

## Buyang Huanwu Decoction ameliorates ischemic stroke by modulating multiple targets with multiple components: *In vitro* evidences

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**[ABSTRACT]** Buyang Huanwu Decoction (BYHWD) is a well-known traditional Chinese medicine prescription which is used to treat ischaemic stroke and stroke-induced disabilities. However, the exact mechanism underlying BYHWD's amelioration of ischaemic stroke and its effective constituents remain unclear. The present study aimed to identify the effective constituents of BYHWD and to further explore its action mechanisms in the amelioration of ischaemic stroke by testing the activities of 15 absorbable chemical constituents of BYHWD with the same methods under the same conditions. The following actions of these 15 compounds were revealed: 1) Ferulic acid, calycosin, formononetin, astrapterocarpan-3-*O*- $\beta$ -D-glucoside, paeonol, calycosin-7-*O*- $\beta$ -D-glucoside, astraisoflavan-7-*O*- $\beta$ -D-glucoside, ligustrazine, and propyl gallate significantly suppressed concanavalin A (Con A)-induced T lymphocyte proliferation; 2) Propyl gallate, calycosin-7-*O*- $\beta$ -D-glucoside, paeonol, and ferulic acid markedly inhibited LPS-induced apoptosis in RAW264.7 cells; 3) Propyl gallate and formononetin significantly inhibited LPS-induced NO release; 4) Hydroxysafflor yellow A and inosine protected PC12 cells against the injuries caused by glutamate; and 5) Formononetin, astragaloside IV, astraisoflavan-7-*O*- $\beta$ -D-glucoside, inosine, paeoniflorin, ononin, paeonol, propyl gallate, ligustrazine, and ferulic acid significantly suppressed the constriction of the thoracic aorta induced by KCl in rats. In conclusion, the results from the present study suggest that BYHWD exerts its ischaemic stroke ameliorating activities by modulating multiple targets with multiple components.

**[KEY WORDS]** Buyang Huanwu Decoction; Chemical constituents; Ischemic stroke; Anti-inflammatory-immunity; Neuroprotection; Vasodilation

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### Introduction

Stroke is the second leading cause of mortality and a major cause of disability<sup>[1]</sup>. Ischemic stroke accounts for approximately 85% of all stroke cases<sup>[2]</sup>. Studies have shown

that various mechanisms, including inflammation, immune modulation, and apoptosis and mediators, such as excitatory amino acids and nitric oxide, contribute to the development of ischemic stroke<sup>[3]</sup>. The major therapeutic approach for ischemic stroke is thrombolysis; however, this treatment is not sufficient to prevent damage in all patients<sup>[4]</sup>.

Buyang Huanwu Decoction (BYHWD) is a well-known traditional Chinese medicine (TCM) prescription in “Correction on Errors in Medical Classics”, which was written in the Qing dynasty, and recommended for use in patients with ischemic stroke. Many researches show that BYHWD has notable curative effectiveness in ischemic stroke and other vascular diseases<sup>[5-7]</sup>. It is composed of the following seven crude drugs: Astragali Radix (120 g) [the dried roots of *As-*

