Effects of ginsenoside CK pretreatment on oxidative stress and inflammation in rats with cerebral ischemia/reperfusion injury

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ABSTRACT

The prevention and treatment of cerebral ischemia/reperfusion injury has become a key link in the treatment of ischemic cerebrovascular diseases. In this study, Wistar rats were randomly divided into Sham, low-dose ginsenoside (L-CK), high-dose ginsenoside (H-CK) and nimodipine groups, and the rats in the L-CK, H-CK, and nimodipine groups were intragastrically given the corresponding agents successively once a day for 15 days before surgery. At 1 h after the last administration, a rat cerebral ischemia/reperfusion model was established by suture-occluded method and the effects of ginsenoside CK on the neurological scores, brain tissue water content, brain infarct volume, oxidative stress and inflammation were investigated. The results showed that, compared with those in the model group, the neurological behaviour scores in the L- and H-CK groups, the brain tissue water content in the H-CK group, and the brain infarct volume ratios in the L- and H-CK groups were significantly reduced. Compared with those in the model group, the superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities increased, whereas the malondialdehyde (MDA) content decreased significantly in the brain tissue of the rats in the L- and H-CK groups. Compared with that in the model group, the content of TNF-α in the H-CK group, the content of IL-1β and the expression of HMGB1 protein in the L- and H-CK groups were significantly reduced. These results suggest that ginsenoside CK can protect against cerebral ischemia reperfusion injury in rats, which may be related to its anti-oxidative, anti-inflammatory and HMGB1-expression inhibitory activity.

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Introduction

Cerebrovascular disease (CVD) is one of the most common diseases currently recognized as a hazard to human health [1]. Ischemic CVD (ICVD) accounts for 60–80% of CVDs, characterized by a high incidence rate, high disability rate and high recurrence rate [2]. One of the principles for the treatment of ischemic CVD in clinic is to restore the blood reperfusion as early as possible, which is conducive to reducing the ischemic cerebral injury, and at least to the functional recovery of some reversible injuries [3]. However, it has been found in recent years that the recovery of blood flow after ischemia can lead to further injury and dysfunction of tissues in some cases, namely the cerebral ischemia/reperfusion injury [4]. That is why the prevention and treatment of cerebral ischemia/reperfusion injury has become a key link in the treatment of ischemic CVDs. The pathological process of cerebral ischemia/reperfusion injury is very complex, and it is generally believed that multiple aspects are involved in it, such as the free-radical damage, inflammation, metabolic disorder, toxicity of excitatory amino acids, intracellular calcium overload, cytotoxicity of NO, abnormal blood–brain barrier opening, and energy metabolic disorder in brain tissues [5]. These aspects interact and influence each other to lead to some pathological processes, such as the adhesion of multiform nuclear leukocytes to the endothelial cells, destruction of the blood–brain barrier, effusion of vascular contents, and edema and necrosis of neurons [6]. It is due to the pathogenesis of cerebral ischemia/reperfusion injury involving multiple pathophysiological links that no ideal effective drug for the

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prevention and treatment of cerebral ischemia/reperfusion injury has been found in clinic yet.

Ginsenoside is the main active ingredient of ginseng, and has shown good protection against the cerebral ischemia reperfusion injury, as well as anti-tumour, memory improving, anti-aging and anti-inflammation activity [7]. However, Akao et al. [8] found that a little ginsenoside could be absorbed through the gastrointestinal tract and it only existed in the form of a ‘natural active precursor’ in the body; however, it is well known that the active product that is actually responsible for its role in the body is ginsenoside CK. Currently, the research on the activity of ginsenoside CK and the development of drugs from it have been a hot topic, but no related study on whether ginsenoside CK has a protective effect against cerebral ischemia/reperfusion injury has been reported to the best of our knowledge. In view of many reports on the protective effect of the other ginsenosides, such as Rg1, Rb1 and Rd, against cerebral ischemia-reperfusion injury [9–12], and the crucial role of oxidative stress and inflammation in the occurrence and development of cerebral ischemia/reperfusion injury [13], in this study, we investigated the effects of the pretreatment with ginsenoside CK on the cerebral neurobehavioural scores, brain water content, brain infarct volume, level of oxidative stress, inflammatory reaction and HMGB1 protein expression in rats with cerebral ischemia/reperfusion injury, in order to provide experimental evidence for the further research and application of ginsenoside CK.

Materials and methods

Animals

Male Wistar rats (specific pathogen free), weighing 180–220 g, were purchased from The Experimental Animal Center of Jilin University (China).

