other pro-apoptotic factors which are instead promoted by the activity of NF-κB (64, 65). Nrf2 reduces ROS levels and affects the redox-sensitive NF-κB signaling pathway involved in neuroinflammation. Moreover, in a recent study, it has been reported that Nrf2-/- mice have greater NF-κB activation and generation of pro-inflammatory cytokines in the brain and spinal cord injury compared to their wild-type Nrf2+/+counterparts (66). Relevant to our study is the fact that chronic TS exposure dampened Nrf2 activity in TBI mice. Thus, the cascading effect of Nrf2 downregulation well fit the slowed recovery and overall, worse outcome observed in TBI animals chronically exposed to TS when compared to TBI mice that were not exposed to smoke.

An additional risk factor for TBI patients that is associated with chronic smoking may be derived from the impact of smoking on blood hemostasis. Thrombomodulin is a key component of the anticoagulant protein C pathway and is tightly regulated to maintain blood homeostasis and to ensure control over the process of blood-coagulation (by blocking the activity of the prothrombinase complex and promoting fibrinolysis) following activation of the coagulation cascade in response to vessel injury and/or inflammation (67). Although the mechanism is largely unknown, thrombomodulin is potently inhibited by inflammatory cytokines, thus blocking NF-kB activation effectively prevents cytokine-induced downregulation of thrombomodulin. TS-exposure has been previously observed to promote the downregulation of thrombomodulin at the vascular endothelial level (through its pro-inflammatory activity), thus increasing the risk of blood clot formation and stroke (24). Therefore, the downregulation of thrombomodulin by TS exposure can provide an additional risk for TBI patients were traumatic injury is likely to impact the integrity of the blood vessel and trigger the activation (now poorly controlled) of the coagulation cascade.

Although premature at this stage, we plan to revolve the focus on potential treatment intervention to reduce or eliminate the add-on risk of TS exposure on post-TBI injuries and improve outcomes. This is mostly relevant for patients that recently quit smoking since many of these TS-related harmful effects persist for months or years after quitting, thus remaining at risk of smoking-related comorbidities which may still aggravate the outcome of neurological disorders associated with cerebrovascular impairments (e.g., stroke) and TBI.

Conclusion

Chronic cigarette smoking is modifiable health risks, representing the third leading cause of preventable mortality in the United States (3). at the cerebrovascular level, chronic smoking can contribute to oxidative damage, trigger a strong inflammatory cascade and severely impair endothelial physiology, thus leads to the onset and/or progression of several main cerebrovascular diseases. Regarding TBI, a better understanding of cigarette smoking potential influence on neurocognitive and neurobiological recovery from TBI may inform the design of targeted pharmacological interventions to facilitate maximum rate and magnitude of recovery after brain injury. In this study, the effect of TS on the exacerbation of TBI and retardation of post TBI recovery was investigated using a weight-drop model. Considering all the studies presented, we showed that TS leads to TBI exacerbation and retardation of pathophysiological recovery after TBI. The effects of TS are consequential to impairments of the BBB, including the loss of BBB integrity, OS damage, and inflammation. Based on previous studies by us and others, exacerbated pathophysiological mechanism related to TS seems to depend upon downregulation of Nrf2 expression. Although outside the scope of this work, for future experiments, we plan to explore in more detail the underlying pathogenic mechanisms and evaluate the feasibility of targeting Nrf2 to prevent TBI exacerbation and additional vascular comorbidities induced by TS for patients that either cannot quit smoking or those that quit recently but still remain at high risk of developing CNS disorders.

List Of Abbreviations

- **ARE** - Antioxidant Response Element
- **BBB** - Blood-Brain Barrier
- **CDC** - Centers for Disease Control and Prevention
- **CNS** - Central nervous system
- **CS** - Cigarette Smoke
- **2DM** - Type 2 Diabetes Mellitus
- **FTC** - Federal Trade Control
- **HO-1** - Heme Oxygenase 1
- **ISO** - International Organization for Standardization
- **Keap1**- Kelch-like ECH-associated protein 1
- **NF-κB**- Nuclear factor kappa-light chain-enhancer of activated B cells
- **NQO-1** - NAD(P)H: Quinone reductase I
- **Nrf2** - Nuclear factor erythroid 2-related factor
- **OS** - Oxidative stress
- **PECAM-1** - Platelet Endothelial Cell Adhesion Molecule-1
- **ROS** - Reactive Oxygen Species
- **SCSM** - Single Cigarette Smoking Machine

- **TBI** -Traumatic Brain Injury
- **TJ** - Tight Junction
- **TS** - Tobacco smoke
- **ZO-1** - Zonulae occludentes-1

Declarations

Ethics approval and consent to participate

All animals were housed in facilities fully accredited by AAALAC in accordance with the applicable portions of the Animal Welfare Act and the" Guide for the Care and Use of Laboratory Animals". All studies were performed with regard to the alleviation of pain and suffering under protocols approved by the TTUHSC Institutional Animal Care and Use Committee.

