Protection by the gross saponins of *Tribulus terrestris* against cerebral ischemic injury in rats involves the NF-κB pathway

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**Abstract**  
The aim of this study was to investigate whether the gross saponins of *Tribulus terrestris* (GSTT), a traditional Chinese herbal medicine, have neuroprotective effects on rats subjected to middle cerebral artery occlusion (MCAO), through nuclear factor-κB (NF-κB) pathway and inflammatory mediators. Cerebral ischemia was produced by MCAO in either untreated (control) or GSTT-pretreated rats, and the animals were examined for infarct volume, cerebral edema, neuro-behavioral abnormality and pathological changes. Meanwhile, the expression of NF-κB protein in brain tissue was analyzed on Western blots and the serum levels of TNF-α and IL-1 were determined by ELISA. The experimental results demonstrated that, compared with the control MCAO group, GSTT-pretreated MCAO group had significantly reduced infarct volume, cerebral edema and lesser degree of pathological changes in the brain, as well as had lower levels of serum TNF-α and IL-1, and higher levels of brain NF-κB (P<0.05). Furthermore, treatment with an NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) abolished the protective effects of GSTT against MCAO-induced cerebral ischemic injury. These results...
indicated that GSTT’s ability to protect against cerebral ischemic injury was mediated through the NF-κB signaling pathway, and that GSTT may act through inhibition of the production of inflammatory mediators.

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1. Introduction

Cerebral ischemia is one of the common diseases that have the most devastating effects on human beings. During cerebral ischemia, inflammation is closely interrelated with neuronal cell death and thereby promotes the neurological deficit. Inflammation and apoptosis both depend largely on alterations in gene expression and share key regulators, transcription factor nuclear factor-κB (NF-κB) being a prominent example. NF-κB includes at least five DNA-binding protein subunits. The classic form of NF-κB consists of two subunits, p50 and p65, which form homo- and heterodimers. NF-κB, which is well known for its antiapoptotic function, plays an important role in the protection against cerebral ischemia injury. The protective effect of ischemic preconditioning has been shown to be mediated by NF-κB. NF-κB can protect neurons from the ischemic injury induced by middle cerebral artery occlusion (MCAO). Cytokines, such as tumor necrosis factor α (TNF-α) and IL-1β, can activate NF-κB in response to ischemia. Some evidence indicates that TNF cytotoxicity can be dependent on the loss of NF-κB activity and that under-expression of NF-κB in the ischemic cerebral cortex can influence neuronal sensitivity to TNF-induced apoptosis.

The use of herbal medicine for the treatment of cerebral ischemia has a long history in China. Traditional Chinese medicines, such as Panax notoginseng, Scutellaria baicalensis, Pu-erariae, and Nardostachys, have been used to treat cerebral ischemia for thousands of years in China. Tribulus terrestris is an annual plant found around the world. It has been used for the treatment of several problems, including cardiovascular diseases, edema, abdominal distention, kidney troubles, and sexual dysfunction. The total saponins of T. terrestris (GSTT) are a group of the effective ingredients extracted from the whole plant of caltrop. It is a mixture of over 10 kinds of saponins, mainly steroid saponins. These saponins have been commercially available as active compounds in traditional Chinese medicine formulations, such as “Xin-nao-shutong”, which has been used for the treatment of cardiovascular disease.

Our previous research has shown that GSTT (at 30 mg/kg) can protect against cerebral ischemia injury in rats. However, the underlying protection mechanisms of GSTT, including the possible involvement of NF-κB, have not been fully explored. The primary objective of this study was to investigate whether GSTT acted through the NF-κB pathway in protecting against cerebral ischemia injury.

2. Materials and methods

2.1. Materials

GSTT (purity >96%) was purchased from Aodong Taonan Pharmaceuticals Co. (Jilin, China). DAB kit was provided by Beijing Zhongshan Golden Bridge Biotechnology Co. (Beijing, China). NF-κB p65 antibody and secondary antibody were purchased from Santa Cruz. TNF-α kit and IL-1β ELISA kit were purchased from R&D Systems. All other chemicals used were of biochemical grade.