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*tragalus membranaceus* var. *mongholicus* (Bunge) P. K. Hsiao], Angelicae Sinensis Radix (6 g) [the dried lateral roots of *Angelica sinensis* (Oliv.) Diels], Chuanxiong Rhizoma (3 g) [the dried rhizomes of *Ligusticum chuanxiong* S. H. Qiu, Y. Q. Zeng, K. Y. Pan, Y. C. Tang & J. M. Xu], Paeonia Radix Rubra (4.5 g) [the dried roots of *Paeonia lactiflora* Pall.], Persicae Semen (3 g) [the dried seeds of *Prunus persica* (L.) Batsch], Carthami Flos (3 g) [the dried flowers of *Carthamus tinctorius* L.], and Pheretima (3 g) [the dried bodies of *Pheretima aspergillum* (Perrier)]. It has been reported that BYHWD can protect neurons from ischemic injury<sup>[8-9]</sup>, improve the recovery of neurological function, and stimulate neural proliferation<sup>[10]</sup>. Furthermore, BYHWD can repair injured blood vessels and lesioned tissues<sup>[11]</sup> and reduce infarction volumes in the ischemic brains of rats<sup>[10]</sup>. However, the exact mechanism underlying BYHWD's ability to ameliorate the injury of ischemic stroke and its effective chemical constituents remain unclear. TCM exerts its effects by releasing various chemical constituents into the blood circulation. Therefore, we try to find the effective constituents of BYHWD by identifying the chemical constituents that can enter the body. With the rat everted gut sac and Caco-2 cell monolayer models, the *in vitro* absorption of seven constitutive crude drugs of BYHWD are studied, and we find that 14 constituents, including 12 isoflavones, such as calycosin, calycosin-7-*O*- $\beta$ -D-glucoside, formononetin, ononin, (6a*R*, 11a*R*)-3-hydroxy-9, 10-dimethoxypterocarpan, (6a*R*, 11a*R*)-3-hydroxy-9, 10-dimethoxypterocarpan-3-*O*- $\beta$ -D-glucoside, (3*R*)-7, 2'-dihydroxy-3', 4'-dimethoxyisoflavan, (3*R*)-7, 2'-dihydroxy-3', 4'-dimethoxyisoflavan-7-*O*- $\beta$ -D-glucoside, and (6a*R*, 11a*R*)-3-hydroxy-9, 10-dimethoxypterocarpan-3-*O*- $\beta$ -D-glucoside-6''-*O*-malonate of Astragali Radix [the dried roots of *Astragalus membranaceus* var. *mongholicus* (Bunge) P. K. Hsiao, voucher sample No. 2688], 13 constituents (paeoniflorin, oxypaeoniflorin, 8-debenzoylpaeoniflorin, albiflorin, etc.) of Paeonia Radix Rubra (the dried roots of *Paeonia lactiflora* Pall., voucher sample No. 2401), 13 constituents (ferulic acid, senkyunolide H/I, senkyunolide R/S, and senkyunolide F, etc.) of Chuanxiong Rhizoma (the dried rhizomes of *Ligusticum chuanxiong* S. H. Qiu, Y. Q. Zeng, K. Y. Pan, Y. C. Tang & J. M. Xu, voucher sample No. 2684), six constituents (the same to those of Chuanxiong Rhizoma) of Angelicae Sinensis Radix [the dried lateral roots of *Angelica sinensis* (Oliv.) Diels, voucher sample No. 2685], five constituents (inosine, hypoxanthine, xanthine, uracil, and uridine) of Pheretima [the dried bodies of *Pheretima aspergillum* (Perrier), voucher sample No. 2405], three constituents (hydroxysafflor yellow A, safflomin-A, and kaempferol-3-*O*- $\beta$ -rutoside) of Carthami Flos (the dried flowers of *Carthamus tinctorius* L., voucher sample No. 2584), and two constituents (amygdalin and prunasin) of Persicae Semen [the dried seeds of *Prunus persica* (L.) Batsch,

voucher sample No. 2687] can be absorbed<sup>[12]</sup>. In total, 48 constituents of seven crude drugs are absorbable *in vitro*. All seven crude drugs were authenticated by Prof. CAI Shao-Qing (School of Pharmaceutical Sciences, Peking University, Beijing, China). The voucher samples were deposited in the Herbarium of Pharmacognosy, School of Pharmaceutical Sciences, Peking University. Furthermore, after thoroughly reviewing the results of our previous research and related literature, we can deduce that more than 100 compounds (including the constituents and their metabolites) originated from BYHWD may be identified *in vivo*<sup>[13-23]</sup>. However, these compounds are mainly produced from about 30 parent compounds in BYHWD. Based on an overall consideration of various factors (such as the availability of these parent compounds, their reported pharmacological actions, their contents in crude drugs and BYHWD, and their blood brain barrier permeability), in the present study, we chose 15 of them and studied their anti-inflammatory, immune-related, neuroprotective, and vascular tension-regulating effects simultaneously with the same methods and under the same conditions to explore the mechanisms by which BYHWD ameliorates the injury of ischemic stroke. The selected 15 components were 7 constituents [astragaloside IV, calycosin, calycosin-7-*O*- $\beta$ -D-glucoside, formononetin, ononin, (3*R*)-7, 2'-dihydroxy-3', 4'-dimethoxyisoflavan-7-*O*- $\beta$ -D-glucoside (astraisoflavan-7-*O*- $\beta$ -D-glucoside), (6a*R*, 11a*R*)-3-hydroxy-9, 10-dimethoxypterocarpan-3-*O*- $\beta$ -D-glucoside (astrapterocarpan-3-*O*- $\beta$ -D-glucoside)] of Astragali Radix, which is the monarch drug of BYHWD, ferulic acid from Angelicae Sinensis Radix (the ministerial drug of BYHWD), ligustrazine from Chuanxiong Rhizoma (the adjuvant drug of BYHWD), 3 constituents (paeoniflorin, paeonol, propyl gallate) of Paeonia Radix Rubra (the adjuvant drug of BYHWD), amygdalin from Persicae Semen (the adjuvant drug of BYHWD), hydroxysafflor yellow A from Carthami Flos (the adjuvant drug of BYHWD), and inosine from Pheretima (the adjuvant drug of BYHWD).

