Protection of PC12 Cells against Superoxide-induced Damage by Isoflavonoids from *Astragalus mongholicus*[1]

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Objective To further investigate the neuroprotective effects of five isoflavonoids from *Astragalus mongholicus* on xanthine (XA)/ xanthine oxidase (XO)-induced injury to PC12 cells. **Methods** PC12 cells were damaged by XA/XO. The activities of antioxidant enzymes, MTT, LDH, and GSH assays were used to evaluate the protection of these five isoflavonoids. Contents of Bcl-2 family proteins were determined with flow cytometry. **Results** Among the five isoflavonoids including formononetin, ononin, 9, 10-dimethoxypterocarpan-3-O-β-D-glucoside, calycosin and calycosin-7-O-glucoside, calycosin and calycosin-7-O-glucoside were found to inhibit XA/ XO-induced injury to PC12 cells. Their EC_{50} values of formononetin and calycosin were 0.05 μg/mL. Moreover, treatment with these three isoflavonoids prevented a decrease in the activities of antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), while formononetin and calycosin could prevent a significant deletion of GSH. In addition, only calycosin and calycosin-7-O-glucoside were shown to inhibit XO activity in cell-free system, with an approximate IC_{50} value of 10 μg/mL and 50 μg/mL. Formononetin and calycosin had no significant influence on Bcl-2 or Bax protein contents. **Conclusion** Neuroprotection of formononetin, calycosin and calycosin-7-O-glucoside may be mediated by increasing endogenous antioxidants, rather by inhibiting XO activities or by scavenging free radicals.

Key words: *Astragalus mongholicus*; Neuroprotection; Xanthine/xanthine oxidase; PC12 cells

INTRODUCTION

Oxidative stress refers to the imbalance between the production and removal of reactive oxygen species (ROS). It is well known that, due to the reactions between ROS and macromolecules, generation of ROS can lead to damage or death of cells in various tissues[1]. Compared with all other tissues, brain tissue is particularly vulnerable to oxidative stress due to its high glucose metabolism rate and low antioxidant defense enzyme level[2-3]. Since ROS and oxidative stress have been implicated in a variety of neuronal disorders, including Alzheimer's disease (AD), atherosclerosis, cerebral ischemia, seizures, amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD)[4], various antioxidants have become a promising candidate for application in neurodegenerative diseases such as AD, PD and stroke-induced ischemic brain damage[5].

Astragalus mongholicus Bunge, known as Huangqi in China, has been widely used as one of the primary Chinese tonic herbs for thousands of years. We have reported the neuroprotective effects of five isoflavonoids from *Astragalus mongholicus* on PC12 cells damaged by glutamate[6]. The present study further investigated how to protect PC12 cells against xanthine (XA)/ xanthine oxidase (XO)-induced injury with these compounds.

MATERIALS AND METHODS

The plant materials used in this study were dried roots of *Astragalus mongholicus* Bunge obtained from Dalian Pharmacy (Dalian, China) and identified by Dr. Xian Lan (Pharmacology College, Shandong University). A voucher specimen was deposited at Dalian University of Technology. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum, penicillin and streptomycin were obtained from Gibco BRL Life Technology, Inc

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(Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT), xanthine, xanthine oxidase, poly-L-lysine, N, N-dimethylfor-mamide and sodiumdo- decylsulfate (SDS) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Monoclonal antibodies to Bcl-2 and Bax were obtained from Backman Coulter Inc (IM, USA). Kits used in enzyme assays were from Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were of analytic grade.

Preparation of Isoflavonoids

Isoflavonoids, including formononetin, ononin, calycosin, calycosin-7-O-glucoside and 9, 10-dimethoxypterocarpan-3-O-β-D-glucoside, were isolated, purified and identified using chemical and spectroscopic methods as previously reported (Figs. 1 and 2)[7]. HPLC analysis showed that their purity was over 96.8%.

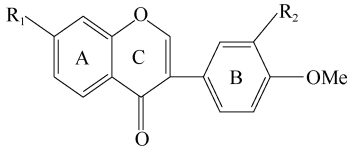

FIG. 1. Structures of formononetin, ononin, calycosin, and calycosin -7-O-glucoside (formononetin, R_1=OH, R_2=H; ononin, R_1=Oglc, R_2=H; calycosin, R_1=R_2=OH; calycosin -7-O-glucoside, R_1=Oglc, R_2=OH).

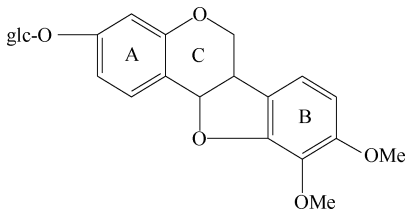

FIG. 2. Structure of 9, 10-dimethoxypterocarpan-3-O-β-D-glucoside.

