Protective multi-target effects of DL-3-n-butylphthalide combined with 3-methyl-1-phenyl-2-pyrazolin-5-one in mice with ischemic stroke

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Abstract. DL-3-n-butylphthalide (NBP) and 3-methyl-1-phenyl-2-pyrazolin-5-one (edaravone) are acknowledged neuroprotective agents that protect against ischemic stroke. However, the underlying mechanisms of a combination therapy with NBP and edaravone have not yet been fully clarified. The aim of the present study was to explore whether the co-administration of NBP and edaravone had multi-target protective effects on the neurovascular unit (NVU) of mice affected by ischemic stroke. Male C57BL/6 mice were randomly divided into the following three groups: i) Sham operation control, ii) middle cerebral artery occlusion (MCAO) and reperfusion, iii) and MCAO/reperfusion with the co-administration of NBP (40 mg/kg) and edaravone (6 mg/kg) delivered via intraperitoneal injection at 0 and 4 h after reperfusion (NBP + edaravone). After ischemia and reperfusion, infarct volumes and neurological deficits were evaluated. The immunoreactivity of the NVU, comprising neurons, endothelial cells and astrocytes, was determined using immunofluorescence staining of neuronal nuclei (NeuN), platelet and endothelial cell adhesion molecule 1 (CD31) and glial fibrillary acidic protein (GFAP). Western blotting was used to detect the expression levels of apoptosis-related proteins. The infarct volume, neurological function scores and cell damage were increased in the MCAO group compared with the sham operation group. Furthermore, the MCAO mice had reduced NeuN and CD31 expression and increased GFAP expression compared with the sham group. By contrast, the NBP + edaravone group exhibited reduced cell damage and consequently lower infarct volume and neurological deficit scores compared with the MCAO group. The NBP + edaravone group exhibited increased NeuN and CD31 expression and decreased GFAP expression compared with the MCAO group. Furthermore, the expression levels of Bax and cleaved caspase-3 in the NBP + edaravone group were decreased significantly compared with the MCAO group, while the expression levels of Bcl-2 and mitochondrial cytochrome *c* were increased. In conclusion, the results of the present study demonstrated that NBP and edaravone effectively prevented ischemic stroke damage with multi-target protective effects. In addition, NBP + edaravone may be a promising combination therapy for ischemic stroke.

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Abbreviations: BBB, blood-brain barrier; CD31, platelet and endothelial cell adhesion molecule 1; HE, hematoxylin-eosin; HRP, horseradish peroxidase; MCAO, middle cerebral artery occlusion; NBP, DL-3-n-butylphthalide; NeuN, neuronal nuclei; NVU, neurovascular unit; PSD95, post synaptic density protein 95; SYP, synaptophysin; TTC, 2,3,5-triphenyltetrazolium chloride

Key words: ischemic stroke, NBP, 3-methyl-1-phenyl-2-pyrazolin-5-one, combination therapy, NVU

Introduction

Strokes are the main cause of long-term disabilities, the second leading cause of cardiovascular disease-related deaths in the United States and the fifth leading cause of death among all residents in the country according to a report from the American Heart Association in 2019 (1). Ischemic stroke, a brain injury caused by insufficient blood supply, accounted for 79.1 and 64.9% of the global prevalence and incidence, respectively, of all strokes in 2017 (2). Although a previous investigation revealed that there were a maximum of 430 potentially useful stroke drug candidates between 1995 and 2015, only 19 (4%), including aspirin, dipyridamole, atenolol, ramipril, hydrochlorothiazide, Polycap and simvastatin, have been used clinically worldwide (3). Notably, a combination of dipyridamole and aspirin has been reported to decrease ischemic stroke recurrence (4). The combination drug Polycap, containing aspirin, hydrochlorothiazide, ramipril, atenolol and simvastatin, might prevent stroke in high-risk subjects (5,6). Therefore, combination therapy for ischemic stroke requires further research.

DL-3-n-butylphthalide (NBP) is a natural product extracted from celery, which acts as a neuroprotective agent against ischemic brain damage (3). It is currently in clinical trials, registered and approved by the China Food and Drug Administration (3,7,8). Furthermore, as a well-known free radical scavenger, 3-methyl-1-phenyl-2-pyrazolin-5-one (edaravone) also works as a neuroprotective agent and is recommended for patients with acute cerebral strokes in Japan, China, India and other countries, such as those in Europe (9-12). Both NBP + edaravone and a hybrid compound of a ring-opening derivative of NBP + edaravone (compound 10b) exhibit increased protective effects against brain damage compared with NBP or edaravone alone in rats with ischemia-reperfusion (13). These drugs may be used for ischemic stroke treatment (13,14). The results of previous research indicate that compound 10b exerts neuroprotective effects by improving mitochondrial function (13). However, the mechanisms underlying the neuroprotective effects of NBP combined with edaravone have not yet been fully elucidated.

Recently, the neurovascular unit (NVU) has attracted the attention of researchers as it emphasizes the importance of communication among neurons, endothelial cells and astrocytes, instead of only blood vessels or neurons, in ischemic injuries (15). The NVU contributes to disease development and responses; therefore, it could be a therapeutic target (16,17). To the best of our knowledge, whether NBP combined with edaravone targets the whole NVU and protects against multiple cell death is unknown. Therefore, to investigate the effects of NBP combined with edaravone on various neurological aspects, the present study evaluated brain infarct volume using 2,3,5-triphenyltetrazolium chloride (TTC) staining, the neurological deficits in mice, and cell damage using hematoxylin-eosin (HE) and Nissl staining. The dysfunction of the major NVU components, including neurons, endothelial cells and astrocytes, was also studied via immunofluorescence analysis in the mice models with middle cerebral artery occlusion (MCAO) and reperfusion. Furthermore, the effects of NBP combined with edaravone on apoptosis-related proteins were determined using western blotting.

Materials and methods

Ethics statement. Ethical permission was obtained from the Animal Ethics Committee of North China University of the Science and Technology (approval no. 2019068; Tangshan, China), which records and regulates all research activities. The approval from the Animal Ethics Committee included the permission to use mice under anesthesia or euthanasia, and all experimental procedures were conducted in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (18). Pre- and post-surgery pain management was maintained via anesthesia with 3% isoflurane in all animals to ensure compliance with guidelines set forth by the North China University of the Science and Technology (Tangshan, China).

Mouse groups and drug administration. Male C57BL/6 mice (weight, 20-25 g; age, 12 weeks) were purchased from Shanghai Jiesijie Experimental Animal Co., Ltd. (http://www.jsj-lab.com/; Grade II; certificate no. 2020027). Mice (n=3 per cage) were raised under controlled conditions with a 12-h light/dark cycle at room temperature (21-23˚C) and 40-60% humidity with free access to water and food. A total of 39 mice were randomly divided into three groups (n=13 for each group): i) Sham operation control; ii) MCAO and reperfusion; and iii) NBP + edaravone MCAO (NBP + Edaravone). NBP (40 mg/kg; batch no. 2019122001; CSPC Pharmaceutical Co. Ltd. (http://en.e-cspc.com/index.html) and edaravone (6 mg/kg; batch no. 2020010702; China National Medicines Guorui Biomedical Technology Co., Ltd. (https://www.guorui.com.cn/wzsy) were administered by intraperitoneal injection at 0 and 4 h after reperfusion. The dosage was determined according to the manufacturers' instructions and previous experiments (19,20). Briefly, from each of the three groups (n=13 per group), 4 mice were used for neurological deficit scores and infarct volume, while 3 mice each were used for HE/Nissl staining, immunofluorescence analysis and protein extraction for western blotting.