The detailed standards for selecting them were as follows: (1) they could be absorbed from intestine or detected in blood and/or cerebrospinal fluid after oral administration; (2) they were well-known representative bioactive constituents of each crude drug; and (3) they were commercially available or could be prepared easily.

## Materials and Methods

### Reagents and chemicals

Concanavalin A (Con A, Lot No. C2272), lipopolysaccharide (LPS, Lot No. 011M4008V), glutamate and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Lot No. Ameresco 0793) were purchased from Sigma (St. Louis, MO, USA). Foetal bovine serum (FBS, Lot No. 16000044) and horse serum (HS, Lot No. 16050130) were purchased from Gibco (Grand Island, NY, USA). Dulbecco's

Modified Eagle Medium (DMEM, Lot No. CM10013), RPMI 1640 medium, F-12K medium (Lot No. B3004120), Hanks solution (Lot No. A1909110), red blood cell lysis buffer (Lot No: B1009120), phosphate buffer solution (PBS, Lot No. C1401130), and dimethyl sulfoxide (DMSO, Lot No. 9H009628) were purchased from M&C Gene Technology Ltd. (Beijing, China). The nitric oxide (NO) assay kit (Lot No. E1030) was purchased from Applygen Technologies Inc. (Beijing, China).

#### Animals

The experiments were performed on male BALB/c (weighing 18–22 g) mice and Sprague-Dawley rats (weighing 250–300 g) that were provided by the Department of Laboratory Animal Science of Peking University Health Science Center. The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Peking University (ethics approval number: LA2014189, approval date: 2/27/2014), and all animal procedures were performed according to IACUC policies. All efforts were made to minimize animal suffering and to reduce the number of animals used. All the rats were housed under standard animal housing conditions (i.e., a 12 h/12 h light/dark cycle at  $23 \pm 1$  °C and relative humidity of  $55\% \pm 5\%$ ) with free access to commercial rat diet and tap water.

#### Compound preparation

The 15 chemical components of BYHWD were provided and identified by the authors at State Key Laboratory of Natural and Biomimetic Drugs, Peking University. They included astragaloside IV (Sanleng biotech Co., Ltd., Guilin, China, Lot No. 20090914); ferulic acid, propyl gallate, and inosine (Sinopharm Chemical Reagent Co., Ltd., Lot Nos. 20100303, 20090707, 20080516); ligustrazine (Tengzhou Runlong Perfume Co., Ltd., Shandong, China, Lot No.: 20090225); hydroxysafflor yellow A (Lvye natural medicine research and Development Co., Ltd., Shandong, China, Lot No. 20080116), calycosin, calycosin-7-*O*- $\beta$ -D-glucoside, formononetin, ononin, (3*R*)-7, 2'-dihydroxy-3', 4'-dimethoxyisoflavan-7-*O*- $\beta$ -D-glucoside (astraisoflavan-7-*O*- $\beta$ -D-glucoside), (6*aR*, 11*aR*)-3-hydroxy-9, 10-dimethoxypterocarpan-3-*O*- $\beta$ -D-glucoside (astrapterocarpan-3-*O*- $\beta$ -D-glucoside) (isolated from *Astragali Radix*, the dried roots of *Astragalus membranaceus* var. *mongholicus* (Bunge) P.K.Hsiao)<sup>[24]</sup>, paeoniflorin (isolated from *Paeoniae Radix Rubra*, the dried roots of *Paeonia lactiflora* Pall.), paeonol (isolated from Moutan Cortex, the dried root barks of *Paeonia*  $\times$  *suffruticosa* Andrews), and amygdalin (isolated from *Persicae Semen*, the dried seeds of *Prunus persica* (L.) Batsch). The reference compounds prepared by the authors were identified by modern spectral methods, such as NMR and MS. The purities of all 15 compounds were tested with HPLC-DAD-ELSD, and all the purities exceeded 98%. All water-soluble compounds (i.e., astragaloside IV, propyl gallate, inosine, hydroxysafflor yellow A, paeoniflorin, ononin, and amygdalin) were dissolved in PBS, and all lipid-soluble compounds (i.e., ferulic

