Effects of naoshuning on the plasma TNF-α, IL-1β and ICAM-1 levels in a rat brain ischemia-reperfusion injury model

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Accepted 26 January, 2011

To investigate the effects of naoshuning on plasma TNF-α, IL-1β and ICAM-1 levels in a rat brain ischemia-reperfusion injury model. 72 rats were randomly divided into four groups (n=18): Sham group, ischemia-reperfusion (IR) group, naoshuning group and ginton group. Middle cerebral artery occlusion (MCAO) was performed in rats for 2 h followed by 1, 7 and 14 days of reperfusion. Animals were sacrificed and the plasma levels of TNF-α, IL-1β and ICAM-1 were detected by ELISA and radioimmunoassay. After brain ischemia-reperfusion injury, the contents of TNF-α, IL-1β and ICAM-1 in the plasma of rats in IR group were significantly higher than that in sham group (p<0.05); after the treatment with naoshuning, the contents of TNF-α, IL-1β and ICAM-1 in the plasma was significantly decreased when compared with those in the remaining groups (P<0.05). When compared with the MCAO group, the TNF-α, IL-1β and ICAM-1 levels in naoshuning group was decreased after injury (P<0.05). Naoshuning can decrease the secretion of inflammatory cytokines (TNF-α, IL-1β) and adhesion molecules (ICAM-1), improving brain ischemia reperfusion injury.

Key words: Naoshuning, brain ischemia reperfusion, tumor necrosis factor-α, interleukin-1β, intercellular adhesion molecule-1.

INTRODUCTION

Brain ischemia-reperfusion (IR) injury is a common complication in clinical practice, and its mechanism is complex. Studies have demonstrated acute inflammation plays an important role in the secondary lesions caused by reperfusion (Sui and Zhou, 2007; Jean et al., 1998). A large number of experiments show a lot of cytokines are expressed and numerous inflammatory cells infiltrate in the ischemic lesions after brain IR injury, and over-expression of cytokines may aggravate the secondary injury. Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), two important inflammatory cytokines, are produced by neuron, astroglial cells and microglial cells during brain IR injury (Wang et al., 2001). TNF-α and IL-1β not only directly promote the aggregation of neutrophils in the ischemic lesions, mediate inflammatory response, cause hemorheological change, cerebral edema and eventually result in neuron death (Saito et al., 1996), but also increase the expression of intercellular adhesion molecule-1 (ICAM-1), facilitate leukocytes to migrate and adhere to endothelialcytes, and subsequently infiltrate the brain aggravating brain IR injury. So, interfering with the expressions of inflammatory cytokines and/or adherence factors may be beneficial for the improvement of brain IR injury. The present study aimed to investigate the effects of naoshuning on the plasma levels of TNF-α, IL-1β and ICAM-1 in a rat middle cerebral artery occlusion (MCAO) and reperfusion model to evaluate the mechanisms underlying the
neuroprotective effects of naoshuning.

MATERIALS AND METHODS

Experimental animals

Male SD rats weighing 260±20 g were purchased from the Experimental Animal Center of Chinese People's Liberation Army (PLA), Academy of Military Medical Science. The license No is SCXK-(army)2007-004. This study was approved by the Ethics Committee of the Chinese PLA General Hospital.

Experimental drugs and reagents

Chinese traditional medicine naoshuning is extracted from Leech, Panax Powder, Motherwort, Cyathula, and purchased from Chinese medicine dispensary of PLA; Ginaton (Ginko biloba extract) was produced by Dr. Willmar Schwabe GmbH and Co., Germany (batch number: H20040335). Radioimmunoassay kits for rat TNF-α, IL-1β and enzyme-linked immunosorbent assay (ELISA) kit for rat sICAM-1 were provided by the development center of science and technology of PLA.

