RESEARCH ARTICLE

Inhibiting endogenous tissue plasminogen activator enhanced neuronal apoptosis and axonal injury after traumatic brain injury

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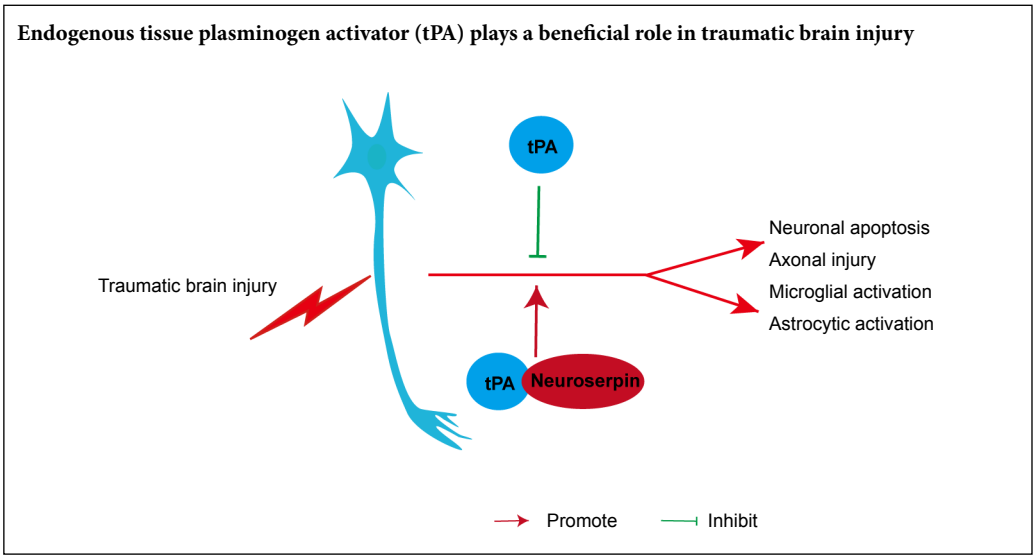
Graphical Abstract



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Abstract

Tissue plasminogen activator is usually used for the treatment of acute ischemic stroke, but the role of endogenous tissue plasminogen activator in traumatic brain injury has been rarely reported. A rat model of traumatic brain injury was established by weight-drop method. The tissue plasminogen activator inhibitor neuroserpin (5 μL, 0.25 mg/mL) was injected into the lateral ventricle. Neurological function was assessed by neurological severity score. Neuronal and axonal injuries were assessed by hematoxylin-eosin staining and Bielschowsky silver staining. Protein level of endogenous tissue plasminogen activator was analyzed by western blot assay. Apoptotic marker cleaved caspase-3, neuronal marker neurofilament light chain, astrocyte marker glial fibrillary acidic protein and microglial marker Iba-1 were analyzed by immunohistochemical staining. Apoptotic cell types were detected by immunofluorescence double labeling. Apoptotic cells in the damaged cortex were detected by terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP-biotin nick-end labeling staining. Degenerating neurons in the damaged cortex were detected by Fluoro-Jade B staining. Expression of tissue plasminogen activator was increased at 6 hours, and peaked at 3 days after traumatic brain injury. Neuronal apoptosis and axonal injury were detected after traumatic brain injury. Moreover, neuroserpin enhanced neuronal apoptosis, neuronal injury and axonal injury, and activated microglia and astrocytes. Neuroserpin further deteriorated neurobehavioral function in rats with traumatic brain injury. Our findings confirm that inhibition of endogenous tissue plasminogen activator aggravates neuronal apoptosis and axonal injury after traumatic brain injury, and activates microglia and astrocytes. This study was approved by the Biomedical Ethics Committee of Animal Experiments of Shaanxi Province of China in June 2015.

Key Words: *apoptosis; astrocytes; axonal injury; inflammation; microglia; nerve regeneration; neural regeneration; neuronal injury; neurons; neuroserpin; tissue plasminogen activator; traumatic brain injury*

Chinese Library Classification No. *R453; R363; R741*

Introduction

Traumatic brain injury (TBI) is a common and critical disease that most often affects young adults (Maa et al., 2008). It causes a great financial burden on families and society (Rudolfson et al., 2018). A survey in America showed that over 1.7 million people suffer from TBI every year and 5.7 million people are living with TBI-related complications (Roozenbeek et al., 2013; Peeters et al., 2017). Pathophysiology of TBI is divided into primary damage caused by mechanical force and secondary damage induced by primary damage, including glutamate dysfunction, inflammatory response, mitochondrial dysfunction and apoptosis (Maas et al., 2008; Johnson et al., 2013; Qin et al., 2015; Wang et al., 2019). All of these secondary effects contribute to neural injury or death, but the molecular and cellular mechanisms have not been completely elucidated.

