Original Article

Herbal compound Naoshuantong capsule attenuates retinal injury in ischemia/reperfusion rat model by inhibiting apoptosis

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Abstract: Objectives: Ischemic ophthalmopathy threatens people’s lives and health. The herbal compound medication, Naoshuantong capsule, plays a critical role in the treatment of cardiac-cerebral vascular diseases; however, the roles and mechanisms of action of Naoshuantong capsule in ischemic ophthalmopathy is unknown. The objective of the present study was to determine the effect and mechanism of action of Naoshuantong capsule on ischemic ophthalmopathy in rats. Methods: In this study a rat model of ischemic ophthalmopathy was constructed using a high intra-ocular pressure-induced ischemia/reperfusion model. The effects of Naoshuantong capsule on ischemic ophthalmopathy were detected using electroretinography, and changes in retinal ultrastructure were examined by HE staining and electron microscopy. The mechanism of action of Naoshuantong capsule on ischemic ophthalmopathy was explored by immunofluorescence and real-time PCR. Results: Rat models of ischemic ophthalmopathy were successfully constructed by intra-ocular hypertension, which presented decreased amplitudes of the electroretinogram (ERG-b) wave and total retinal thickness, intracellular damage, increased expression of Bax and caspase 3, and decreased expression of Bcl-2. Treatment with Naoshuantong capsule attenuated the changes and damage to the ischemic retina in the rat model, inhibited the over-expression of Bax and caspase 3, and increased the expression of Bcl-2. Conclusion: Our study indicated that Naoshuantong capsule attenuates retinal damage in rat models of ischemic ophthalmopathy, possibly by inhibiting apoptosis.

Keywords: Naoshuantong capsule, ischemic ophthalmopathy, ERG-b wave, caspase 3, apoptosis

Introduction

Ischemic ophthalmopathy is an ischemic disease with obstructed blood supply in eye tissues, especially the optic nerve and retina, which is caused by various factors, such as vascular diseases, metabolic abnormalities, trauma, surgeries, and ocular hypertension [1-4]. With the rapid development of the economy and changes in lifestyle, the morbidity rate of ischemic ophthalmopathy is increasing worldwide, which severely threatens people’s lives and health. Once ischemic ophthalmopathy, such as ischemic optic neuropathy, central retinal artery or vein occlusion, or glaucoma, occurs, there will be permanent damage to eye tissues, especially the optic nerve and retinal neuronal cells, and finally damage to vision.

Naoshuantong capsule is a compound herbal medicine, which consists of extracts of five herbs (Cattail pollen, Radix paeoniae rubra, Rhizoma gastrodiae, Radix Rhapontic and Curcuma aromatic). Naoshuantong capsule has been shown to be effective in the treatment of various ischemic diseases, such as cerebral arterial thrombosis, myocardial infarction, and angina pectoris [5, 6]. It is considered that Naoshuantong capsule may have potential treatment effects on ischemic ophthalmopathy of the optic nerve or ischemic retinal injury; however, the effect of Naoshuantong capsule on ischemic ophthalmopathy is still unknown and needs to be studied. In this present study, we applied a high intra-ocular pressure-induced ischemic/reperfusion rat model, which can be considered as an ischemic ophthalmopathy...
Naoshuantong capsule in ischemic ophthalmopathy

model, to investigate the effects of Naoshuantong capsule on ischemic ophthalmopathy.

Studies have found that apoptosis exists in the retina of ischemia/reperfusion injury animals, such as rat and rabbit models [7, 8]; however, whether or not Naoshuantong capsule has effects on apoptosis in ischemic ophthalmopathy is unclear. In the current study we also detected several apoptosis-related factors to elucidate the potential mechanism underlying the Naoshuantong capsule effect on ischemic ophthalmopathy.