Assay for Inhibition of XO Activity

XO inhibitory activity was measured as previously reported with some minor modifications[8]. A 100 mmol/L XA solution (980 μL) in sodium phosphate (pH 7.8) and 10 U/mL XO solution (10 μL) were incubated for 10 min at 25 ℃ either in the presence or absence of test compounds (10 μL) at various concentrations (5, 10, 50, and 100 μg/mL) dissolved in DMSO. The absorption at 295 nm, indicating the formation of uric acid, was measured with a spectrophotometer (Jasco V-560). The enzyme was replaced with a blank containing the same volume of phosphate buffer.

PC12 Cell Culture

The PC12 cell line was obtained from the Shanghai Institute of Cell Biology and maintained in DMEM supplemented with 10% fetal bovine serum and 50 μg/mL penicillin/streptomycin in a humidified incubator containing 5% CO_2 at 37 ℃. For measurement of cell viability and enzyme activity, the cells were seeded in 24- or 96-well plates at 1×10^5 cells/mL and used in experiments after 24 h.

Drug Treatment and MTT Assay for Cell Survival

To assess the protective effect of the five isoflavonoids from Huangqi against oxidative damage, XA (100 μmol/L)/XO (25 mU/mL) was used to induce apoptosis as previously described[9]. The cultures were pretreated with different concentrations of isoflavonoids (dissolved in DMSO, with final culture concentration less than 0.1%), and incubated for 9 h with exposure to XA (100 μmol/L)/XO (25 mU/mL). Cell viability was assessed by MTT assay[10] and calculated as 100×(OD of drug-treated and XA/XO cultures-OD of XA/XO-insulted cultures)/(OD of control cultures ― OD of XA/XO-insulted cultures). All experiments were performed in triplicate and the results were shown graphically as $\bar{x} \pm s$ (Fig. 3). The cell survival was also evaluated by morphological observation (Fig. 4).

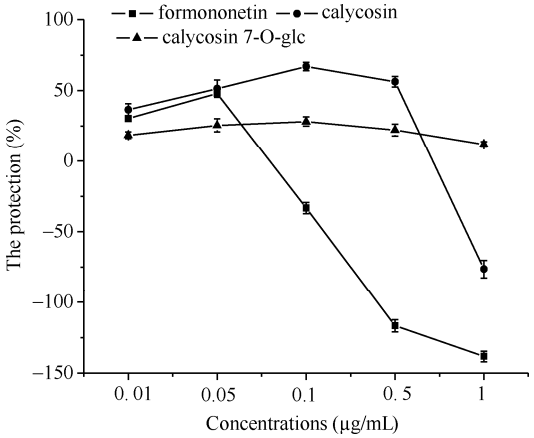

FIG. 3. Effects of formononetin, calycosin and calycosin-7-O-glucoside on toxicity induced by XA/XO to PC12 cells. Data are presented as $\bar{x} \pm s$ (n=3).

Measurement of LDH, SOD, GSH-Px, and GSH

At the end of treatment, the medium was collected for LDH assay to assess cellular integrity[11]. The cells were washed twice with ice-cold phosphate buffered saline (PBS, pH 7.4), pooled in 1 mL of 0.1 mol/L PBS (pH 7.4) and homogenized. The homogenate

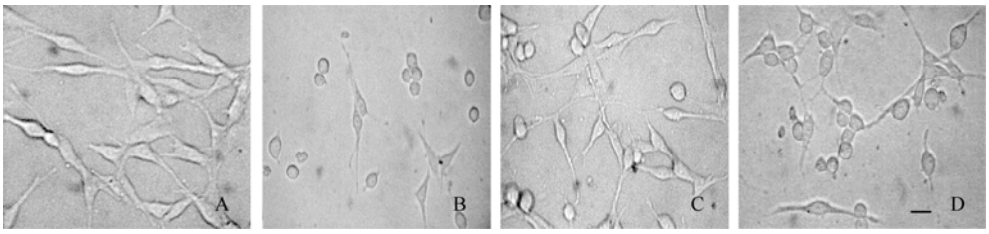

FIG. 4. Protective effects of formononetin and calycosin on PC12 cell injury induced by XA/XO. A: control; B: PC12 cells exposed to XA (100 μmol/L)/XO (25 mU/mL) for 9 h; C: PC12 cells pro-incubated with 0.1 μg/mL calycosin for 12 h and exposed to XA/XO for 9 h; D: PC12 cells pro-incubated with 0.05 μg/mL formononetin for 12 h and exposed to XA/XO for 9 h. Scale bar= 10 μm.

was centrifuged at 3000×g for 30 min at 4 ℃ and the supernatant was used in GSH-Px, SOD enzyme and GSH assays according to the manufacturer's instructions. All experiments were performed in triplicate and the results were expressed as $\bar{x} \pm s$.