Tissue plasminogen activator (tPA) is a common fibrinolytic drug for acute ischemic stroke (Nagai et al., 2017; Lee et al., 2018; Saber et al., 2019; Simon et al., 2019). It is synthesized and secreted not only by endothelial cells, but by all cells in the central nervous system, including neurons, astrocytes, microglia and oligodendrocytes (Yepes et al., 2009; Vivien and Ali, 2012). Besides its feature as a plasminogen activator, tPA plays an extensive role in the central nervous system and takes part in many physiological and pathological processes (Melchor and Strickland, 2005; Yepes et al., 2009). However, the role of tPA in central nervous system injury is controversial. On one hand, tPA has been shown to play a harmful role in ischemic brain injury, even though tPA is a common treatment of acute stroke patients (Yepes et al., 2009). On the other hand, exogenous tPA has been shown to play a neuroprotective role in TBI (Stein et al., 2009; Meng et al., 2014). However, the role of endogenous tPA in TBI is not clear and has been rarely examined. We hypothesized that endogenous tPA may play a protective role in TBI. In this study, we detected the expression of endogenous tPA after TBI and explored the effect of endogenous tPA on pathological changes in a rat model of TBI.

Materials and Methods

Animals

A total of 84 adult male Sprague-Dawley rats weighing 250–300 g, aged 8–10 weeks, were provided by the Experimental Animal Center of Xi'an Jiaotong University, China (SYXK [Shaan] 2015-002). The rats were raised in a room with a constant temperature of 22°C, under a 12-hour light/dark cycle. Rats were housed five per cage. Food and water was available ad libitum. All protocols and procedures were approved by the Biomedical Ethics Committee of Animal Experiments of Shaanxi Province in China in June 2015 and complied with the principles and procedures of the Guidance Suggestions for the Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology of PRC (The Ministry of Science and Technology of the People's Republic of China 1988). Rats were randomly divided into a sham group (n = 18), TBI 6-hour group (n = 12), TBI 1-day group (n = 12), TBI 3-day group (n = 18), TBI 7-day group (n = 12), vehicle group (n = 6; treatment similar to the TBI 3-day group but received sterile water), and neuroserpin group (n = 6).

Animal model establishment of TBI

The TBI model was established as previously described (Feeney et al., 1981). Briefly, rats were anesthetized with 1% pentobarbital sodium (35 mg/kg). Core temperature was maintained at 37.5°C measured via rectal temperature probe. A 5-mm diameter craniotomy was performed in the left parietal bone using a high-speed electric drill (Xiyi, Tianjin, China). The injury was performed by dropping a 40-g weight from a height of 20 cm on a 4.5-mm diameter impact tip. After injury, the bone flap was replaced and the scalp was sutured. The sham group received anesthesia and a craniotomy without the dropping injury.

Stereotactic injection of neuroserpin

Neuroserpin (a selective inhibitor of tPA; Sino Biological Inc., Beijing, China) was diluted to 0.25 mg/mL with sterile water. Neuroserpin (5 μL) was injected into the lateral ventricle using a Hamilton microsyringe (Gaoge, Shanghai, China) under the guidance of a stereotaxic instrument (Kent Scientific Co., Torrington, CT, USA) within 20 minutes before TBI. After each rat was anesthetized, the scalp was incised and the bregma was exposed, then a burr hole with a diameter of less than 1.5 mm was formed. The stereotaxic coordinates were 0.8 mm posterior, 1.5 mm lateral to the bregma, and 4 mm ventral to the surface of the skull (Paxinos and Watson, 2007). The needle was held in place for 5 minutes after injection (Sherchan et al., 2016). The craniotomy was sealed with bone wax, and the scalp was closed with sutures. Core temperature was maintained at 37.5°C during the operation. Rats in the vehicle group received the same operation with 5 μL sterile water.

Neurological behavioral assessment

Neurological assessments were performed as previously described (Germanò et al., 1994). Neurological functions were assessed by neurological severity score and were graded using a scale of 0–18 (normal, 0; mild injury, 1–6; moderate injury, 7–12; severe injury, 13–18). Neurological severity scoring was performed in rats of the sham group, the TBI 3-day group, the vehicle group and the neuroserpin group. An investigator blinded to the groups conducted all assessments and analysis of neurological severity scores.

Hematoxylin-eosin staining and Bielschowsky silver staining

The rats were euthanized with an intraperitoneal injection of 5% pentobarbital sodium (100 mg/kg) and perfused with normal saline, and 4% paraformaldehyde. The brain was removed, fixed, embedded, and cut into sections (5 μm) in the coronal plane. Hematoxylin-eosin staining and silver staining were used to assess the neuronal and axonal injury. Tissue sections were stained with hematoxylin and eosin,

followed by dehydration, hyalinization and mounting.

Bielschowsky silver staining was performed as previously described (Ng et al., 1994; Huang et al., 2018). First, brain sections were rehydrated in a graded ethanol series. Second, sections were immersed in a 4% (w/v) silver nitrate solution for 30–60 minutes at 37°C in the dark and were washed three times. Third, sections were deoxidized with 10% (v/v) formaldehyde and were washed three times. Fourth, sections were immersed in ammoniacal silver solution for 10 minutes and in 4% (w/v) formalin for 10 minutes. Finally, the sections were fixed in 5% (w/v) sodium thiosulfate for 5 minutes. The sections were dried in the open air, cleared in xylene, and covered by coverslip. Slides were scanned by a scanning microscopy imaging system (Leica SCN400, Solms, Germany) at 200× magnification. Three sections per animal were processed for hematoxylin-eosin staining and silver staining. Images were acquired at three defined areas from the peri-lesion cortex. Microscopic observations were performed by an investigator blinded to the experimental groups.