Consent for publication

Not applicable

Availability of data and material

The datasets included in this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

F.S. planned and performed the experiments, analyzed the data and prepared the drafting of the manuscript. F.A setup of the weight drop model, contributed to the planning and execution of the

experiments and data analysis. A.S. contributed to the planning of the experiments and behavioral/motor analysis. L.C. conceived the study, assisted with data interpretation, drafting of the manuscript and preparation of the figures. L.C. also oversaw the research study and provided funding. All authors reviewed the manuscript.

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Not applicable

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Figures

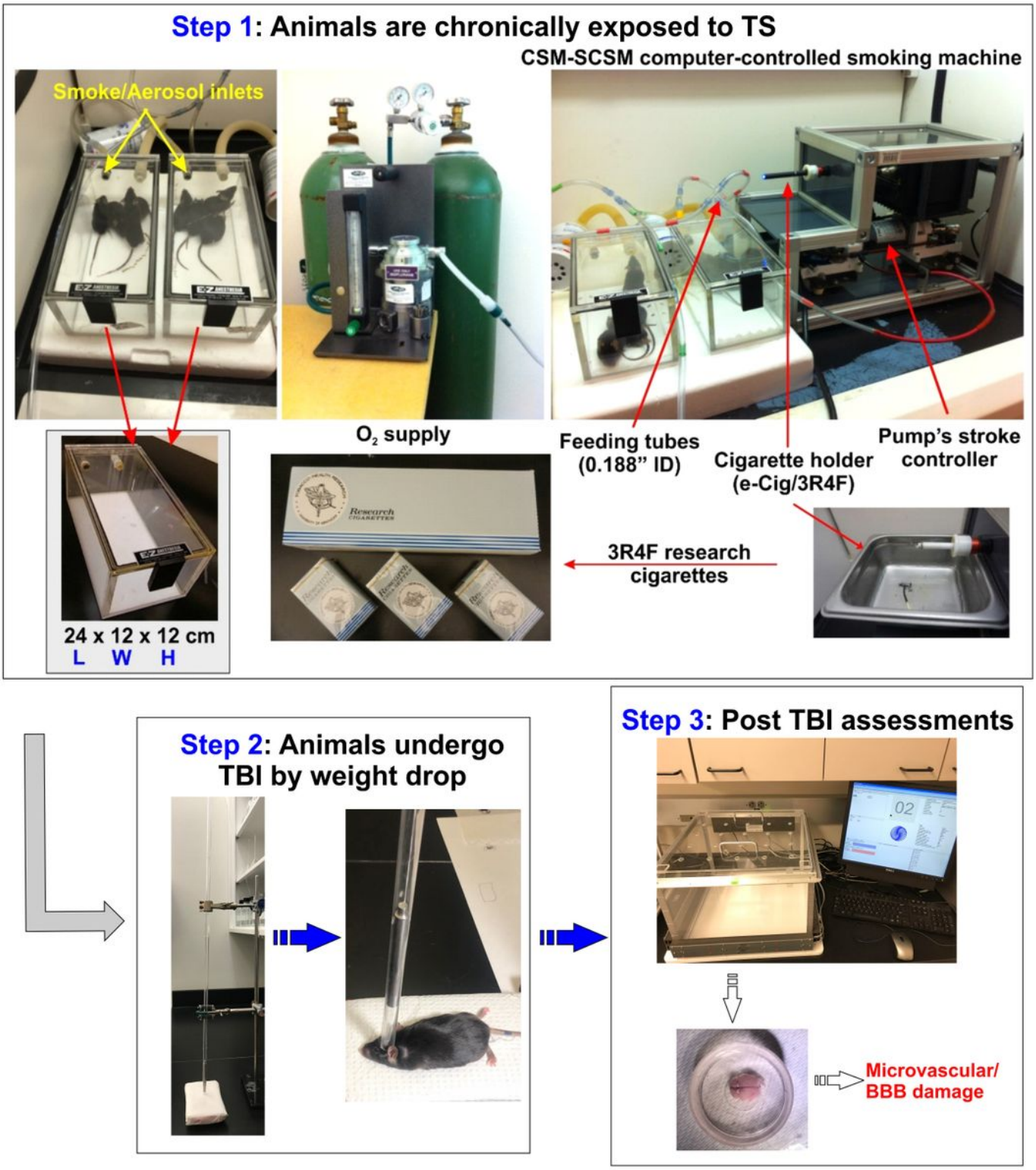

Figure 1

Experimental flow and set up including cigarette smoke generation, animal exposure, induction of traumatic brain injury and post traumatic assessment of motor activity. C57BL/6J mice, were chronically exposed to TS (full body side stream exposure) for three weeks using a computer controlled single cigarette smoking machine using the FTC approved smoking protocol. Test animals were subjected to TBI by head weight (30g) drop from an 80 cm height through pre-positioned vertical guide. Physical

activity and weight of the mice were analyzed before TBI and at 1h, 24h and 3 days after TBI using an open-field test. Finally, mice were sacrificed to collect blood and brain samples for subsequent biochemical and molecular analysis.

