Original Article
Combination treatment with breviscapine and netrin-1 suppresses permanent focal cerebral ischemic injury by activating PGE2 receptor

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Received November 28, 2015; Accepted February 15, 2016; Epub June 15, 2017; Published June 30, 2017

Abstract: Our study investigated the effect of combination treatment of experimental stroke with Breviscapine, an extract from an important traditional herb-plant of China, Erigeron breviscapus (vant.) Hand-Mazz., and Netrin-1 which is a secreted molecule related with laminin that has been perceived as a neuronal guidance cue, on functional outcome, and the pathological lesions in the cerebral ischemic rats. Middle cerebral artery occlusion (MCAO) can induce cerebral ischemia in Wistar rats. Adult male rats were randomly separated into 5 groups: Netrin-1, Sham, Breviscapine, MCAO and combinational treatment groups. The effects of multi-drug on neurological deficits, water content of brain, size of cerebral infarction, and the malondialdehyde (MDA), the levels of Ca²⁺ and Na⁺-K⁺-ATPase and SOD activities in the brain tissue were analyzed. Expression of prostaglandin EP2 & EP4 were detected as well. Furthermore, the analysis of Bcl 2 expression was performed by using western blotting. Both the neurological deficit score and the infarct size were greatly decreased and the water content of brain was reduced from 83.11 to 80.26% (P<0.05) with both pretreatment with Breviscapine, Netrin-1 and combination. Intervention drugs remarkably increased the activities of superoxide dismutase and Na⁺-K⁺-ATPase and reduced Ca²⁺ and MDA levels in the brain tissue (P<0.05) in comparison with those in the MCAO group. Pretreatment with combinational drugs increased the protein expression level of Bcl-2 in comparison with that in the MCAO group. The histo-morphological study revealed that Breviscapine & Netrin-1 has protective effect on ischemic injury. The experimental results showed the protective effects of combinational therapy on rats with ischemic injury. The mechanism might have something to do with the inhibition of oxidative stress and apoptosis, and inducing prostaglandin EP2 & EP4 receptor activation.

Keywords: Breviscapin, netrin-1, cerebral ischemia, bcl-2, prostaglandin, PGE2

Introduction

Ischemic stroke is identified as one of the primary factor causing disability and mortality in adults all over the world, the poor prognosis for stroke is a great challenge for the shortage of effective therapeutic strategies [1]. Application of traditional Chinese medicine for stroke treatment is an effective way [2].

Breviscapine is the extract from an important traditional herb-plant of China, Erigeron breviscapus (vant.) Hand-Mazz., which is a member of the compositae family. It grows mainly in southwest China. Literatures have indentified the neuroprotective and anticoagulation effects of breviscapine [3, 4]. Its preparations have been widely used in clinic treatment of cerebral insufficiency and problems of peripheral circulatory [5]. High-performance capillary electrophoresis with electrochemical detection method has determined the exact nine pharmacologically active ingredients of breviscapine, among which Scutellarin, a well-known flavone 7-O-glucuronide with a molecular weight of 462.21, is regarded as the main active ingredient of breviscapine. However, in clinic application we usually could not separate them individually, so breviscapine is still used as a compound preparation yet [6].

Netrin-1, a laminin-related secreted molecule that has been perceived as a neuronal guidance cue, directs neurons and their axons to...
targets during the nervous system development. It has been stated that Netrin-1 is in possession of anti-stress, anti-diabetic and antitumor effects [7, 8]; in addition, recent study has reported its renal-protective effects in kidney ischemia-reperfusion injury [9]. As known, in the central nervous system (CNS), injured axons not only degenerate but also are incapable of regenerating and re-establishing lost connections and their ability to sprout is restrained as well [10, 11]. Hence, axonal damage often leads to long-term disability [11]. Following the immediate primary central nervous system injury, there are many downstream events known as “secondary injury” that ends up with progressive axonal degeneration [12, 13]. Latest observations have reported the combination therapeutic regimen with netrin-1 and nitric oxide/NO scavenger and it was cardioprotective against ischemia reperfusion (I/R) injury [14]; so we hypothesized that breviscapine possess anti-oxidative stress, anti-inflammation and inhibition of apoptosis effects in ischemic injury [15, 16]; whether the combination therapy with breviscapine and netrin-1 on cerebral ischemia is effective or not? It has not been elucidated yet.

Our study investigated the effect of combination treatment with breviscapine and netrin-1 on a middle cerebral artery occlusion (MCAO) cerebral ischemic rat model, we unraveled the protective effects of combinational therapy and we investigated some related potential mechanism.