Western blot assay

In each group, rats were anesthetized with 1% pentobarbital sodium (35 mg/kg; Merck, Darmstadt, Germany) and the hemisphere ipsilateral to the injury was immediately separated and stored in liquid nitrogen until processing. Brain tissue from the peri-lesion region was collected and total protein was purified using radioimmunoprecipitation assay (Sigma, Darmstadt, Germany). Protein samples (10 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide electrophoresis gels, and were electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Darmstadt, Germany). The membranes were blocked with 5% skim milk powder and incubated with rabbit anti-tPA antibody (1:1000; Abcam, Cambridge, UK) at 4°C overnight. Membranes were washed, followed by incubation with goat anti-rabbit IgG-horseradish peroxidase secondary antibody (1:10,000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour at room temperature. Horseradish peroxidase was detected using an enhanced chemiluminescence substrate. β-Actin (1:1000; Santa, Dallas, TX, USA) was used as an internal control to ensure equivalent amounts of protein. Results were analyzed by optical density using ImageJ software (version 1.29x: NIH, Bethesda, MD, USA).

Immunohistochemical staining

Immunohistochemical staining was used to detect apoptosis (cleaved caspase-3), axonal injury (neurofilament protein light chain), activated astrocytes (glial fibrillary acidic protein, GFAP) and activated microglia (ionized calcium binding adapter molecule 1, Iba-1) from the peri-lesion cortex after TBI. Immunohistochemistry was performed as described in a previous protocol (Zhao et al., 2016). Sections were dewaxed, hydrated, and incubated in 3% H_2O_2 deionized water for 20 minutes to block endogenous peroxidase, followed by antigen retrieval. The sections were placed in 0.01 M citrate buffer and heated in a microwave oven over medium-high heat for 8 minutes and over medium-low heat for 12 minutes. Non-specific protein binding was blocked by normal goat serum at room temperature for 20 minutes, then sections were incubated with primary antibodies, including rabbit monoclonal anti-cleaved-caspase-3 (1:1000; Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal anti-neurofilament protein light chain (1:1000; Proteintech, Rosemont, IL, USA), mouse monoclonal anti-GFAP (1:400; Cell Signaling Technology) and rabbit monoclonal anti-Iba-1 (1:400; Wako, Chuo-ku, Osaka, Japan) at 4°C overnight. The sections were then incubated with goat anti-rabbit or anti-mouse IgG-biotin (1:300; ZSGB-BIO, Beijing, China) at room temperature for 1 hour and streptavidin-horseradish peroxidase (ZSGB-BIO) for 30 minutes at 37°C. Diaminobenzidine was used as the chromogen, and hematoxylin was used as the counterstain. Finally, the sections were dehydrated in ethanol, cleared in xylene, and covered with coverslips. The results were observed under a microscope (OX41; Olympus, Tokyo, Japan) at 100× and 400× magnification or scanned with a scanning microscopy imaging system at 200× magnification. Images were acquired from the peri-lesion cortex. The number and density of positive cells were counted at three defined areas using Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). Immunohistochemical scores were calculated by multiplying quantity scores and intensity scores. Quantity scores were: 0, no staining; 1, 1–10% positive cells; 2, 11–50%; 3, 51–80%; and 4, 81–100%. Intensity scores were: 0, negative; 1, weak; 2, moderate; and 3, strong. The average was used for statistical analysis. Microscopic observations were performed by an investigator blinded to the experimental groups (Hilton et al., 2008; Zhao et al., 2016).

Immunofluorescence staining

Immunofluorescent double labeling was used to detect apoptotic cell types. An apoptotic marker (cleaved caspase-3) was combined with a neuronal marker (neuronal nuclear antigen, NeuN), astrocytic marker (GFAP) and microglial marker (Iba-1). The brain tissue processing methods were the same as those described above. Sections were incubated with primary antibodies, including rabbit monoclonal anti-cleaved-caspase-3 (1:200; Cell Signaling Technology), mouse monoclonal anti-rat NeuN (1:200; Millipore), mouse monoclonal anti-rat GFAP (1:200; Cell Signaling Technology), and goat polyclonal anti-Iba-1 (1:200; Novus, Centennial, USA) at 4°C overnight. After washes in phosphate buffered saline, sections were incubated with donkey anti-rabbit, donkey anti-mouse, or donkey anti-goat secondary antibodies labeled with Alexa-Fluor 488 and Alexa-Fluor 647 (1:200; Jackson ImmunoResearch) for 1 hour at room temperature. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/mL; Roche, Basel, Switzerland) for 10 minutes. Stained sections were covered with coverslips, and final images were acquired from the peri-lesion cortex using a fluorescence microscope (Nikon Eclipse Ti-S, Tokyo, Japan).

TUNEL assay

A DeadEnd™ Fluorometric Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP-biotin nick-end la-

beling (TUNEL) System (Promega, Madison, WI, USA) was used to confirm apoptosis in the rat cortex. TUNEL was performed according to the manufacturer's instructions. Briefly, 100 μL of 20 μg/mL proteinase K was added to each slide to cover the tissue sections, and the sections were incubated for 10 minutes at room temperature. Then, the nuclei were stained with DAPI for 10 minutes. Finally, the sections were examined from the peri-lesion cortex with the fluorescence microscope (Nikon Eclipse Ti-S). Data were expressed as the ratio of TUNEL to total nuclei.