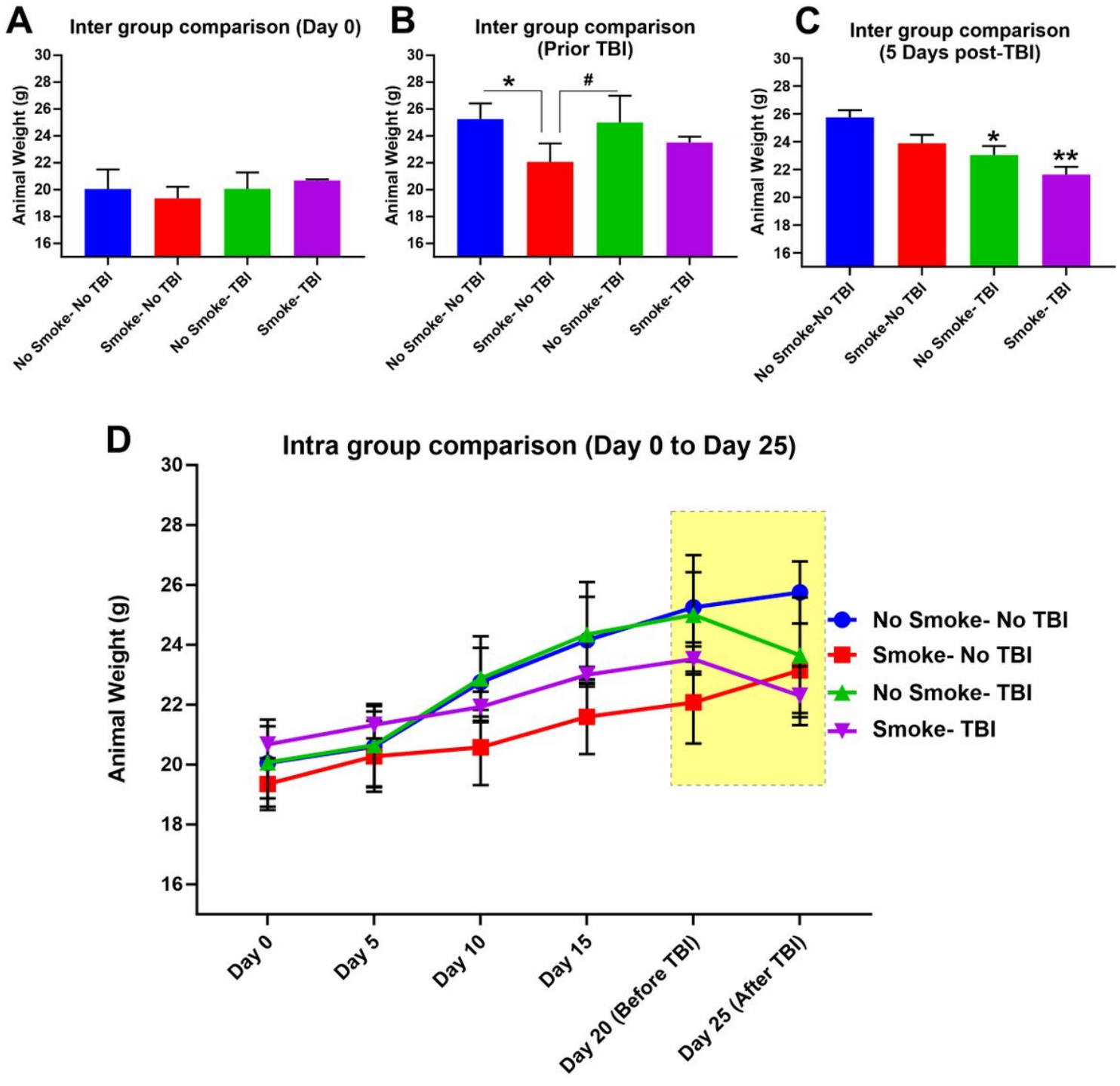

Figure 2

Effect of TS-exposure and TBI on body weight in vivo. (A) Measurements of animals' body weight does not show any significant difference between the tested groups at Day 0, however, at the end of the three weeks exposure prior TBI (B), animal exposed to TS showed a decreased body weight when compared to controls. (C) 3 days after TBI animals showed a significant decreased body weight in compared to

controls. (D) Longitudinal assessment of animals' body weight (all groups). n = 4 biological replicates. *p < 0.05 versus control. # p < 0.05 0001 versus smoked group.

