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ORIGINAL PAPER

Tetramethylpyrazine Nitrone Reduces Oxidative Stress to Alleviate Cerebral Vasospasm in Experimental Subarachnoid Hemorrhage Models

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Abstract

Cerebral vasospasm is one of the deleterious complications after subarachnoid hemorrhage (SAH), leading to delayed cerebral ischemia and permanent neurological deficits or even death. Free radicals and oxidative stress are considered as crucial causes contributing to cerebral vasospasm and brain damage after SAH. Tetramethylpyrazine nitrone (TBN), a derivative of the clinically used anti-stroke drug tetramethylpyrazine armed with a powerful free radical scavenging nitrone moiety, has been reported to prevent brain damage from ischemic stroke. The present study aimed to investigate the effects of TBN on vasospasm and brain damage after SAH. Two experimental SAH models were used, a rat model by endovascular perforation and a rabbit model by intracisternal injection of autologous blood. The effects of TBN on SAH were evaluated assessing basilar artery spasm, neuronal apoptosis, and neurological deficits. TBN treatment significantly attenuated vasospasm, improved neurological behavior functions and reduced the number of apoptotic neurons in both the SAH rats and rabbits. Mechanistically, TBN suppressed the increase in 3-nitrotyrosine and 8-hydroxy-2-deoxyguanosine immuno-positive cells in the cortex of SAH rat brain. Western blot analyses indicated that TBN effectively reversed the altered expression of Bcl-2, Bax and cytochrome C, and up-regulated nuclear factor erythroid-derived 2-like 2 (Nrf2) and hemeoxygenase-1 (HO-1) protein expressions. In the in vitro studies, TBN inhibited H_2O_2-induced bEnd.3 cell apoptosis and reduced ROS generation. Additionally, TBN alleviated the contraction of rat basilar artery rings induced by H_2O_2 ex vivo. In conclusion, TBN ameliorated SAH-induced cerebral vasospasm and neuronal damage. These effects of TBN may be attributed to its anti-oxidative stress effect and up-regulation of Nrf2/HO-1.

Keywords Subarachnoid hemorrhage · Cerebral vasospasm · Tetramethylpyrazine nitrone · Anti-oxidative stress · Nrf2 · HO-1

Introduction

Subarachnoid hemorrhage (SAH) is a disastrous subtype of stroke accounting for 5–10% of all strokes (Macdonald et al. 2013). Approximately 10/100,000 people suffer from an aneurismal SAH each year (Chen et al. 2014a). Cerebral vasospasm is the most serious complication after SAH and thought to be the main factor that leads to high mortality and morbidity. Despite the recent progress in microsurgical and endovascular surgical techniques, the outcome of patients who suffer a SAH remains unsatisfactory (O'Neill et al. 2017). Traditional medical treatment for SAH, including triple H (hypervolemia, hemodilution, and hypertension) therapy and oral nimodipine prophylaxis, was reported to have a poor efficacy in improving cerebral blood flow and

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clinical outcomes and have systemic hypotension adverse event (Al-Mufti et al. 2017; Hanggi et al. 2017). Recently, emerging medical strategies including neuroprotective and vasoactive agents were extensively investigated in SAH animal experiments and clinical research (Li et al. 2019).

The role of free radical generation and oxidative stress in the pathogenesis of cerebral vasospasm has been demonstrated by numerous clinical (Huang et al. 2018) and experimental studies (Aladag et al. 2017). There are several sources for the excessive free radicals generation in SAH, including mitochondrial oxidative stress, oxyhemoglobin free radical generation, enzymatic sources of free radicals, and disrupted anti-oxidant protection (Yang et al. 2017). Cerebral vasospasm is commonly induced by the presence of blood clot, especially oxyhemoglobin in the subarachnoid space. The oxyhemoglobin released into the cerebrospinal fluid (CSF) following SAH is a major producer of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Luo et al. 2011). Excessive generation of free radicals following SAH resulted in neuronal apoptosis and the altered contractile response of cerebral vessels (Li et al. 2016b). Therefore, an anti-oxidative therapeutic strategy using free radical scavengers is a reasonable approach for the treatment of SAH.

Tetramethylpyrazine, an active ingredient of the Chinese herbal medicine *Chuanxiong*, could alleviate cerebral vasospasm and neuronal apoptosis in rats after SAH (Gao et al. 2008; Shao et al. 2018). Two-[[(1, 1-dimethylethyl) oxidoimino]-methyl]-3, 5, 6-trimethylpyrazine (tetramethylpyrazine nitrone, TBN) is a novel nitrone derivative of tetramethylpyrazine and a potent free radical scavenger with multi-functional neuroprotective effects. In our previous studies, TBN effectively scavenged free radicals such as $O_2^{\cdot-}$, $^{\cdot}OH$, $ONOO^-$ in vitro (Zhang et al. 2018). TBN exerted promising therapeutic potential in the experimental models of stroke (Sun et al. 2008, 2012), Parkinson's disease (Guo et al. 2014), traumatic brain injury (Zhang et al. 2016b), as well as chronic cerebral hypoperfusion (Zhang et al. 2017a), attributing to its strong free radical scavenging effect, reducing oxidative damage, inhibiting Ca^{2+} overload, and preventing neuronal apoptosis properties. However, the therapeutic effect of TBN on models of SAH is still unknown. In this study, we investigated the protective effect of TBN against SAH and explored its underlying mechanism of action.

Materials and Methods

Animals

A total of 40 male Sprague–Dawley rats (300–350 g, specific pathogen free animals) and 20 male New Zealand rabbits (2.0–3.0 kg, clean-grade animals) were purchased from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). All procedures were approved by the experimental animal care and use of Jinan University (Guangzhou, China). All rabbit experiments were performed in accordance with the guidelines and regulations of Laboratory animal center of Guangzhou University of Chinese Medicine (Guangzhou, China). The animals were maintained on a 12-h light/dark cycle under controlled temperature and humidity conditions and conformed to internationally accept ethical standards (Guide for the Care and Use of Laboratory Animals. US NIH Publication 86-23, revised 1985). All efforts were made to minimize the numbers of animals used and ensure minimal suffering.

Rat Endovascular Perforation SAH Model

Rats were randomly divided into three groups. (1) In the TBN group, rats were treated via tail vein injection of TBN (60 mg/kg) at 3 h and 6 h after surgery and then twice daily with a 6 h interval at 9:00 AM and 15:00 PM for a total of seven consecutive days; (2) in the vehicle group, rats were treated via tail vein injection of equal volume of saline; (3) in the sham group, rats were administrated with equal volume of saline.

The rat subarachnoid hemorrhage model was performed by endovascular perforation as described in previous study (Park et al. 2004). Briefly, rats were anesthetized with 2–3% isoflurane by animal anesthesia ventilator system (RWD Life Science, Shenzhen, China). After anesthesia, the right carotid artery and its branches were exposed. The right external carotid artery was transected distally and reflected caudally in line with the internal carotid artery (ICA). A blunted 4-0 monofilament nylon suture was placed in the external carotid artery and advanced through the ICA until resistance was felt, approximately 18–20 mm from the common carotid artery bifurcation. The suture was further advanced for approximately 3 mm to perforate the ICA near its intracranial bifurcation. The suture was withdrawn after approximately 15 s. Rats in the sham surgery groups underwent the procedures except for the suture perforation. After surgical procedures, animals were transferred to a temperature and humidity-controlled chamber.