Materials

Ginsenoside CK was purchased from Shanxi Yangling Ciyuan Biological Technology Co., Ltd (purity >95%, China); nimodipine was purchased from Tianjin Central Pharmaceutical Co., Ltd; triphenyltetrazolium chloride (TTC) was purchased from Shanghai Chaorui Biological Technology Co., Ltd (China). Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) test kits were purchased from Shanghai Chaorui Biological Engineering Institute (China). TNF-α and IL-1β test kits were purchased from R&D Company (USA). HMGB1 and GAPDH antibodies and horseradish peroxidase-labelled second antibody were purchased from Cell Signaling Company (USA).

Animal grouping, administration and model preparation

Wistar rats that had acclimated to the laboratory environment for 1 week were randomly divided into six groups: sham operation group (Sham), ischemia/reperfusion model group (I/R), low-dose ginsenoside CK group (L-CK, 30 mg/kg/day), high-dose ginsenoside CK group (H-CK, 60 mg/kg/day) and nimodipine group (nimodipine, 15 mg/kg/day), 18 rats in each group. The rats in the L-CK, H-CK and nimodipine groups were intragastrically given the corresponding agents successively for 15 days before surgery, once a day, and those in the sham operation group and model group were given the same volume of solvent in the same way. At 1 h after the last administration, the cerebral ischemia model was established by suture-occlusion method; at 2 h after the myocardial ischemia, the occluded suture was pulled out, and then the 24 h reperfusion was carried out.

Neurological behaviour assessment and brain water content measurement

After the 24 h reperfusion, six rats from each group were randomly selected and scored according to the neurological behaviour assessment method proposed by Bederson et al. [14]. A flexed forepaw with resistance to lateral pressure, accompanied by circling to the left, was recorded as 3 points; a flexed forepaw with resistance to lateral pressure, but not accompanied by circling to the left, as 2 points; an incomplete extension of the contralateral forepaw as 1 point; no symptoms of nerve system injury as 0 point. At the end of the neurological behavioural assessment, the rats were decapitated and the brain water content was measured by the dry-wet method.

Brain infarct volume measurement

After the 24 h reperfusion, another six rats from each group were randomly selected and anaesthetized with sodium pentobarbital (60 mg/kg) and sacrificed by decapitation to obtain brain samples. The brains were placed in a −20 °C refrigerator for 20 min and then cut into 2-mm continuous coronal slices. The slices were stained with 2% TTC phosphate buffer at 37 °C in darkness for 20 min, and then rapidly moved to paraformaldehyde solution (4%) which had been pre-
cooled to 4°C and were fixed in it for 30 min. The slices were photographed and recorded, in which the white area was considered an infarcted area and the red one, a normal brain tissue. The collected images were analyzed by Image Pro Plus 6.0 image processing software for the calculation of the infarct volume ratio.

Detection of biochemical indexes in the brain tissue

The remaining six rats were decapitated after anesthesia to take their brains. An accurately weighed part of the brain was placed in normal saline that had been pre-cooled to 4°C in a proportion of 1:9, and was homogenized to obtain a supernatant. The supernatant was sub-packaged and frozen for use. According to the operation steps of the kits, the SOD and GSH-Px activities and the MDA content in the brain tissue were measured by spectrophotometry. The TNF-α and IL-1β content was determined by radioimmunoassay.

Detection of HMGB1 protein expressions in the brain tissue

The remaining brain tissue was prepared into a brain tissue homogenate by repeated grinding at low temperatures. The homogenate was added an appropriate amount of RIPA lysis buffer and centrifuged at 3000 rpm for 5 min to obtain a supernatant, and the concentration of total proteins in the supernatant was measured by the BCA method. Twenty grams of the total proteins were loaded on a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) apparatus, and transferred onto a nitrocellulose membrane after the electrophoresis. After the blocking and rinsing, the first anti-HMGB1 (diluted at 1:1000) was added onto the membrane, which was kept in a refrigerator at 4°C overnight, and then the horseradish peroxidase-labelled second antibody (diluted at 1:2000) was added onto the membrane. The membrane was washed, developed by ECL, exposed and photographed. Image Plus 6 Pro image processing software was used to analyze the images, in which the gray ratios of HMGB1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein bands represented the expression levels of HMGB1 protein.

Statistical analysis

The data were represented as mean values with standard deviation (mean ± SD) and were analyzed using SPSS 13 statistical software. One-way analysis of variance was used for the repeated comparison among multi groups and LSD was applied for the pairwise comparison between two groups. \( P < 0.05 \) or \( P < 0.01 \) was considered a statistically significant difference.