Materials and methods

Antibodies and reagents

Goat anti-rabbit immunoglobulin (IgG) conjugated with horseradish peroxidase (GAR-HRP), goat anti-mouse immunoglobulin (IgG) conjugated with horseradish peroxidase (GAM-HRP), and FITC-conjugated goat anti-mouse IgG (FITC-GAM) were purchased from Biosource (Camarillo, CA, USA). Antibodies against Bax, Bcl-2, and caspase-3 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Naoshuantong capsule, which consists of extracts of five herbs (Cattail pollen, Radix paeoniae rubra, Rhizoma gastrodiae, Radix Rhapontic, and Curcuma aromatic), was provided by Guangdong Southern China Pharmaceutical. The experimental dose was calculated according to the dose based on weight; the adult dosage of the Naoshuantong capsule was 1.2 g/day, and the dose for experimental rats of the Naoshuantong capsule was 3.6 g/kg/day. The dose of Nimodipine was 10 mg/kg/day, which served as the positive drug. All the drugs were dissolved in 2% sodium carboxymethylcellulose.

Animals

The present study was approved by the Ethics Committee of Zhongshan Ophthalmic Center at Sun Yat-sen University. All male healthy SD rats were purchased from the Animal Center of Southern Medical University in China. Rats were maintained on a 12-hour light-dark cycle and were allowed free access to standard rodent chow and water ad libitum. All procedures were performed carefully and gently to ameliorate any extra suffering. According to the existing literature and our previous experience, a low concentration of chloral hydrate (450 mg/kg) is safe for use in eye research involving rats [9, 10]. Therefore, chloral hydrate was used intraperitoneally for anesthesia in this present study. Rats were placed on a heating pad to keep warm during anesthesia. After all of the procedures and eyeball collections, rats were sacrificed using cervical dislocation under anesthesia.

Construction of a rat model of ischemic ophthalmopathy

A rat model of ischemic ophthalmopathy was constructed using high intra-ocular pressure induction [11]. Briefly, rats were anaesthetized with chloral hydrate (450 mg/kg) intraperitoneally. The right eyes of the rats were selected and tropicamide eye drops were used twice to fully dilate the pupils. An intravenous needle was used to puncture the anterior chamber. Equilibrium liquid was perfused into the anterior chamber with the liquid level, at which a 110 mmHg intra-ocular pressure was produced and an ischemic retina was induced. When the intra-ocular pressure was enhanced, the cornea exhibited fog opacity, the fundus oculi was pale, and the red light reflection disappeared. The liquid level was decreased gradually to the same plane of the eyeball after 1 h and the puncture needle was removed. Then, the operative eye was treated with tetracycline eye ointment.

Grouping and administration of the rat model of ischemic ophthalmopathy

The rat models of ischemic ophthalmopathy were randomly divided into the following 4 groups: normal control group; model group; positive drug (Nimodipine) group; and Naoshuantong capsule group. Eye tissues were collected on the 1st, 3rd, 7th, 14th, and 28th days after ischemia/reperfusion. Each time point included 15 rats. Animals in the Naoshuantong capsule group were treated with Naoshuantong capsule by gavage. Animals in the positive drug group were treated with Nimodipine by gavage. Animals in the normal and model control groups received 2% sodium carboxymethylcellulose by gavage.

Monitoring method of ERG

The ERG-b wave was recorded using the ERG recording method, as previously described [12]. Briefly, rat animals were put in a dark room overnight. After the administration of anesthe-
Naoshuantong capsule in ischemic ophthalmopathy

Figure 1. Effects of Naoshuantong capsule on ERG.

sia, pupils were dilated using cyclopentolate (1%) and atropine (1%). An electrode was placed in the tail of the rat and another reference electrode was placed in the buccal mucosa. The gold electrodes on the corneas of rats were employed to record the ERG responses of the eyes of the rats at the same time with flash intensities of 1000 cd.s/m² and 2000 cd.s/m². The amplitudes of the b-wave were examined and analyzed in the analysis of retinal function.

HE staining and retinal thickness

The eyeball samples were collected by cutting the median in the vertical direction through the optic nerve head for the following paraffin sections. The collected paraffin sections were then dewaxed, rehydrated, and incubated with the commonly used hematoxylin and eosin (HE staining) according to previous reports [12]. Then, the HE stained samples were observed under light microscopy. Under 20X magnification, pictures of three consecutive microscopic fields from the optic nerve head to the peripheral retina were taken and the retinal thickness through the center point of each field was measured using ImageJ software. The average thickness of these 3 points was calculated and considered as the retinal thickness of this sample.

