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Troxerutin cerebroprotein hydrolysate injection ameliorates neurovascular injury induced by traumatic brain injury – via endothelial nitric oxide synthase pathway regulation

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ABSTRACT

Background: Neurovascular dysfunction caused by traumatic brain injury (TBI) is characterized by cerebral vascular damage, blood–brain barrier (BBB) breakdown, brain edema, etc. This study was designed to assess the protective role of 5 days troxerutin cerebroprotein hydrolysate (TCH) injection treatment against TBI, as well as the potential mechanism.

Methods: The weight-drop model of TBI in male Sprague-Dawley rats was chosen to induce TBI model, rats either with TCH or a vehicle via intraperitoneal injection were examined 3 days after TBI.

Results: TCH resulted in alleviation of neurological deficits, reduction of infarct volume, improvement of regional cerebral blood flow (rCBF), amelioration of neuronal death, astrocyte proliferation, endothelial cell loss, and BBB dysintegrity. These effects of TCH treatment against TBI were through endothelial nitric oxide synthase (eNOS) coupling/decoupling status adjustment, which not only increased nitric oxide (NO) level, but also decreased peroxynitrate level expression.

Conclusions: All the results indicated that TCH injection has multifaceted protective effects of neurovascular unit (NVU) against TBI via eNOS pathway regulation.

Introduction

Traumatic brain injury (TBI) is the leading cause of mortality and disability in people under the age of 45 years [1]. It is estimated that around 10 million people are affected by TBI worldwide each year [2]. Neurovascular unit (NVU) is considered a structural and functional unit of the nervous system. The structural basis of the NVU includes neurons, astrocytes, brain endothelial cells, pericytes, vascular smooth muscle cells, etc. [3]. Amounting evidence indicates that NVU plays an important role in physiological functions, as well as pathogenesis during many central nervous system diseases [4]. During TBI, nearly all components of the NVU are destroyed [5], resulting in ultrastructural changes of brain tissues [6], the disruption of regional cerebral blood flow (rCBF) [7], the increased blood–brain barrier (BBB) permeability, the development of brain edema [8], and so forth. All these pathophysiological changes are considered as frequent causes of neurobehavioral deficits and mortality in patients with TBI. As a result, it is convinced widely that protecting NVU is the most potential strategy to treat TBI [9].

Nitric oxide (NO), a mediator of biological effects in the brain, is produced by three different isoenzymes: the endothelial form (endothelial nitric oxide synthase, eNOS), the neuronal form (nNOS), and the inducible form (iNOS). NO and nitric oxide synthase (NOS) are found to be dysregulated induced by TBI, although the clear conclusions are still lacking. For example, NO production is shown increased following TBI [10], furthermore, expression of eNOS has also been considered increased after TBI [11]. After TBI, NO can react with superoxide and produce peroxynitrite, which reduces NO bioavailability [12]. This process could be responsible for the decrease in CBF and the consequent BBB leakage as well as cell death [9].

Troxerutin is a derivative of the natural bioflavonoid rutin which has been reported to possess important...
biological activities [13–15]. For years, evidence has shown that flavonoids have many biological effects, such as anti-oxidative, anti-inflammatory, antiplatelet, neuroprotective, and vasoprotective effects) and have protective effects against many cerebral vascular diseases [16–21]. Till recently, Kumar et al. have shown their possible NO modulation in the protective effects against experimental head trauma [22].

Our study was designed with the aim to investigate the protective effects of troxerutin cerebroprotein hydrolysate (TCH) injection against TBI inducing NVU dysfunction and the possible involvement of eNOS pathway in this effect.

Methods

Animal

Ten-week-old adult male Sprague-Dawley rats, aged 10 weeks (280 ± 20 g) were purchased from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The room in which rats were housed was 21 ± 2°C and 55 ± 5% relative humidity with artificial light-dark cycles. All experimental procedures were carried out according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the local Ethical Committee at the Army General Hospital of PLA.

Drug administration

The animals were randomly divided into three groups: (i) Sham-operated group treated with vehicle (Sham), (ii) TBI + treatment with vehicle (TBI), and (iii) TBI + TCH treatment (TBITCH). The number of animals used in each experiment is 18. As TCH was confirmed to be effective when administered daily for five consecutive days before experimental intervention [23], we chose a dose of 1.5 ml/kg TCH intraperitoneal injection each day before TBI.