acid, ligustrazine, calycosin, astrapterocarpan-3-*O*- $\beta$ -D-glucoside, calycosin-7-*O*- $\beta$ -D-glucoside, formononetin, astraisoflavan-7-*O*- $\beta$ -D-glucoside, and paeonol) were dissolved in DMSO.

#### T cell proliferation assay

The spleens of normal BALB/c mice were isolated and minced in a glass homogenizer with PBS solution under aseptic conditions. Next, the suspension was passed through a fine steel mesh, and the erythrocytes were depleted with red blood cell lysis buffer. The lymphocytes were washed twice with PBS. The final splenic lymphocyte suspension was resuspended in 5 mL of RPMI1640 medium (containing 10% FBS), and cell numbers were determined with a haemocytometer via the trypan blue dye exclusion technique. Cell viability exceeded 95%. 100  $\mu$ L of splenic lymphocyte suspensions ( $2 \times 10^6$  cells/mL) were then seeded into 96-well flat-bottom microtiter plates and cultured with 100  $\mu$ L of RPMI 1640 medium containing Con A ( $2.5 \mu\text{g}\cdot\text{mL}^{-1}$ ) and the 15 compounds in BYHWD ( $30 \mu\text{mol}\cdot\text{L}^{-1}$ ). In the pre-experiment, we tested the compounds with the concentrations of 10, 30, 50, and 100  $\mu\text{mol}\cdot\text{L}^{-1}$  in suppressing T lymphocyte proliferation, and found that the best effect of some compounds appeared at the concentration of 30  $\mu\text{mol}\cdot\text{L}^{-1}$ , so we chose 30  $\mu\text{mol}\cdot\text{L}^{-1}$  as the test concentration to evaluate the effect of each compound of BYHWD. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> for 48 h.

#### MTT assay

20  $\mu$ L of MTT solution ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) was added, and the plates were incubated for 4 h at 37 °C prior to the end of culture. The plates were centrifuged ( $1\ 400 \times g$ , 6 min), and the untransformed MTT was carefully removed by pipetting. Finally, 100  $\mu$ L of DMSO was added to each well to fully dissolve the colored materials. The absorbance at 570 nm was measured with a microplate reader (Model iMark-680, Bio-RAD Instruments, Hercules, California, USA). The inhibition rate of cell proliferation was calculated by the following formula: inhibition (%) =  $(OD_{\text{ConA}} - OD_{\text{compound}})/OD_{\text{ConA}} \times 100$ . Each experiment was performed in triplicate.

#### LPS-induced cytotoxicity and NO production

Murine RAW264.7 monocyte macrophages were obtained from the American Type Culture Collection (Manassas, VA, USA), and cultured in DMEM supplemented with 10% foetal bovine serum, 100 U·mL<sup>-1</sup> penicillin, and 100  $\mu\text{g}\cdot\text{mL}^{-1}$  streptomycin at 37 °C in a humidified incubator containing 5% CO<sub>2</sub>. The RAW264.7 cells were seeded into 96-well plates at a density of  $6 \times 10^5$  cells/mL and were treated with the 15 compounds found in BYHWD ( $30 \mu\text{mol}\cdot\text{L}^{-1}$ ) or culture medium (containing equivalent amounts of PBS or DMSO). The pre-experiment (with the concentration of 10, 30, 50, and 100  $\mu\text{mol}\cdot\text{L}^{-1}$ ) showed that the best effect of some compounds appeared at 30  $\mu\text{mol}\cdot\text{L}^{-1}$ . The cells in the LPS group and the compounds group were stimulated with 1  $\mu\text{g}\cdot\text{mL}^{-1}$  of LPS after 12 h of incubation, and the incubation was then continued for another 24 h. Nitric oxide levels in the culture media

were measured with a NO assay kit according to the manufacturer's instructions. Cell viabilities were assessed using the MTT assay as described above. Cell viabilities (%) were calculated by the following formula: Cell viabilities (%) =  $OD_{LPS}$  or  $OD_{compound}/OD_{control} \times 100$ .