Anesthesia and euthanasia. Mice were anesthetized using box induction with 3% isoflurane (cat. no. R510-22-8; batch no. 20191222; RWD Life Science Co., Ltd. (https://www.rwdstco.com/) for ~3 min and then maintained on 1.5% isoflurane in medical grade oxygen via a facemask from a small animal anesthesia machine (cat. no. R500; RWD Life Science Co., Ltd.). The body temperatures of the mice were measured using a laser Doppler flowmeter (moorVMS-LDF2; Moor Instruments, Ltd.) during the whole surgical procedure, and this was required to be maintained at ~37±0.5˚C with a heating blanket, and the blood pressure was monitored with a small animal blood pressure monitor (BP-2010A; RWD Life Science Co., Ltd.).

Before euthanasia, the mice were carefully examined for the last health checkup and then exposed to 5% isoflurane for 5 min. Finally, the mice were sacrificed with dislocation of cervical vertebra, and the criteria to judge the death of animals were continuous absence of spontaneous breathing for 2-3 min, with no blink reflex and no blood pressure.

Development of mice ischemia-reperfusion model. The MCAO operation was performed by skilled experimenters and lasted for ~10 min for each animal. After the mice were anesthetized, the right common carotid artery was inserted with a rounded tip 4-0 surgical monofilament nylon suture (MSMC21B120PK50; RWD Life Science Co., Ltd.), and then the suture was carefully advanced ~11 mm (from the bifurcation of the common carotid artery) into the middle cerebral artery origin. A laser Doppler flowmeter (moorVMS-LDF2) was adopted to confirm the decrease of the middle cerebral artery blood flow immediately after the occlusion to <70% of the basic cerebral blood flow. Those mice with reduced blood flow to <70% of pre-ischemia levels were used for further study. After a 1-h occlusion, the suture was removed to obtain blood reperfusion, the wound was sutured and the animal was kept breathing in pure medical oxygen for ~5 min to recover from anesthesia and then finally transferred to the individual home cage with the heating blanket. After the operation, the animals were observed and recorded every 2 h, including breathing,

activity, diet and body temperature measured by a portable infrared remote sensing thermometer (DKHIRT; Wuhan Dikai Optoelectronics Technology Co., Ltd.). The sham mice were similarly treated, although without focal cerebral ischemia-reperfusion. There were no animal mortalities during the formal experiment, while 2 of 10 mice died at ~24 h after the operation due to brain edema in the pre-experiment. All results of the pre-experiment were excluded from statistical analysis.

Neurological deficit scores. The neurological deficit scores were assessed for 24 h following reperfusion. The mice were scored according to the method described by Bederson *et al* (21): Mice with no neurological symptoms were scored as 0; when unable to flex the left forepaw fully, as 1; when rotating while crawling and unable to move the contralateral side, as 2; if unable to walk unaided, as 3; and if unconscious, as 4. The mice were euthanized as aforementioned at 24 h after reperfusion.

Measurements of the infarct volume. The mice were euthanized as aforementioned at 24 h after reperfusion and the brains were extracted and stored at -20˚C for 10 min. Subsequently, the brain tissues were cut into 2-mm-thick slices and maintained in 0.25% TTC for 30 min at 37˚C. The slices were then fixed with 4% paraformaldehyde at 4˚C for 24 h. The brain infarct volumes were measured by an experienced rater who was blinded to the design of this experiment.

HE staining and Nissl staining. Parts of the brain tissues were used for HE staining. The brains were fixed with 4% paraformaldehyde overnight at 4˚C, then embedded in paraffin, sliced at 5 μm and stained in hematoxylin aqueous solution for 10 min and alcohol eosin staining solution for 2 min at 25˚C. Morphological changes and histological observations were performed using an IX81 light microscope (Olympus Corporation). Some brain tissue sections were placed in 60˚C incubator and dyed with 1% toluidine blue for 40 min for the Nissl staining after the same preparatory process.

Immunofluorescence analysis. Although actual cell activity cannot be revealed using immunofluorescence, markers for different protein activity can be assessed. Therefore, an astrocyte marker glial fibrillary acidic protein (GFAP), a neuronal marker neuronal nuclei (NeuN), and an endothelial marker platelet and endothelial cell adhesion molecule 1 (CD31) were analyzed using immunofluorescence staining.

At room temperature, 4% paraformaldehyde-fixed, paraffin-embedded sections at 10-μm thickness were placed on pre-cleaned and positively charged microscope slides and were heated in a tissue-drying oven for 45 min at 60˚C, and then deparaffinization and rehydration procedures were conducted. The slides were washed twice in xylene for 3 min each time and in xylene 1:1 with 100% ethanol for 3 min, twice in 100% ethanol for 3 min each, and twice in 95% ethanol for 3 min each, in 70% ethanol for 3 min and in 50% ethanol for 3 min. Slides were rinsed gently with running distilled water for 5 min at room temperature, and then antigen retrieval was conducted. Slides were boiled in 0.01 M sodium citrate buffer (pH 6.0) at 100˚C for 15-20 min, then the slides were removed from the heat and allowed to stand at room temperature in buffer for 20 min. Then, the slides were rinsed twice with TBS-Tween-20 (TBST, with 20% Tween 20) for 5 min at room temperature. Slices were blocked using 5% normal goat serum (cat. no. ab7481; Abcam) for 2 h at room temperature, followed by overnight incubation at 4˚C with the following primary antibodies: Mouse anti-GFAP (dilution, 1:200; cat. no. ab7260; Abcam), rabbit anti-NeuN (dilution, 1:500; cat. no. ABN78; MilliporeSigma) and mouse anti-CD31 (dilution, 1:500; cat. no. ab24590; Abcam). Subsequently, after rinsing with PBS, the slices were incubated with fluorescein isothiocyanate-labeled anti-rabbit IgG (dilution, 1:800; cat. no. ab7171; Abcam) and tetramethylrhodamine-conjugated anti-mouse IgG (dilution, 1:800; cat. no. ab6668; Abcam) at room temperature for 2 h. The nuclei were counterstained with 1 μg/ml DAPI (MilliporeSigma) 10 min at room temperature before mounting, and images were obtained with an Olympus light microscope and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Western blotting. The animals were re-anesthetized as aforementioned at 24 h after reperfusion, and brain tissues were obtained from the ipsilateral hemisphere. The cortex tissues were cut into small samples and mixed on ice. Subsequently, ~150 mg of the tissue mass was collected for mitochondrial protein extraction, while the rest was used for total protein extraction. The protein extraction of both the total and the mitochondrial fractions was performed according to the instructions of the Mitochondrial Protein Extraction kit (cat. no. AR0156; Wuhan Boster Biological Technology, Ltd.) and the Total Protein Extraction kit for Animal Cultured Cells/Tissues (cat. no. BB-3101; BEST BIO Technical Co., Ltd.; http://www.bestbio.com.cn/search?q=BB-3101). The protein concentration was quantified using the BCA method (BCA Protein Assay kit; cat. no.P0010S; Beyotime Institute of Biotechnology), and an equal amount of protein (20 μg) was loaded for 10% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane, followed by blocking in 10% non-fat milk at room temperature for 30 min. Subsequently, the membrane was incubated overnight at 4˚C with gentle shaking with the following primary antibodies: rabbit anti-synaptophysin (SYP; dilution, 1:1,000; cat. no. 4329; Cell Signaling Technology, Inc.), rabbit anti-post synaptic density protein 95 (PSD95; dilution, 1:1,000; cat. no. 2507; Cell Signaling Technology, Inc.), anti-zonula occludens-1 (ZO-1; dilution, 1:1,000; cat. no. ab190085; Abcam), rabbit anti-Bcl-2 (dilution, 1:1,000; cat. no. 15071; Cell Signaling Technology, Inc.), rabbit anti-Bax (dilution, 1:1,000; cat. no. 2774; Cell Signaling Technology, Inc.), rabbit anti-cleaved caspase-3 (dilution, 1:1,000; cat. no. 9664; Cell Signaling Technology, Inc.), rabbit anti-cytochrome *c* (Cyt-c; dilution, 1:1,000; cat. no. ab133504; Abcam) and anti-GAPDH (dilution, 1:5,000; cat. no. ab8245; Abcam). The cleaved caspase-3 (Asp175) (5A1E) rabbit monoclonal antibody can detect endogenous levels of the large fragment (17/19 kDa) of activated caspase-3, resulting from cleavage adjacent to Asp175, and the antibody does not recognize full length caspase-3 or other cleaved caspases. After rinsing with TBST (with 20% Tween-20), the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG

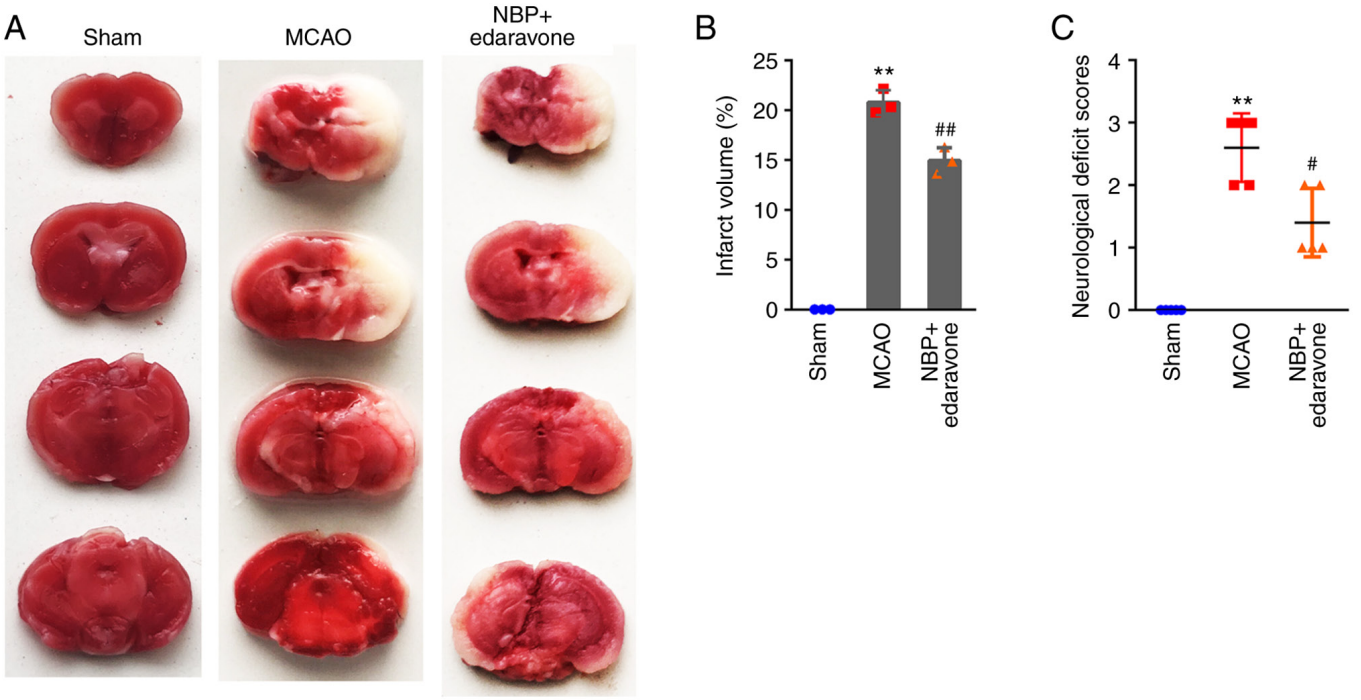

Figure 1. NBP combined with edaravone reduces infarct volumes and neurological deficit scores. Effects of NBP combined with edaravone on infarct volumes and neurological deficit scores in mice following 1 h of MCAO and 24 h of reperfusion. (A) Representative 2,3,5-triphenyltetrazolium chloride staining images of the brain slices. (B) Percentage of infarct volumes in the whole brain (n=4 for each group). (C) Neurological deficit scores in the groups (n=4 for each group). **P<0.01 vs. Sham; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. MCAO. NBP, DL-3-n-butylphthalide; edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one; MCAO, middle cerebral artery occlusion.

(dilution, 1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) and HRP-conjugated anti-mouse IgG (dilution, 1:2,000; cat. no. 7076; Cell Signaling Technology, Inc.) for 1 h at room temperature. After washing the slices with TBST, the protein levels were determined with HRP-ECL method (BeyoECL Star Kit, cat. no. P0018AS; Beyotime Institute of Biotechnology), and detected by the ChemiDoc™ XRS + Imaging System (Bio-Rad Laboratories, Inc.). The immunoblot density was analyzed using ImageJ software V1.49 (National Institutes of Health).

Statistical analysis. Each test was repeated at least three times, and all experimental data are presented as the mean ± standard deviation. Using SPSS version 20.0 (IBM Corp.), an unpaired Student's t-test was performed to determine the statistical significance of the differences between pairs of groups, while one-way ANOVA with post hoc Bonferroni's multiple comparison tests was used to compare multiple group means. P<0.05 was considered to indicate a statistically significant difference.

Results

NBP combined with edaravone attenuates infarct volumes and neurological deficit scores. Infarct volumes were determined using TTC staining. The control mice in the sham group had no obvious infarcts, while the average volumes of the NBP + edaravone and MCAO groups were 14.92±1.33 and 20.75±1.23%, respectively (Fig. 1A and B). The NBP + edaravone mice exhibited significantly lower infarct volumes compared with the MCAO group. Consistent with the TTC staining results, NBP combined with edaravone significantly attenuated the neurological deficit scores compared with the MCAO only group (Fig. 1C). The data revealed that treatment of NBP combined with edaravone could ameliorate the reduced neurological scores and the infarction volumes resulting from cerebral ischemia.

NBP combined with edaravone attenuates cerebral cell damage. Cerebral cell damage was determined using Nissl and HE staining. As presented in HE stained tissue sections, the structures of cerebral cortical neurons were clear and complete, the cells were arranged densely and orderly without edema, and the cytoplasm of neurons was lightly stained in sham group (Fig. 2A, sham). In the MCAO group, the structures of cortical neurons were disordered, including cell swelling, nuclear pyknosis, fragmentation and dissolution (Fig. 2A, MCAO). The neuronal damage in NBP combined with edaravone group was less than that in the MCAO group, with the images displaying that most of the cell structures were complete, but there were scattered necrotic and slight edema between cells (Fig. 2A, NBP + edaravone). For Nissl stained tissue sections, there were no marked morphological changes in the sham group, the Nissl bodies were uniformly stained; neurons were arranged orderly without necrosis and edema (Fig. 2B, sham). By contrast, significant morphological changes were detected in the peri-infarct zone of the MCAO model mice, including neuronal loss, nuclei shrinkage and dark staining of neurons (Fig. 2B, MCAO), while the neuronal loss, nuclear shrinkage and morphological changes were significantly reduced in the NBP combined with edaravone treated mice compared with the MCAO group (Fig. 2B, NBP + edaravone). These results indicated that NBP combined with edaravone could ameliorate