Materials and methods

Chemicals and reagents

We purchased Malondialdehyde (MDA), superoxide dismutase (SOD), NO, NOS, calcium (Ca²⁺) and Na⁺-K⁺-ATP assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). We obtained Antibodies against Bcl-2 from Cell Signaling Technology, Inc. (Beverly, MA, USA), while purchasing antibodies against β-actin from Tianjin Jinmai Gene Mapping Technology Co., Ltd. (Tianjin, China). All other chemicals were analytical reagent.

Animal model and experimental groups

Wistar male rats (weight, 270-300 g) were obtained from the Experimental Animal Center of Tongji University (Shanghai, China). All animals were treated following the Guide for Care and Use of Laboratory Animals which is published by the US National Institutes of Health. The ethics committee of local academy (Shanghai, China) approved the study. Transient right middle cerebral artery occlusion (MCAO) was induced for 2 hours by advancing a 4-0 surgical nylon suture (18.5-19.5 mm) depending on the weight of the animal, its tip was rounded by heating near a flame to block the origin of the MCA, using a method of intraluminal vascular occlusion modified in our laboratory [17]. 2 hours after MCAO, reperfusion was carried out by withdrawing the suture. Experimental groups consist of rats subjected to 2 hours of MCAO. 24 hours after MCAO rats were randomly divided into 5 groups (each group has 12 rats): 1) Sham group: where the rats was treated with physiological saline by intraperitoneal (i.p.) injection at 2 ml/kg dose; 2) MCAO group: rats received MCAO and then saline was administered to the rats by i.p. injection at a dose of 2 ml/kg; 3) MCAO + Breviscapine group: rats received MCAO and then breviscapine was injected i.p. at 50 mg/kg dose; 4) MCAO + Netrin-1 group: rats received MCAO and then netrin-1 was injected i.p. at a dose of 50 mg/kg; 5) MCAO + combinational group: rats received MCAO and then breviscapine (50 mg/kg) & recombinant netrin-1 (5 μg/rat) were both injected i.p.

Functional tests

A modified neurological severity score (mNSS) evaluation and Foot-fault tests were conducted before MCAO, and at 1, 7, 14 days after MCAO by a researcher who knew nothing about the experimental groups [17].

Measurement of infarct size

The 2,3,5-triphenyltetrazolium chloride (TTC) method was adopted to evaluate the infarct size was evaluated, as previously described [18]. The rats were sacrificed and their brains were extracted after the neurological deficit score was measured. Each brain was cut into five coronal slices, and the brain slices were stained with 2% TTC solution at 37°C for half an hour. The cerebral infarction areas were determined with the different color-tones following staining (white and red for ischemic cerebral tissue and non-ischemic cerebral tissue, respectively). The infarct size was calculated as a percentage fraction of the ischemic cerebral tissue in the whole brain.
Measurement of brain water content

The wet-dry method was used to measure the content of brain water [19]. The rats were anesthetized using chloral hydrate (350 mg/kg, i.p.) and then sacrificed following the measurement of the neurological deficit score, ahead of the rat brains being rapidly extracted. The brains' wet weight was measured using an electronic balance after removing the olfactory bulb and pons, and then the brains were dried for 24 hours at 100°C with the purpose of acquiring the dry weight. The calculating formula of BW was shown as below: $\text{BW} = \left(\frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}}\right) \times 100$, and BW can be used as an indicator for brain edema.

Analysis of MDA and Ca²⁺ levels and the activities of SOD and Na⁺-K⁺-ATPase in the brain tissue

Following the collection of the blood samples, the brains were removed, weighed and homogenized in ice-cold phosphate-buffered saline (PBS). The homogenate was centrifuged at 2,500×g for 15 minutes at 4°C, and then the supernatant was acquired to measure the SOD activities and Na⁺-K⁺-ATPase and MDA and Ca²⁺ levels using assay kits and a spectrophotometer (7202B; Unico (Shanghai) Instrument Co., Ltd., China) according to the kit manufacturer's guidances.

Histopathological assay

The rats' brains were fixed by transcardial perfusion with saline at 14 days after MCAO, followed by perfusion and immersion in 4% paraformaldehyde before being embedded in paraffin. Hematoxylin and eosin (H&E) was used to process and stain the seven coronal sections of tissue in order to calculate the cerebral infarct size [20]. The indirect lesion area, in which the intact ipsilateral hemisphere area was subtracted from the contralateral hemisphere area, was calculated by using the Global Lab Image analysis system (Data Translation, Malboro, MA). The histological changes after MCAO was captured using a light microscope (×400).