Results and discussion

Effects of ginsenoside CK on the neurobehavioural score and brain tissue water content in rats

Common modelling methods for cerebral ischemia/reperfusion injury include ligation, suture-occlusion and photochemically induced thrombosis [15]. In 1986, Koizumi et al. [16] first successfully established a middle cerebral artery occlusion (MCAO) model through common carotid artery, not by craniotomy, and the result was affirmed and it was recognized that the ischemia/reperfusion time could be well controlled by this method. After Longa et al. [17] modified it, the method has been widely used. In this study, a modified suture method was used to establish a rat cerebral ischemia/reperfusion model. As shown in Figure 1A, the rats did not present neurological deficits in the sham operated group. Compared with those in the sham operation group, the neurobehavioural scores of the rats in the
model group increased significantly ($P < 0.01$). Compared with those in the model group, the neurobehavioural scores in the low- and H-CK groups and the nimodipine group were significantly lowered ($P < 0.01$), suggesting that ginsenoside CK can reduce some nerve functional defects in rats. As shown in Figure 1B, the brain tissue water content of the rats in the model group increased significantly ($P < 0.01$) compared with that in the sham operation group. Moreover, the brain tissue water content in the low- and H-CK groups and the nimodipine group was reduced to a certain extent compared with that in the model group, the reduction being significant in the H-CK group and the nimodipine group ($P < 0.05$). These results suggest that ginsenoside CK can reduce the brain tissue water content in rats.

**Effect of ginsenoside CK on the infarct volume ratio in rats**

The TTC staining results (Figure 2) showed that, compared with that in the sham operation group, the brain infarct volume ratio of the rats in the model group increased significantly ($P < 0.01$). When compared with that in the model group, the brain infarct volume ratio of the rats in the L- and H-CK groups and the nimodipine group were significantly reduced ($P < 0.05$, $P < 0.01$), indicating that ginsenoside CK could have some protection against the cerebral ischemia/reperfusion injury in rats.

**Antioxidant effect of ginsenoside CK in the brain of rats**

So far, the pathogenesis of cerebral ischemia/reperfusion injury has not been elucidated, but the theory about the role of reactive oxygen species (ROS) in cerebral ischemia/reperfusion injury has attracted more and more attention [18]. The oxygen consumption of brain tissues is high, accounting for about 20% of the total oxygen consumption of the body, and more ROS can be produced in the brain. Nervous tissues themselves are relatively short of antioxidant substances, and rich in polyunsaturated fatty acids sensitive to ROS, so that brain cells are most susceptible to the invasion and damaging action of ROS [19]. SOD, one of the most important antioxidant enzymes scavenging oxygen free radicals in the body, widely exists in various organisms to protect cells from oxidative injury. GSH-Px can catalyze the transformation of hydrogen peroxide into water and oxidized GSH, thereby reducing the hydrogen peroxide level in the body to prevent cells from oxidative damage and maintain the normal structure and function [20, 21]. MDA is the end product of lipid peroxidation and can indirectly reflect the severity of the damage in the body's cells attacked by ROS [22]. Li et al. [23] confirmed that resveratrol could play a protective effect against cerebral ischemia reperfusion injury by inhibiting the oxidative damage of brain tissues in rats. Due to the above reasons, we determined the SOD and GSH-Px activities and the MDA content in the brain tissue of rats to evaluate the antioxidant effect of ginsenoside CK. The results (Figure 3) showed that the SOD and GSH-Px activities were reduced and the MDA content increased in the brain tissue of the rats in the model group, with significant differences compared with those in the sham operation group ($P < 0.01$). Compared with those in the model group, the SOD and GSH-Px activities increased significantly, whereas the MDA content decreased significantly in the brain tissue of the rats in the L- and H-CK groups and the nimodipine group ($P < 0.05$, $P < 0.01$). This suggests that ginsenoside CK can lower the level of oxidative stress in the brain tissue of rats with cerebral

![Figure 2. Effect of ginsenoside CK on the infarct volume ratio of rats.](image)

**Note:** Values are means ± SD ($n = 6$). Compared with the Sham group, **$P < 0.01$; compared with the I/R group, # $P < 0.05$, ## $P < 0.01$.**
ischemia/reperfusion injury. Shao et al. [24] found that ginsenoside CK could increase the activities of SOD and GSH-Px, and decrease the content of MDA in diabetic rats, to play an antioxidant effect, consistent with the results of this study.