Flow Cytometric Analysis of Bcl-2 and Bax Protein

The level of Bcl-2 and Bax protein was measured by flow cytometry as previously described[12]. Briefly, PC12 cells were collected and washed with ice-cold PBS. After fixation with 2% paraformaldehyde for 20 min and permeabilization with 0.5% Triton-X 100, the cells were incubated with primary antibodies against Bcl-2 or Bax for 30 min, respectively, then with corresponding fluorescent isothiocyanate (FITC)-conjugated secondary antibodies for 30 min at room temperature in the dark. After the cells were washed with PBS, the antigen density was analyzed with a FACScan flow cytometer (BD FACScantoTM).

Protein Assay

Protein content was measured with Bradford method using bovine serum albumin as a standard[13].

Statistical Analysis

Results obtained are expressed as $\bar{x} \pm s$ ($n \geqslant 3$). Statistical analysis was performed according to Student's *t*-test by one-way analysis of variance. $P < 0.05$ was considered statistically significant. Statistics and graphs were obtained using the software Microcal™ Origin 7.5 (Microcal Software, Inc., Northampton, MA, USA).

RESULTS

Effects of Formononetin, Calycosin and Calycosin-7-O-glucoside on XO Activity

Calycosin and calycosin-7-O-glucoside inhibited XO activity in the formation of uric acid from XA *in vitro*. Calycosin inhibited 52.1% of XO activity at a concentration of 10 μg/mL and calycosin-7-O-glucoside inhibited 44.6% of XO activity at 50 μg/mL. However, formononetin, 9, 10-dimethoxypterocarpan-3-O-β-D-glucoside and ononin had no effect on XO activity at the concentrations of 10 μg/mL and 50 μg/mL.

Formononetin, Calycosin and Calycosin-7-O-glucoside Protected PC12 cells against XA/XO-induced Cytotoxicity

Formononetin and calycosin inhibited oxidative cell injury induced by XA/XO with an estimated EC_{50} of 0.05 μg/mL and 0.01 μg/mL, respectively (Fig. 3). The protection rate of formononetin against oxidative cell injury induced by XA/XO was 48.04% at the concentration of 0.05 μg/mL which was at the concentration of 0.1 μg/mL. Calycosine increased the cellular viability of 36.4%, 53.1%, and 67.1% at the concentration of 0.01 μg/mL, 0.05 μg/mL, and 0.1 μg/mL, respectively. Formononetin and calycosin at the concentration of over 0.5 μg/mL deteriorated the cell damages. Calycosin-7-O-glucoside slightly inhibited the damage induced by XA/XO even at the concentration of 1 μg/mL. The protective effects of formononetin and calycosin on PC12 cell injuries induced by XA/XO were also observed (Fig. 4). Furthermore, the data on LDH release demonstrated similar results as evaluated by MTT (Fig. 5). Formononetin and calycosin dramatically increased LDH release at the concentration of 0.1 μg/mL or 1 μg/mL, indicating that they have no neuroprotective effect on PC12 cell injuries. In addition, 9, 10-dimethoxypterocarpan-3-O-β-D-glucoside and ononin demonstrated no protective activity. These findings demonstrate that calycosin is the most potent neuroprotective compound among the five isoflavonoids.

Effects of Formononetin, Calycosin and Calycosin-7-O-glucoside on Activities of SOD, GSH-Px and GSH in XA/XO-treated PC12 Cells

GSH, SOD, and GSH-Px play a very important role

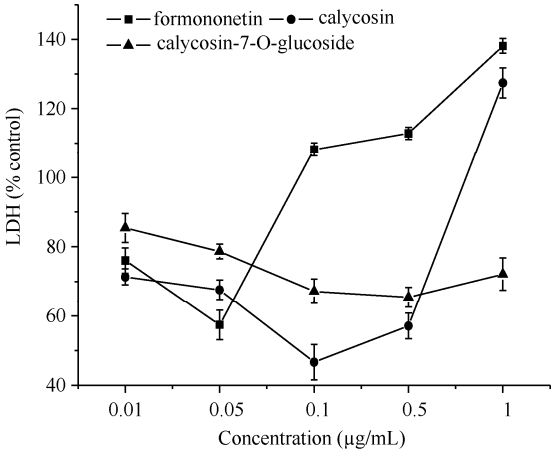

FIG. 5. Inhibition of LDH release induced by XA/XO in PC12 cells in the presence of formononetin, calycosin-7-O-glucoside and calycosin under XA/XO. Data were calculated as percentage of XA/XO-treated LDH activity. Each point represents $\bar{x} \pm s$ (n=3).

in protecting PC12 cells from oxidative stress. The present study investigated the effects of these three isoflavonoids on the antioxidant defense system. As shown in Table 1, treatment of PC12 cells with XA/XO significantly decreased the activities of SOD, GSH-Px and GSH. However, pre-incubation of PC12 cells with formononetin and calycosin prevented the depletion of GSH and markedly increased the activities of SOD and GSH-Px in XA/XO-damaged PC12 cells. Calycosin-7-O-glucoside also increased the activities of SOD and GSH-Px. However, it had no significant effects on the content of GSH in the damaged cells.