Western blotting

The analysis of Bcl-2 protein expression in the rats was performed using western blotting. To put it simply, before being centrifuged (1,200×g, 15 minutes) at 4°C, the brains were dissected and homogenized with a lysis buffer [1X PBS, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate and phenylmethylsulfonyl fluoride (PMSF)] in order to extract the cellular proteins. The bicinchoninic acid (BCA) method was adopted to determine the protein concentration. The same amounts of protein were separated using 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. Subsequent to blocking with 5% non-fat milk in PBS-Tween-20 (PBST) for one hour, the membranes were cultured overnight at 4°C with β-actin and anti-Bcl-2. The membranes were then cultured with horseradish peroxidase (HRP)-conjugated secondary antibody (Tianjin Jinmai Gene Mapping Technology Co., Ltd.) against rabbit for one hour at room temperature. An enhanced chemiluminescence (ECL) detection system using X-ray film enabled the immunoactive bands to be visualized. β-actin was served as an internal control.

Immunostaining

Brain tissue was harvested, processed, and immunostained as described previously [21]. Primary antibodies included anti-EP2, -EP4 receptor polyclonal antibodies (1:1000; Cayman Chemicals, Ann Arbor, MI), Neu N monoclonal antibody (1/1000; Chemicon, Temacula, CA), and anti-GFAP monoclonal antibody (1/2000; Dako, Carpenteria, CA). Secondary detection reagents and antibodies included donkey anti-mouse Alexa 555, Alexa-555 streptavidin and anti-rabbit Alexa 486. Nikon E400 with an Orca ER CCD camera was used to take images which were digitized by Volocity software (Improvision). Negative controls include omission of the primary antibody or blocking peptide.

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**Table 1. Physiological parameters measured in MCAO and different groups 30 min after the start of MCAO**

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>CO₂ (mmHg)</th>
<th>O₂ (mmHg)</th>
<th>Glucose (mg/dl)</th>
<th>MABP (mmHg)</th>
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<tbody>
<tr>
<td>MCAO</td>
<td>7.28±0.029</td>
<td>47.8±2.9</td>
<td>97.3±15</td>
<td>137±19</td>
<td>68±3.7</td>
</tr>
<tr>
<td>MCAO + Breviscapine</td>
<td>7.29±0.028</td>
<td>48.6±3.2</td>
<td>98.5±8.6</td>
<td>133±16</td>
<td>67±3.6</td>
</tr>
<tr>
<td>MCAO + Netrin-1</td>
<td>7.28±0.027</td>
<td>50.2±3.0</td>
<td>99.1±9.3</td>
<td>134±18</td>
<td>68±3.9</td>
</tr>
<tr>
<td>MCAO + combinational</td>
<td>7.27±0.029</td>
<td>49.3±2.9</td>
<td>98.2±9.6</td>
<td>136±17</td>
<td>69±3.7</td>
</tr>
</tbody>
</table>

There were no significant differences noted.
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Statistical analysis

Data were presented as the mean ± standard deviation and analyzed by the SPSS (SPSS Inc. Chicago, IL). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett’s test. In all cases, a p-value of <0.05 was perceived to indicate a statistically significant difference.

Results

Neurological outcome

No prominent differences was observed in the physiological parameters between the MCAO, MCAO + Breviscapine, MCAO + Netrin-1 group, MCAO + combinational group 30 min after the start of MCAO (Table 1).

The differences in functional recovery among the 4 groups (MCAO control, Breviscapine treatment, Netrin-1 treatment and combinational treatment) was detected through GEE statistical analysis to determine what effects the combination treatment of stroke rats has on functional outcome. The 4 groups equaled at the bottom-line and possess no individual functions (P=0.99 based on the Global Test). On day 14, it was observed that there is a marginal significant interaction based on the Global Test.
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Figure 3. Effects on oxidative stress induced by cerebral ischemia in brain tissue. The levels of (A) superoxide dismutase (SOD), (B) malondialdehyde (MDA), (C) Ca²⁺ and (D) Na⁺-K⁺-ATPase were measured respectively following 24 h ischemia. Data are presented as the mean ± standard deviation. *P<0.05 compared with sham group; **P<0.05 compared with middle cerebral artery occlusion (MCAO) group.

Figure 4. Effects on Bcl-2 expression in the brain. The protein expression level of Bcl-2 was assessed using immunoblotting. The level of β-actin was used as the internal control.