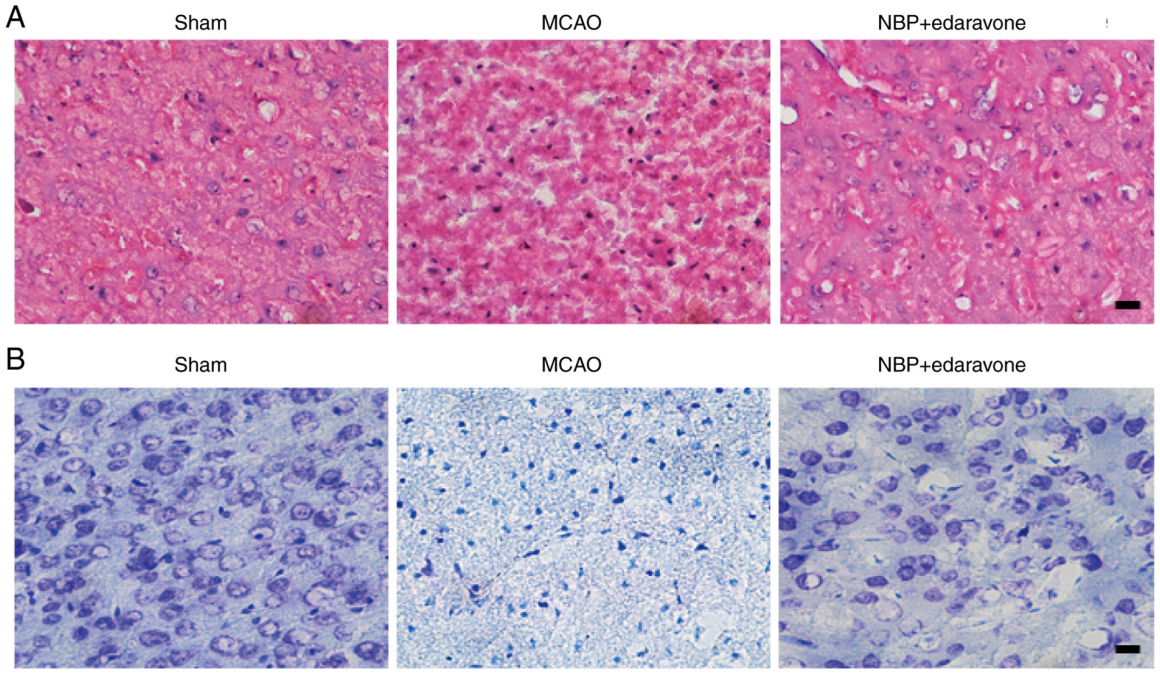

Figure 2. Morphological cell changes in the three groups. Representative images of (A) hematoxylin-eosin staining and (B) Nissl staining in the sham operation, MCAO and NBP + edaravone groups (n=3 in each group). Magnification, x200. MCAO, middle cerebral artery occlusion; NBP, DL-3-n-butylphthalide; edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one.

neuronal damage caused by cerebral ischemia-reperfusion injury.

Effects of NBP combined with edaravone on the NVU cell immunoreactivity. Astrocytes, neurons and vascular endothelial cells are the main components of the NVU (15). Therefore, the effects of NBP combined with edaravone on the NVU were evaluated using the cell immunoreactivity of astrocytes, neurons and vascular endothelial cells, visualized using immunofluorescence staining.

Astrocyte immunoreactivity was assessed using GFAP immunofluorescence staining. GFAP marks the cytoskeleton of astrocytes (22), and thus indicates the astrocyte-positive and soma structure in the single images. The GFAP expression of the MCAO group was significantly increased compared with that of the sham group (Fig. 3). However, NBP combined with edaravone significantly weakened the GFAP expression compared with the MCAO group.

Neuron immunoreactivity was assessed by NeuN immunofluorescence staining. As presented in Fig. 4, the MCAO group exhibited significantly decreased NeuN expression compared with the sham operation group. By contrast, NBP combined with edaravone significantly increased the NeuN expression compared with the MCAO group.

CD31 is a marker of vascular endothelial cells. Vascular endothelial cell immunoreactivity was determined using CD31 immunofluorescence staining. CD31 protein expression indicated that the two markers are closely associated with the basic structure of cells in the merged image (23). CD31 expression was significantly decreased in the MCAO group compared with the sham operation group (Fig. 5), whereas NBP combined with edaravone significantly increased the CD31 expression compared with the MCAO group.

These results indicated that GFAP, NeuN and CD31 were expressed in neurons in mice, and also suggested that cerebral ischemia can increase the expression of GFAP, but decrease the expression of both NeuN and CD31, while treatment of NBP combined with edaravone could decrease the expression of GFAP and increase the expression of both NeuN and CD31 in MCAO model mice.

Effects of NBP combined with edaravone on the blood-brain barrier (BBB). To explore the effects of NBP combined with edaravone on the BBB, western blotting was performed to measure the expression levels of PSD95, the synaptic protein SYP and the tight junction protein ZO-1. The expression levels of PSD95 and SYP were significantly reduced in the MCAO model mice, while NBP + edaravone significantly increased their expression levels compared with the MCAO group (Fig. 6A and B). ZO-1 expression was significantly decreased in the MCAO group compared with the sham operation group, while NBP + edaravone significantly increased ZO-1 protein expression compared with the MCAO group (Fig. 6C). These results suggested that cerebral ischemia could decrease the protein expression levels of PSD95, SYP and ZO-1 in the MCAO groups at 24 h, but administration of NBP + edaravone significantly increased the expression levels of these three proteins compared with the MCAO group at the same time.

Effects of NBP combined with edaravone on the expression levels of apoptosis-related proteins. The expression levels of apoptosis-related proteins were detected using western blotting. The expression levels of cleaved caspase-3 and pro-apoptotic protein Bax were significantly increased, while anti-apoptotic protein Bcl-2 expression was significantly