Induction of TBI

The TBI model used in this study was a modified version of the weight-drop model [24,25]. Rats were anesthetized by Intraperitoneal injection of 10% chloral hydrate (1 ml/300 g), and a 6-mm diameter hole was made through the exposed skull using a dental drill on the left (parietal) side; the center of the hole was 1.5 mm posterior and 2.5 mm lateral to bregma. The dura was left intact during the operation. TBI was induced by a 40 g weight dropped from a height of 25 cm along a stainless steel string, which translated into 1000 × g/cm. Sham rats were subjected to identical treatment but without injury. The rats were returned to their cages after the operation.

Assessment of neurological deficits

The neurologic status of the rat was evaluated on third day after TBI using the neurologic severity score (NSS) [26]. The investigators evaluated the ability of each rat to perform 10 different tasks that demonstrated motor function, balance, and alertness. One point was given for failing to perform each of the tasks; thus, 0 = the minimum deficit, and 10 = the maximum deficit.

MRI acquisition and data analysis

The rats were subjected to MRI examination 3 days after TBI. MRI was conducted on a 7.0 T magnet (Bruker, Pharma Scan, Ettlingen, Germany), equipped with an actively shielded gradient system (14 cm inner diameter). Rats were anesthetized using facemask inhalation of 1.8% isoflurane by isoflurane anesthesia system (JD Medical Dist. Co. Inc., Phoenix, AZ).

Evaluation of cerebral infarct volume

T2-weight MRI was used for measuring cerebral infarct volume [27]. T2-weight images of coronal slices were acquired with a fast spin-echo pulse sequence, repetition time (TR) = 4400 ms, echo time (TE) = 45 ms, field-of-view (FOV) = 3 × 3 cm, matrix size = 256 × 256 and slice thickness = 0.7 mm. The hyperintense areas in each coronal slice were analyzed by ImageJ software. The cerebral infarct volume was determined as the sum of the hyperintense areas in each slice multiplied by the slice thickness [28].

Measurement of cerebral perfusion

CBF images are ideal to evaluate the extent of cerebral perfusion after TBI. CBF images were obtained from continuous Arterial spin labeling (ASL) with echo-planar imaging fluid-attenuated inversion recovery (EPI-FLAIR) sequences. CBF images were reconstructed with Paravision version 5.1 software (Bruker, PharmaScan, Ettlingen, Germany). Acquisition parameters were TR/TE = 18,000/25 ms, FOV = 3.0 × 3.0 cm, matrix size = 128 × 128, and the number of excitations (NEX) = 1. CBF was measured in the ipsilateral and contralateral hemispheres. Then, the relative cerebral
blood flow (rCBF) was calculated using the equation: 
\[ \text{rCBF} = \frac{\text{ipsilateral CBF} / \text{contralateral CBF}}{C2} \% \] [27].

**Enzyme-linked immunosorbent assay (ELISA)**

The concentration levels of NO in the injured side of the brain were assayed by ELISA (6 rat per group). Three days after TBI, a piece of the rat brain with a diameter of 4.5 mm across the injured area was harvested. Brain homogenates were centrifuged at −4 °C and 14,000 rpm for 30 min. The supernatants were transferred to new Eppendorf tubes and assayed in duplicate using NO assay kit (Beyotime, Haimen, China) according to the manufacturer’s instructions.

**Nissl and immunofluorescent staining**

The rats were sacrificed 3 days after TBI under anesthesia with 10% chloral hydrate (1 ml/300 g, i.p.). After, transcardial perfusion with PBS, the brains were harvested and post-fixed for 24 h in 4% paraformaldehyde. The post-fixed brains were cryoprotected in PBS containing 25% sucrose. The brains were then sectioned into 10 mm thick sections with a cryostat (CM1900, Leica).

For Nissl staining, the method was performed according to procedures described previously [28]. The sections were mounted with neutral balata and blotted on to slides before being processed through different baths as follows: chloroform (30 min), acetone (15 min), 100% ethanol (30 s), 95% ethanol (30 s), 70% ethanol (30 s), distilled water (30 s, twice), cresyl violet (20 min), distilled water (30 s, three times), 70% ethanol (1 min), 95% ethanol (1 min), 100% ethanol (1 min), chloroform (5 min), differentiator (95% ethanol added with glacial acetic acid till pH 4.1, 6 min), 95% ethanol (2 min), 100% ethanol (3 min, twice), xylene (2 min), and xylene (3 min, twice); then, the sections were covered with a coverslip. After Nissl staining, the neuronal cells were identified under a high-magnification (×400) light microscope and counted by an investigator blinded to the grouping. Only intact neurons with a clearly defined cell body and nucleus were counted.