Instruments

800-1 centrifuge (Jiangsu Jintan Medical Instrument Company), TW8 thermostatic waterbath (You Lebo company; Germany); Sn-695B immune counter (Shanghai hesuo ri huan guang dian instrument company) and Wellscan MKS Atomatic Microplate Reader (Finland Wellsscan MK3 microplate reader devices company) were used in the present study.

Grouping and treatment

Rats were housed in the Animal Center for 4 days for accommodation and then randomly divided into sham group, IR group, IR+Naoshuning group and IR+Ginaton group. Furthermore, each group was subdivided into 1 d group, 7 and 14 days group, with 6 rats in each subgroup. In naoshuning group, rats received Naoshuning (1.5 g/100 g body weight) intragastrically. In the ginaton group, rats were given Ginaton (5 mg/100 g body weight) intraperitoneally. Rats in sham group and IR group were administered with normal saline (1 ml/100 g body weight). Treatment was performed twice daily for consecutive 3 days. On the 4th day, 1 h after treatment, MCAO model was established and treatment was performed once 6 h later and then once daily. After operation, all rats were fasted for 12 h, and then given ad libitum access to food and water. Rats in each group were sacrificed at 1, 7 and 14 days after reperfusion.

Establishment of the brain IR model

The MCAO and subsequent reperfusion were performed according to previously described (Harada et al., 2009). Briefly, rats were intraperitoneally anesthetized with 10% choral hydrate (0.40 ml/100 g body weight), and fixed on a table. After skin preparation, a 2 cm incision was made in the middle line of neck, the subcutaneous fascia and muscles were separated and the left common carotid artery was exposed. After exposure of common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA), CCA and ECA were ligated with 10 cm stitches (2/0 nylon thread, 4-0 suture). The ICA was clamped and a small hole was made in the bifurcation of CIA followed by insertion of a nylon monofilament into ICA. Then, the clamp was released and the monofilament was inserted forward until feeling a slight resistance, which indicates the nylon monofilament reached the starting point of anterior cerebral artery. The suture in ICA was fastened and occlusion was performed for 2 h followed by removal of the monofilament. Then, the wound was closed. For rats in sham group, operation was performed without occlusion.

Detection of plasma levels of TNF-α, IL-1β and ICAM-1

At the end of reperfusion, rats were anesthetized, the heart was exposed, and 3 ml of blood sample was obtained from the right atrium. The blood was centrifuged at 3500 r/min for 15 min and plasma was collected and stored at -20°C. The contents of TNF-α and IL-1β were determined by radioimmunoassay, and that of ICAM-1 detected by ELLSA.

Statistical analysis

Data were expressed as means ± standard deviation (X ± s) and statistical analysis was performed with SPSS version 11.0 statistic software package. One way analysis of variance was applied for comparison among different groups.

RESULTS

Modeling

The MCAO model was successfully established or after testing of neurological functions, animals with brain injury were subjected to the subsequent experiment.

Plasma TNF-α level

When compared with sham group, the TNF-α level in the other three groups was significantly increased at different time points (except at 14 days after reperfusion in naoshuning group) and gradually decreased with the prolongation of reperfusion. When compared with the IR group, the TNF-α level in Naoshuning group was markedly decreased (except at 7 days after Naoshuning treatment), but that in ginaton group was only slightly decreased. Furthermore, the TNF-α level in naoshuning group was lower than that in ginaton group but without significant difference (Table 1).

Plasma IL-1β level

When compared with sham group, the content of IL-1β in the other three groups was significantly increased at different time points (P<0.05), especially at 1 and 7 days after reperfusion in the IR group, and gradually decreased with the prolongation of reperfusion. When compared with the IR group, the IL-1β content in the Naoshuning group at different time points and that in the ginaton group at 7 and 14 days after reperfusion was
Table 1. Content of TNF-α in the plasma of rats (n=6, s).