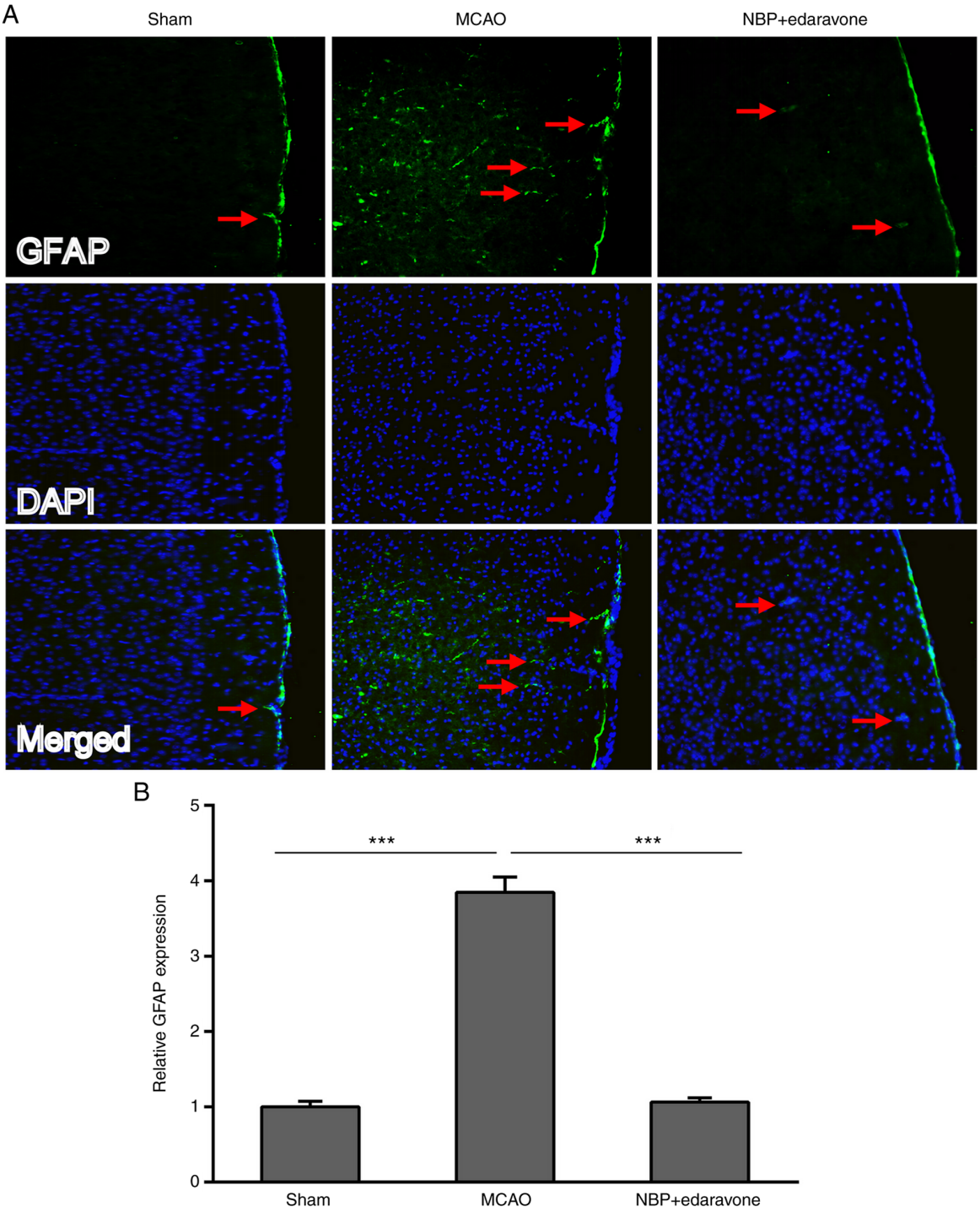

Figure 3. GFAP immunofluorescence staining to evaluate astrocyte immunoreactivity. The merged image indicates that the structure of GFAP was closely connected with the nucleus stained by DAPI to demonstrate that this was an astrocyte. Immunofluorescence staining for GFAP (arrows suggest the localization of GFAP) in the ischemic cortex of mice following 1 h of MCAO and 24 h of reperfusion. (A) Representative GFAP immunofluorescence staining images of brain slices. Magnification, x100. (B) Quantification of GFAP expression. The GFAP expression in the MCAO group was stronger compared with that in the sham operation group, while it was significantly decreased in the NBP combined with edaravone group compared with that in the MCAO group (n=3 in each group). ***P<0.001. GFAP, glial fibrillary acidic proteins; MCAO, middle cerebral artery occlusion; NBP, DL-3-n-butylphthalide; edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one.

decreased in the MCAO model mice compared with the sham group. NBP + edaravone significantly decreased cleaved caspase-3 and pro-apoptotic protein Bax expression, and significantly increased anti-apoptotic protein Bcl-2 expression compared with the MCAO mice (Fig. 7A-C). In the sham group, the expression levels of Cyt-c in the mitochondria were significantly increased compared with those in the MCAO mice. In the NBP + edaravone group, Cyt-c expression was significantly increased compared with that in the MCAO group (Fig. 7D). These data indicated that NBP + edaravone treatment may play a key role in preventing apoptosis during cerebral ischemia-reperfusion injury.

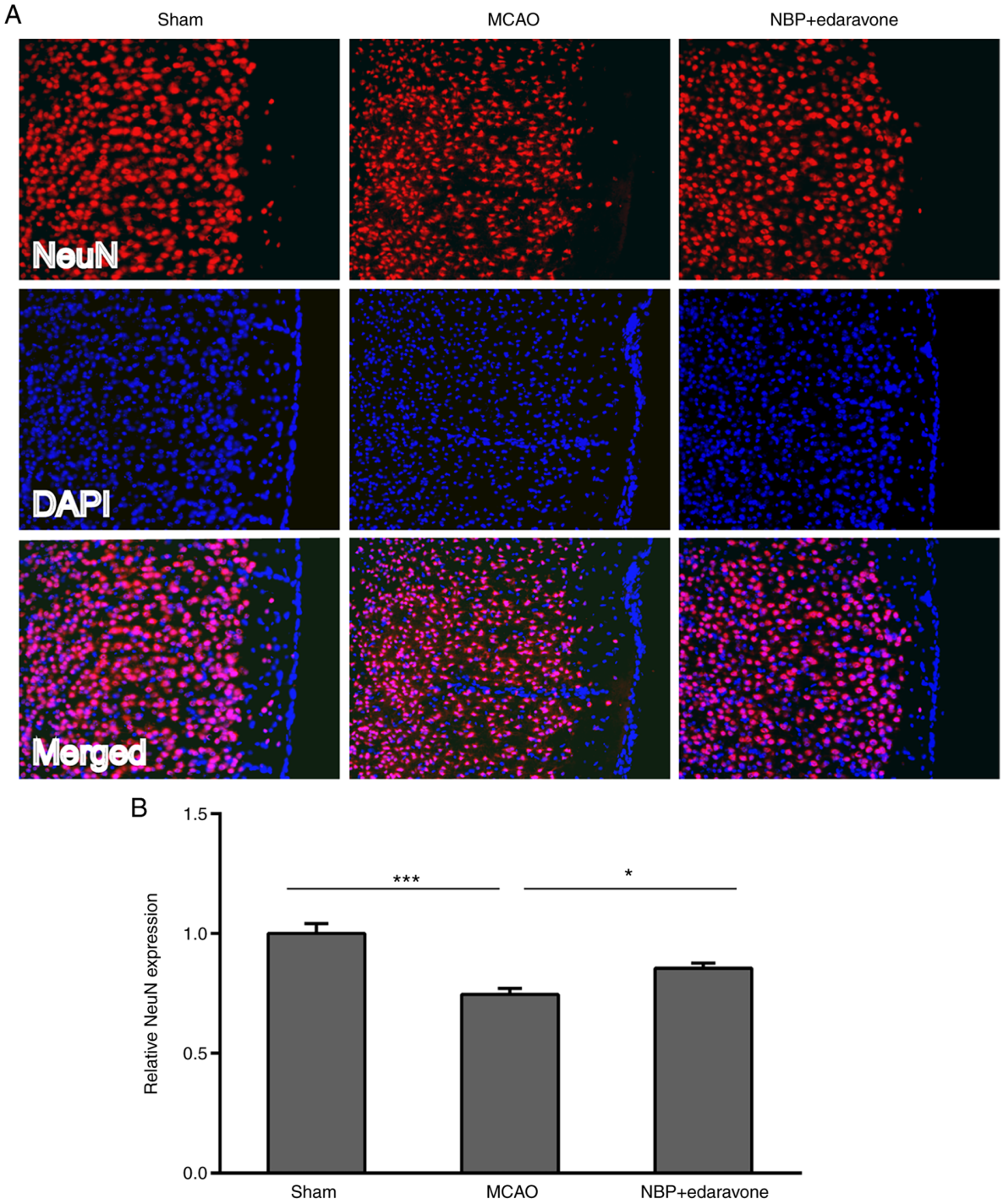

Figure 4. NeuN immunofluorescence staining to appraise neuron immunoreactivity. Immunofluorescence staining for NeuN in the ischemic cortex of mice following 1 h of MCAO and 24 h of reperfusion. (A) Representative NeuN immunofluorescence staining images of brain slices. Magnification, x100. (B) Quantification of NeuN expression. NeuN expression in the MCAO group was reduced compared with that in the sham operation group, while NBP combined with edaravone significantly increased NeuN expression compared with that in the MCAO group (n=3 in each group). *P<0.05, ***P<0.001. NeuN, neuronal nuclei; MCAO, middle cerebral artery occlusion; NBP, DL-3-n-butylphthalide; edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one.