Bcl-2 Family Proteins Content

Since Bcl-2 gene family is closely associated with mitochondrial function, the level of Bcl-2 and Bax proteins was measured in this study, by flow cytometric analysis in the presence or absence of formononetin or calycosin. The results revealed that XA/XO treatment decreased Bcl-2 protein by 26.7% and slightly increased Bax protein (data not shown). On the contrary, the level of Bcl-2 protein in PC12 cells pre-incubated with 0.05 μg/mL formononetin or 0.1 μg/mL calycosin was not significantly elevated, while the level of Bax protein remained unchanged (data not shown).

TABLE 1

Effects of Formononetin, Ononin and Calycosin on the Activities of SOD, GSH-Px, and GSH in XA/XO-injured PC12 Cells ($\bar{x} \pm s$)

	SOD (U/mL)	GSH-Px (μmoL/mg pro.)	GSH (μmoL/mg pro.)
Control	10.44±1.96	73.77±4.11	6.74±1.12
XA/XO-insulted	6.99±1.0$^{\#\#}$	56.25±3.92$^{\#\#}$	4.7±1.06$^{\#\#}$
XA/XO+ Ⅰ [1]	11.27±3.47*	82.92±1.96**	7.01±1.35*
XA/XO+ Ⅱ [2]	13.38±4.33**	80.33±1.12*	6.51±1.48*
XA/XO+Ⅲ[3]	11.26±1.87**	78.64±3.63*	6.01±2.33

Note. $^{*}P$<0.05;$^{**}P$<0.01 *vs* XA/XO-injured cells; $^{\#\#}P$<0.01 *vs* control. [1]Ⅰ : formononetin; [2]Ⅱ : calycosin; [3]Ⅲ: calycosin-7-O-glucoside.

DISCUSSION

There is evidence that oxidative stress plays a major role in the pathogenesis of neurodegeneration. Oxidant production that exceeds the endogenous antioxidant capabilities, leads to oxidative molecular damage to tissues. Thus, protecting neurons from oxidative injuries may prevent or treat oxidative stress-induced neurodegenerative disorders[14].

Our previous study showed that the five isoflavonoids have different antioxidant actions on the cell-free system[7]. The present study showed that calycosin was the most effective compound among the five isoflavonoids, followed by calycosin-7-O-glucoside and formononetin. 9, 10-dimethoxypterocarpan-3-O-β-D-glucoside and ononin demonstrated no ability to scavenge free radicals. However, the neuroprotective activities of the five isoflavonoids in PC12 cells were not consistent with their direct antioxidant actions. Calycosin-7-O-glucoside showed almost no protective activity in damaged PC12 cells although its antioxidant activity was better than that of formononetin PC12 cells. It was reported that the more lipophilic a compound is, the better it penetrates cell membranes[14-15]. Tirosh *et al.* (1999) also pointed out that a lipophilic compound may have stronger antioxidant effects by scavenging peroxide in cytosol or by interacting with enzymes such as GSH-Px[16], which is supported by the findings of the present study that formononetin and calycosin had better neuroprotective effects than ononin and calycosin-7-O-glucoside.

The antioxidant system in the body consists of high levels of antioxidant compounds and antioxidant enzymes. GSH is a major endogenous antioxidant in

cells and plays an important role in protecting the brain from oxidative stress. The present study showed that formononetin, calycosin and calycosin-7-O-glucoside could prevent a decrease in the activities of antioxidant enzymes (SOD and GSH-Px), whreas formononetin and calycosin could prevent the excessive depletion of GSH in XA/XO-damaged PC12 cells.

It has been shown that Bcl-2, a negative regulator of cell death in the Bcl-2 family members, protects cells against apoptosis[17]. In the present study, XA/XO treatment decreased Bcl-2 protein level and increased Bax protein level. Formononetin and calycosin played no significant role in changoffing the Bcl-2 and Bax level, suggesting that their protection does not result from Bcl-2 family. In addition, the effective concentrations of the three isoflavonoids were much lower in the cell system than in the cell-free system, indicating that they act mainly by increasing endogenous antioxidant enzymes to inhibit the cell damage.

Formonotine and calycosin, also known as phytoestrogens, act as estrogens and have neuroprotective and antioxidant capabilities[18-19]. Zeng *et al.* (2004) showed that genistein, a soy isoflavone, protects neurons from damage *via* an estrogen receptor-mediated pathway at nanomolar level[20]. Further study is needed to show whether isoflavonoids from *Astragalus mongholicus* have the same mechanisms.

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