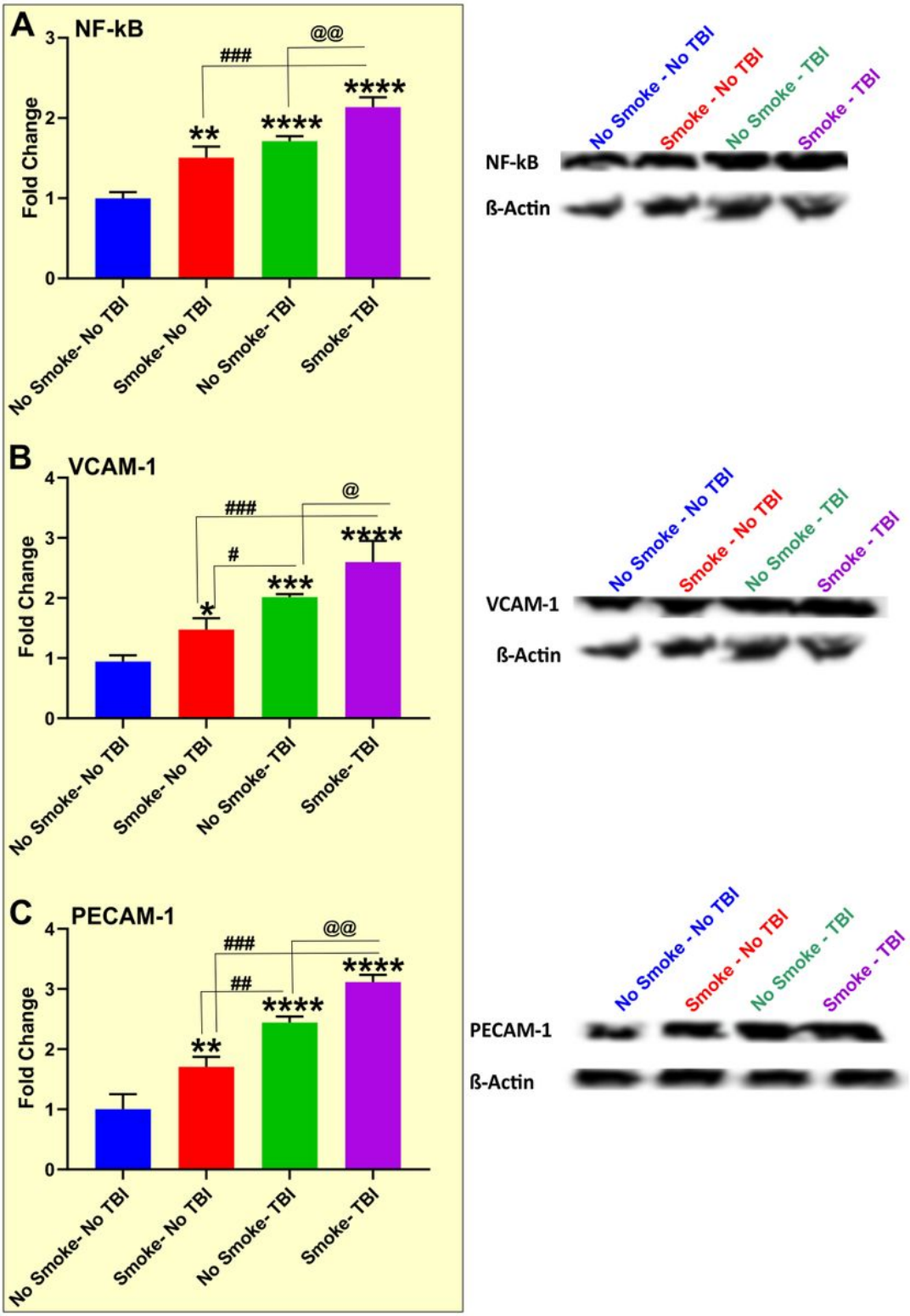

Figure 3

Effect of TS-exposure and TBI on vascular inflammatory responses. (A) Expression level of the inflammatory marker NF-KB and (B) inflammatory adhesion molecules VCAM-1 and (C) PECAM-1 which was upregulated by TS-exposure and potentiated by TBI. *p < 0.05, **p < 0.01, ***p < 0.001, ****p <

0.0001 versus control. #p < 0.05, ##p < 0.01, ###p < 0.001 versus smoked group. @p < 0.05, @@p < 0.01 versus TBI-induced group. WB analyses report protein/β-actin ratios.