Flouro-Jade B staining

Flouro-Jade B staining was performed by removing paraffin in a series of xylene, immersed twice in 100% ethanol and 1% sodium hydroxide for 90 seconds, and then 70% ethanol for 30 seconds. Sections were washed in 0.06% potassium permanganate and distilled water. The sections were immersed in Flouro-Jade B solution (Histo-Chem Inc., Jefferson, AR, USA) for 30 minutes at room temperature (Simon et al., 2018). Images were acquired from the peri-lesion cortex with a fluorescence microscope (Nikon Eclipse Ti-S).

Statistical analysis

All results are presented as the mean ± SD. All statistical analyses were performed with SPSS 18.0 (SPSS, Chicago, IL, USA). One-way analysis of variance was used to compare numerical data in more than two groups, followed by least significant difference *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Neuroserpin deteriorates neurological behavior of TBI rats

All rats subjected to TBI exhibited dramatic neurological deficits, and rats in the neuroserpin group exhibited moderate to severe injury. Neurological severity score was significantly higher in the TBI 3-day group (12.50 ± 0.92, $n = 6$) than in the sham group (0, $n = 6$) ($P < 0.0001$). There was no significant difference in neurological severity score between the TBI 3-day group (12.50 ± 0.92, $n = 6$) and the vehicle group (12.83 ± 0.65, $n = 6$) ($P = 0.77$). However, neurological severity score was significantly higher in the neuroserpin group (14.83 ± 0.31, $n = 6$) than in the TBI 3-day group (12.50 ± 0.92, $n = 6$) ($P < 0.05$), which suggests a negative role of neuroserpin in neurological behavior (**Figure 1**).

Histological changes in the brain tissue of TBI model rats

Hematoxylin-eosin staining and Bielschowsky silver staining showed pathological changes around the cerebral cortex lesions in rats, such as nuclear pyknosis, nuclear margination, colliquative necrosis, axonal varicosities, axonal swelling and axonal disconnection (**Figure 2B** and **D**). The normal brain tissues were regularly intact and arranged (**Figure 2A** and **C**).

Expression of tPA in the cortex of TBI rats

The expression of tPA was detected by western blot assay.

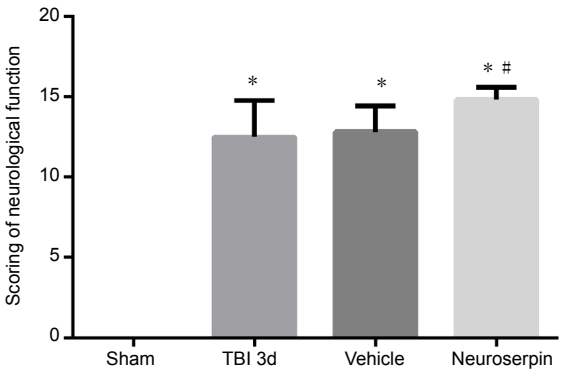

Figure 1 Effects of neuroserpin on neurological behavior of TBI rats. Data are presented as the mean ± SD ($n = 6$). *$P < 0.0001$, *vs.* sham group; #$P < 0.05$, *vs.* TBI 3d group (one-way analysis of variance followed by least significant difference test). TBI: Traumatic brain injury.

The expression level of tPA increased 6 hours after TBI (0.71 ± 0.01, $n = 6$) ($P < 0.0001$). The high expression level continued for 3 days (0.87 ± 0.01, $n = 6$) ($P < 0.0001$) and decreased to a normal level 7 days (0.64 ± 0.06, $n = 6$) after TBI ($P = 0.38$; **Figure 3**).

Expression and cellular localization of cleaved caspase-3 in the cortex of TBI rats

Apoptosis occurs following TBI (Raghupathi et al., 2000; Chen et al., 2018; Wu et al., 2018). Thus, immunochemistry and immunofluorescence were used to detect the expression and the cellular localization of cleaved caspase-3, an important molecule in the apoptosis pathway (Glushakova et al., 2018). Cleaved caspase-3 was highly expressed in the cortex 6 hours to 7 days after TBI ($P < 0.0001$ for all time points; **Figure 4A** and **C**, **Tables 1** and **2**), and was coexpressed with NeuN but not with Iba-1 or GFAP (**Figure 4B**), which indicates that apoptosis after TBI affects neurons but not microglia or astrocytes.

Neuroserpin enhances apoptosis induced by TBI and exacerbates neuronal and axonal injury after TBI

Neuroserpin is a serine protease inhibitor that inhibits tPA (Sashindranath et al., 2012; Reumann et al., 2017). TUNEL results showed that the number of apoptotic cells in the TBI 3-day group (31.50 ± 1.26, $n = 6$) was significantly increased

Table 1 Quantity percentage (scores) of cleaved caspase-3 positive cells

	Groups				
Number	Sham	TBI 6 h	TBI 1 d	TBI 3 d	TBI 7 d
1	0	53 (3)	40 (2)	28 (2)	10 (1)
2	0	60 (3)	38 (2)	30 (2)	9 (1)
3	0	56 (3)	43 (2)	25 (2)	9 (1)
4	0	57 (3)	35 (2)	35 (2)	8 (1)
5	0	48 (2)	33 (2)	53 (3)	10 (1)
6	0	43 (2)	42 (2)	52 (3)	6 (1)

TBI: Traumatic brain injury.

