Calycosin Alleviates Oxidative Injury in Spinal Astrocytes by Regulating the GP130/JAK/STAT Pathway

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Abstract: Spinal injury is a complicated disease and is reported to be associated with damages on spinal astrocytes induced by oxidative injury. Astragali Radi, a famous traditional Chinese medicine, is reported to have promising efficacy in protecting injuries in the central nervous system. This study aims to investigate the effect of calycosin, an isoflavone phytoestrogens isolated from Astragali Radi, on oxidative injury in spinal astrocytes induced by H₂O₂ and the underlying mechanism. Primary rat spinal astrocytes were pretreated with 5, 10, and 20 μM calycosin and subjected to H₂O₂ treatment for 24 h to establish an oxidative injury model. Cell viability was detected using the CCK-8 assay to screen the optimized concentration of calycosin. Flow cytometry was used to evaluate the apoptotic rate and cell cycle. The expression level of BrdU was visualized using the immunofluorescence assay. Western blotting was used to measure the expression levels of p-JAK2, p-STAT3, p-AKT, GP130, and IL-6 in spinal astrocytes. We found that proliferation was inhibited and that apoptosis was induced by the stimulation of H₂O₂. The expression levels of p-JAK2, p-STAT3, p-AKT, GP130, and IL-6 were significantly elevated in H₂O₂-treated astrocytes. After the treatment of calycosin, proliferation was facilitated, and apoptosis was suppressed. These phenomena were accompanied by the downregulation of p-JAK2, p-STAT3, p-AKT, GP130, and IL-6, which were abolished by the co-administration of PI3K (ly294002) or STAT3 (stattic) inhibitor. Overall, calycosin alleviated oxidative injury in spinal astrocytes by mediating the GP130/JAK/STAT pathway.

Key words: spinal astrocytes, calycosin, oxidative injury, GP130/JAK/STAT pathway

1 Introduction

Recently, the morbidity of spinal injury increases annually globally¹. Spinal injury, a complicated disease induced by multiple factors, is divided into primary spinal and secondary injuries. As an important neurogliocytes in the central nerve system with the highest amounts, astrocytes maintain the structure and physiological function of the central nerve system². The activation and proliferation of astrocytes play a critical role in the repair process of spinal injury². H₂O₂ is one of the main reactive oxygen species (ROS) that induces the apoptosis of astrocytes. Currently, H₂O₂ is widely used for the establishment of the oxidative injury model in astrocytes in vitro³,⁴.

Astragali Radi, a famous traditional Chinese medicine and an important component of the Buyang Huanwu Decoction, is reported to play an important role in treating injuries in the central nerve system⁶,⁷. Calycosin, an isoflavone phytoestrogen isolated from Astragali Radi, is widely reported to have potential pharmacological activities. Calycosin exerts a neuroprotective property by downregulating the Ras dexamethasone-induced protein 1 and upregulating miR-375 in cerebral ischemia/reperfusion rats⁸. In addition, in the co-cultural system of primary neurons and astrocytes stimulated by LPS, calycosin significantly reverses the declined number of dopaminergic neurons induced by LPS. Moreover, the inhibitory effect of calycosin against superoxide radicals is higher than those of TNF-α and nitric oxide⁹. Therefore, we suspect that calycosin may exert a promising effect on the proliferation of spinal astrocytes. In addition, the PI3K/AKT and JAK2/STAT3 pathways are closely associated with the proliferation of astrocytes¹⁰,¹¹. In the present study, the effect of calycosin
on oxidative injury in spinal astrocytes induced by H$_2$O$_2$ and the underlying mechanism will be investigated to explore the potential therapeutic effect of calycosin on spinal injury.

2 Materials and Methods

2.1 Isolation of rat primary spinal astrocytes

After sacrificing the animals through euthanasia, the tibia and humerus tissues were collected under aseptic conditions and washed using PBS buffer with 10% streptomycin and penicillin. After excising the metaphyseal ends to expose the marrow cavity, the 10% completed medium was used to wash the marrow cavity, and the bone marrow was blown out repeatedly by using a sterile syringe. A unicellular suspension of spinal cells was prepared, centrifuged, resuspended with the complete medium, and further incubated at 37°C and 5% CO$_2$.

