The protective effect of butyphthalide on the cerebral ischemia-reperfusion injury of rats and the related molecular mechanism

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Objective: To study the protective effect of butyphthalide on the cerebral ischemia-reperfusion injury in rats and the related molecular mechanism. Methods: Male SD rats were selected as the experimental animals and randomly divided into Sham group, ischemia-reperfusion group (I/R group) and butyphthalide group (NBP group), the ischemia-reperfusion models were established by suture method and then received 6 mg/kg butyphthalide sodium chloride injection before reperfusion for intervention. 12, 18 and 24 h after reperfusion, the number of apoptotic cells in brain tissue as well as the levels of cell apoptosis molecules and oxidative stress molecules were determined. Results: 12, 18 and 24 h after reperfusion, analysis of the number of apoptotic cells in brain tissue of three groups of rats was as follows: the number of apoptotic cells in brain tissue of I/R group was significantly more than that of Sham group (P<0.05), and the Fas, FasL, BNIP3, Caspase-3 and Caspase-9 protein expression as well as reactive oxygen species (ROS) and malondialdehyde (MDA) levels were significantly higher than those of Sham group (P<0.05); the number of apoptotic cells in brain tissue of NBP group was significantly less than that of I/R group, and the Fas, FasL, BNIP3, Caspase-3 and Caspase-9 protein expression as well as ROS and MDA levels were significantly lower than those of I/R group (P<0.05). Conclusion: Butyphthalide can inhibit the apoptosis and oxidative stress reaction to reduce the cerebral ischemia-reperfusion injury in rats.

1. Introduction

Cerebral infarction is the cerebrovascular disease with high mortality and morbidity, thrombolytic therapy and interventional therapy are the common means for clinical treatment of cerebral infarction, and they can make ischemic brain tissue obtain perfusion and interrupt the ischemia hypoxia damage to brain tissue. Although reperfusion therapy can restore blood perfusion to brain tissue and alleviate the nerve injury caused by ischemia hypoxia, ischemia-reperfusion process, however, can aggravate the damage to brain tissue structure and function, and affect the outcome of cerebral infarction[1,2]. Therefore, inhibiting ischemia-reperfusion injury is regarded as the important target for improving the effect of cerebral infarction reperfusion therapy. Butyphthalide (NBP) is self-developed cerebral protection drug in our country, and is widely used in the treatment of ischemic cerebrovascular disease[3,4]. Animal study has shown that NBP has protective effects on cerebral ischemic reperfusion damage[5], but the specific molecular mechanism has not yet been elucidated. In the following study, ischemia-reperfusion injury model rats were selected as the research objects, the protective effect of NBP on the cerebral ischemia-reperfusion injury in rats and the related molecular mechanism were analyzed.

2. Materials and methods

2.1. Experimental materials

NBP sodium chloride injection was from CSPC NBP.
2.2. Experimental animals and groups

Experimental animals were a total of 18 SPF male rats that were 250–300 g in body mass, 8 to 10 weeks old and bought from Shanghai Jiesijie Laboratory Animal Co., LTD., and the license was SCXK (Shanghai) 2013-0006. The SD rats were randomly divided into Sham group, ischemia-reperfusion group (I/R group) and NBP group, six in each group.

2.3. Experimental methods

2.3.1. Model making and drug intervention methods

Rats received intraperitoneal injection of 10% chloral hydrate according to the dose of 0.3 mL/100 g, and after anesthesia, the I/R group and NBP group were made into cerebral ischemia-reperfusion injury models according to the following method: the right common carotid artery was separated, the telecetric end of the external carotid artery was clipped, the proximal end of internal carotid artery and the telecetric end of the common carotid artery were clipped, the nylon line with thread head was inserted into the common carotid artery, pushed upward through internal carotid artery, stopped after a slight resistance and pulled out after 2 h of ischemia to form reperfusion, and then the incision was sutured; for sham group, the right common carotid artery was separated after anesthesia, no suture operation was conducted, and the incision was sutured after 2 h. NBP group received intraperitoneal injection of 6 mg/kg butyphthalide sodium chloride injection after ischemia for 2 h, and the Sham group and I/R group received intraperitoneal injection of same dose of saline after ischemia for 2 h. 12, 18 and 24 h after reperfusion, the brain tissue was separated and divided into two, one was fixed with 4% paraformaldehyde and then made into paraffin sections and store them at room temperature, and the other was frozen with liquid nitrogen and then stored in -80℃ refrigerator.

2.3.2. Apoptotic cell number detection methods

Paraffin sections of brain tissue were collected for antigen retrieval, then stained with TUNEL staining kits for 1 hour, incubated with POD transforming agent for 40 min, washed with PBS for DAB development and hematoxylin counterstaining, dehydrated, sealed and observed under a microscope, the apoptotic cell number in each field of vision was counted under high power lens.