Electron microscopy

The collected rat eye samples were subjected to transmission electron microscopic analysis, as described previously [12]. Briefly, the eye samples were fixed with glutaraldehyde at a concentration of 2.5% for 36 h. The vitreous, lens, iris, and cornea in the anterior parts of the eyes were removed. The eyecup samples were cut into pieces and immersed in osmium tetroxide. After dehydration in an ethanol solution, the samples were embedded in epon. Ultrathin
sections of samples were dyed with lead citrate and uranyl acetate. Finally, the prepared sections were observed under a transmission electron microscope.

**Real-time quantitative fluorescence polymerase chain reaction (PCR)**

Extraction of tissue miRNA was performed as described previously using the RNA extraction kit, according to the directions of the manufacturer [12]. cDNA of each retinal sample was produced using designed primers and reverse transcriptase. The real-time PCR system was used in the real-time RT-PCR analysis with the same dose of cDNA. GAPDH simultaneously served as an internal control. Analysis of mRNA expression was performed using a standard PCR curve with GAPDH as a control.

The sequences of the primers used in the current study were as follows (primer sequences of 5’ to 3’): Bax (NM_017059.2), CTGCAGAGGATGATCTGA and GATCGCTGGGGCAGTCTTAG; GAPDH (NM_017008.3), TGCCACTCAGAGACTGTGG and TTCAGCTCTGGGATGACCTT; caspase 3 (NM_012922.2), GGACCTGTGGACTGTGG and TTCAGCTCTGGGATGACCTT; caspase-3 (Santa Cruz Biotechnology, Inc.) at a dilution ratio of 1:1000. After washing with PBS, the retinal sections were stained with secondary antibodies and observed under a fluorescence microscope.

**Statistical method**

All data are presented as the mean ± standard deviation (X ± SD). One-way analysis of variance (ANOVA) was used for comparisons between > 3 groups. A two samples t-test was used to compare two groups. A P < 0.05 indicated that the difference was statistically significant.

**Results**

**Effects of Naoshuantong capsule on ERG-b**

To demonstrate the effects of Naoshuantong capsule on ischemic ophthalmopathy in rats, an ischemic ophthalmopathy rat model was constructed. As illustrated in **Figure 1** and **Table 1**, the amplitude of the ERG-b wave in rats with ischemic ophthalmopathy induced by intra-ocular hypertension were significantly lower than the control group on the 1st, 3rd, 7th, 14th, and 28th days, indicating that ischemic ophthalmopathy rat models were constructed successfully. Therefore, the ischemic ophthalmopathy rats were treated with Naoshuantong capsule, and the results demonstrated that Naoshuantong capsule enhanced the amplitudes of the ERG-b wave in the rats of ischemic ophthalmopathy in a time-dependent manner, and there was no significant difference in the amplitude of the ERG-b wave between the normal control and Naoshuantong capsule groups on the 28th day, suggesting that the Naoshuantong capsule significantly improved the amplitude of the ERG-b wave. The improvement on ERG-b by Naoshuantong capsule was better than the positive drug group at each time point, indicating that the effect of Naoshuantong capsule may be better than the positive drug (Nimodipine).