Discussion

The neurons, BBB, microglial cells and extracellular matrix that maintain the integrity of brain tissue are the structural basis of the NVU (15,16). The experimental results indicated that NBP + edaravone protected the brain against ischemic stroke damage by targeting the whole NVU in the mouse MCAO model. NBP + edaravone alleviated brain injury in MCAO mice, as demonstrated by the reduced infarct volumes and neurological deficit scores. These neuroprotective effects might be due to these drugs attenuating the cerebral cell damage, positively affecting the expression of some associated neuroproteins, such as PSD95, SYP, ZO-1, claudin-5 and CD31, for cell activity in the NVU, improving the BBB function and inhibiting apoptosis by altering the expression levels of apoptosis-related proteins.

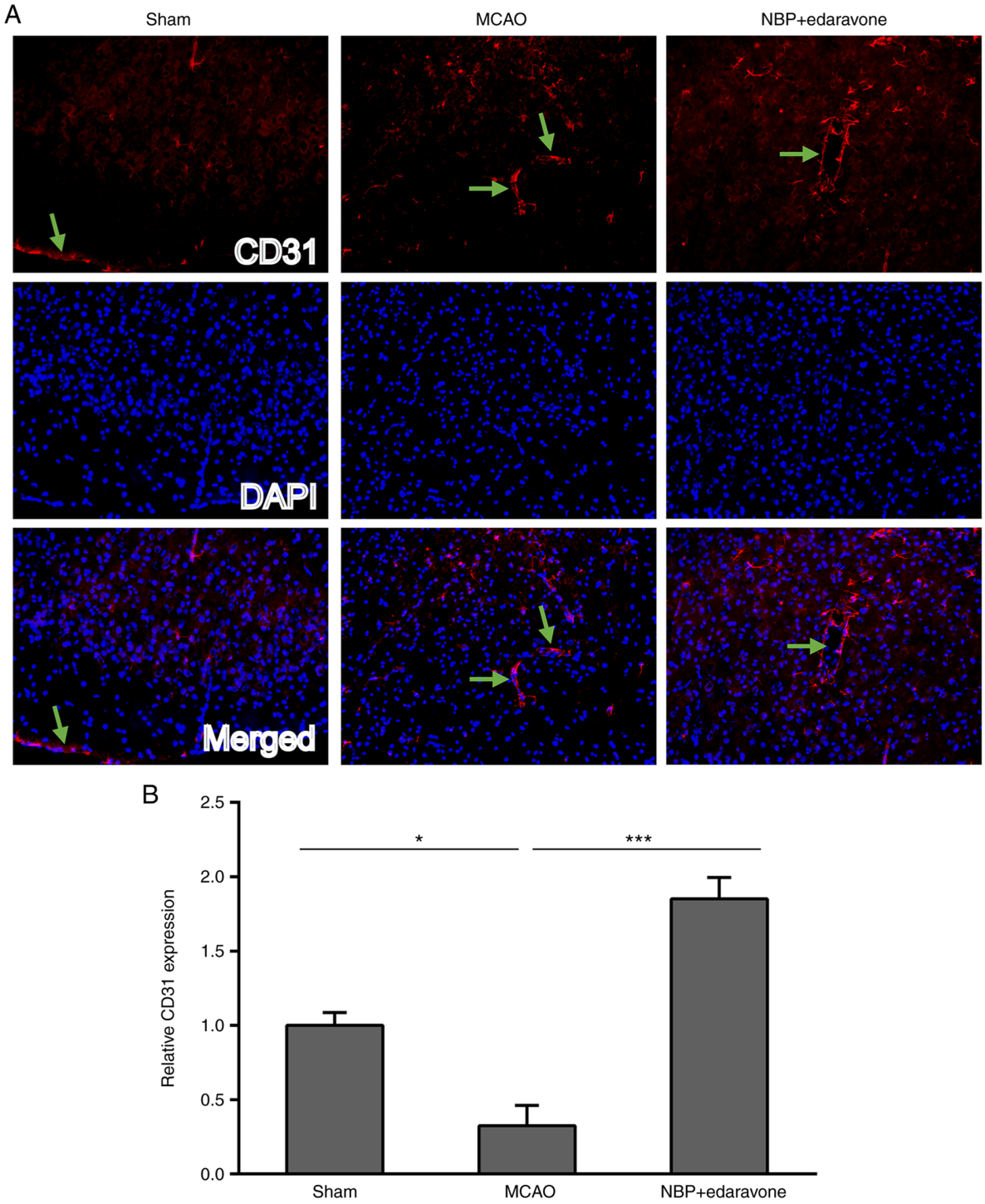

Figure 5. Relationship between CD31 and vascular endothelial cell immunoreactivity. Immunofluorescence staining for CD31 (arrows suggest the localization of CD31) in the ischemic cortex of mice following 1 h of MCAO and 24 h of reperfusion. (A) Representative CD31 immunofluorescence staining images of brain slices. Magnification, x100. (B) Quantification of CD31 expression. CD31 expression was significantly decreased in the MCAO group compared with the sham operation group, while NBP combined with edaravone significantly increased CD31 expression compared with both the sham and the MCAO groups (n=3 in each group). *P<0.05, ***P<0.001. CD31, platelet and endothelial cell adhesion molecule 1; MCAO, middle cerebral artery occlusion; NBP, DL-3-n-butylphthalide; edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one.

NBP reportedly attenuates neuronal injuries following both *in vitro* and *in vivo* ischemic strokes (24,25). NeuN immunosignals are decreased in the neocortex and striatum of mice following 24-h ischemia (26). Additionally, the NBP derivative, (S)-ZIM-289, reportedly protects neurons in the brain from ischemic damage by decreasing the brain infarct area, improving the neurological function and preventing neuronal loss and apoptosis in a rat MCAO model (27). In the present study, NBP + edaravone increased NeuN expression compared with that in MCAO model mice, demonstrating their neuronal protective effect against ischemic brain injury.

GFAP is a major mature astrocyte filament and is considered a promising serum biomarker to differentiate between intracerebral hemorrhage and acute ischemic stroke (28).