Rabbit Cisterna Magna Single-Injection SAH Model

Rabbits were randomly divided into three groups. (1) In the TBN group, rabbits were treated via ear vein injection of TBN (30 mg/kg) at 3 h and 6 h after surgery and then twice daily with a 6 h interval at 9:00 AM and 15:00 PM for a total of three consecutive days; (2) in the vehicle group, rabbits were treated via ear vein injection of equal volume of saline; (3) in the sham group, rabbits were administrated with equal volume of saline.

The single blood injection rabbit SAH model was preformed according to a previously described procedure (Duan et al. 2016). Rabbits were anesthetized with sodium pentobarbital (250 mg/kg i.v.) and were then placed on a heated pad to keep the body temperature at 37 °C during the experimental procedure. A 23-gauge butterfly needle was inserted into the cisterna magna. Cerebrospinal fluid (CSF) of 1.0 mL/kg and equal volume of fresh non-heparinized autologous arterial blood which was obtained from the ear artery were injected into the cisterna magna within 1 min. After injection, the rabbits were placed in a head-down position at an approximately 30 °C angle for 30 min. The rabbits were returned to their feeding room after waking up from anesthesia. The sham group was subjected to a procedure similar to the vehicle group, but without autologous blood injection.

Neurological Scoring

The neurological scores were blindly evaluated at 7 days after SAH using the previously described scoring system (Sugawara et al. 2008). The evaluation consists of six tests that can be scored 0–3 or 1–3. These six tests include spontaneous activity, spontaneous movement of four limbs, forepaw outstretching, climbing, body proprioception and the response to vibrissae touch.

Open Field Test

The open field test is commonly used for detecting the exploring behaviors, spontaneous activities, anxiety, and other behavioral or emotional characteristics in animals in an open environment. Animal was placed in the center of the apparatus (50 × 50 × 50 cm) with a division of nine equal rectangles on the ground. After 7 days of surgery, the animal behavior was recorded for 5 min by a video camera positioned above the apparatus. The apparatus was thoroughly cleaned by 75% alcohol solution after each test animal.

SAH Grading Scale

The severity of SAH was evaluated by investigators blinded to the groupings according to a previously published grading scale (Sugawara et al. 2008). The rats were killed under deep anesthesia with pentobarbital sodium (100 mg/kg) at 7 days after surgery, and the brains were removed rapidly. The basal cistern was divided into six segments, and each segment was scored from 0 to 3 depending on the amount of subarachnoid blood clot. Grade 0: no subarachnoid blood; Grade 1: minimal subarachnoid blood; Grade 2: moderate blood clot with recognizable arteries; Grade 3: blood clot obliterating all arteries within the segment.

Histopathological Detection and Vasospasm Measurement

Animals were deeply anesthetized with pentobarbital sodium (100 mg/kg) and were perfused by intracardial puncture with ice-cold PBS and 4% paraformaldehyde. The brainstems were removed and immersed overnight in 4% paraformaldehyde solution with 30% sucrose. The brainstem regions were examined via coronal section (10 mm thick) through the basilar artery at the same point, about two-thirds of the distance from the proximal side to avoid arterial branches. The degree of cerebral vasospasm was blindly evaluated by using measurements of the luminal inner perimeter and wall thickness of basilar artery.

Western Blot Analysis

Proteins were extracted from the corresponding region of the right cortex and were measured by the BCA protein assay kit (Fdbio science, Hangzhou, China). The extract (30 μg of protein) was separated by 12% SDS-PAGE gel. The separated proteins were transferred to a PVDF membrane. The membrane was then blocked with 5% skimmed milk for 2 h at room temperature and followed by incubation overnight at 4 °C with the appropriate primary antibodies against Bax, Bcl-2, Cyt-c, HO-1, GAPDH (1:1000, Cell Signaling Technology, MA, USA) and Nrf2 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed thrice for 10 min each in PBST and were then incubated with anti-rabbit IgG (1:2000, Cell Signaling Technology, USA) conjugated to horseradish peroxidase for 90 min at room temperature. Protein expression was visualized by an ECL advanced Western blotting detect kit (Fdbio science, Hangzhou, China). Quantitative analysis of protein bands was based on the mean pixel density of target protein using a Carestream software (Rochester, NY, USA) and was presented as expression ratio of the protein GAPDH.

Immunofluorescence Staining

Rats were deeply anesthetized with pentobarbital sodium (100 mg/kg) and killed by perfusion with PBS followed 4% ice-cold paraformaldehyde (pH 7.4). The formalin-fixed brain tissues were embedded in paraffin and sectioned at 5 μm thickness. For TUNEL and NeuN co-staining, the sections were incubated with NeuN antibody (1:1000, Millipore, MA, USA) at 4 °C overnight and were then subjected to TUNEL staining with an in situ cell death detection kit (Beyotime Biotechnology, Shanghai, China). Finally, cell nuclei were stained with DAPI (1:1000, Beyotime Biotechnology, Shanghai, China). For 3-NT/NeuN and 8-OHdG/NeuN co-staining, after deparaffinization and rehydration, antigen was retrieved by heating the sections for 15 min in

0.01 mmol/L sodium citrate (pH 6.0). Sections were permeabilized with 0.3% Triton X-100 for 30 min and then incubated with 10% fetal bovine serum, 1% bovine serum albumin and 0.1% Triton X-100 for 60 min. Primary antibodies were monoclonal anti-NeuN (1:1000, Millipore, MA, USA) and mouse anti-8-OHdG (1:500, Santa Cruz Biotechnology, CA, USA) or 3-NT (1:500, Abcam, Cambridge, UK) overnight at 4 °C. Secondary antibodies were Alexa Fluor 568 conjugated goat anti-mouse for 8-OHdG or 3-NT (1:1000; Thermo Fisher, Kalamazoo, MI, USA) and Alexa Fluor 488 conjugated goat anti-rabbit for NeuN (1:1000, Sigma, St. Louis, MO, USA). Finally, cell nuclei were stained with DAPI (1:1000, Beyotime Biotechnology, Shanghai, China). All sections were analyzed using inverted fluorescence microscope (Olympus, Tokyo, Japan). Immunofluorescent positive cells were counted in six sections per rat, which performed by a viewer blinded to the experimental group. Results were expressed as the average numbers of positive cells in unit area per section of four rat brains.