2.2 Identification of astrocytes by using immunofluorescence assay

The isolated astrocytes were seeded into 6-well plates, fixed using 4% paraformaldehyde for 15 min, and washed thrice by using PBS buffer. After incubating with 0.5% Triton X-100, astrocytes were blocked with 5% BSA solution, washed with PBS buffer, and incubated with the primary antibody against GFAP (1:200, CST, Boston, USA) at room temperature for 2 h. After washing with PBS buffer, cells were incubated with the secondary antibodies conjugated with Cy3 and DAPI. The samples that were not incubated with the primary antibody were used as the negative control. Finally, fluorescence microscopy (Olympus, Tokyo, Japan) was used to take images of the fluorescence in each group.

2.3 Grouping

Astrocytes were treated with different concentrations of calycosin (5, 10, and 20 μM) for 12 h and stimulated with 100 μM H$_2$O$_2$ for 24 h to establish the oxidative injury model.

2.4 CCK-8 assay

The CCK-8 assay kit (Jiangcheng, Nanjing, China) was used to detect the cell viability of astrocytes treated with different strategies in accordance with the instructions of the manufacturer. In brief, cells were seeded into 96-well plates, added with 10 μL CCK-8 solution, and incubated at 37°C for 3 h. The absorbance at 450 nm was detected using the PerkinElmer microplate reader (PerkinElmer, Massachusetts, USA).

2.5 Apoptosis analysis

About 2 × 10$^5$ cells were collected and centrifuged at 1500 rpm for 3 min. Cells were resuspended using 300 μL 1× Binding Buffer, added with 3 μL Annexin V–FITC and 5 μL PI-PE, and incubated at room temperature for 10 min. Lastly, samples were added with 500 μL precooled 1× Binding Buffer and analyzed using flow cytometry (BD, New Jersey, USA).

2.6 Cell cycle analysis

Astrocytes were centrifuged at 1500 rpm for 3 min and resuspended using 1 mL PBS. Subsequently, cells were centrifuged at 1500 rpm for 3 min and resuspended with 1 mL DNA staining solution and 10 μL permeabilization solution. After vortex oscillation for 5–10 s and incubation at room temperature for 30 min, samples were analyzed using flow cytometry (BD, New Jersey, USA).

2.7 Western blotting assay

Proteins from astrocytes were obtained using the lysis solution (Biorbyt, Cambridge, UK). The BCA kit (Biorbyt, Cambridge, UK) was used to quantify the isolated proteins, which were loaded on a gel, separated by 12% SDS–PAGE, and transferred onto the PVDF membrane (Merck, New Jersey, USA). The membrane was further incubated in 5% skim milk and incubated at 4°C overnight with primary antibodies against p-JAK2 (1:800, Biorbyt, Cambridge, UK), p-STAT3 (1:800, Biorbyt, Cambridge, UK), p-AKT (1:800, Biorbyt, Cambridge, UK), GAPDH (1:800, Biorbyt, Cambridge, UK), or GP130 (1:800, Biorbyt, Cambridge, UK). Then, the membrane was incubated with secondary antibody (1:4000, Biorbyt, Cambridge, UK) for 2 h. Lastly, following exposure, bands were analyzed using the Image J software (Bio-Rad, California, USA) with GAPDH as the loading control.

2.8 Statistical analysis

Data were expressed as mean ± SD and analyzed using one-way ANOVA for the test of differences between groups. The Dunnett’s T3 was used to analyze data with unequal variances. P < 0.05 was considered statistically significant.

3 Results

3.1 Identification of spinal astrocytes

Primary spinal astrocytes were isolated from rats and identified using immunofluorescence assay to measure the expression of GFAP. As shown in Fig. 1A, GFAP was found to be positively expressed in isolated cells, indicating the successful isolation of spinal astrocytes.

3.2 Screening of the concentration of calycosin

Cell viability was detected after astrocytes were treated with different concentrations of calycosin to obtain the ap-
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Appropriate concentration of calycosin. As shown in Fig. 2, cell viability was significantly suppressed by the stimulation of H$_2$O$_2$ and remarkably elevated by the treatment of 20 μM calycosin (*p<0.05 vs. control, #p<0.05 vs. H$_2$O$_2$). Therefore, 20 μM calycosin was utilized in the subsequent experiments.