**Table 1. Amplitude of the ERG-b wave on dark adaptation of the mixed potential in various groups (X ± SD)**

<table>
<thead>
<tr>
<th>Time point</th>
<th>Normal control group</th>
<th>Model control group</th>
<th>Positive drug control group</th>
<th>Naoshuantong capsule group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st day</td>
<td>102.2 ± 23.2</td>
<td>39.2 ± 14.9</td>
<td>34.3 ± 18.1</td>
<td>35.8 ± 21.1</td>
</tr>
<tr>
<td>3rd day</td>
<td>103.9 ± 35.8</td>
<td>30.9 ± 12.3</td>
<td>38.4 ± 27.3</td>
<td>43.6 ± 20.2</td>
</tr>
<tr>
<td>7th day</td>
<td>116.4 ± 35.1</td>
<td>45.3 ± 24.3</td>
<td>44.9 ± 23.6</td>
<td>65.4 ± 36.2</td>
</tr>
<tr>
<td>14th day</td>
<td>116.4 ± 28.8</td>
<td>37.6 ± 23.0</td>
<td>67.3 ± 18.2</td>
<td>80.8 ± 28.5</td>
</tr>
<tr>
<td>28th day</td>
<td>128.1 ± 35.8</td>
<td>54.0 ± 17.6</td>
<td>76.3 ± 16.9</td>
<td>107.4 ± 42.9</td>
</tr>
</tbody>
</table>

*Compared with the normal control group, P < 0.05. **Compared with the model control group, P < 0.05. Compared with the positive drug control group, P < 0.05.
Effects of Naoshuantong capsule on total retinal thickness

Rats with ischemic ophthalmopathy exhibited abnormal retinal morphology and function. To further verify the possible therapeutic effects of Naoshuantong capsule on ischemic ophthalmopathy rats, HE staining was used to observe the morphologies of the ischemic ophthalmopathy eyes. As shown in Figure 2 and Table 2, the total retinal thicknesses in the model groups after reperfusion were significantly decreased from the 7th to the 28th day when compared to the normal control group. Further, the effects of Naoshuantong capsule on the total retinal thickness of the rat model of ischemic ophthalmopathy were examined. The data in Figure 2 and Table 2 suggested that retinas in the Naoshuantong capsule treatment group were significantly thicker than the ischemic ophthalmopathy model group on the 7th, 14th, and 28th days, although still significantly thinner than the normal control group. The total retinal thickness in the Naoshuantong group was slightly higher (P > 0.05) than the positive drug control at each time point, especially on the 28th day, which indicated that the improvement resulting from Naoshuantong capsule was at least as good as or even better than the positive drug control group.

Effects of Naoshuantong capsule on morphology by electron microscopy

The role of Naoshuantong capsule in retinal structure and morphology was further verified.
Table 2. Total retinal thickness between various groups (X ± SD)

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Normal control group</th>
<th>Model control group</th>
<th>Positive drug group</th>
<th>Naoshuantong capsule group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st day</td>
<td>172.5 ± 15.4</td>
<td>173.7 ± 12.1</td>
<td>171.0 ± 10.6</td>
<td>171.1 ± 31.9</td>
</tr>
<tr>
<td>3rd day</td>
<td>178.1 ± 11.7</td>
<td>168.7 ± 12.8</td>
<td>165.4 ± 8.4</td>
<td>167.4 ± 28.2</td>
</tr>
<tr>
<td>7th day</td>
<td>171.6 ± 6.5</td>
<td>121.4 ± 14.2</td>
<td>133.0 ± 11.7</td>
<td>146.5 ± 15.5</td>
</tr>
<tr>
<td>14th day</td>
<td>170.0 ± 13.6</td>
<td>90.7 ± 11.3</td>
<td>122.2 ± 19.5</td>
<td>123.6 ± 11.6</td>
</tr>
<tr>
<td>28th day</td>
<td>171.2 ± 24.0</td>
<td>91.7 ± 10.5</td>
<td>118.4 ± 8.3</td>
<td>132.1 ± 25.0</td>
</tr>
</tbody>
</table>

*Compared with the normal control group, P < 0.05. #Compared with the model control group, P < 0.05. Compared with the positive drug control group, P < 0.05.

Effects of Naoshuantong capsule on expression of apoptosis-related genes

Apoptotic proteins, including anti-apoptotic proteins, such as Bcl-xL and Bcl-2, and pro-apoptotic proteins, such as Bax and Bak, play critical roles in apoptosis [13-16]. Caspase-3 activation is the final step and gold-standard of apoptosis [17]. To investigate whether or not apoptosis is involved in ischemic ophthalmopathy and Naoshuantong treatment, real-time PCR was used to determine the expression of apoptosis-related genes, such as Bax, Bcl-xL, and caspase-3. Thus, there were significant changes in ERG-b and morphology in the model group on the 28th day of model construction, and the Naoshuantong group demonstrated significantly effective results, therefore we selected the samples of the 28th day for this experiment.