Artery Rings Preparation and Isometric Force Measurement

Sprague–Dawley rats (300–350 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (100 mg/kg). The rats were killed, and brains were removed. The endothelium-intact basilar arteries were dissected out and placed in ice-cold oxygenated physiological saline solution (PSS). Physiological saline solution contained the following compositions: NaCl (119 mM), KCl (4.7 mM), KH_2PO_4 (1.18 mM), $MgSO_4$ (1.17 mM), $CaCl_2$ (1.5 mM), $NaHCO_3$ (24.9 mM), EDTA (0.01 mM), and glucose (11 mM); pH 7.4. Basilar arteries rings (approx. 2 mm in length) were mounted in a Multi Myograph System (Danish Myo Technology A/S, Denmark), bathed in PSS bubbled with 95% O_2–5% CO_2 and maintained at 37 °C as described previously (Santiago et al. 2013). Basilar artery rings were set to an optimal tension of 4 mN and stabilized for 90 min. High K^+ solution (KPSS: equivalent to PSS except that NaCl was exchanged for KCl on an equimolar basis, giving a final concentration of 123.7 mM K^+) was used to induce steady and maximal contraction tone in endothelium-intact rings. H_2O_2 (1–300 μM) was cumulatively added in 30 mM K^+ solution after pre-incubation with TBN (100 μM) or vehicle (ddH_2O) for 20 min.

Cell Viability and Intracellular ROS Measurement

Cerebrovascular endothelial cells (bEnd.3 cells) were cultured in complete medium (high-glucose Dulbecco modified Eagle medium plus 10% fetal bovine serum) at 37 °C in a humidified atmosphere in the presence of 5% CO_2. Complete medium was changed every 2 days. Cultured bEnd.3 cells were divided into eight groups as follows: (1) control group, cultured in completed media, (2) control + TBN (30 μM) group, (3) control + TBN (100 μM) group, (4) control + TBN (300 μM) group, (5) H_2O_2 (500 μM) group, cultured in completed media with H_2O_2 (500 μM) for 24 h, (6) H_2O_2 (500 μM) + TBN (30 μM) group, (7) H_2O_2 (500 μM) + TBN (100 μM) group, (8) H_2O_2 (500 μM) + TBN (300 μM) group. At 24 h after H_2O_2 treatment, 3-(4, 5-dimethylthia-zol-2-yl)-2,5-biphenyl tetrazolium bromide (MTT, 10 μL) was added to each well and was incubated for 4 h, followed by replacing with DMSO (150 μL) to dissolve the formazan crystals. The absorbance was measured by a BioTek microplate reader (San Diego, CA, USA.) at 570 nm. Cell viability was expressed as the percentage of control.

The level of the intracellular ROS in all groups was measured by using a fluorescent probe, 2′, 7′-dichlorodihydrofluorescein diacetates (DCF-DA; Beyotime Biotechnology, Shanghai, China). The bEnd.3 cells (1×10^6 cells/ml) were seeded in the plate and treated with H_2O_2 or/and TBN for 24 h. Then, bEnd.3 cells were treated with 5 μM DCF-DA for 30 min at 37 °C. After washing with HBSS, the fluorescence was measured by an inverted fluorescence microscope (Olympus, Tokyo, Japan). All images were captured under a microscope with the same light intensity, and only one camera setting was used.

Data and Statistical Analysis

The neurological scores, cerebral bleeding severity scores, quantitative analyses of immunohistochemistry, and immunofluorescence were all conducted by investigators blinded to the experimental groups and drug treatment. All results were expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by the Tukey's test, using the GraphPad Prism 6 software (La Jolla, CA, USA). $P < 0.05$ was considered to be statistical significance.

Results

TBN Ameliorated Behavioral Dysfunction in SAH Rats

There was no significant difference on mortality rate between the vehicle and the TBN groups. The mortality rate was 15.4% (2/13 rats) and 13.3% (2/15 rats) in the vehicle and TBN groups, respectively. All animals survived in sham-operated group (Fig. 1a).

Neurobehavioral function was assessed by neurological scores and open field test at 7 days after SAH. As shown in Fig. 1b–e, the neurological scores in the vehicle group were significantly lower than that in the sham group. TBN

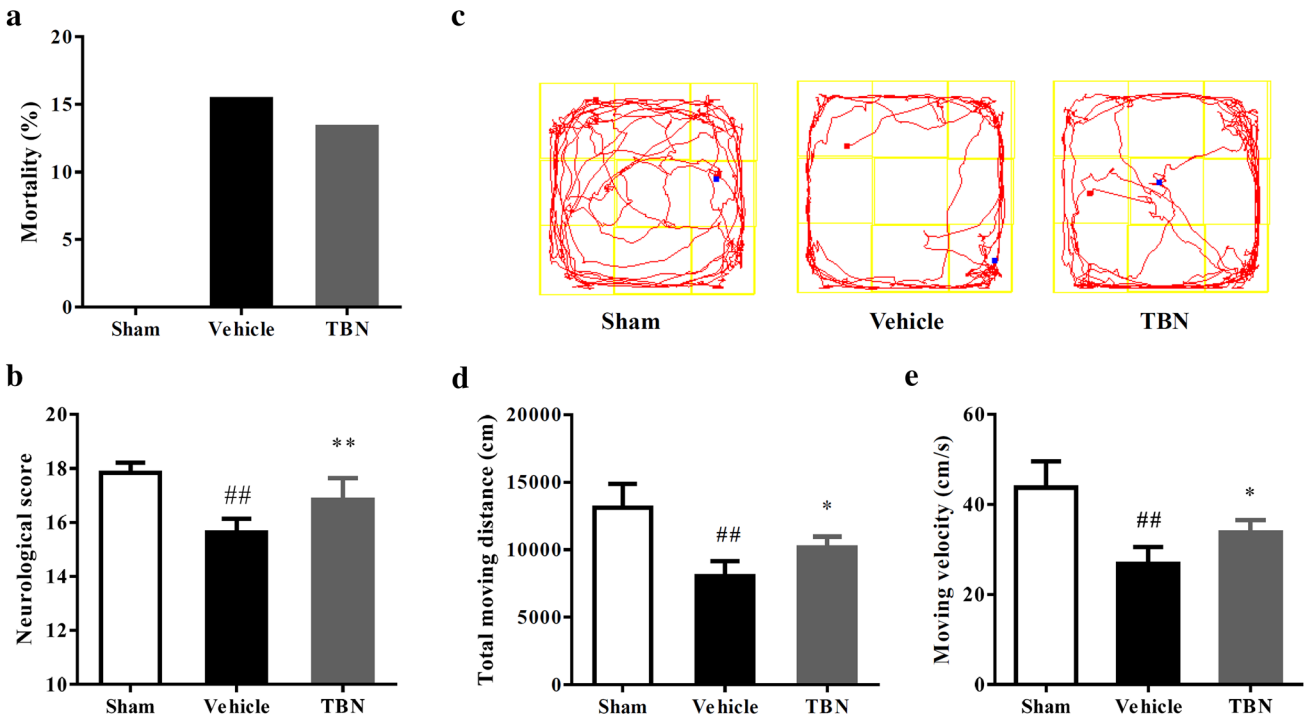

Fig. 1 Effect of TBN on mortality and behavioral impairments of rats at 7 days after SAH. **a** Mortality of SAH rats. **b** Neurological score. **c** Representative motion route of rats in open field test. **d** The total distance moved. **e** The average moving velocity. The results were mean ± SEM ($n = 12$ for sham group, $n = 11$ for vehicle group, $n = 13$ for TBN group). $^{\#\#}P < 0.01$ versus sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle group

treatment effectively improved neurological scores compared with the vehicle treatment at 7 days after SAH (Fig. 1b). In the open field test, the total moving distance and average moving velocity were main indicators of locomotion activities. TBN treatment significantly improved the performance of total moving distance and average moving velocity compared with vehicle treatment (Fig. 1c–e).