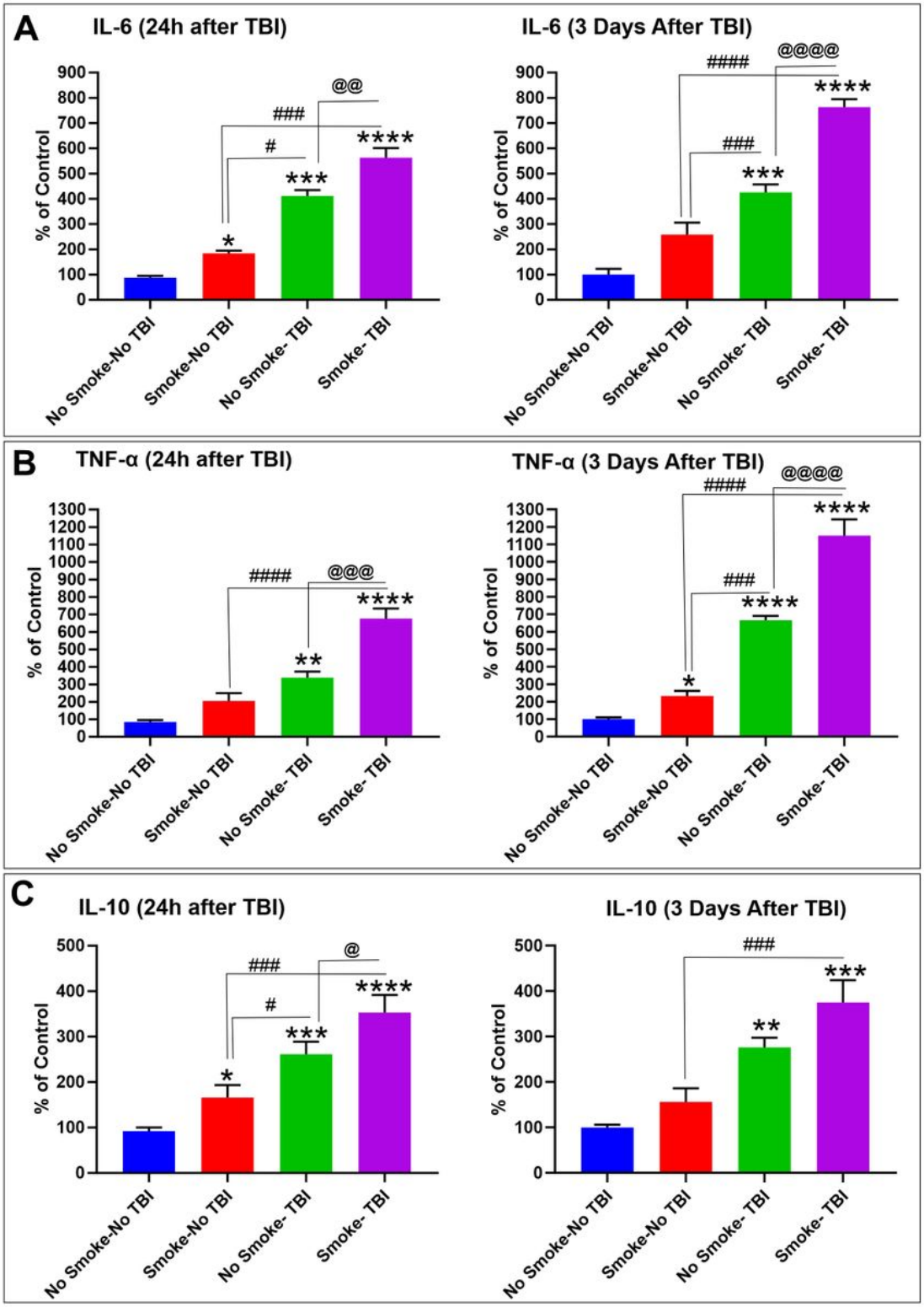

Figure 4

Effect of TS-exposure and TBI on pro-inflammatory cytokines IL-6, IL-10 and TNF-α. ELISA results of pro-inflammatory cytokines (A) IL-6, (B) TNF-α. and (C) IL-10 24h and 3 days after TBI demonstrated upregulation by TS-exposure which synergistically potentiated upregulation by TBI. n = 4 biological

replicates. #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001 versus smoked group. @p < 0.05, @@p < 0.01, @@@p < 0.001, @@@@p < 0.0001 versus TBI-induced group.