<table>
<thead>
<tr>
<th>Group</th>
<th>Content of TNF-α (ng/mL)</th>
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<tbody>
<tr>
<td></td>
<td>1 d</td>
<td>7 d</td>
<td>14 d</td>
<td></td>
</tr>
<tr>
<td>Sham group</td>
<td>1.89±0.45**</td>
<td>1.83±0.38**</td>
<td>1.91±0.16**</td>
<td></td>
</tr>
<tr>
<td>IR group</td>
<td>3.75±0.81△△</td>
<td>3.74±0.86△△</td>
<td>3.27±0.78△△</td>
<td></td>
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<tr>
<td>Naoshuning group</td>
<td>2.80±0.26△△</td>
<td>2.79±0.62△△</td>
<td>2.39±0.68*</td>
<td></td>
</tr>
<tr>
<td>Ginaton group</td>
<td>3.05±1.09△△</td>
<td>2.76±0.69△△</td>
<td>2.58±0.97△</td>
<td></td>
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</tbody>
</table>

Note: *P<0.05, **P<0.01 vs IR group; △P<0.05, △△P<0.01 vs sham group.

Table 2. Content of IL-1β in the plasma of rats (n=6, s).

<table>
<thead>
<tr>
<th>Group</th>
<th>Content of IL-1β (ng/ml)</th>
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<tbody>
<tr>
<td></td>
<td>1 d</td>
<td>7 d</td>
<td>14 d</td>
<td></td>
</tr>
<tr>
<td>Sham group</td>
<td>0.12±0.02**</td>
<td>0.11±0.03**</td>
<td>0.13±0.02**</td>
<td></td>
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<tr>
<td>IR group</td>
<td>0.30±0.09△△</td>
<td>0.27±0.05△△</td>
<td>0.26±0.04△△</td>
<td></td>
</tr>
<tr>
<td>Naoshuning group</td>
<td>0.18±0.05△△</td>
<td>0.15±0.04**</td>
<td>0.14±0.03**</td>
<td></td>
</tr>
<tr>
<td>Ginaton group</td>
<td>0.20±0.06△△</td>
<td>0.17±0.05**</td>
<td>0.15±0.04**</td>
<td></td>
</tr>
</tbody>
</table>

Note: *P<0.05, **P<0.01 vs IR group; △P<0.05, △△P<0.01 vs sham group.

Plasma ICAM-1 level

When compared with the sham group, the content of ICAM-1 in the other three groups was significantly increased at different time points (P<0.05), especially at 1 and 7 days after reperfusion in the IR group and the naoshuning group (P<0.01). When compared with the IR group, the content of ICAM-1 in the Naoshuning group and the ginaton group was remarkably reduced (P<0.05), especially at 14 days after reperfusion in the naoshuning group and at all time points in the ginaton group (P<0.01). Moreover, the ICAM-1 level in the naoshuning group was markedly higher than that in the ginaton group (P<0.05) except at 14 after reperfusion. (Table 3)

DISCUSSION

Dysfunction of cerebral microvessels induced by acute inflammatory injury represents an important pathological event in secondary injury (Wagers and Weissman, 2004). The histological features are characterized by infiltration of leukocytes further causing increased permeability of microvessels and leakage of plasma protein to the brain in the alveolar cavities. Inflammatory response in ischemic cortex is characterized by leukocyte aggregation, lysosome dysfunction, synthesis of hydrolase, excessive production of free radicals, and generation of inflammatory mediators. A large number of leukocytes especially polymorphonuclear leukocyte (PMN) infiltrate into parenchyma, and produce a variety of free radicals, inflammatory cytokines and lipid metabolites further aggravating the brain injury. At the early stage of brain ischemia, a large number of PMNs migrate and adhere to the vascular endothelial cells in the injured brain, then cross the blood brain barrier and infiltrate into the lesions exerting pro-inflammatory effects. Once bind to the endothelial cells, the PMNs can mechanically block the microvessels and deteriorate the microcirculatory disturbance influencing the blood supply of the brain. In addition, activated leukocytes produce a lot of free radicals and proteases damaging the cerebral vessels and compromising vascular permeability, which eventually deteriorate the brain edema. At the same time, these leukocytes secrete a variety of inflammatory mediators and cytokines which recruit more leukocytes to infiltrate into the lesions aggravating the inflammatory response.