TBN Attenuated SAH Bleeding Severity and Cerebral Vasospasm in Rats After SAH

The severity of cerebral vasospasm's pathology is due to the amount of blood lost and the location of that blood in the basal cisterns. This classification of severity of a SAH patient's condition has been widely utilized in clinic. We used a similar rating system previously described in the literature (Sugawara et al. 2008) to evaluate the severity of bleeding in our rat models. As shown in Fig. 2a, b, the sham group displayed no bleeding. The basal cisterns of the vehicle-treated SAH model group, however, were full of blood clots. In the TBN treatment group, the bleeding severity score decreased significantly.

As shown in Fig. 2c, the histological images of H&E staining for the rats' basilar arteries indicated severe cerebral vasospasm in the SAH model group compared with the sham operation group. The morphology of cerebral vasoconstriction is characterized by a corrugated internal elastic lamina, and a thickened vessel wall. These morphological changes were attenuated by TBN treatment. Next, cross sections of basilar arteries were semi-quantitatively analyzed. TBN treatment was found to profoundly increase the perimeter and decrease the thickness of the arteries (Fig. 2d, e).

TBN Attenuated Cerebral Vasospasm in Rabbits After SAH

We further assessed the efficacy of TBN in a rabbit SAH model, which was produced by a single blood injection into cisterna magna. The basilar artery was visibly constricted and the arterial wall thickened after the SAH event. TBN treatment resulted in an obvious relaxation of the basilar artery spasm (Fig. 3a). Semi-quantitative analysis of the cross-sectional basilar artery indicated that TBN significantly increased the perimeter and attenuated the arterial wall thickness of basilar artery when compared with the untreated SAH model group (Fig. 3b, c).

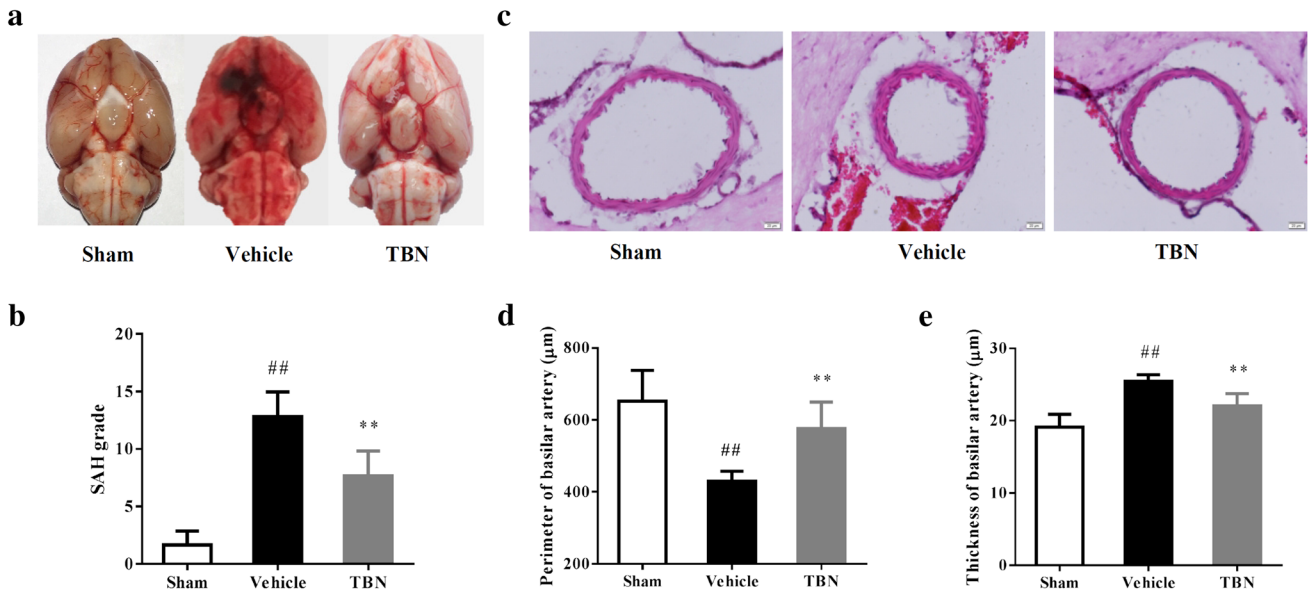

Fig. 2 TBN attenuated cerebral bleeding severity score and cerebral vasospasm in rats after SAH. **a** Representative SAH photography after endovascular perforation in rats. **b** Effect of TBN on the SAH bleeding grade. **c** Representative H&E-stained cross sections of rat basilar arteries after SAH. Scale bars: 20 μm. Quantification of the luminal inner perimeter (**d**) and the wall thickness (**e**) of basilar arteries in rats after SAH. The results were mean ± SEM ($n = 6$ per group). $^{\#\#}P < 0.01$ versus sham group; $^{**}P < 0.01$ versus vehicle group

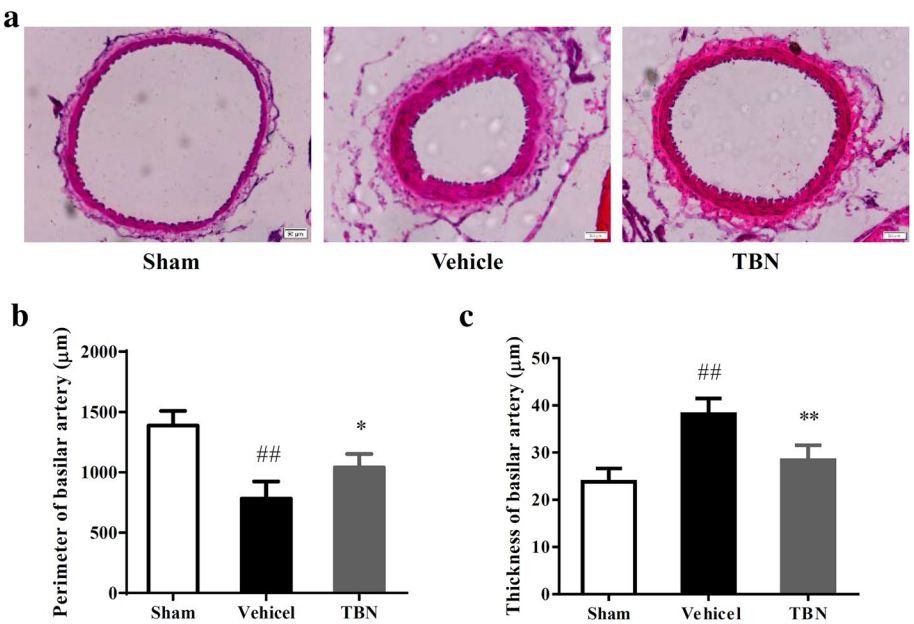

Fig. 3 TBN attenuated cerebral vasospasm in rabbits after SAH. **a** Representative images of H&E-stained cross-sectional basilar arteries of SAH rabbits. Quantitative analysis of the luminal inner perimeter (**b**) and wall thickness (**c**) of basilar arteries in SAH rabbits. The results were mean ± SEM ($n = 6$ for sham and vehicle group, $n = 5$ for TBN group) $^{\#\#}P < 0.01$ versus sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle group. Scale bars: 50 μm