Astrocyte and endothelial cells were examined by Immunofluorescent staining (4 rats per group). The brain sections were washed three times with 0.01 mol/l PBS containing 0.3% Triton X (wash buffer, pH 7.4) for 30 min and blocked with 3% normal goat serum (Servicebio, Wuhan, China) in wash buffer for 60 min at room temperature. The sections were then incubated in blocking solution with one of the following primary antibodies: rabbit anti-GFAP antibody (1:000; Abcam, Shanghai, China); rabbit anti-vWF antibody (1:50; Santa Cruz, Shanghai, China), rabbit anti-Occludin antibody (1:200, Santa Cruz, Shanghai, China) at 4 °C overnight. The sections were subsequently incubated with secondary antibody 488-conjugated goat anti-rabbit IgG (1:500, Beyotime, Haimen, China) at room temperature for 2 h. Then, the sections were incubated with DAPI dying kit (1:500, Solarbio, Beijing, China) for 15 min. The sections were covered with coverslips for microscope observation after antibody incubation. Fluorescent microscopic images were obtained with a computer-driven microscope (Leica DMLB, Leica Corporation, Solms, Germany) using Leica Qwin V3 software (Cambridge, UK). The immune-positive cells were counted using a high magnification (×400) fluorescent microscope by an observer blinded to the individual treatments.

**Transmission electron microscopy (TEM)**

TEM method was according to the procedures described previously [29]. Frontal cortex specimens were immediately placed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, sectioned to 1 mm², and incubated in the same glutaraldehyde solution for 12 h at room temperature. Samples were post-fixed in 1% osmium tetroxide for 1.5 h, dehydrated in increasing concentrations of alcohol, immersed in propylene oxide, and embedded in Araldite 502 resin at 60 °C. Ultrathin sections were placed on grids and stained with uranyl acetate and lead citrate. Thick sections were examined on a light microscope (Axioscop; Zeiss, Jena, Germany) and the grids with thin sections were evaluated under a transmission electron microscope (TEM, JEOL 1010; JEOL, Akishima, Japan).

**Western blotting**

For Western blotting, the prefrontal cortex tissue was homogenized in RIPA buffer (Beyotime, Haimen, China) containing a protease inhibitor cocktail (Complete Mini, Roche, Basel, Switzerland). The supernatant was collected, and the total protein concentration was determined using a BCA Protein Assay Kit (Beyotime, Haimen, China). The total amount of proteins from the tissue was subjected to 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Servicebio, Wuhan, China). The membranes were blocked with 5% (w/v) nonfat dried milk in Tris-buffered saline with Tween-20 (TBST; Beyotime, Haimen, China) and were subsequently incubated with primary antibodies at 4 °C overnight. The following primary
antibodies were used: rabbit anti-3-NT (1:1000; Sigma-Aldrich, St. Louis, MO), rabbit anti-Claudin-5 (1:1000; Abcam; Cambridge, MA), and rabbit anti-eNOS (1:1000; Signalway Antibody, Baltimore, MD). The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Abmart, Shanghai, China) for 2 h at room temperature. The film was scanned, and the protein levels were quantified using ImageJ software. The protein band signal intensity comparison was obtained by following the method described by Du et al. [30]. The GAPDH (1:1000; Servicebio, Wuhan, China) was used as the internal reference protein.

**Statistics analysis**

All statistical analyses were carried out by using SPSS version 19.0 (Data Editing for Windows, IBM Corp., Armonk, NY). Data are expressed as means ± standard error of the mean (SEM). The one-way analysis of variance (ANOVA) followed by a Fisher’s LSD test was used for within-group comparisons. *p* < .05 was considered to indicate a statistically significant difference.

**Results**

**Effect of TCH on neurobehavioral scores after TBI**

As shown in Figure 1, on third day after TBI, the NSS score of the TBI group rats was statistically lower relative to sham group rats (*p* < .001). Furthermore, TCH/TBI rats have a significant higher NSS score than TBI group rats (*p* < .01). These results demonstrated that TCH is useful to alleviate neurological deficits after TBI.