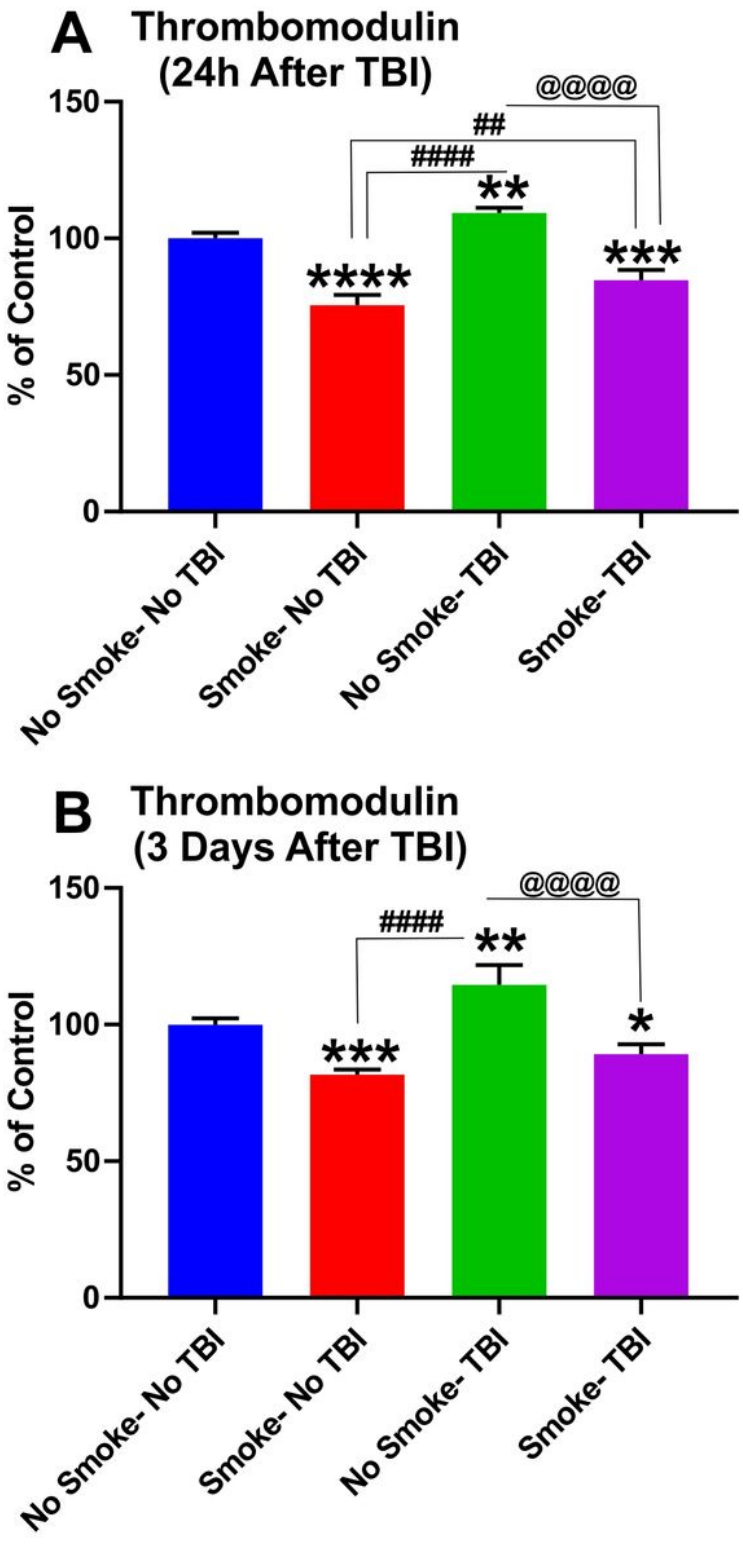

Figure 5

Effect of TS-exposure and TBI on Plasma Level of Thrombomodulin. ELISA results of thrombomodulin in the blood plasma (A) 24h after TBI and (B) 3 days after TBI demonstrated downregulation by TS-exposure. n = 4 biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus control. ##p

< 0.01, ####p < 0.0001 versus smoked group. @@@@p < 0.0001 versus TBI-induced group. WB analyses report protein/β-actin ratios.

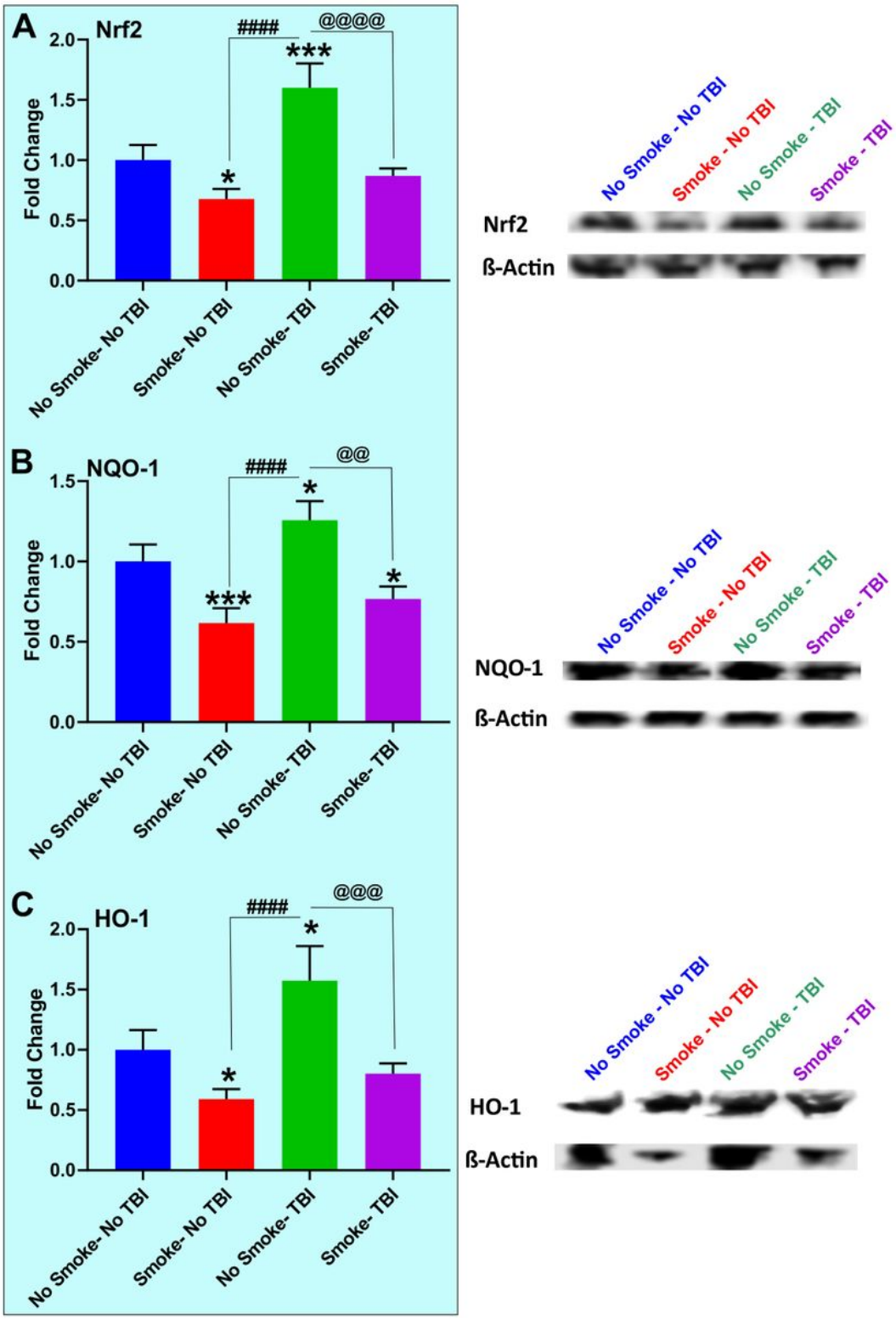

Figure 6

Effect of TS-exposure and TBI on oxidative stress. (A)Western blotting analysis emphasizing the effect of chronic TS-exposure on Nrf2 expression level as a standalone factor and in synergism with TBI. (B)&C) Changes in Nrf2 expression levels were paralleled by corresponding changes of its downstream