TBN Decreased Cell Apoptosis in the Cerebral Cortex of SAH Rats

Neuronal cell apoptosis contributes to early brain injury after SAH (Zhang et al. 2017b). The TUNEL/NeuN co-staining directly reflected the occurrence of neural cell apoptosis in the cortex. As shown in Fig. 4a, b, very few TUNEL-positive neurons were found in sham group. An obvious increase in the number of TUNEL-positive neurons was detected in the vehicle-treated group. However, compared with the vehicle group, TBN treatment significantly reduced the number of apoptotic neurons.

Furthermore, we examined the expression level of the apoptosis-related proteins Bcl-2, Bax, and cytochrome c (Cyt-c) by Western blotting. The anti-apoptotic factor Bcl-2 was significantly down-regulated, while pro-apoptotic factors Bax and Cyt-c were significantly up-regulated in vehicle-treated SAH model group as compared with the

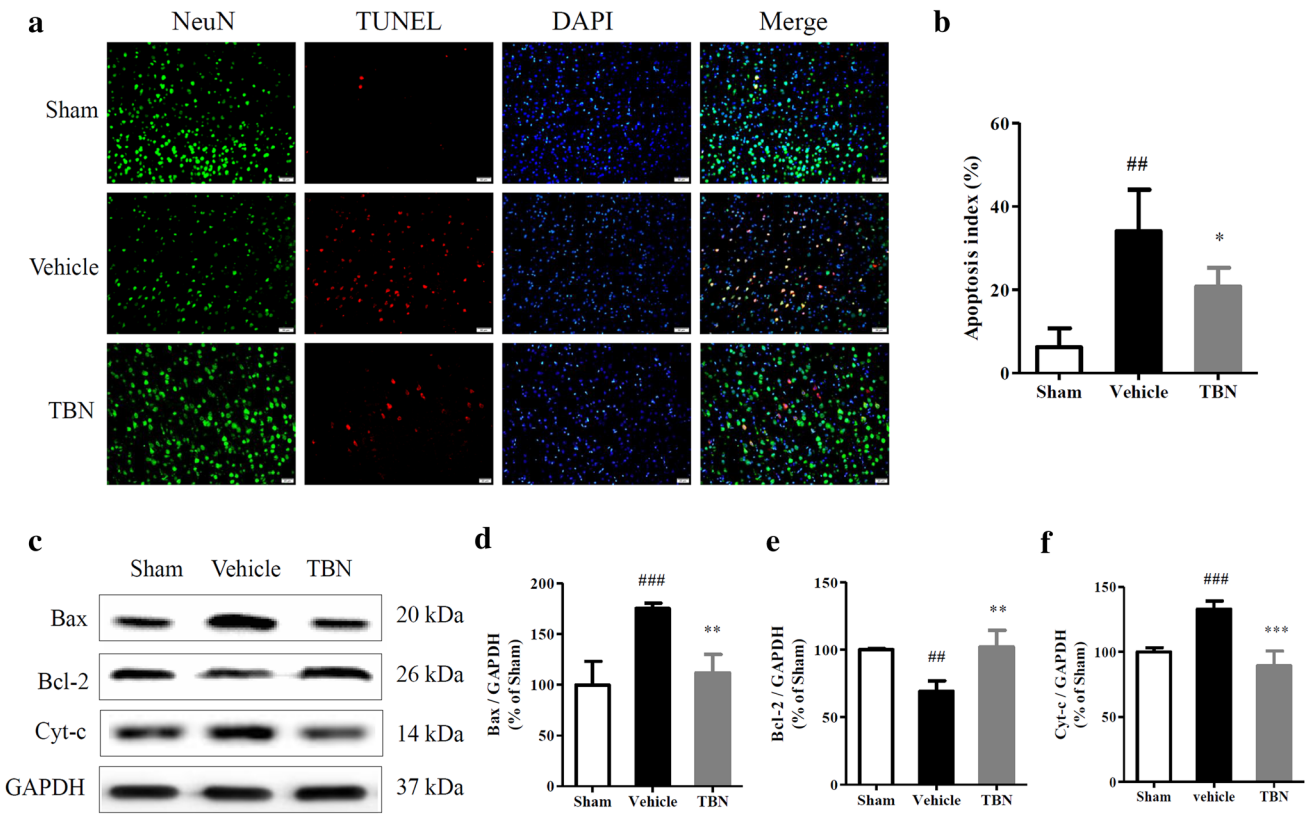

Fig. 4 TBN attenuated cell apoptosis of cerebral cortex in SAH rats. **a** Representative photomicrographs of neuronal apoptosis in the ipsilateral cerebral cortex. Scale bars: 50 μm. **b** Quantification of TUNEL-positive neurons. **c** Representative blots of the protein expression of Bcl-2, Bax, and cytochrome c (Cyt-c). Densitometry analysis of Bax (**d**), Bcl-2 (**e**), and Cyt-c (**f**) expression. The results were mean ± SEM ($n = 4$ per group). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus vehicle group

sham group. TBN treatment almost completely reversed the altered expression of Bcl-2, Bax, and Cyt-c after SAH (Fig. 4c–f).

TBN Reduced Cell Apoptosis in the Cerebral Cortex and Hippocampus of SAH Rabbits

Similar to the findings in the rat model, there were significant increases in apoptotic cells in the rabbit cortex and hippocampus CA1 of the vehicle-treated SAH group, when compared with sham group. TBN treatment resulted in remarkable decreases in the percentage of apoptotic cells compared with the vehicle group (Fig. 5).

TBN Attenuated the Oxidative Damage Products Through Activation of Nrf2/HO-1 Signaling Pathway in Rats After SAH

Oxidative stress is one of the key factors contributing to post-hemorrhagic vasospasm (Kim et al. 2002). Three-nitrotyrosine (3-NT) and 8-hydroxy-2-deoxyguanosine (8-OHdG) are oxidative products of protein and DNA (Zhang et al. 2015). To determine if oxidative damage occurred in neurons, the cortex slices were double-stained by 3-NT/NeuN and 8-OHdG/NeuN at 7 days after SAH. The percentage of 3-NT/NeuN and 8-OHdG/NeuN double-positive cells significantly increased in the vehicle-treated model group compared with sham group. However, TBN treatment effectively attenuated the percentage of 3-NT/NeuN and 8-OHdG/NeuN double-positive cells (Fig. 6a–d).