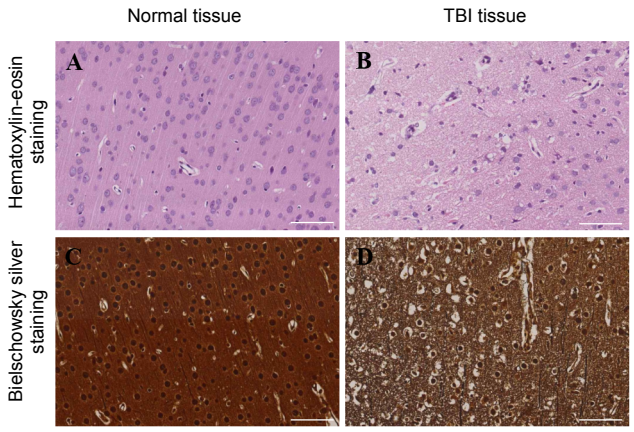

Figure 2 Histological assessment of TBI model in rats.
Obvious pathological changes around the cerebral cortex lesions of the TBI rats were nuclear pyknosis, nuclear margination, colliquative necrosis, axonal varicosities, axonal swelling and axonal disconnection. Scale bars: 73 μm. TBI: Traumatic brain injury.

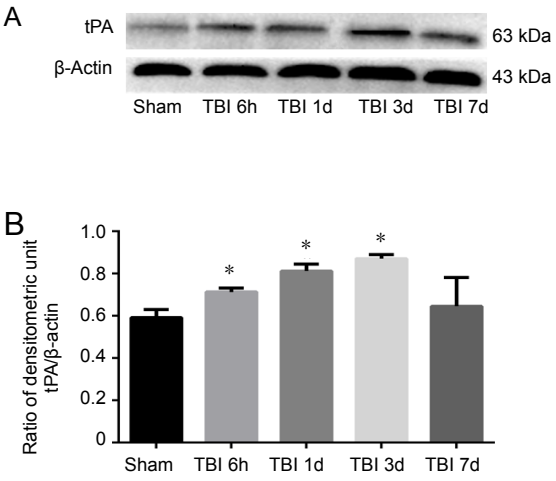

Figure 3 Protein expression of tPA in the peri-lesion cortex detected by western blot assay.
(A) Expression of tPA increased at 6 hours, 1 day and 3 days after TBI. (B) Quantitative results of tPA expression. Data are presented as the mean ± SD (n = 6). *P < 0.0001, *vs.* sham group (one-way analysis of variance followed by least significant difference test). TBI: Traumatic brain injury; tPA: tissue plasminogen activator.