Our results showed that these three genes (Bax, Bcl-xL, and caspase 3) had altered levels of expression, indicating that the mechanism of action of Naoshuantong capsule might be associated with these apoptosis-related genes (Table 3).

Specifically, the level of Bax in the model control group was higher than the normal control group, indicating that Bax might be involved in the mRNA levels of the ischemic ophthalmopathy rat model. In comparison, the level of Bax mRNA following the administration of Naoshuantong capsule was lower than the rat model group, although there was no significant difference. There was no difference in the Bcl-xL mRNA levels in various groups. The expression of caspase-3 in the model group was higher than the normal control group. Surprisingly, although there was no significant difference, the expression of caspase-3 following the administration of Naoshuantong capsule was not only lower than the model group, but also the normal control group, suggesting that caspase-3 might be involved in the therapeutic effects of Naoshuantong capsule.

Effects of Naoshuantong capsule on the expression of apoptosis-related proteins by immunofluorescence

After real-time PCR experiments, we applied immunofluorescence to further investigate apoptosis-related factors, including Bax, Bcl-2, and caspase-3, on protein levels. Samples were obtained at multiple time points, including the 1st, 3rd, 7th, 14th, and 28th days. Both Bax and caspase-3 were strongly expressed in the retinal ganglion cell layer, and Bcl-2 was weakly...
Naoshuantong capsule in ischemic ophthalmopathy

Table 3. mRNA expressions of Bax, Bcl-xL and Caspase-3 in the 28th day after reperfusion by real-time PCR (X ± SD)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normal group</th>
<th>Model group</th>
<th>Positive drug group</th>
<th>Naoshuantong capsule group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>1.03 ± 0.33</td>
<td>3.11 ± 1.68</td>
<td>4.88 ± 3.59*</td>
<td>1.84 ± 0.19</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>1.01 ± 0.22</td>
<td>2.36 ± 1.27</td>
<td>2.29 ± 1.39</td>
<td>0.89 ± 0.53</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>1.03 ± 0.31</td>
<td>1.60 ± 1.60</td>
<td>1.73 ± 0.80</td>
<td>0.64 ± 0.60</td>
</tr>
</tbody>
</table>

*Compared with the normal control group, P < 0.05.  ○Compared with the model control group, P < 0.05.  ◊Compared with the positive drug control group, P < 0.05.

expressed in the model group compared to the normal control group at each of the above time points, especially the 1st and 3rd days, indicating that apoptosis was involved in the ischemic ophthalmopathy rat model. In the Naoshuantong capsule group, the expression of Bax and caspase-3 was lower than the model group, and even lower than the positive drug (Nimodipine) group, while the expression of Bcl-2 was higher when compared to the model control group and similar to the positive drug (Nimodipine) group, confirming that Naoshuantong capsule has a critical role in the ischemic ophthalmopathy rat model by inhibiting apoptosis (Figures 4-6).

Discussions

Ischemic ophthalmopathy is a common medical condition manifested as decreased visual acuity, defect in the visual field, and decreased vision or blindness [1-4]. There has been little research involving Naoshuantong capsule on the treatment of ischemic ophthalmopathy. Therefore, the present investigation explored the effect and potential mechanism of Naoshuantong capsule on ischemic ophthalmopathy.

In the present investigation we found that the amplitude of the ERG-b wave was significantly lower and the total retinal thicknesses was decreased in rats with ischemic ophthalmopa-
Naoshuantong capsule in ischemic ophthalmopathy

thy induced by intra-ocular hypertension compared to the control group at each time point, and retinal tissues and cells showed abnormal morphology, indicating that the rat model of ischemic ophthalmopathy was constructed successfully. The rat model was then used to explore the possible effect of Naoshuantong capsule, and the results demonstrated that Naoshuantong capsule effectively and significantly enhanced the amplitude of the ERG-b wave, increased the total retinal thicknesses, and improved the cellular morphology in retinas when compared to the model group. Our results showed that the effect of Naoshuantong capsule was at least as good as, or even better than the positive drug, suggesting that Naoshuantong capsule is effective in improving the morphology and function of retinas in rats with ischemic ophthalmopathy.