It has been reported that activation of nuclear factor erythroid-derived 2-like 2 (Nrf2)/heme oxygenase 1 (HO-1) pathway exerts strong neuroprotection against oxidative insults after SAH (Khurana et al. 2004). Herein, the expression of Nrf2 and HO-1 proteins was significantly down-regulated after 7 days of SAH. By contrast, TBN treatment significantly up-regulated the expression of Nrf2 and HO-1 compared with the vehicle group (Fig. 6e–g).

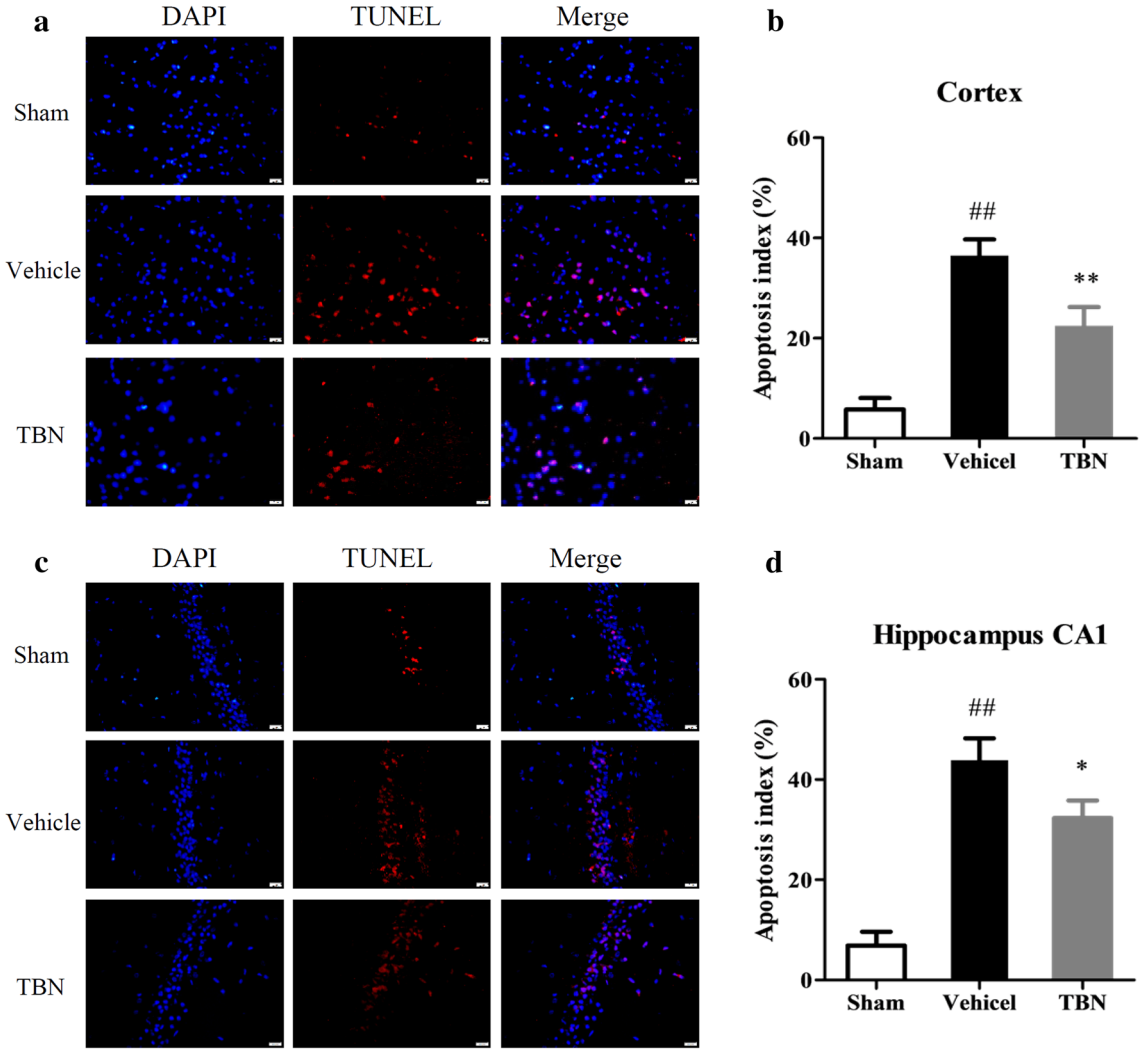

Fig. 5 TBN reduced cell apoptosis in the cortex and hippocampus of SAH rabbits. Representative images of TUNEL staining showed cell apoptosis in the cortex (**a**) and the hippocampus (**c**) after SAH. Scale bars: 20 μm. Quantitative analysis of the TUNEL-positive cells in the cortex (**b**) and hippocampus CA1 (**d**) of SAH rabbits. The results were mean ± SEM ($n = 4$ per group). $^{\#\#}P < 0.01$ versus sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle group

TBN Attenuated H_2O_2-Induced Endothelial Cell Oxidative Damage In Vitro and H_2O_2-Induced Vasoconstriction in the Isolated Basilar Artery Rings Ex Vivo

As oxidative stress-induced endothelial cell damage is closely connected to cerebral vasospasm (Park et al. 2004), we tested the effect of TBN on H_2O_2-induced injury of cerebrovascular endothelial cell line bEnd.3. Exposure to H_2O_2 (500 μM) caused a significant decrease in cell viability of the bEnd.3 cells. TBN substantially increased cell viability of bEnd.3 compared with the H_2O_2 (500 μM) group. TBN (30–300 μM) treatment had no significant effect on the cell viability of normal endothelial cells (Fig. 7b). DCF-DA staining showed that H_2O_2 treatment obviously increased intracellular reactive oxygen species (ROS) level, which was significantly attenuated by TBN treatment in a concentration dependent manner (Fig. 7a, c).

ROS including superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) enhanced vasoconstriction in both physiological and pathophysiological conditions (Santiago et al. 2013). Herein, as shown in Fig. 7d, H_2O_2 concentration-dependently induced vasoconstriction of the isolated basilar artery rings. Compared with vehicle group, TBN (100 μM) significantly attenuated H_2O_2-induced vasoconstriction.