2.3.3. Protein content detection methods

Brain tissue frozen in liquid nitrogen was taken, added in protein lysis buffer and grinded, the tissue suspension was collected, enzyme-linked immunosorbent assay kits were used to determine Fas, FasL, BNIP3, Caspase-3 and Caspase-9 contents, ROS detection kits, MDA detection kits, SOD detection kits and CAT detection kits were used to determine the contents of ROS, MDA, CAT and SOD, and the BCA protein quantification kits were used to determine total protein content. The total protein content was used as the reference to calculate Fas, FasL, BNIP3, Caspase-3, Caspase-9, ROS, MDA, CAT and SOD contents per mg total protein.

2.4. Statistical analysis

SPSS20.0 software was used to input data of apoptosis count as well as Fas, FasL, BNIP3, Caspase-3, Caspase-9, ROS, MDA, CAT and SOD levels, comparison of above measurement data among three groups was by variance analysis, and P<0.05 indicated statistical significance in differences.

3. Results

3.1. Number of apoptotic cells

12, 18 and 24 h after reperfusion, analysis of the number of apoptotic cells in brain tissue of three groups of rats was as follows: the number of apoptotic cells in brain tissue of I/R group was significantly more than that of Sham group, and the number of apoptotic cells in brain tissue of NBP group was significantly less than that of I/R group. Differences in pair-wise comparison of the number of apoptotic cells in brain tissue were statistically significant among four groups of rats (P<0.05) (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>12 h</th>
<th>18 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham group</td>
<td>3.59±0.61</td>
<td>3.78±0.58</td>
<td>3.62±0.69</td>
</tr>
<tr>
<td>I/R group</td>
<td>35.1±5.96</td>
<td>49.29±6.74</td>
<td>67.57±8.16</td>
</tr>
<tr>
<td>NBP group</td>
<td>19.32±3.26 *</td>
<td>28.51±4.59 *</td>
<td>39.41±5.78 *</td>
</tr>
</tbody>
</table>

*: compared with Sham group, P<0.05; #: compared with I/R group, P<0.05.

3.2. Apoptosis–related molecule expression

12, 18 and 24 h after reperfusion, analysis of apoptosis–related molecules Fas, FasL, BNIP3, Caspase-3 and Caspase-9 expression in brain tissue of three groups of rats was as follows: the Fas, FasL, BNIP3, Caspase-3 and Caspase-9 protein expression in brain tissue of I/R group were significantly higher than those of Sham group, and the Fas, FasL, BNIP3, Caspase-3 and Caspase-9 protein expression in brain tissue of NBP group were significantly lower than those of I/R group. Differences in pair-wise comparison of the Fas, FasL, BNIP3, Caspase-3 and Caspase-9 protein expression in brain tissue were statistically significant among three groups of rats (P<0.05) (Table 2).
brain tissue damage through oxidative stress and cell apoptosis. Reactive oxygen species in ischemic brain tissue, which will cause brain damage, and the number of apoptotic cells increases significantly in local tissue. Further analysis of the protective effect of NBP intervention on cerebral ischemia-reperfusion injury showed that the number of apoptotic cells in brain tissue of NBP group was significantly lower than that of I/R group ($P<0.05$). This means that NBP intervention can inhibit apoptosis during cerebral ischemic reperfusion, and reduce ischemia-reperfusion injury to brain tissue.

In the process of cerebral ischemia hypoxia injury and ischemia-reperfusion injury, the cell apoptosis process is significantly activated. Fas and FasL are the molecules regulating death receptor ligand-receptor can activate caspase cascade activation reaction, and after a series of cascade activation reactions, it finally activates caspase-3, causes cellular morphological and biochemical reaction, and finally leads to apoptosis[11,12]. In the study, analysis of the above apoptosis molecule expression in brain tissue confirmed that Fas, FasL, BNIP3, Caspase-3 and Caspase-9 protein expression in brain tissue of I/R group were significantly higher than those of Sham group ($P<0.05$) (Table 3).