3.3 Facilitated effects of calycosin on the proliferation of H$_2$O$_2$-treated astrocytes abolished by ly294002 or stattic

Astrocytes were treated with calycosin in the presence of 50 μM ly294002 or 5 μM stattic to investigate the potential mechanism underlying the protective effects of calycosin on H$_2$O$_2$-treated astrocytes. As shown in Fig. 3A, compared with that in control samples, the cell viability in H$_2$O$_2$-treated astrocytes was dramatically suppressed and remarkably elevated by the treatment of calycosin. Compared with calycosin, the co-incubation of ly294002 or stattic reversed the promoted cell viability (*p<0.05 vs. control, #p<0.05 vs. H$_2$O$_2$, @p<0.05 vs. H$_2$O$_2$ + calycosin).

We further detected the expression level of Brdu by using the immunofluorescence assay. As shown in Fig. 3B, Brdu was downregulated in the H$_2$O$_2$ group and significantly upregulated by the administration of calycosin. After the co-administration of ly294002 or stattic, the expression level of Brdu was significantly reversed. These data indicated that ly294002 or stattic abolished the facilitated effects of calycosin on the proliferation of H$_2$O$_2$-treated astrocytes.

3.4 Effects of calycosin on the apoptosis and cell cycle of H$_2$O$_2$-treated astrocytes abolished by ly294002 or stattic

We further explored the effects of calycosin on the apoptosis and cell cycle by using flow cytometry. As shown in Fig. 4, compared with that in control cells, the apoptotic rate in H$_2$O$_2$-treated astrocytes was significantly elevated and significantly suppressed by the treatment of calycosin. After the co-culture of ly294002 or stattic, the apoptotic rate of astrocytes was significantly reversed (*p<0.05 vs. control, #p<0.05 vs. H$_2$O$_2$, @p<0.05 vs. H$_2$O$_2$ + calycosin), indicating that the effects of calycosin on the apoptosis of H$_2$O$_2$-treated astrocytes were related to the activation of PI3K/Akt and JAK2/STAT3 signal pathways. Calycosin had no significant effect on the proportion of G0/G1 phase astrocytes. In addition, compared with the calycosin treatment, the co-treatment of ly294002 or stattic did not significantly affect the proportion of G0/G1 phase astrocytes.

3.5 Inhibition of the GP130/JAK/STAT pathway by calycosin

As shown in Fig. 5, compared with those in control cells, the expression levels of p-JAK2, p-STAT3, p-AKT, GP130, and IL-6 were significantly elevated in H$_2$O$_2$-treated astrocytes and significantly suppressed in the calycosin + H$_2$O$_2$, calycosin + H$_2$O$_2$ + ly294002, and calycosin + H$_2$O$_2$ + stattic groups (*p<0.05 vs. control, #p<0.05 vs. H$_2$O$_2$). Compared with the calycosin + H$_2$O$_2$ treatment, the calycosin + H$_2$O$_2$ +...
Fig. 3  After pretreatment with 20 μM calycosin for 24 h, cells were incubated with 100 μM H₂O₂ for 24 h for modeling and treated with inhibitor LY294002 or Stattic for 24 h. A. Cell viability was used to measure the cellular proliferation of astrocytes (⁎p < 0.05 vs. control, #p < 0.05 vs. H₂O₂, @p < 0.05 vs. H₂O₂ + calycosin). B. Brdu expression was visualized by immunofluorescence assay.

Fig. 4  After pretreatment with 20 μM calycosin for 24 h, cells were incubated with 100 μM H₂O₂ for 24 h for modeling and treated with inhibitor LY294002 or Stattic for 24 h. A. Apoptosis was determined by flow cytometry. B. Cell cycle was analyzed using flow cytometry. C. Apoptotic rate of each group was counted. D. Percentage of cells at different phases of cell cycle was counted (⁎p < 0.05 vs. control, #p < 0.05 vs. H₂O₂, @p < 0.05 vs. H₂O₂ + calycosin).
ly294002 and calycosin + H2O2 + stattic treatments had no significant effect on the expression levels of p-JAK2, p-STAT3, p-AKT, GP130, and IL-6.