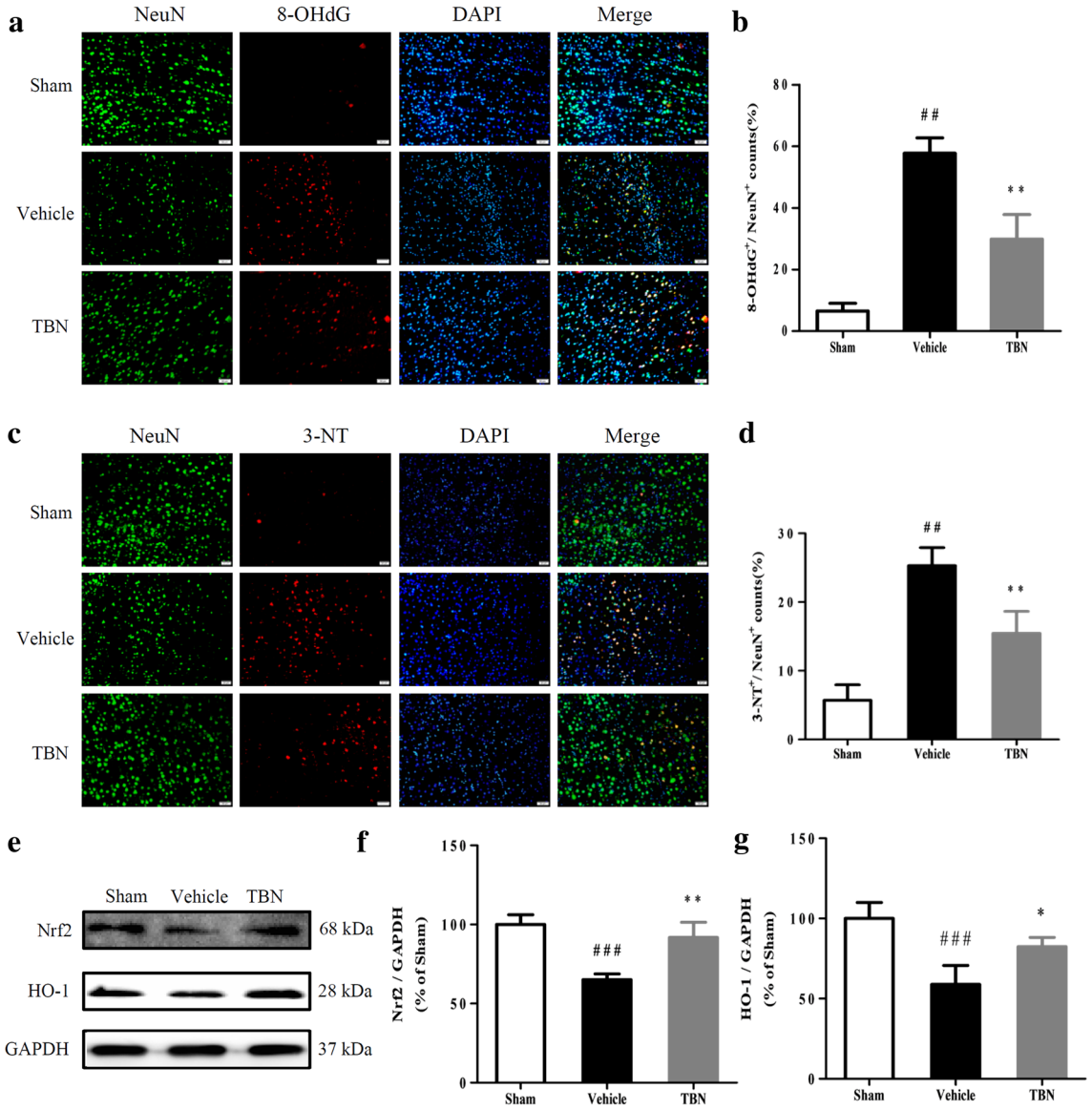

Fig. 6 TBN suppressed oxidative stress through the Nrf2/HO-1 signaling pathway after SAH. Representative photomicrographs of double immunofluorescence staining of 8-OHdG/NeuN (**a**) and 3-NT/NeuN (**c**) in the cerebral cortex at 7 days following SAH. Scale bars: 50 μm. Quantification of 8-OHdG-positive (**b**) and 3-NT-positive (**d**) neurons. **e** Representative blots of the protein expression of Nrf2 and HO-1. Densitometry analysis of Nrf2 (**f**) and HO-1 (**g**) expression. The results were mean ± SEM ($n = 4$ per group). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle group

Discussion

Cerebral vasospasm is considered a major cause of disability and death after SAH (Sun et al. 2018). Approximate two-thirds of SAH patients develop cerebral vasospasm within 3–14 days, which could increase the risk of delayed cerebral ischemia (Macdonald 2014). Cerebral vasospasm is characterized by significant morphological changes in the basilar arterial wall, such as luminal narrowing, vascular smooth muscle cell proliferation, and vascular endothelial apoptosis (Cheng et al. 2014; Cui et al. 2016). Oxidative stress and over-production of free radicals play a crucial role in the development of cerebral vasospasm after SAH (Macdonald and Weir 1994). Therefore, free radical scavenger and anti-oxidants may provide protective effects against cerebral vasospasm after SAH. In the present study, we demonstrated the protective effect of TBN against cerebral vasospasm after SAH in both the rat and rabbit models. TBN treatment attenuated SAH bleeding severity, cerebral vasospasm, neuronal apoptosis, and improved the neurological outcome after SAH.