### 4. Discussion

Ischemia-reperfusion injury is the important pathological link that affects the effect of cerebral infarction reperfusion therapy, and blood reperfusion can cause the massive production of reactive oxygen species in ischemic brain tissue, which will cause brain tissue damage through oxidative stress and cell apoptosis activation[6,7]. NBP is the cerebral protection drug with independent intellectual property rights in our country, and it can improve the cerebral microcirculation and reduce ischemia hypoxia injury to brain tissue. Related animal study has confirmed that NBP has protective effects on cerebral ischemic reperfusion damage[5], but the specific molecular mechanism is not yet clear. In the study, cerebral ischemia reperfusion model rats were selected as the research objects to specifically analyze the protective effect of NBP on cerebral ischemia-reperfusion injury and the molecular mechanism. First of all, analysis of the apoptotic cell number in ischemia reperfusion model rats proved that the number of apoptotic cells in brain tissue of I/R group was significantly higher than that of Sham group ($P<0.05$). This means that ischemia-reperfusion process can cause brain damage, and the number of apoptotic cells increases significantly in local tissue. Further analysis of the protective effect of NBP intervention on cerebral ischemia-reperfusion injury showed that the number of apoptotic cells in brain tissue of NBP group was significantly lower than that of I/R group ($P<0.05$). This means that NBP intervention can inhibit apoptosis during cerebral ischemic reperfusion, and reduce ischemia-reperfusion injury to brain tissue.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time point</th>
<th>ROS (µmol/mg)</th>
<th>MDA (µmol/mg)</th>
<th>CAT (U/mg)</th>
<th>SOD (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham group</td>
<td>12 h</td>
<td>1.03±0.15</td>
<td>2.21±0.36</td>
<td>65.52±9.35</td>
<td>78.76±8.59</td>
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<tr>
<td></td>
<td>18 h</td>
<td>1.12±0.18</td>
<td>2.09±0.41</td>
<td>68.12±10.25</td>
<td>79.12±9.25</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>1.08±0.14</td>
<td>2.27±0.45</td>
<td>67.31±8.95</td>
<td>77.87±9.62</td>
</tr>
<tr>
<td>I/R group</td>
<td>12 h</td>
<td>3.96±0.64</td>
<td>8.39±1.04</td>
<td>32.52±5.75</td>
<td>48.76±6.26</td>
</tr>
<tr>
<td></td>
<td>18 h</td>
<td>5.52±0.79</td>
<td>14.42±1.86</td>
<td>27.64±4.25</td>
<td>30.25±5.62</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>8.21±1.02</td>
<td>22.31±3.52</td>
<td>21.59±3.64</td>
<td>21.45±3.76</td>
</tr>
<tr>
<td>NBP group</td>
<td>12 h</td>
<td>1.87±0.26</td>
<td>5.28±0.93</td>
<td>49.52±6.76</td>
<td>60.22±7.87</td>
</tr>
<tr>
<td></td>
<td>18 h</td>
<td>2.52±0.33</td>
<td>7.82±0.91</td>
<td>55.12±8.75</td>
<td>56.54±6.91</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>2.92±0.41</td>
<td>9.31±1.18</td>
<td>52.19±7.59</td>
<td>58.76±7.42</td>
</tr>
</tbody>
</table>

*: compared with Sham group, $P<0.05$; #: compared with I/R group, $P<0.05$. 

### 3.3. Oxidative stress molecule levels

12, 18 and 24 h after reperfusion, analysis of oxidative stress molecules ROS, MDA, CAT and SOD levels in brain tissue of three groups of rats was as follows: ROS and MDA levels in brain tissue of I/R group were significantly higher than those of Sham group while CAT and SOD levels were significantly lower than those of Sham group; ROS and MDA levels in brain tissue of NBP group were significantly higher than those of I/R group were significantly higher than those of I/R group while CAT and SOD levels were significantly lower than those of I/R group. Differences in pair-wise comparison of ROS, MDA, CAT and SOD levels in brain tissue were statistically significant among three groups of rats ($P<0.05$) (Table 3).
of Sham group (P<0.05), and Fas, FasL, BNIP3, Caspase-3 and Caspase-9 protein expression in brain tissue of NBP group were significantly lower than those of I/R group (P<0.05). It means that ischemia-reperfusion could increase the apoptosis molecule expression in brain tissue and promote cell apoptosis, and NBP intervention can reduce the apoptosis molecule expression and inhibit cell apoptosis.

Oxidative stress damage is an important link for ischemia-reperfusion to cause brain tissue damage, and the ROS produced during oxidative stress can not only directly cause the local tissue structure and function injury, but can also activate cell apoptosis process through different channels[13,14]. The ROS produced during cerebral ischemic reperfusion can directly have peroxide reaction with the lipid composition in the neurons and glial cells, which causes cellular structure and function damage while produces a large number of oxidation product MDA[15,16]. SOD and CAT are the important antioxidant enzymes in brain tissue, the former can have reduction reaction with ROS and generate H2O2, and the latter can further reduce and clear H2O2; constantly generated ROS in local tissue will massively consume SOD and CAT, resulting in a decrease in SOD and CAT levels[17,18]. In the study, analysis of oxidative stress molecule levels in ischemia-reperfusion brain tissue proved that ROS and MDA levels in brain tissue of I/R group were significantly higher than those of Sham group while CAT and SOD levels were significantly lower than those of Sham group (P<0.05). It means that ischemia-reperfusion can activate oxidative stress response in brain tissue and cause the increased generation of ROS and MDA as well as the increased consumption of CAT and SOD. Further analysis of NBP intervention effect on oxidative stress reaction in ischemia-reperfusion brain showed that ROS and MDA levels in brain tissue of I/R group were significantly lower than those of Sham group while CAT and SOD levels were significantly higher than those of I/R group (P<0.05). This means that NBP has inhibiting effect on oxidative stress reaction in ischemia-reperfusion brain tissue, and can reduce the generation of ROS and MDA as well as the consumption of CAT and SOD.

To sum up, it can be preliminarily concluded in the research that NBP has protective effect on cerebral ischemia-reperfusion injury of rats, and the molecular mechanisms of NBP to exert protective effect is inhibiting the apoptosis and oxidative stress during ischemia-reperfusion. In future study, cytological study can be adopted to further verify the signaling pathways of NBP to inhibit neuron apoptosis and oxidative stress.

References