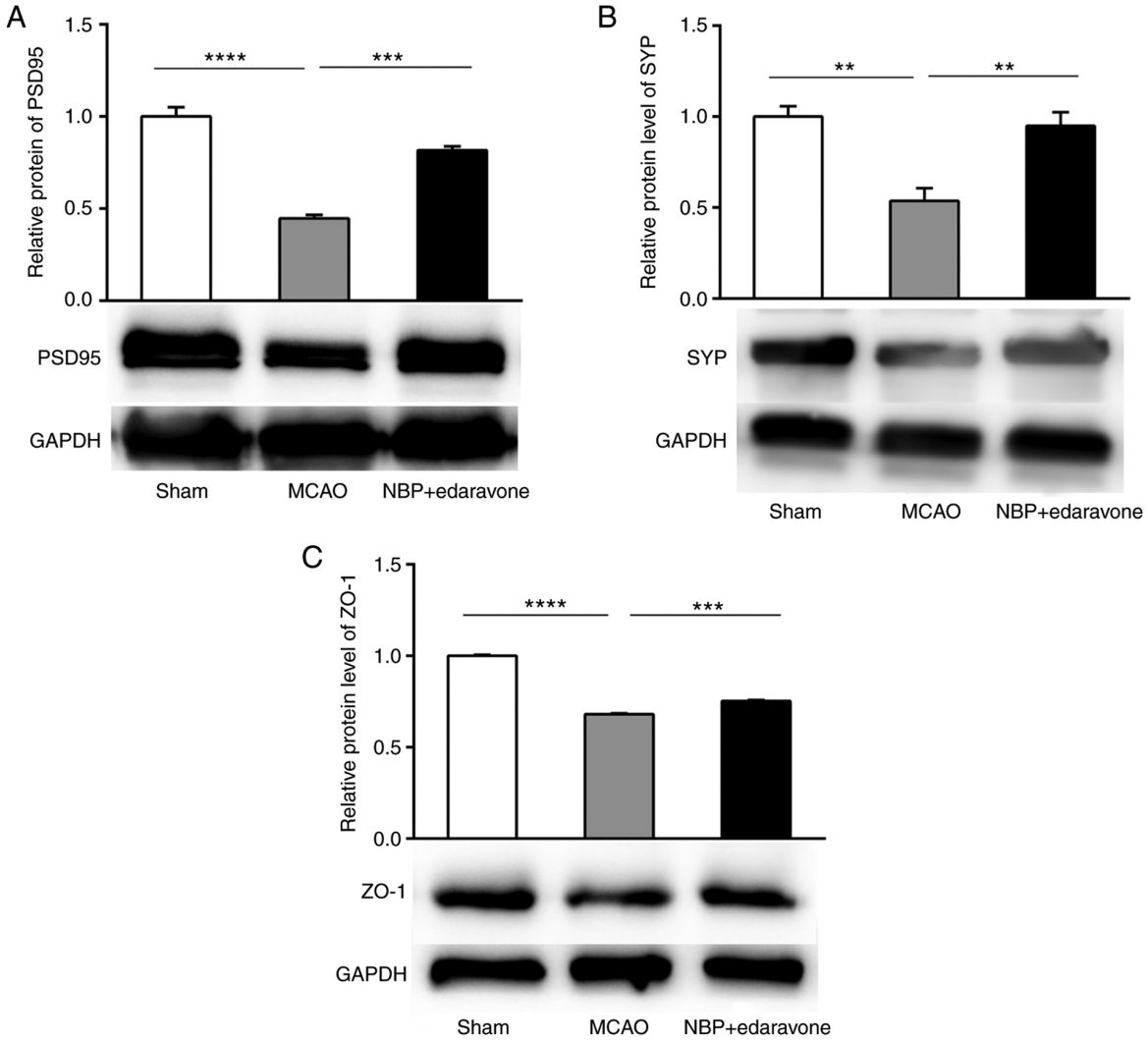

Figure 6. NBP combined with edaravone increases the expression levels of PSD95, SYP and ZO-1. Expression levels of (A) PSD95, (B) SYP and (C) ZO-1 in the different groups (n=3 in each group). **P<0.01, ***P<0.001 and ****P<0.0001. MCAO, middle cerebral artery occlusion; NBP, DL-3-n-butylphthalide; edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one; PSD95, post synaptic density protein 95; SYP, synaptophysin; ZO-1, zonula occludens-1.

GFAP expression is low in the healthy mouse cortex (29,30). However, GFAP expression levels are high in various neurological dysfunction diseases, including ischemic stroke (31). High GFAP expression is considered a marker of reactive astrogliosis, a process in which astrocytes respond to various neurological dysfunction diseases (31-33). High serum GFAP levels are associated with poor outcomes in patients with acute ischemic stroke (34). Several studies have demonstrated that the expression levels of GFAP may increase at different time intervals after unilateral MCAO (35,36); however, some neuroprotective agents can reduce the expression levels of GFAP (37,38). Similarly, in the present study, the MCAO mice exhibited significantly increased GFAP expression compared with the sham mice. NBP + edaravone significantly decreased GFAP expression, indicating their suppressive effects on superabundant gliogenesis. These results suggested that the combination of butylphthalide and edaravone would not cause damage to the NVU, and the combination of the two drugs should have protective effects on the NVU due to the decrease of GFAP expression at 24 h after reperfusion in mice.

NBP has been reported to promote angiogenesis and improve the BBB function following ischemic injury in a rat MCAO model (39-41). NBP markedly increases the levels of circulating endothelial progenitor cells in patients with acute ischemic stroke, improving the clinical prognosis (42). CD31 is associated with angiogenesis (43,44). Physical exercise reduces the brain infarct area, improves neurological function and promotes angiogenesis by increasing CD31 expression and protein expression in a rat MCAO model (45). In the present study, CD31 expression was decreased in mice following ischemic stroke injury, while NBP + edaravone significantly increased CD31 expression compared with the MCAO model mice. The positive effects of NBP + edaravone in individuals with ischemic stroke may be partly due to the promotion of angiogenesis.

Zhang *et al* (46) reported an improvement in neurological function and survival rate in a mouse MCAO model with non-erythropoietic mutant erythropoietin injected intraperitoneally, which may be due to erythropoietin promoting angiogenesis and neurogenesis, while suppressing superabundant gliogenesis. Based on the GFAP, NeuN and CD31 immunofluorescence staining findings of the present study, it