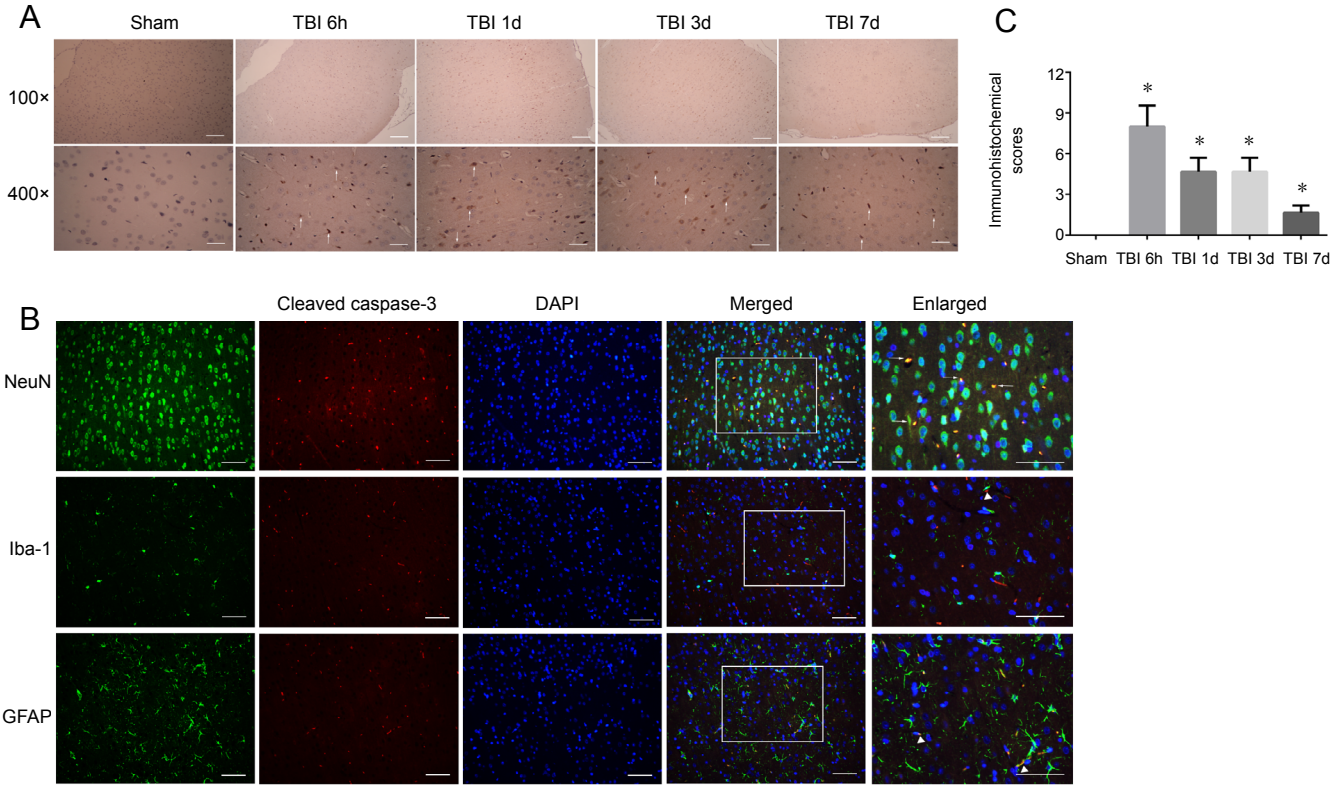

Figure 4 Immunoreactivity of cleaved caspase-3, NeuN, Iba-1 and GFAP in the peri-lesion cortex detected after TBI.
(A) Immunoreactivity of cleaved caspase-3 in cortex after TBI. White arrowheads indicate positive cells. (B) Cellular localization of cleaved caspase-3, NeuN, Iba-1 and GFAP in TBI 3d group. Cells were stained with NeuN, Iba-1 and GFAP (green), cleaved caspase-3 (red) and DAPI (blue). White arrowheads indicate immunopositive cells and white triangles indicate immunonegative cells. Scale bars: 100 μm in A upper, 25 μm in A lower, 50 μm in B. (C) Quantity scores of cleaved caspase-3 expression. Data are presented as the mean ± SD (n = 6). *P < 0.0001, *vs.* sham group (one-way analysis of variance followed by least significant difference test). GFAP: Glial fibrillary acidic protein; TBI: traumatic brain injury.

Table 2 Intensity scores of cleaved caspase-3 positive cells

	Groups				
Number	Sham	TBI 6 h	TBI 1 d	TBI 3 d	TBI 7 d
1	0	3	3	2	1
2	0	3	3	2	2
3	0	3	2	2	1
4	0	3	2	2	2
5	0	3	2	3	2
6	0	3	2	3	2

TBI: Traumatic brain injury.

compared with the sham group (0, $n = 6$) ($P < 0.0001$). Furthermore, there was a greater number of apoptotic cells in the neuroserpin group (49.33 ± 1.86, $n = 6$) than in the TBI 3-day group (31.50 ± 1.26, $n = 6$) ($P < 0.0001$). Combined with results that cleaved caspase-3 was localized to neurons, these findings suggest that neuroserpin exacerbated neuronal apoptosis (**Figure 5A** and **D**).

The effect of neuroserpin on neuronal injury was investigated next. Flouro-Jade B staining showed that injured neurons were obvious in the cortex around the lesion (27.17 ± 1.25, $n = 6$) ($P < 0.0001$) and the number of injured cells of the neuroserpin group (41.67 ± 0.88, $n = 6$) was greater than that of the TBI 3-day group (27.17 ± 1.25, $n = 6$) ($P < 0.0001$; **Figure 5B** and **E**). This indicated that neuroserpin exacerbated neuronal injury.

Neurofilament protein light chain is used to mark axonal injury (Rohrer et al., 2016; Bagnato et al., 2017; Korley et al., 2018). In the sham group, neurofilament protein light chain staining displayed normal axon morphology. However, in the TBI 3-day group, there were some signs of axonal injury, such as axonal fracture, tortuousness and retraction balls. In the neuroserpin group, there were more injured axons than that in the TBI 3-day group (**Figure 5C**). This indicated that neuroserpin exacerbated axonal injury.

Neuroserpin increases microglial and astrocytic activation in the cortex of TBI rats

Microglial and astrocyte activation in the cortex were detected by immunohistochemistry after TBI. The number of activated microglia and astrocytes increased, their somata were enlarged and their processes were decreased and thickened significantly in the TBI 3-day group (5.67 ± 0.33, $n = 6$) (**Tables 3** and **4**) compared with the sham group (3.33 ± 0.42, $n = 6$) ($P = 0.0015$). The number of activated astrocytes increased, their somata were enlarged and their processes were extending significantly in the TBI 3-day group (4.17 ± 0.40, $n = 6$) (**Tables 5** and **6**) compared with the sham group (1.17 ± 0.17, $n = 6$) ($P < 0.0001$). By intracerebroventricular injection of neuroserpin, microglia and astrocytes were significantly activated in the neuroserpin group (8.50 ± 0.92, $n = 6$ and 9.17 ± 0.60, $n = 6$) compared with the TBI 3-day group (5.67 ± 0.33, $n = 6$, and 4.17 ± 0.40, $n = 6$) ($P = 0.0161$ and $P < 0.0001$) and the sham group (3.33 ± 0.42, $n = 6$ and 1.17 ± 0.17, $n = 6$) ($P = 0.0005$ and $P < 0.0001$) (**Figure 6**).

Table 3 Quantity percentage (scores) of Iba-1 positive cells

	Groups			
Number	Sham	TBI 3 d	Vehicle	Neuroserpin
1	12 (2)	38 (2)	35 (2)	65 (3)
2	14 (2)	43 (2)	40 (2)	61 (3)
3	15 (2)	35 (2)	37 (2)	72 (3)
4	13 (2)	41 (2)	35 (2)	58 (3)
5	10 (1)	48 (2)	31 (2)	56 (3)
6	9 (1)	33 (2)	37 (2)	63 (3)

TBI: Traumatic brain injury.