detoxifying effector molecules NQO-1 and HO-1. n = 4 biological replicates. *p < 0.05, ***p < 0.001 versus control. ####p < 0.0001 versus smoked group. @@p < 0.01, @@@p < 0.001, @@@@p < 0.0001 versus TBI-induced group. WB analyses report protein/β-actin ratios.

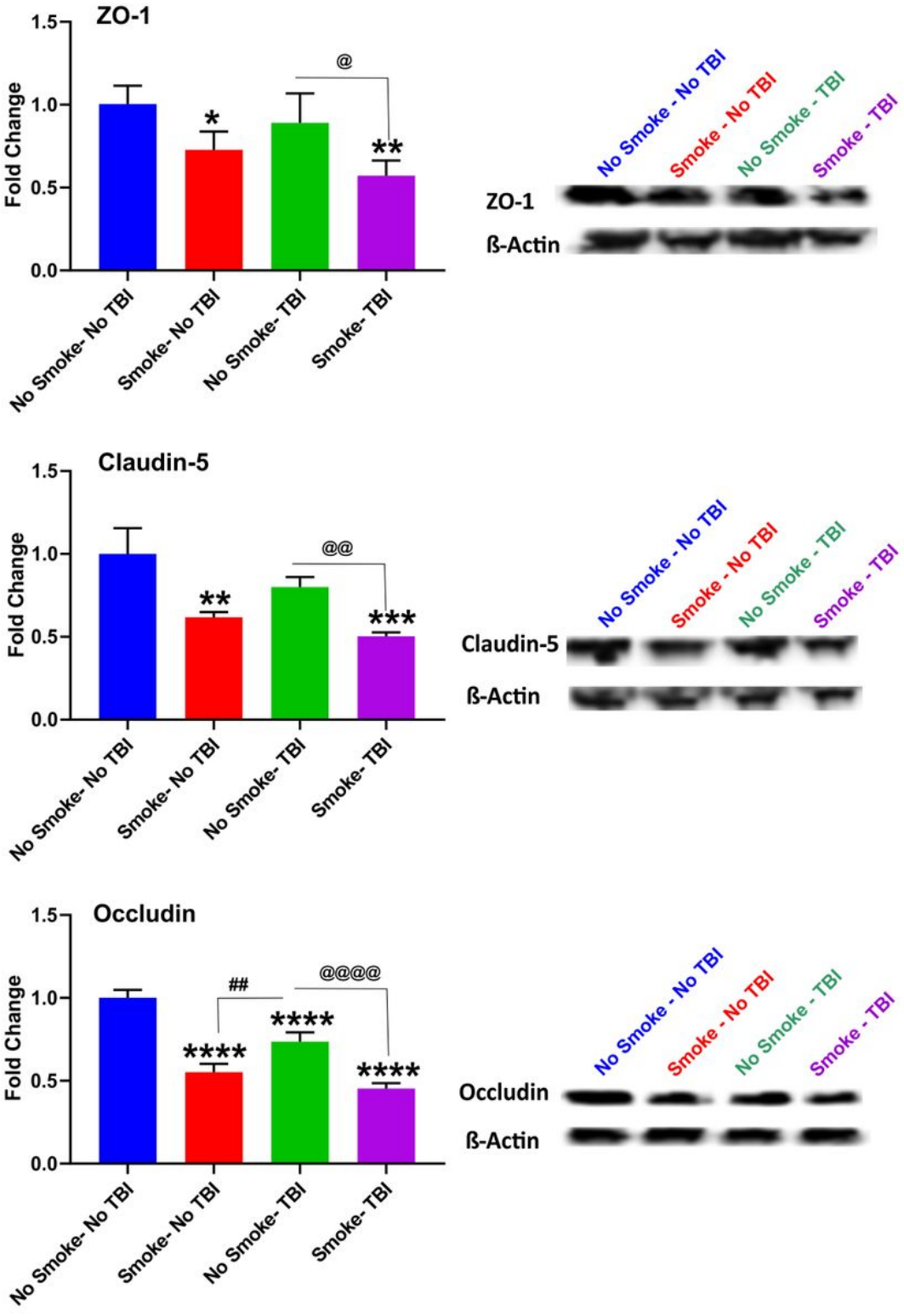

Figure 7

Effect of TS-exposure and TBI on Blood-Brain Barrier integrity. Western blotting analysis demonstrating downregulation of accessory and TJ proteins (A) ZO-1 and (B) Claudin-5 and (C) Occludin in mice

exposed to TS and/or TBI. Note that TBI per se has a more marginal effect on these TJ proteins when compared to TS alone, thus suggesting that TS is the main factor responsible for TJ regulation observed in TBI mice chronically exposed to cigarette smoke. n = 4 biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus control. ##p < 0.01 versus smoked group. @p < 0.05, @@p < 0.01, @@@@p < 0.0001 versus TBI-induced group. WB analyses report protein/β-actin ratios.