However, the pathogenesis of ischemic ophthalmopathy is not completely understood, and uncovering the mechanism underlying ischemic ophthalmopathy is undoubtedly helpful in the development of new methods for treatment. It has been shown that there is excess production of ROS in the ischemic ophthalmopathy model, which might induce apoptosis [18, 19]. In the current study, we explored whether or not apoptosis was involved in the pathogenesis of the ischemic ophthalmopathy rat model. Real-time PCR was used to analyze the expression of

Figure 4. Effects of Naoshuantong capsule on Bax in retinal tissue observed by fluorescence microscopy.
the pro-apoptotic gene, Bax, the anti-apoptotic factor, Bcl-xL and the apoptosis executor, caspase 3. Although there was increased expression of Bax and caspase 3 in the rat model of ischemic ophthalmopathy, there was no significant difference between the normal control and model control groups; however, Naoshuantong capsule decreased the level of Bax and caspase 3 in the model group, indicating that Naoshuantong capsule may have some effects during apoptosis. In the following immunofluorescence experiments, there were significantly increased levels of Bax and caspase 3, and decreased levels of Bcl-2 in retinas of the rat model group, especially in the GCL, confirming that apoptosis is involved in the rat model of ischemic ophthalmopathy. At each time point, Naoshuantong capsule significantly inhibited apoptosis by preventing the over-expression of the pro-apoptosis factor, Bax, and the apoptosis executor, caspase 3, while increasing the expression of the anti-apoptosis factor, Bcl-2, suggesting that the effect of Naoshuantong capsule on ischemic ophthalmopathy may be partly dependent on inhibiting apoptosis.

As we described above, Naoshuantong capsule is a compound herbal medicine and consists of extracts of five herbs (Cattail pollen, Radix paeoniae rubra, Rhizoma gastrodiae, Radix Rha pontici, and Curcuma aromatic). Studies have shown that cattail pollen can inhibit platelet
aggregation, reduce blood lipid levels, prevent atherosclerosis, decrease blood pressure, impart anti-oxidative and protective effects to damaged vascular endothelial cells, and has a key role in anti-cerebral ischemia reperfusion injury [20, 21]. Radix paeoniae rubra can inhibit platelet aggregation, protect vascular endothelium, prevent coagulation and thrombosis, and provide anti-oxidative effects, such as reducing MDA and NO levels and increasing SOD activity [22, 23]. Curcuma aromatic can decrease blood lipid levels, thus inhibiting atherosclerosis and improving hemorrhology [24, 25]. Rhizoma gastrodiae can inhibit platelet aggregation, reduce blood pressure, enhance intelligence, improve microcirculation, and resist ischemia and hypoxia [26, 27]. Radix rhapontici has antioxidant effects, reduces blood lipid levels, attenuates atherosclerosis, and can prevent hypoxia [28, 29]. As the compound of these herbals, Naoshuantong capsule may have a role in the treatment of ischemia-reperfusion injuries, and is used in the treatment of ischemic stroke [5, 6]. Although in our study we did not detect hemorheologic changes in the ischemic ophthalmopathy and Nanshuantong capsule-treated rats, we suggest that Naoshuantong capsule might improve the microvascular circulation of retinas, ameliorate retinal ischemia, and therefore inhibit retinal cell apoptosis.
apoptosis, protect rats against ischemia-induced retinal morphologic damage, and attenuate visual function, such as ERG results.

In this present study we show that Naoshuantong capsule is effective in protecting retinas from ischemia-induced damage, in part by inhibiting apoptosis; however, additional studies are needed to further elucidate the detailed mechanisms on how ischemia induces retinal damage and how Naoshuantong capsule is involved in ischemia-induced apoptosis.

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Disclosure of conflict of interest

None.

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Naoshuantong capsule in ischemic ophthalmopathy