(P=0.09), which demonstrates a trend of synergism between the two treatments. A remarkable effect was observed in the groups treated with Breviscapine (P=0.0127), Netrin-1 (P=0.0014) and the combination (P=0.0028) in comparison with the MCAO control group (Figure 1A, 1B). On day 7, based on the Global Test (P=0.27), no treatment interaction was observed, which
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Displays additivity of the two treatments and a notable effect was detected in the group treated with Breviscapine (P=0.038), Netrin-1 (P=0.027) and the combination (P=0.0087) in comparison with the MCAO control group (Figure 1A, 1B). Therefore, the data suggest that the combination treatment decreases an additive up to 14 days after stroke.

Effects on brain infarcted area and the content of brain water

To determine the effect of combinational treatment on cerebral ischemia, the infarcted volume and the content of brain water were measured. It can be seen from Figure 2A that no infarcted volume was observed in the sham group. The infarcted volume in the Breviscapine, Netrin-1 and combinational groups and MCAO group were 27.5±3.77, 26.9±3.49 and 18.72±2.53%, 33.4±5.48%, respectively, which means that Breviscapine, Netrin-1 and combinational groups have lower infarcted volume than the MCAO group. The infarcted volume of the combinational treatment group is the smallest among all the groups.

The content of brain water following 24 hours ischemia can be seen in Figure 2B. The content of brain water in the sham group was 77.68±3.19%. Ischemia resulted in a great increase in content of brain water in the MCAO group in comparison with that in the sham group (83.11±2.58%, P<0.05). However, in the Breviscapine, Netrin-1 and combinational treatment groups, the content of brain water was greatly reduced. And the mean contents of brain water were 80.69±1.07, 80.54±1.62 and 80.26±2.85% in the three groups, respectively.

Effects on MDA and Ca\(^{2+}\) levels and the activities of SOD and Na\(^{+}\)-K\(^{-}\)-ATPase in brain tissue

To describe the effect of combinational therapy on the oxidative stress induced by MCAO, MDA and Ca\(^{2+}\) levels, the activities of SOD and Na\(^{+}\)-K\(^{-}\)-ATPase were measured. MDA and Ca\(^{2+}\) levels are higher in the MCAO group than those in the sham group (Figure 3). Breviscapine, Netrin-1 and combinational treatment greatly decreased MDA and Ca\(^{2+}\) levels in comparison with those in the MCAO group. On the contrary, the activities of SOD and Na\(^{+}\)-K\(^{-}\)-ATPase were noticed to have experienced great reductions in the MCAO group, which were largely attenuated by our treatment.

Effect on Bcl-2 expression

To look into the apoptotic signaling, the anti-apoptotic protein Bcl-2 expression was analyzed. In contrast with the sham group, the MCAO group has lower level of Bcl-2 protein expression, which was in line with previous studies [22]. Breviscapine, Netrin-1 and combinational treatment markedly increased Bcl-2 expression in comparison with that in the MCAO group, indicating that apoptosis inhibition may be able to mediate the protective effects of combinational treatment (Figure 4).

Histopathological examination of the brain tissues

As can be seen in Figure 5, plenty of pyramidal neurons were noticed in the group with sham-surgery, while marked morphological changes like loss of neuronal cell, neuronal vacuolization, nuclear shrinkage and dark staining of the neurons were observed in the model group. Treatment with Breviscapine, Netrin-1 and
Figure 6. EP2 and EP4 receptors are expressed in neurons under basal conditions (400× magnification). Co-localization studies with the neuron-specific marker NeuN demonstrates staining of (A) EP2 and (B) EP4 in cortical neurons in frontal cortex. Both EP2 and EP4 were broadly expressed in neurons of cerebral cortex, striatum, and hippocampus. EP2 and EP4 were increased by MCAO treatment; however, combinational treatment further promoted the expression of EP2 (C) and EP4 (D). *P<0.05 and ***P<0.001, vs. the MCAO group.
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combination all significantly attenuated these pathological changes.

Effect on prostaglandin EP2 & EP4 receptor expression

To figure out the cellular mechanism of combination therapy, we investigated the temporal dynamics of EP2 and EP4 receptor expression in sham and MCAO-RP rats at 4 and 24 h after MCAO. EP2 and EP4 receptors co-localized with NeuN in neurons in rats in sham group (Figure 6A and 6B; layers II-IV of frontal cortex), suggesting that neurons and endothelial cells are where the receptors of EP2 and EP4 are basally expressed. At 4 hours of reperfusion, neuronal EP2 staining and NeuN in neurons in the ischemic core both reduced, but the expression of EP2 adhered to peri-infarct NeuN positive neurons (Figure 6B).