TNF-α and IL-1β are two important inflammatory cytokines and play an important role in pro-inflammation after brain IR injury. Reperfusion can stimulate the transcriptions and expressions of TNF-α and IL-1β. Both cytokines can exert chemotactic effect mediating the rolling, adherence, and penetration of neutrophils toward endothelial cells and promoting the migration, proliferation and infiltration of peripheral inflammatory cells toward ischemic tissues. These processes then finally result in brain edema and acute inflammation. Among numerous cytokines, TNF-α is an initiator. It can be secreted by macrophages, astrocytes, microglial cells and neurons and have multiple biological functions. TNF-
α can stimulate the high expression of ICAM-1 and induce the rolling and adherence of leukocytes to microvessels resulting in microvascular obstruction and subsequent delayed microvascular hyperperfusion. This process also destructs the basement membrane which leads to the infiltration of inflammatory cells causing brain edema (Kostulas et al., 2003; Abillerira et al., 2003). TNF-α also promotes the synthesis of endothelin, activates Von Willebrand factor and platelet activation factor (PAF), resulting in contraction of vessels and subsequent aggravation of brain injury. IL-1β is an important mediator triggering the immune response and inflammation and can be secreted by neurons, astrocytes, oligodendrocytes and endothelial cells. As an inflammatory mediator and an immune-derived cytokines, IL-1β cannot only promote the activation of B cells and T cells by cooperating with other cytokines, but stimulate the proliferation of astrocytes and the secretion of TNF-α, IL-6, nervous growth factor (NGF), granulocyte colony-stimulating factor (GCSF) and NO by astrocytes. IL-1β plays dual roles in nervous system and can promote the production of ICAM-1 and vascular cell adhesion molecule (VCAM-1) facilitating the inflammation (Gabryel et al., 2004). IL-1β also promotes the production of NO by NOS, induce the secretion of excitatory amino acid and generation of free radicals, which further aggravate the brain IR injury. Under normal condition, ICAM-1 is at a low level in endothelial cells. During the ischemic injury, the ICAM-1 expression is markedly increased in the presence of TNF-α and IL-1β. The secreted ICAM-1 then mediates the adherence between leukocytes and endothelial cells and the permeation of leukocytes accelerating the occurrence and development of inflammation. The present study aimed to use Chinese Traditional Medicine to inhibit the expressions of inflammatory cytokines and adhesion molecule and subsequently interfere with the inflammatory cascade, improving the inflammatory response and protecting the brain IR injury. Hirudo nipponica and pseudo-ginseng are the two main effective ingredients. Hirudo nipponica plays important roles in anticoagulation, thrombolysis and anti-inflammation. Liang et al. (2006) have found that Hirudo nipponica injection could significantly decrease the expression of TNF-α, IL-1β and ICAM-1 in the ischemic lesions and subsequent alleviated the inflammatory response after R. Previous studies show Panax Notoginseng Saponins (PNS) can inhibit the expression of ICAM-1 on the endothelial cells after brain IR injury, which then decreases the recruiting of the neutrophils, inhibits the release of inflammatory mediators, and declines the permeability of blood brain barrier finally alleviating the brain edema and protecting the brain injury (He and Zhu, 2005). Our results showed the contents of TNF-α, IL-1β and ICAM-1 were increased after brain IR injury and the high levels of these cytokines was maintained for at least 7 days, and then decreased gradually 14 days after reperfusion. In the Naoshuning group, the contents of TNF-α, IL-1β and ICAM-1 decreased over time. Our study demonstrated that naoshuning could decrease the expressions of inflammatory cytokines and adhesion molecule, which alleviated the post-IR inflammation, and brain ischemia resulting in improvement of brain injury.

### REFERENCES