Table 4 Intensity scores of Iba-1 positive cells

	Groups			
Number	Sham	TBI 3 d	Vehicle	Neuroserpin
1	2	3	3	3
2	2	3	3	3
3	2	3	3	3
4	2	3	3	2
5	1	3	2	2
6	1	2	2	4

TBI: Traumatic brain injury.

Table 5 Quantity percentage (scores) of glial fibrillary acidic protein positive cells

	Groups			
Number	Sham	TBI 3 d	Vehicle	Neuroserpin
1	10 (1)	25 (2)	56 (3)	55 (3)
2	9 (1)	30 (2)	59 (3)	60 (3)
3	9 (1)	31 (2)	45 (2)	65 (3)
4	8 (1)	38 (2)	38 (2)	83 (4)
5	15 (2)	37 (2)	30 (2)	85 (4)
6	10 (1)	54 (3)	33 (2)	81 (4)

TBI: Traumatic brain injury.

Table 6 Intensity scores of glial fibrillary acidic protein positive cells

	Groups			
Number	Sham	TBI 3 d	Vehicle	Neuroserpin
1	1	2	2	3
2	1	2	2	3
3	1	2	2	3
4	1	2	2	3
5	1	3	2	2
6	1	1	2	2

TBI: Traumatic brain injury.

Discussion

This study investigated the expression of tPA in cortex of rats subjected to TBI and the role of tPA in neuronal and axonal injury by using its inhibitor, neuroserpin. Expression of tPA was increased at 6 hours to 3 days after TBI, and decreased

at 7 days. Apoptosis in the TBI groups was remarkably enhanced compared with that in the sham group. Inhibiting tPA noticeably exacerbated neuronal apoptosis and axonal injury in the neuroserpin group compared with the TBI group. Neuroserpin also enhanced activation of microglia and astrocytes and had a negative effect on neurological behavior after TBI.

The role of tPA in the brain parenchyma is highly controversial. There is evidence that tPA plays both beneficial and detrimental roles in central nervous system injury. There is no doubt that tPA has a wide application in thrombolytic therapy after cerebral ischemia (Hacke et al., 2008). Recombinant tPA promotes cell survival in cerebral cortical neurons, and mice overexpressing tPA have less ischemic damage and better neurological outcomes than wild-type mice (Wu et al., 2012). tPA has also been shown to promote neurite elongation and neuronal survival in cultured neurons (Lee et al., 2007). In contrast, harmful effects of tPA on the ischemic brain, which were plasminogen-independent, have been widely reported. tPA interacts with the N-methyl-D-aspartate receptor, low-density lipoprotein receptor-related protein and annexin-II in glial cells and neurons, causing cerebral edema and cell death (Nicole et al., 2001; Siao and Tsirka, 2002; Yepes et al., 2003). Results from this study showed that inhibition of tPA exacerbated neuronal injury and neurological severity score, indicating a protective role of tPA in TBI, which is in part consistent with the notion of tPA's positive effect on ischemic brain injury. In addition, we found that endogenous inhibition of tPA exacerbated axonal injury, which has rarely been shown (Meng et al., 2014; Xia et al., 2018).

Currently there are different opinions about the relationship between tPA and neuronal apoptosis. Some previous studies showed that tPA promoted neuronal apoptosis in mouse cerebral ischemic model (Liu et al., 2004), and promoted microtubule destabilization and apoptosis via Erk1/2 in primary hippocampal neurons (Medina et al., 2005). In contrast, other previous studies showed that tPA protected cultured cortical neurons against apoptosis by reducing caspase-3 activation (Liot et al., 2006) and demonstrated anti-apoptotic effects to promote neuronal survival by activating epidermal growth factor receptor in mouse primary cortical neuron cultures (Bertrand et al., 2015). Here, we detected the high expression of tPA and enhancement of apoptosis 6 hours to 3 days after TBI. Neuroserpin, an inhibitor of tPA, promoted neuronal apoptosis in the cortex after TBI. These findings indicate that apoptosis is regulated, at least in part, by tPA. Additionally, the enhanced activation of microglia and astrocytes induced by tPA inhibition in this study was consistent with a previous study that showed low-dose tPA reduced microglial and astrocyte activation (Fan et al., 2017). Therefore, enhanced activation of microglia and astrocytes by tPA may contribute to the deterioration of neuronal injury and neurological behavior.

There are two shortcomings of this study. First, we detected the role of endogenous tPA in TBI using an indirect method in which we used neuroserpin to inhibit tPA, but did not directly examine the role of tPA using knock out or knock down. Second, we did not explore mechanisms underlying the effect of endogenous tPA inhibition on neuronal apoptosis and axonal injury.

In conclusion, endogenous tPA played a beneficial role in a rat model of TBI. The findings provide experimental evidence for the clinical effect of endogenous tPA on TBI, suggesting that it inhibits neuronal apoptosis, neuronal injury, axonal injury and activation of microglia and astrocytes.