![Figure 1. Neurological deficits of rats on third day after TBI. Values are means ± SEM. **p < .01, ***p < .001 vs. the TBI group.](image)

**TCH reduced the cerebral infarct volume and increased the rCBF**

T2-weighted images showed abnormal hyperintensity in the ipsilateral hemisphere of trauma involving related cortical regions after TBI (Figure 2(a)). TCH significantly decreased the cerebral infarct volume relative to the TBI group. In the ipsilateral hemisphere, CBF images showed a decrease of rCBF in the TBI group rats (*p* < .001). The reduction of rCBF was mitigated by TCH on the third day (*p* < .05) (Figure 2(b)). The statistical analysis revealed that rCBF was below 85% on the third day in the TBI group and there was a significant difference in comparison with the Sham group. TCH treatment elevated rCBF to more than 90% in the TBI brain area, suggesting TCH could improve hypoperfusion after TBI.

**TCH both protected neurons and up-regulated the expression of vWF, but down-regulated GFAP in pericontusional area**

Figure 3 shows that neurons (stained by Cresyl violet), vWF-positive endothelial cells and GFAP-positive astrocytes were decreased in the pericontusional area of the TBI group on the third day after injury. However, TCH treatment increased neurons and generated vWF-positive capillaries in the pericontusional region on the third day after TBI (Figure 3(b,d)), suggesting that TCH not only alleviated the neuronal injury, but also protected endothelial damage. Furthermore, the expression levels of GFAP in the pericontusional cortex were obviously reduced in TCH-treated rats (Figure 3(c)), suggesting that TCH might inhibit the astrocyte proliferation and migration. These findings demonstrated that TCH had multi-target NVU protection against TBI.

**TCH mitigated tight junction protein expression decline and protected BBB dysfunction induced by TBI**

We investigated BBB structure through TEM. Tight Junctions were continuous and adjacent of endothelial cells was closely aligned in sham group rats. In contrast, tight junctions of the TBI group rats were obviously damaged and adjacent was open widely, while TCH could attenuate these BBB abnormalities (Figure 4(a)). As BBB integrity depended on the expression of tight junction protein, we detected whether TCH improves BBB integrity via Claudin-5 expression using western blot (Figure 4(b,c)) and Occludin expression using immunofluorescence.
TBI group rats showed a significant Claudin-5 expression decline in comparison with sham group rats \( (p < .05) \); while, TCH could mitigate this descending expression \( (p < .05) \). The same trend was found in Occludin expression.

**Effectiveness of TCH on eNOS coupling/decoupling**

We further tested the hypothesis that TCH would maintain physiological eNOS function at the third day after TBI by measuring eNOS dimer to monomer ratio. The ratio of eNOS dimer to monomer was significantly down-regulated in TBI group rats when compared with sham group rats \( (p < .05) \), while, TCH treatment could up-regulate eNOS dimer to monomer ratio significantly \( (p < .05) \) (Figure 5(a,b)).

As eNOS effectiveness was determined by the status of coupling/uncoupling, we further detected NO levels and 3-NT levels so as to confirm our hypothesis. The expression of 3-NT, a reactive oxygen species formed by eNOS in monomeric state and implicated in cerebral vascular dysfunction and the consequent BBB disruption [31], was measured at third day following TBI in the injury region using western blot (Figure 5(c,d)). TBI led to a significant elevation of 3-NT expression \( (p < .05) \); while TCH treatment significantly reduced the TBI-mediated increased expression of 3-NT \( (p < .05) \). At the same time, NO, a CBF regulator produced by eNOS in dimeric state, was found reduced significantly in TBI group rats relative to sham group rats \( (p < .05) \); yet, this reduction was ameliorated by TCH treatment \( (p < .05) \) (Figure 5(e)). All these findings implied that TCH might adjust eNOS coupling/decoupling status, through attenuating 3-NT increasing and NO level decreasing induced by TBI.

**Discussion**

This study demonstrated that, for the TBI rats, 5 days TCH treatment could improve the neurological deficits, reduce infarct volume, maintain cerebral perfusion, and protect NVU through modulating eNOS coupling. Importantly, TBI not only decreased NO level, but also raised 3-NT expression, while TCH could reverse these deleterious roles of eNOS decoupling. This might underpin the mechanism of TCH treatment.

Rutin has been proved to show neuronal protective effects against TBI and other diseases [32–34]. In our study, behavioral findings reflected by NSS resulted from TBI was improved by TCH, a new injection composed of troxerutin and hydrolysate.

Neurons as well as astrocytes labeled by GFAP antibody and endothelial cells labeled by vWF antibody are the NVU members [35]. Researchers nowadays consider these NVU components in pericontusional area as key factors for interventions, as
they could be effectively converted with proper treatment after traumatic injuries [36–38]. On the third day after TBI, we found that traumatic damages induced neuronal death, astrocytes increasing, and endothelial cells decreasing significantly in pericontusional area. All these changes were reversed by TCH significantly.