**Glutamate-induced cytotoxicity assay**

PC12 cells were obtained from the Shanghai Cell Culture Center (Shanghai, China) and maintained in F-12K with L-glutamine medium containing 5% FBS, 15% HS, 100 U·mL<sup>-1</sup> of penicillin and 100 mg·mL<sup>-1</sup> of streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. The cell pellets were resuspended in the culture medium and seeded onto 96-well culture plates at  $1 \times 10^5$  cells/mL per well. At 20 h after seeding, the cells were cultured with the 15 compounds in BYHWD (30 μmol·L<sup>-1</sup>) or culture medium (containing equivalent PBS or DMSO) for 4 h prior to exposure to glutamate (12 mmol·L<sup>-1</sup>). The pre-experiment (with the concentration of 10, 30, 50 and 100 μmol·L<sup>-1</sup>) indicated that the best effect of some compounds appeared at 30 μmol·L<sup>-1</sup>. After incubation for 24 h, cell viabilities were determined by MTT assay as described above. Cell viabilities (%) were calculated by the following formula: Cell viabilities (%) =  $OD_{glu}$  or  $OD_{compound}/OD_{control} \times 100$ .

**Assay of KCl-induced contraction of isolated thoracic aortic rings**

The rats were sacrificed by cervical dislocation, and the thoracic aortas were rapidly removed and dissected in ice-cold Krebs solution (pH 7.4, containing [in mmol·L<sup>-1</sup>] 118.0 NaCl, 5.4 KCl, 41.0 NaH<sub>2</sub>PO<sub>3</sub>, 1.2 MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.9 CaCl<sub>2</sub>, 25.0 NaHCO<sub>3</sub>, and 11.1 glucose) and cut into 3 mm-wide ring segments. Each ring was suspended with two L-shaped steel wires in a 15-mL organ bath filled with Krebs solution and maintained at 37 °C. The upper wire was connected to a force displacement transducer (Grass Instruments, West Warwick, RI, USA), and the lower wire was fixed to the bottom of the organ bath. The bath solution was continuously bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The mounted aortic rings were subse-

quently allowed to equilibrate for 60 min under a baseline load of 1.0 g. The bath solution was replaced every 15 min with pre-warmed and oxygenated Krebs solution. After equilibration, all aortic rings were exposed to KCl (60 mmol·L<sup>-1</sup>), and maximal contraction responses were obtained. Afterward, each solution of 15 compounds in BYHWD (final concentration:  $2.5 \times 10^{-4}$ – $10 \times 10^{-4}$  mol·L<sup>-1</sup>) or the vehicle was added to the bath solution. According to the literature and pre-experiment results, we chose  $2.5 \times 10^{-4}$ – $10 \times 10^{-4}$  mol·L<sup>-1</sup> as the test concentration of the compounds [25–27]. Changes in the tensions of the aortic rings were recorded, and the relaxation ratio was defined as the maximum compound-evoked relaxation relative to the maximum KCl-evoked contraction.