2.2. Animals

The experiments were performed on adult male Wistar rats (n=50) weighing 220–260 g, clean grade, and purchased from the Laboratory Animal Center of Bethune Medical College of Jilin University. Rats were housed under 12 h light-dark cycles, with room temperature of 23±1 °C and relative humidity of 55–65%. The animal handling procedures in the experiment process complied with the China National Institutes of Healthy Guidelines for the Care and Use of Laboratory Animals.

2.3. Experimental protocol

Animals were divided into five groups, each with ten rats. The first group served as sham, and saline was given intraperitoneally (sham group); the second group was subjected to MCAO for 24 h (MCAO group); the third group was pretreated for 7 days with GSTT (30 mg/kg, i.p., once daily) followed by MCAO for 24 h (MCAO+GSTT group); the fourth group was pretreated with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC, 120 mg/kg, i.p.) at 30 min prior to the onset of the 24-h MCAO (MCAO+PDTC group); and the last group was pretreated for 7 days with GSTT (30 mg/kg, i.p., once daily), followed by treatments with PDTC and MCAO, as for the 4th group (MCAO+GSTT+PDTC group). After the completion of the occlusion period, the animals were assessed for neuro-behavioral activity and then sacrificed for tissue collection and analysis.

Before the operation, all animals were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.). MACO was produced on the right side using an intraluminal filament model. The procedure was done by introducing a nylon filament with a rounded tip, via the external cerebral artery (ECA) stump, into the proximal internal carotid artery (ICA), followed by arterial ligature. The occlusion was allowed to last for 24 h. Successful establishment of the MACO model was confirmed by the induction of Horner’s syndrome, as indicated by circling motion toward the right and flexible right forelimb. In sham-operated animals, the filament was threaded into the ECA stump, but the artery was not ligated.

2.4. Sample collection and management

After producing MCAO, the rat brains were removed and then either immediately frozen for later preparation of tissue
homogenates and nuclear fraction, or fixed with formalin. The fixed tissues were embedded in paraffin and 2–5-mm thick sections were prepared for HE staining. Serum was stored by freezing for later detection of the TNF-α and IL-1β levels.

2.5. Measurement of experimental cerebral infarction and brain water (cerebral edema) and neurological examination

After 24 h of occlusion, rats were sacrificed. Rat brains were quickly removed and immediately sectioned. Tissue was completely covered in 1% 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) solution at 37 °C for approximately 15 min. TTC staining resulted in viable tissue staining a “brick-red,” as the tetrazolium salts reacted with the dehydrogenases in the cells, and the infarcted tissue staining a pale white, since they lacked the enzymes with which the TTC would react. The infarction area was computed using the BI2000 software system. The extent of brain infarction was calculated according to the following equation:

\[
\text{Brain infarction (\%)} = \left( \frac{\text{white infarct area/slice area}}{} \right) \times 100\% 
\]

Brain tissues were immediately weighed to obtain the wet weight and then dried in an oven at 100 °C for 24 h to obtain the dry weight. The water content was determined as (wet weight−dry weight)/dry weight.

Neurological examination was performed on each rat at 24 h after MCAO surgery to assess the neuro-behavioral abnormality score. The test was conducted essentially according to the method of Bederson et al., with minor modification. The scoring was done by suspending the rats 20 cm above the ground. Intact animals extended both forelimbs toward the floor. These animals were assigned a score of 0. Abnormal postures included flexing the contralateral limb and limb medially. Rats displaying abnormal postures were further analyzed by being placed on a sheet of soft, plastic-backed paper that could be gripped by its claws. Lateral pressure was applied from behind the shoulders, so that the forelimbs slid gently to the left and then to the right. Rats that resisted sliding in both directions were graded as 1, while more severely affected animals exhibiting a decreased resistance to the lateral push were scored as 2, and those that circled toward the parietal side consistently were graded as 3.

2.6. HE staining

After deparaffinization and hydration, the sections were stained in hematoxylin solution and then washed in running tap water, followed by differentiation in 1% acid alcohol for 30 s. The sections were washed with running tap water again, and then placed in 0.2% ammonia water, followed by staining in eosin solution for 30 s–1 min. At last, sections were dehydrated through alcohol and cleared in xylene. Sections were examined under an optical microscope.