**Effect of ginsenoside CK on the inflammation of brain tissue in rats**

Inflammation plays an important role in the process of brain injury caused by ischemia/reperfusion [6]. A lot of evidence has demonstrated that after cerebral ischemia/reperfusion, a large number of inflammatory signals are released, which may increase the expression of a lot of inflammatory factors in platelets, cerebral vascular endothelial and glial cells, such as TNF-α and IL-1β. TNF-α, the initial mediator of a systemic inflammatory reaction, can promote the expression and release of intercellular adhesion molecules, IL-1 and IL-6, and then amplify the cascade of inflammatory injury [25]. IL-1β, the major form of existence of IL-1 in the brain, interstitial fluid and blood, can promote the infiltration of leukocytes and induce the expression of chemokines and adhesion molecules in cerebral microvascular endothelial cells [26]. Therefore, the effect of ginsenoside CK on the inflammation of brain tissue of rats was evaluated. The results (Figure 4) showed that compared with that in the sham operation group, the TNF-α and IL-1β content in the brain tissue of the rats in the model group increased significantly ($P < 0.01$). Compared with that in the model group, the TNF-α content in the brain tissue of the rats in the L-CK group was not significantly different ($P > 0.05$), and that in the H-CK and nimodipine groups decreased significantly ($P < 0.05$, $P < 0.01$). This suggests that ginsenoside CK could decrease the level

![Figure 3. Antioxidant effect of ginsenoside CK in the brain tissue of rats.](image)

Note: Values are means ± SD ($n = 6$). Compared with the Sham group, **$P < 0.01$; compared with the I/R group, *$P < 0.05$, **$P < 0.01$.

![Figure 4. Effect of ginsenoside CK on the inflammation of brain tissue of rats.](image)

Note: Values are means ± SD ($n = 6$). Compared with the Sham group, **$P < 0.01$; compared with the I/R group, *$P < 0.05$, **$P < 0.01$. 

$\text{Figure 3.}$ Antioxidant effect of ginsenoside CK in the brain tissue of rats. 
$\text{Figure 4.}$ Effect of ginsenoside CK on the inflammation of brain tissue of rats.
of inflammation of the brain tissue in rats. The results from this study were consistent with those from the study by Joh et al. [27], in which the inhibitory effect of ginsenoside CK on the expression of inflammatory factors, such as TNF-\(\alpha\) and IL-1\(\beta\) was also confirmed.

**Effect of ginsenoside CK on the expression of HMGB1 in the brain of rats**

HMGB1, a member of the highly conservative non-tissue nucleoproteins, exists in the nucleus of most eukaryotic cells, and plays an important role in the process of cerebral ischemia-reperfusion injury as a novel proinflammatory cytokine [28]. In the physiological state, HMGB1 plays the role of nuclear binding protein, but it can enter the intracellular space through a passive release and active secretion to mediate inflammatory reaction when HMGB1 is stimulated by some factors [29]. There are cross-links between HMGB1 and other proinflammatory cytokines, such as TNF-\(\alpha\) and IL-1\(\beta\), and once it is released from macrophages and apoptotic or necrotic cells, HMGB1 can promote the upregulated expression of TNF-\(\alpha\) and IL-1\(\beta\) and other inflammatory cytokines [30]. In addition, some studies have also indicated that oxidative stress is involved in the release of HMGB1. Tsung et al. [31] confirmed that ROS could regulate the release of HMGB1 from cells, and the inhibition of ROS might reduce the expression of HMGB1. Furthermore, diosgenin and parecoxib could reduce the injury caused by cerebral ischemia/reperfusion to a certain extent through inhibiting the expression of HMGB1 [32, 33]. In view of the importance of HMGB1 in the process of cerebral ischemia-reperfusion injury, the effect of ginsenoside CK on the expression of HMGB1 in the brain of rats was observed in this study. As shown in Figure 5, the expression of HMGB1 in the brain tissue of the rats in the model group increased significantly compared with that in the sham group \((P < 0.01)\). Compared with that in the model group, the expression of HMGB1 in the brain tissue of the rats in the low- and H-CK groups and the nimodipine group was significantly reduced \((P < 0.05, P < 0.01)\), indicating that ginsenoside CK can inhibit the expression of HMGB1 in rats with cerebral ischemia/reperfusion injury.

**Conclusions**

The pretreatment with ginsenoside CK could decrease the neurobehavioural score, the brain tissue water content and the cerebral infarct volume ratio of rats with cerebral ischemia/reperfusion injury. Ginsenoside CK pretreatment increased the activity of SOD and GSH-Px and reduced the content of MDA in the brain tissue. Ginsenoside CK also lowered the content of TNF-\(\alpha\) and IL-1\(\beta\), and down-regulated the expression of HMGB1 in the brain tissue of the rats. We suggest the protective effect of ginsenoside CK against cerebral ischemia/reperfusion injury in rats could be attributed antioxidant, anti-inflammatory and HMGB1-expression inhibitory activity.

**Disclosure Statement**

The authors declare that there is no conflict of interest regarding the publication of this paper.

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