To compare the early effects of therapy on EP2 & EP4 receptor expression, we compared EP2 expression and EP4 expression on the 6 h post MCAO by western, and results showed that both EP2 & EP4 were increased by MCAO treatment; however, combinational therapy further promoted the expression of EP2 & EP4 (Figure 6C, 6D).

Discussion

Previous studies have demonstrated that both breviscapine and Netrin-1 could reduce the infarcted volume in acute myocardial infarction in rats [22]. In our study, the effects of breviscapin and Netrin-1 on brain damage following MCAO were examined. The study indicated that neurological function was greatly reduced and both infarcted volume and the content of brain water were increased in the MCAO group in comparison with the sham-operation group. Significant reduction of infarct size and improvements of neurological function and related morphological changes were achieved through the administration of breviscapin & Netrin-1, when the administration happened 3 days ahead of MCAO. The results indicated that there is a chance that breviscapin and Netrin-1 can attenuate rats’ cerebral injury. This effect was related with reduced MDA and Ca<sup>2+</sup> levels and increased SOD and Na<sup>-</sup>-K<sup>-</sup>-ATPase activity. It also seemed that breviscapin and Netrin-1 contributed to the increase of Bcl-2 expression and activating prostaglandin EP2 and EP4 receptors in cerebral ischemia.

Oxidative stress plays a vital role in ischemic injury. It has been revealed that reactive oxygen species like the superoxide anion and the hydroxyl radical are generated in the course of ischemia, which attack proteins, lipids and DNA in tissue of ischemic brain [22]. Reactive oxygen species are scavenged by endogenous antioxidant enzymes like glutathione-S-transferase (GST), superoxide dismutase, catalase (CAT) and Na<sup>-</sup>-K<sup>-</sup>-ATPase [23]. Hence, the measurement of such enzymes levels makes it possible for the estimation of the amount of oxidative stress. The present study suggested that the lipid peroxidation was increased and the activity of endogenous antioxidant enzymes was reduced during cerebral ischemia, which was in line with the previous study results [24].

Pretreatment with breviscapin & Netrin-1 markedly decreased MDA level and increased superoxide dismutase and Na<sup>-</sup>-K<sup>-</sup>-ATPase activities in the tissue of brain, which indicated that the antioxidant properties of breviscapin & Netrin-1 may serve as a protective mechanism to battle the oxidative stress induced by MCAO by way of increasing antioxidant enzymes like superoxide dismutase levels.

Our experiment also suggested that breviscapine & Netrin-1 could attenuate neuro-apoptosis and regulate the expression of protein related to apoptosis after transient focal cerebral ischemia, which might contribute partly to the protective effects on cerebral ischemic damage.

Prostaglandin E2 (PGE2) which is made up of arachidonic acid by the action of cyclooxygenases and PGE2 synthases, was formerly regarded as a pro-inflammatory prostaglandin, but it might turn out to be much more complicated. To a large degree, the functions of PGE2 are performed by binding to 4 kinds of membrane-bound G-protein-coupled receptors: E prostaglandin (EP) 1, 2, 3, and 4 which have different impacts on the production of cyclic adenosine monophosphate (cAMP), phosphoinositol turnover, and regulation of intracellular calcium level. In former studies, it has been reported that the EP2 receptor expression was high in striatum, cerebral cortex and hippocampus. Our study determined the expression profile of EP2 in peri-infarct NeuN positive neurons, and it seems that EP2 activation is sustained as time goes on. That whether such changes are related with neurologic improvements still needs further investigations. According to cur-
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In our studies, the combinational therapy did enhance the expression level of EP2 and EP4, which suggested that the protective effects of breviscapine and netrin-1 combination might be correlated with activating PGE2 receptors; however, since the EP3 receptor has three distinct isoforms which are derived by alternative splicing differentiating at the carboxy terminus that lead to differential downstream signaling, constitutive activity and desensitization, further studies should be conducted [27].

In summary, the present study indicated the protective effect of breviscapine and netrin-1 combinational therapy in a model of rat brain ischemia. The protective mechanisms was achieved by reducing radical formation, calcium overload, lipid peroxidation, increasing expression of antiapoptotic protein and regulating PGE2 receptor pathway. The results suggest that breviscapine and netrin-1 may possess beneficial therapeutic potential to treat cerebral ischemia. Application to clinical practice and defining the underlying mechanism required further studies.

Acknowledgements

The support that the Training Program of Young Doctors Foundation of Shanghai Municipal Health Bureau gave this work is much appreciated (2012-105).

Disclosure of conflict of interest

None.

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References


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