2.7. Western blot analysis of NF-κB p65 protein

Western blot analysis was performed with equal amounts of total protein from tissue homogenate (30 μg) or the nuclear fraction (20 μg), prepared according to Dignam et al. The proteins were separated with 10% SDS-PAGE gels and transferred to PVDF membranes (Millipore, USA). Membranes were blocked with 5% nonfat milk for 2 h at room temperature, washed (with PBS), and then incubated overnight at 4 °C with the primary antibody (rabbit polyclonal anti-rat NF-κB p65, 1:1000 dilution). Then the membranes were washed three times and incubated with secondary antibody (goat anti-rabbit, 1:1000 dilution) for 2 h at room temperature. Immunoreactive bands were visualized using DAB chromogen, quantified by image analyzer (Tanon-2008, China). NF-κB protein levels were normalized to that of β-actin.

2.8. ELISA for TNF-α and IL-1β

TNF-α and IL-1β were measured by ELISA kit according to the manufacturer’s instructions, with a detection limit of 1.0 ng/mL.

2.9. Statistical analysis

Values are presented as mean ± SD. The SPSS 11.0 software was used for data analysis. T-test was performed for the differences between two groups. \( P<0.05 \) was regarded as statistically significant.

3. Results

3.1. Effects of GSTT on MCAO-induced infarction, cerebral edema, and neuro-behavioral abnormality

Twenty-four hours of MCAO injury resulted in a prominent infarction. GSTT (30 mg/kg) significantly reduced the ratio of the percent infarct area to the whole cerebral area as indicated by comparisons between the MCAO+GSTT group and the MCAO group. The protective effect of GSTT was reduced by treatment with the NF-κB inhibitor PDTC (Fig. 1A).

Twenty-four hours of MCAO injury resulted in a prominent increase in brain water content as compared to sham-operated rat brain. In the MCAO+GSTT group, the brain water content was reduced. A significant increase in brain water content was also noted in the MCAO+GSTT+PDTC group in comparison to the MCAO+GSTT group (Fig. 1B).

MCAO rats showed prominent neurological deficits. GSTT (30 mg/kg) significantly reduced the severity of neuro-behavioral abnormality, as indicated by comparisons between the MCAO+GSTT group and the MCAO group. In contrast, the severity of neuro-behavioral abnormality was greater in the MCAO+GSTT+PDTC group than in the MCAO+GSTT group (Fig. 1C).

3.2. Effect of GSTT on MCAO-induced histological changes

As shown in Fig. 2, neurocytes had evenly distributed chromatin, with no mesenchyma edematous, and had normal appearance in the sham-operated group. In the MCAO group, there was edema in the brain tissue and inflammatory cell infiltration in mesenchyma; neurocytes became triangular in shape, and there were also vacuoles. GSTT treatment alleviated edema and inflammatory cell infiltration, but PDTC treatment reduced the effects of GSTT, and caused further aggravation of the cerebral lesion, as shown by the large numbers of necrotic neurocytes and the large area of brain edema.
3.3. Effects of GSTT on the expression of NF-κB p65

To investigate the mechanism of GSTT action, NF-κB expression was examined. There was a significant decrease in brain NF-κB expression in the MCAO group, compared to the sham group. Treatment with GSTT increased the expression of NF-κB, whereas treatment with PDTC reduced the expression of NF-κB and blocked the protective effect of GSTT (Fig. 3).

**Figure 1** Effect of GSTT on various parameters measured 24 h after middle cerebral artery occlusion in rats (n=10). Treatment of GSTT (30 mg/kg) was started as intraperitoneal injections for 7 days, and PDTC (120 mg/kg) was intraperitoneally injected for 30 min, followed by middle cerebral artery occlusion for 24 h. The figure represents the % infarct area (A), brain water content, (B) and the water content (g/g of dry wt) of brain tissue as described in methods and neuro-behavioral abnormality score (C). Neurological score was assessed at 24 h after middle cerebral artery occlusion. Neuro-behavioral abnormality score on a scale of 0–3. Data are expressed as mean±SD: △ △ p<0.01 in comparison with sham group, *p<0.05 in comparison with MCAO group; and #p<0.05 in comparison with MCAO+GSTT group.