4 Discussion
Spinal injury is a difficult miscellaneous disease currently. The treatment of spinal injury by using traditional Chinese medicine has thousands of years of history. The Buyang Huanwu Decoction is the representative for promoting blood circulation and dispelling sputum and has been used for the repair of neurofunction in spinal injury for hundreds of years in China\textsuperscript{12, 13}. However, the underlying mechanism is currently unclear. As the main component of Astragali Radi in Buyang Huanwu Decoction, calycosin is widely reported to exert antitumor property\textsuperscript{14, 15}. Nevertheless, the application of calycosin in the treatment of spinal injury is rarely reported. Astrocytes are the main cell type of neurogliocytes, and in the present study, the effect of calycosin on the oxidative injury of spinal astrocytes is investigated.

In the early stage of spinal injury, the activation and proliferation of astrocytes play an important role in inducing injury. However, in the advanced state of spinal injury, the astrocytes formed glial scar exerts the inhibitory effect against the oxidative injury\textsuperscript{16, 17}. Therefore, the beneficial aspect of astrocytes and the reduction of the unfavorable effects should be exploited for the treatment of spinal injury. Xu reported that the synthesis and secretion of neurotrophic factors can be induced by isoflavones\textsuperscript{18}. In addition, the antitumor property of calycosin is widely reported, indicating that proliferation can be inhibited and that apoptosis can be induced by calycosin in tumor cells\textsuperscript{19}. These reports revealed that different functions of calycosin are observed in different types of cells. In the present study, H2O2 is used to stimulate astrocytes for 24 h to establish oxidative injury after the 12 h pretreatment of calycosin. Significant inhibitory effects on apoptosis and activation effect on proliferation are observed on calycosin and H2O2-treated astrocytes.

PI3K/Akt and JAK/STAT signaling pathways are two important pathways involved in the progression of cellular apoptosis and nerve regeneration\textsuperscript{20, 21}. However, the effect of calycosin on the PI3K/Akt signal pathway is controversial. In human osteosarcoma MG-63 cells, the PI3K/Akt signal pathway can be suppressed by calycosin, which contributes to the inhibited proliferation ability and activated apoptosis in MG-63 cells\textsuperscript{22}. However, in human colorectal cancer cells, calycosin is reported to activate the PI3K/Akt signal pathway to facilitate apoptosis and inhibit cell growth\textsuperscript{23}. Interestingly, in the present study, the expression level of p-Akt is significantly inhibited by calycosin. Chen reported that the JAK/STAT and MAPK pathway in the central nerve system can be activated by IL-6\textsuperscript{24}. GP130 is the common signaling chain of multiple cytokines, such as IL-6, IL-1, and leukemia inhibitory factor\textsuperscript{25}. Buyang Huanwu Decoction is reported to alleviate the neuron dysfunction by inhibiting the apoptosis of neurons through the inhibition of the activation of the JAK2/STAT3 pathway\textsuperscript{26}.

![Graph showing protein relative expression levels](image-url)
In astrocytes, PI3K/Akt and GP130JAK/STAT signaling pathways are significantly suppressed by calycosin. However, the combination treatment of calycosin with the inhibitor of the two pathways does not further inhibit the apoptosis or induce the proliferation of astrocytes. This finding deserves to be further investigated. In our future work, whether the related pathways are activated by the combination of calycosin and inhibitors to affect the apoptosis and proliferation of astrocytes will be further explored.

Overall, in the present study, H_2O_2 treatment is used to induce the oxidative injury model in isolated rat spinal astrocytes. Apoptosis is suppressed, and proliferation is facilitated by the treatment of calycosin by inhibiting the phosphorylation of PI3K/Akt and JAK/STAT signaling. These data revealed that calycosin may be an active component in the therapeutic function of Buyang Huanwu Decoction on spinal injury, which needs to be further verified by comparing the functional mechanism between the Buyang Huanwu Decoction and calycosin.

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Declaration of Conflict of Interest

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

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