BBB is the basic component of NVU [7,39]. BBB breakdown is the core feature of TBI and the main cause of brain edema. In our study, both tight junction protein expression detected by Western blotting as well as Immunofluorescence and TEM findings indicated BBB dysfunction after TBI, which is similar to the research of Thal et al. [40] and Tao et al. [5]. Together with the findings in different brain cells, TCH treatment against BBB damage caused by TBI, indicating that TCH had NVU protection in multifaceted targets.

Two-parametric MRI (T2 weight and ASL) was chosen in this study to monitor the NVU function. The former is accepted to show the infarct volume of TBI [37]. Signal changes in T2 weight MRI are indicative of edema and BBB breakdown both in and around core contusional brain area [37,41]. The latter, ASL, a new measurement to determine the blood perfusion state, has been emphasized by Zhang et al. recently [27]. Demonstrations of T2WI and ASL of MRI, combined with vWF expression, showed the way of TCH treatment against TBI was through improving low rCBF.

We further explored the potential target of TCH treatment to maintain the normal cerebral perfusion after TBI. eNOS might be a possible candidate in consideration of the evidence in hypertension, diabetes, myocardial infarction, etc. [42], despite the lacking of the findings focusing on TBI. eNOS has a complex characteristic in pathogenesis of TBI and other cerebral vascular diseases. On one hand, uncoupled eNOS donates electrons to O₂ instead of L-arginine, producing superoxide (O₂⁻) rather than its usual product NO. O₂⁻ can react with NO producing peroxynitrate (reflected by 3-NT) which damaged BBB; on the other hand, coupled eNOS is an important source of NO of bioavailability, which can mitigate the increased vasoconstriction of artery after TBI [9]. In vitro model of cultured human umbilical vein endothelial cells, Ugusman et al. [42] have demonstrated that rutin promoted NO production by increasing eNOS gene, expression, eNOS protein synthesis, and eNOS activity. Based on our observation in in vivo model, TCH treatment not only raised NO level and maintain its bioavailability, but also decreased the

Figure 3. Nissl and GFAP, vWF immunofluorescence staining on the third day after TBI. (a) Representative images of neurons of the Sham, TBI and TBITCH group rats. (b) Representative images of astrocytes (GFAP-positive) of the Sham, TBI and TBITCH group rats. (c) Representative images of endothelial cells (vWF-positive) of the Sham, TBI and TBITCH group rats. (d) Representative of the numbers of cells of neurons, astrocytes and endothelial cells in the pericontusional area on the third day after TBI. Nuclei were counterstained with DAPI (blue), meanwhile, GFAP or vWF figures were merged with DAPI staining images. Values are means ± SEM. *p < .05, **p < .01, *** p < .001 vs. the TBI group.
expression of 3-NT. All these multifaceted effects were through modulating eNOS coupling/decoupling status, which might be mechanism of TCH treatment against TBI.

There are still some limitations in our study. First, we did not observe the changes of nNOS, and iNOS, both of which are isoforms of NOS. Till recently, the role nNOS and iNOS played after TBI is still far from

Figure 4. TEM, western blotting and immunofluorescence staining of BBB tight junction. (a) TEM showed that tight junctions were continuous and adjacent of endothelial cells was closely aligned in sham group rats. In contrast, tight junctions of TBI group rats were obviously damaged and adjacent was open widely. While, tight junctions of TBITCH group rats were attenuated this clearly. (b) Representative Claudin-5 and GAPDH expressions of the Sham, TBI and TBITCH group rats. (c) Comparison of Claudin-5 expression to GAPDH expression ratio between each group. (d) Representative images of BBB tight junction (Occludin-positive) of the Sham, TBI and TBITCH group rats. *p < .05, ***p < .001 vs. the TBI group.
clear, as a result, we should enlarge our study in the follow-up researches. Second, we chose third day as time point to make comparisons, the reason is that damages were found the most serious at this time point [27], we need to extend more time points to show the effectiveness of TCH treatment against TBI in future.

In summary, TCH treatment after TBI resulted in relieving neurological deficits, retaining of NVU integrity and function. Furthermore, the effectiveness of TCH against TBI was contributed to eNOS coupling/decoupling status regulation.

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**Disclosure statement**

All authors report no actual or potential conflicts of interest.
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