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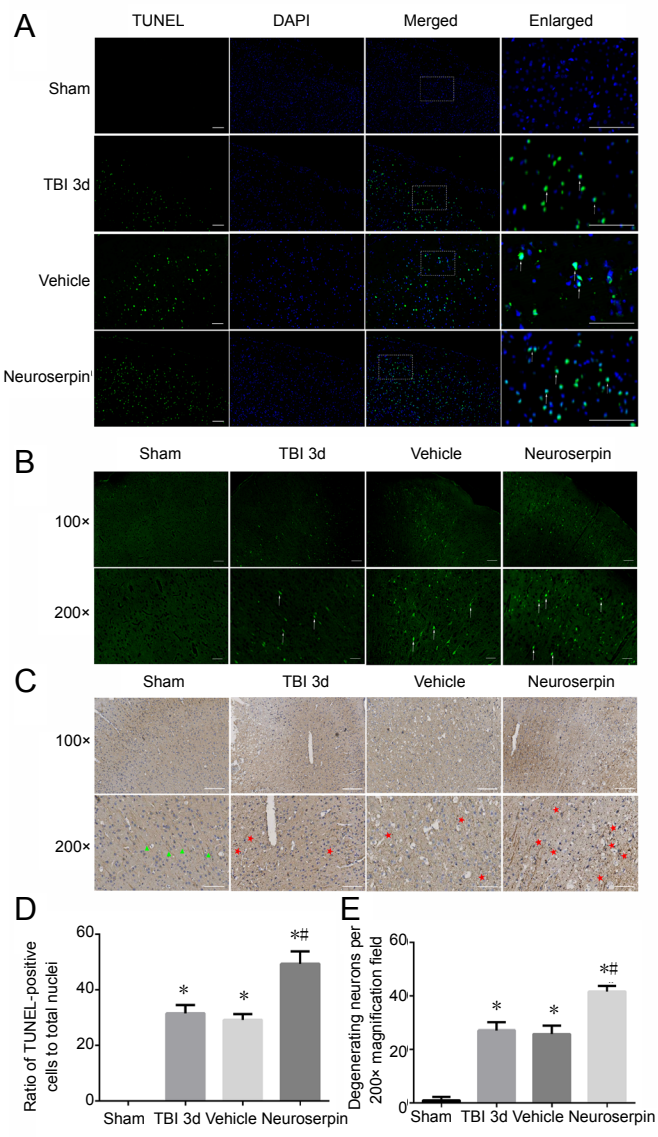

Figure 5 Neuroserpin exacerbates neuronal apoptosis, neuronal injury and axonal injury in the peri-lesion cortex after TBI.
(A) TUNEL staining was used to detect neuronal apoptosis. White arrowheads indicate apoptotic cells. (B) Flouro-Jade B staining indicated degenerating neurons. White arrowheads indicate necrotic cells. (C) Neurofilament protein light chain staining was used to detect injured axons. Green triangles indicate normal axons and red stars indicate injured axons. Scale bars: 50 μm in A, B; 145 μm in C upper, 73 μm in C lower. (D, E) Quantitative results of ratio of TUNEL-positive cells and the number of degenerating neurons. Data are presented as the mean ± SD (n = 6). *P < 0.0001, *vs.* the sham group; #P < 0.0001, *vs.* the TBI 3d group (one-way analysis of variance followed by least significant difference test). TBI: Traumatic brain injury; TUNEL: terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP-biotin nick-end labeling.

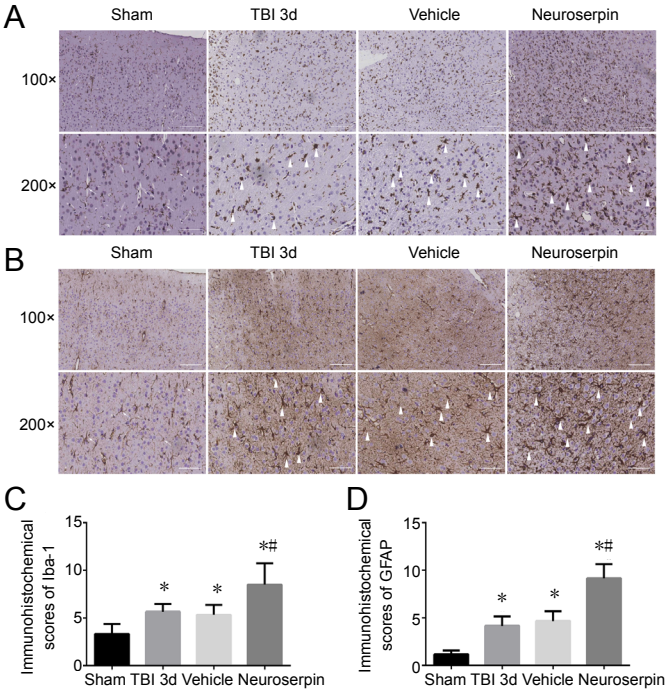

Figure 6 Neuroserpin increases microglial and astrocytic activation in the peri-lesion cortex of TBI rats.
(A) Microglia were stained with Iba-1. White arrowheads indicate normal microglia and white triangles indicate activated microglia. (B) Astrocytes were stained with GFAP. White arrowheads indicate normal astrocytes and white triangles indicate activated astrocytes. Scale bars: 145 μm in upper, 73 μm in lower. (C, D) Quantity scores of Iba-1 and GFAP. Data are presented as the mean ± SD (n = 6). *P < 0.05, *vs.* the sham group; #P = 0.0161, *vs.* the TBI 3d group (one-way analysis of variance followed by least significant difference test). GFAP: Glial fibrillary acidic protein; TBI: traumatic brain injury.

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