**Figure 2** Effect of GSTT on brain HE staining observed 24 h after middle cerebral artery occlusion in rats. Treatment of GSTT (30 mg/kg) was started as intraperitoneal injections for 7 days, and PDTC (120 mg/kg) was intraperitoneally injected for 30 min, followed by middle cerebral artery occlusion for 24 h: (A) sham group, (B) MCAO group, (C) MCAO+GSTT group, (D) MCAO+PDTC group, and (E) MCAO+GSTT+PDTC group. Magnification, 400 ×.
3.4. Effect of GSTT on serum TNF-α and IL-1β levels

Neutrophilic infiltration in cerebral ischemia was mediated by cytokines, including TNF-α and IL-1β. As shown in Fig. 4, TNF-α and IL-1β levels in the MCAO group were simultaneously elevated, compared to the sham group. The MCAO-induced increase in TNF-α and IL-1β levels was inhibited by GSTT. However, treatment with PDTC did not decrease the inhibitory effect of GSTT, and the MCAO+PDTC group did not show reduced TNF-α and IL-1β levels in comparison with the MCAO group.

4. Discussion

Cerebral ischemic injury is a major fatal disease. There is no proven efficient treatment for such a condition; the difficulty is primarily because of the involvement of a multitude of events in the pathogenesis of neuronal injury. In order to use the traditional Chinese medicines to treat cerebral ischemia more reliably and more widely, we have investigated the neuroprotective action of GSTT. In the present work, we have demonstrated that GSTT can prevent ischemia-induced brain injury and inflammation. We show that GSTT can reduce MCAO-induced brain infarction, brain edema, and neurological deficits. At the same time, GSTT can improve MCAO-induced pathologic changes in brain tissue and suppress the increases in TNF-α and IL-1β levels in the serum. Moreover, GSTT can up-regulate the expression of NF-κB; the latter is apparently important for the mechanism of protection by GSTT against cerebral ischemia injury, given the observation that the protective effects of GSTT were inhibited by the NF-κB inhibitor PDTC.

NF-κB is a key transcription factor for many genes involved in cerebral ischemic injury and inflammatory responses, including genes encoding TNF-α and IL-1β. NF-κB activity can support neuronal survival. NF-κB is essential for ischemic preconditioning, after a brief period of brain ischemia, the presence of NF-κB could protect neurons against a subsequent, prolonged period of ischemia.

Furthermore, blocking NF-κB activation pharmacologically with diethylthiocarbamate could inhibit the protective effect of NF-κB in ischemic preconditioning. These and other evidence strongly support the idea that NF-κB plays a physiological role in maintaining neuronal survival. Our Western blot analysis verified that there was a significant decreases in NF-κB expression in MCAO rats compared to the sham group. Treatment with GSTT increased the expression of NF-κB. These results suggest that GSTT’s neuroprotective role might be associated with NF-κB.

The observed increases in TNF-α and IL-1β levels, and the simultaneous decrease in NF-κB levels, in the MCAO rats are consistent with the previous findings by others. Cerebral ischemia is known to induce inflammatory mediators, including TNF-α and IL-1β, at the transcriptional level in both neurons and glial cells. However, cerebral ischemia is also...
known to initiate a “rapid and progressive loss of cortical NF-κB within the ischemic core and nearby penumbra”\(^8\). As pointed out by Botchkina et al.,\(^8\) the simultaneous loss of NF-κB activity and increase in TNF expression can exacerbate cellular damage in both neuronal and glial cells. Thus, the suppression of serum TNF-α and IL-1β and the concurrent enhancement of NF-κB expression both seem to contribute to the observed protection by GSTT against ischemic injury in MCAO rats.

It is interesting that the NF-κB inhibitor PDTC could inhibit the effect of GSTT on NF-κB, but not the effect of GSTT on TNF-α. Previous studies have shown that the ability of TNF to cause cytotoxicity is much dependent on the loss of NF-κB activity\(^21\). Therefore, our results seem to suggest that GSTT reduced TNF-α cytotoxicity in the MCAO rats through the increased expression of NF-κB.

5. Conclusion

In summary, the present study demonstrated that GSTT has a significant protective effect against cerebral ischemia, and that the mechanism of protection appears to include both activation of the NF-κB pathway and inhibition of the production of inflammatory cytokines.

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References