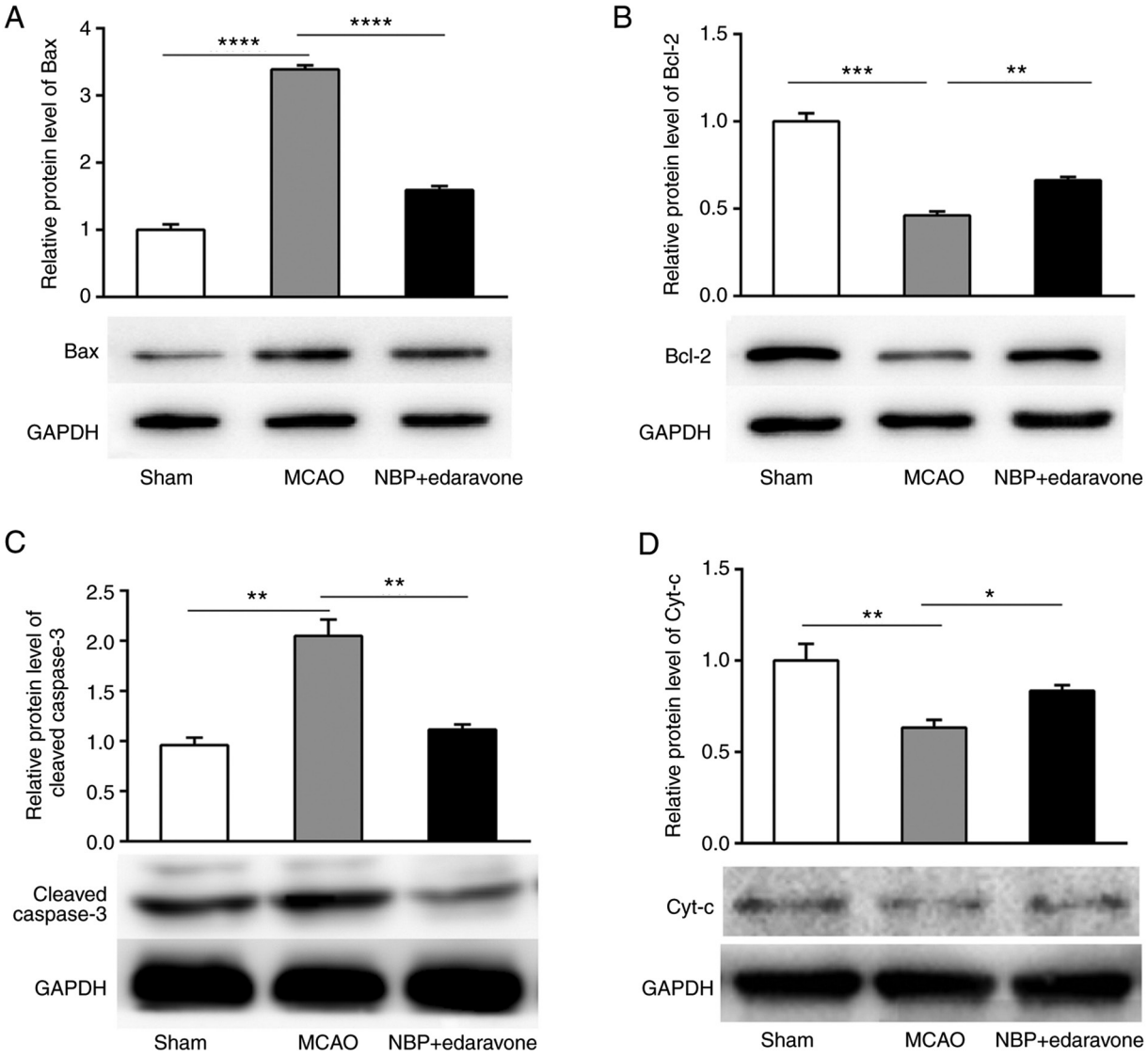

Figure 7. Apoptosis-related protein expression. NBP combined with edaravone inhibited apoptosis. Expression levels of (A) Bax, (B) Bcl-2, (C) cleaved caspase-3 and (D) Cyt-c in the different groups (n=3 in each group). Western blot analysis of Cyt-c was performed only for the mitochondrial fraction. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. MCAO, middle cerebral artery occlusion; NBP, DL-3-n-butylphthalide; edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one; Cyt-c, cytochrome *c*.

was hypothesized that NBP + edaravone might have a similar mechanism by inhibiting superabundant gliogenesis and promoting neurogenesis and angiogenesis.

PSD95, SYN and other proteins, such as microtubule-associated protein 2, which are closely associated with synaptic formation and neurotransmission, can be considered as markers of presynaptic and postsynaptic components. PSD95 and SYP are also markers of synaptic plasticity (47). The decrease of PSD95 and SYP expression indicates a loss of synapses (47,48). Tight junctions are a hallmark of polarized epithelial cells, and ZO-1 is a known key regulator of tight junction formation. ZO-1 is the first confirmed tight junction cytoplasmic protein, and it not only provides a scaffold for connecting adhesion and transmembrane proteins, but is also associated with the increase of BBB permeability when its loss and degradation occur (49). Therefore, ZO-1 serves a notable role in maintaining the continuity and integrity of tight junctions.

An ischemic stroke will first damage the BBB, altering its structure and function and increasing permeability. In a rat ischemic stroke model, the BBB integrity was disrupted following ischemia (50). The deterioration is accompanied by reduced expression levels of the synaptic proteins PSD95 and SYP and the tight junction protein ZO-1 (34). The acute inflammatory disorder drug, ulinastatin, protects the brain from ischemic damage by increasing ZO-1 expression (51). Alogliptin attenuates brain disruption and restores ZO-1 expression in a mouse MCAO model (52). Consistent with the aforementioned reports, the present study revealed that the MCAO mice had significantly increased brain infarct volumes and neurological deficit scores, and significantly reduced expression levels of PSD95, SYP and ZO-1 compared with the sham mice.

The protective effects of NBP or edaravone on ischemia-induced apoptosis have been reported previously (7,8,53). NBP (40 mg/kg; i.p. immediately after ischemia/reperfusion)

markedly increases the ratio of Bcl-2/Bax in the hippocampus of Mongolian gerbils after global cerebral ischemia and reperfusion damage (54). Data has illustrated that a single NBP or edaravone treatment may inhibit the mitochondria-dependent apoptotic cascade after ischemia and reperfusion (7,55); however, to the best of our knowledge, few animal experiments have examined the apoptosis-related effects of both drugs combined. Therefore, it was important to investigate the effects of NBP + edaravone on the release of Cyt-c, the ratio of Bcl-2/Bax and the activation of cleaved caspase-3 after transient focal cerebral ischemia.

A previous study has investigated the effects of NBP on apoptosis induced by transient focal cerebral ischemia in rats and compared the expression levels of Cyt-c in cytosolic and mitochondrial fractions (24). The release of Cyt-c was maximal at 24 h after reperfusion, and NBP markedly inhibited the distributional change of Cyt-c. The levels of Cyt-c in cytosolic and mitochondrial fractions were different at 24 h after reperfusion. There was minimal Cyt-c expression in the cytoplasm of the sham group. By contrast, Cyt-c expression was high in the mitochondria of the same group, and low in both the cytoplasm and mitochondria of the vehicle group. The expression levels of Cyt-c in the cytoplasm and mitochondria of the NBP group were markedly higher compared with those in the vehicle group (24). The results of the present study regarding Cyt-c in mitochondrial protein were consistent with these previous findings. These observations can preliminarily explain how the combination of these two drugs may serve a protective role via Cyt-c regulation. However, the present study only detected Cyt-c levels in the mitochondrial proteins without synchronous data of the cytoplasmic fraction. Therefore, the differences between the two fractions could not be compared, and this absence is a limitation of the present study.

Numerous studies have demonstrated that NBP can improve post-stroke symptoms through several mechanisms and multi-target effects (7,8), including reducing the inflammatory response (56), improving collateral circulation (40-42), protecting mitochondrial function (24,27), inhibiting apoptosis (24,54) and reducing oxidative stress (25). The aim of the present study was to explore whether the co-administration of NBP and edaravone had protective multi-target effects on the NVU of mice with ischemic strokes, and focused on the changes of vascular endothelium in MCAO mice. Although these associated factors were investigated, there are still some deficiencies, and some associated fields require further research.

Numerous studies investigating the effect of butylphthalide on inflammation have been carried out, and the anti-inflammatory effects of butylphthalide have been confirmed (7,8,56). However, not investigating inflammatory targets and proteins is a limitation of the present study, and the anti-inflammatory effect of butylphthalide combined with edaravone remains unclear. Therefore, subsequent research should further clarify the multi-target effects of butylphthalide combined with edaravone involved in anti-inflammatory and antioxidant effects. However, there are still some technical limitations in the present study. Firstly, the lack of low magnification brain slices is a limitation of the pathological study due to some unexpected interference, and future studies should provide these. Secondly, the present study only focused on cleaved caspase-3, and if all types of caspase-3, such as pro-caspase-3 and cleaved caspase-3, had been detected at the same time, the evidence would have been more reliable. Thirdly, the current study would have been improved if the mice groups had been arranged for administration in different dosage (low, middle and high dosage) groups for the combined drugs. It is expected that future studies will be more precise and perfect in experimental design, methods and technologies, in order to make the results more credible and reliable.

Numerous studies have focused on different multi-targets and have revealed some mechanisms associated with the neuroprotective effects of butylphthalide or edaravone; however, few studies specifically involving MCAO models have been carried out to investigate the combined administration of the two drugs.

In conclusion, the present study preliminarily suggested that NBP + edaravone could exert neuroprotective effects in a mouse model by targeting multiple NVU components. These neuroprotective effects mainly included reducing cell damage and apoptosis, increasing the number of neurons and vascular endothelial cells and decreasing astrocyte gliogenesis, thus reducing the brain infarct volume and neurological deficits. Therefore, NBP + edaravone might be a promising future therapy for patients with ischemic stroke.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YC and YG conceived and designed the research. YG and PL conducted all experiments. YL, LG and QW helped conduct experiments. YC and YG confirmed the authenticity of all the raw data. YG and YC wrote the manuscript. All authors revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethical permission was obtained from the Animal Ethics Committee of North China University of the Science and Technology (approval no. 2019068; Tangshan, China), which records and regulates all research activities. The approval from the Animal Ethics Committee included the permission of using mice under euthanasia, and all experimental procedures were conducted in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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