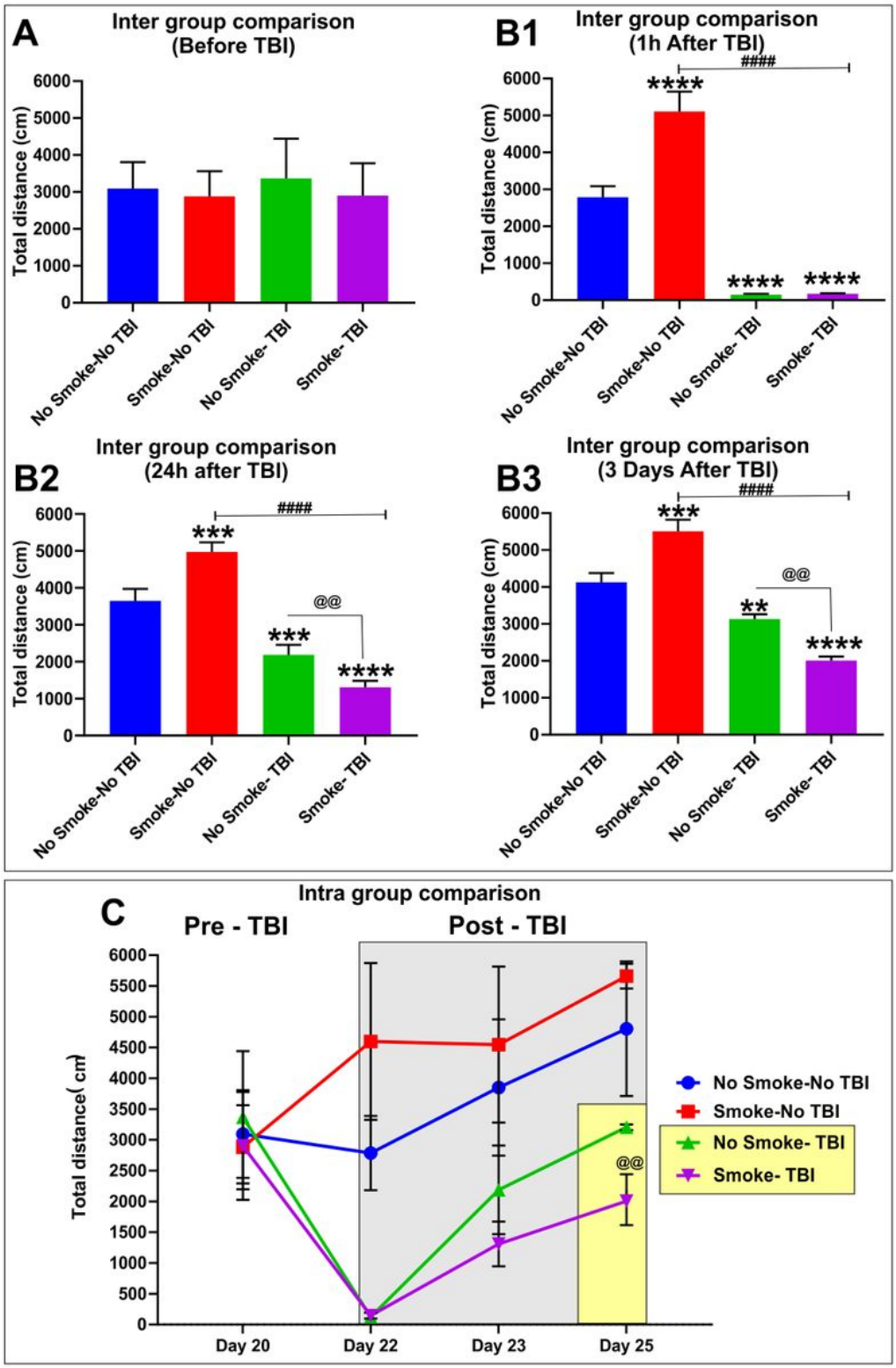

Figure 8

Effect of TS-exposure and TBI on exploratory behavior and general motor activity. (A) Measurements of totaled distance travelled by mice do not show any significant difference between the tested groups at day 0 prior TBI. However, mice undergoing chronic TS exposure w/o TBI demonstrated a significantly higher motor activity (B1) also note that both TS and no TS exposed mice undergoing TBI displayed a significant reduction in motor activity which was further aggravated in TS exposed mice (B1 to B3) up to 3days after TBI. (C) Longitudinal assessment of animals' recovery from TBI shows that animal who were chronically exposed to TS exhibited a significant delay in the recovery of motor activity when compared against other TBI animals that were not exposed to TS. n = 4 biological replicates. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ versus control. ####$p < 0.0001$ versus smoked group. @@$p < 0.01$ versus TBI-induced group.