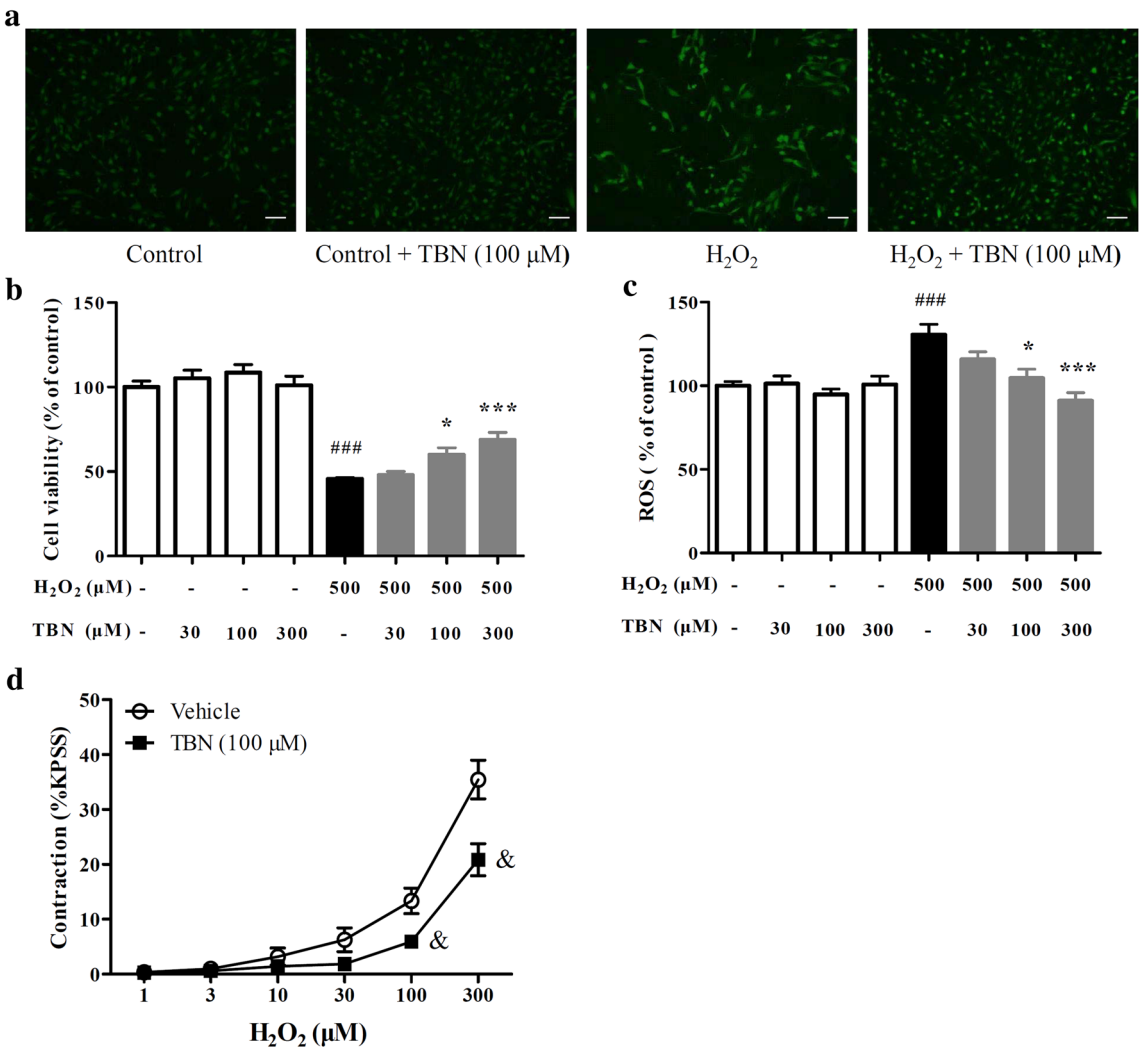

Fig. 7 TBN attenuated H_2O_2-induced oxidative damage of endothelial cell and H_2O_2-induced vasoconstriction in the isolated basilar artery rings. **a** Representative images of intracellular ROS production in bEnd.3 cells induced by H_2O_2 (500 μM). Scale bars: 20 μm. **b** Protective effect of TBN on bEnd.3 cells exposed to H_2O_2 (500 μM). **c** Effect of TBN on H_2O_2-induced ROS production in bEnd.3 cells. **d** TBN alleviated the H_2O_2-induced contractile responses in basal artery rings. Data are shown as mean ± SEM ($n = 6$ per group). $^{###}P < 0.001$ versus control group; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus H_2O_2 (500 μM) group. $^{\&}P < 0.05$ versus vehicle group

Cell death, especially cell apoptosis, occurs in neuron, astrocyte, oligodendrocyte, vascular smooth muscle cell, and endothelial cell after SAH, which plays a vital role in the pathophysiological progression of central nervous system diseases. Therefore, interventions targeting apoptosis seem to be a reasonable way to protect against CNS damage (Fujii et al. 2013). In terms of SAH, apoptosis was detected in cortical, subcortical, or hippocampal neurons. Our previous studies revealed that TBN had potent anti-apoptotic effects in the experimental models of ischemic stroke (Sun et al. 2008, 2012), traumatic brain injury (Zhang et al. 2016b), and chronic cerebral hypoperfusion (Zhang et al. 2017a). In the present study, TBN alleviated the number of TUNEL-positive cells of brain cortex after SAH in both rats and rabbits. Previous studies have reported that the balance of Bcl-2 and Bax has a close link with cell death and survival (Jiang et al. 2012; Love 2003; Willis et al. 2003). As a familiar pro-apoptotic member of the Bcl-2 family, Bax level improved significantly following SAH (Chen et al. 2014b), causing Bax-mediated mitochondrial membranes depolarization, which leading to neuronal apoptosis (Reed 2002). Cyt-c plays a key role in the activation of apoptosis-related proteins, and its release from mitochondria was commonly regulated by the Bcl-2 family of apoptotic proteins and mitochondrial permeability, both of which can be impacted by oxidative stress (Suen et al. 2008). In this work, we observed the increases of Bax and Cyt-c expression and decreases of Bcl-2

expressions after SAH, similar to reported results from previous studies; however, TBN treatment reversed these alterations of protein expression, suggesting that TBN alleviated brain damage after SAH through anti-apoptosis.

Inappropriate over-production of ROS can disrupt the balance between oxidation and anti-oxidation, which contribute to multiple pathological processes involved in organ injuries and biological metabolism, ultimately triggering oxidative damage (Granger and Kvietys 2015). In this study, TBN can inhibit ROS production induced by H_2O_2 in cerebrovascular endothelial cell line bEnd.3. In addition, it has been reported that oxidative stress-induced lipid peroxidation, protein breakdown, and DNA damage, which cause cellular dysfunction and apoptosis, occurring mostly in endothelial cells and neurons (Lewen et al. 2000). Previous studies also suggested that excessive free radicals stimulate the production of 3-NT and 8-OHdG (Sugawara et al. 2002). Three-NT and 8-OHdG are oxidative injury markers of protein and DNA damage, respectively. Current results showed that TBN significantly reduced the number of 3-NT/NeuN and 8-OHdG/NeuN co-staining cells. These data suggested that TBN inhibited oxidative stress and free radicals produced by SAH.

Nrf2 has been shown to be an important anti-oxidant factor in various neurological diseases, including SAH (Wu et al. 2014), traumatic brain injury (Zhang et al. 2016b), and neurodegenerative disorders (Joshi and Johnson 2012). Previous studies showed that the Nrf2 and HO-1 pathways were activated in the cortex during early stage of SAH in rat (Zhang et al. 2010). In the present study, we found that the expression of Nrf2 and HO-1 was decreased at 7 days after SAH. The discrepancy of Nrf2/HO-1 expression may be related to the different time-point after SAH. Importantly, TBN significantly increased the expression of Nrf2 and HO-1. Accumulating evidence shows that oxidative stress is one of the factors contributing to cerebral vasospasm following SAH (Yang et al. 2017). Increased superoxide anion levels in the cerebrospinal fluid have been implicated to vasospasm after SAH (Zheng et al. 2005). The up-regulation of anti-oxidant response elements (ARE) contribute to defense against the toxicity of oxidative stress. Furthermore, ARE-mediated anti-oxidant enzymes, such as HO-1, NQO1, and GPX, regulate redox homeostasis and affect the inflammatory response (Osburn et al. 2006). Therefore, pharmacological activation of the Nrf2/HO-1 signaling pathways is closely associated with the protective effect on neurons exposed to oxidative stress insults induced by SAH. On the other hand, the Nrf2/HO-1 signaling pathway has been reported to inhibit apoptosis based on its effect on mitochondria-related apoptotic proteins such as Bcl-2 and Bax (Li et al. 2016a); hence, the anti-apoptotic property of TBN in SAH rats is partly related to its up-regulation of Nrf2 and HO-1 expression.

In conclusion, TBN treatment effectively attenuated cerebral vasospasm and brain damage in both rat and rabbit SAH models, which may be strongly associated with anti-oxidative stress and up-regulation of Nrf2/HO-1 signaling pathways. Meanwhile, our previous studies have shown that TBN readily penetrates the blood–brain barrier (BBB) and displays adequate pharmacokinetic and safety profiles (Zhang et al. 2016a). Our results suggest that TBN is a promising new therapeutic agent for the treatment of SAH.

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Author contributions YW, GZ, and ZZ designed and supervised the project. GZ, YS, and LZ participated in making experimental rat model. LW and LZ contributed to establish experimental SAH rabbit model. LW and ZS performed the H&E and immunofluorescence staining. ZS performed the vitro experiments. WL, PY, and ZZ analyzed the samples and data. YW, and ZZ wrote the manuscript. All authors have read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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