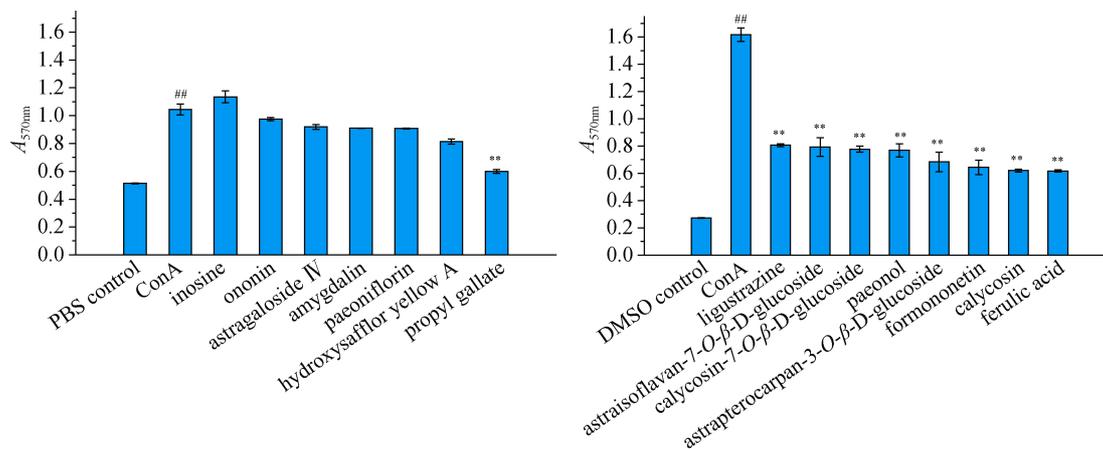
**Statistical analysis**

All data are expressed as the means ± the standard deviations. Data were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) and *P* < 0.05 was used as the threshold for the significance of all statistical tests. In the test of KCl-induced contraction of aortic rings, the LSD post hoc test (post comparison method) was used to calculate the difference between the control group and each concentration group of the compound found in BYHWD.

**Results**

**Effects of 15 compounds found in BYHWD on Con A-induced T lymphocyte proliferation**

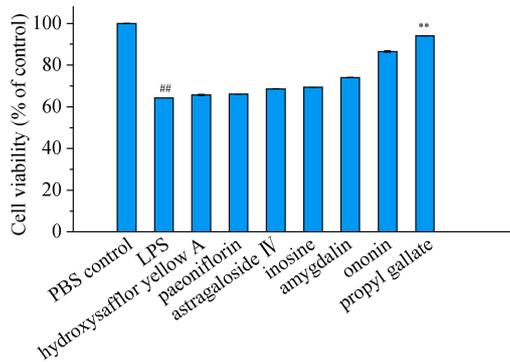
As shown in Fig. 1, the T-cell mitogen Con A induced obvious lymphocyte proliferation compared to the control treatment (*P* < 0.01). Nine tested compounds, including ferulic acid, calycosin, formononetin, astrapterocarpan-3-*O*-β-D-glucoside, paeonol, calycosin-7-*O*-β-D-glucoside, astraisoflavan-7-*O*-β-D-glucoside, ligustrazine, and propyl gallate, significantly decreased the proliferation of lymphocytes (*P* < 0.01, *P* < 0.05), and the inhibition rates were 62%, 62%, 60%, 58%, 52%, 52%, 51%, 50%, and 43%, respectively.



**Fig. 1** Effects of 15 compounds found in BYHWD on T lymphocyte proliferation *in vitro*. Splenocytes were treated with different BYHWD compounds (30 μmol·L<sup>-1</sup>) and Con A (2.5 μg·mL<sup>-1</sup>) for 48 h. After incubation, T lymphocyte proliferation was measured via MTT assay. ##*P* < 0.01 vs control group, \**P* < 0.05 and \*\**P* < 0.01 vs the Con A-treated group. The data are presented as means ± SD of three independent experiments

*Effects of 15 compounds found in BYHWD on LPS-induced cytotoxicity in RAW264.7 cells*

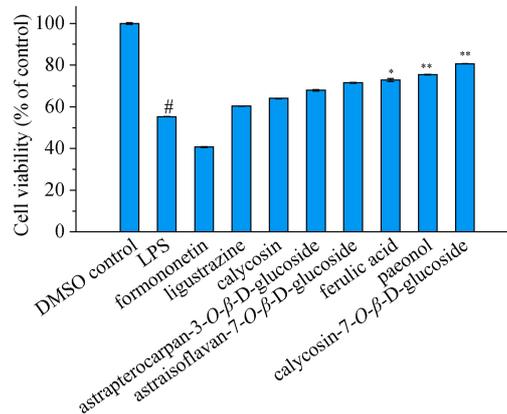
As shown in Fig. 2, treatment with LPS led to cytotoxicity in RAW264.7 cells which resulted in cell survival rates of 55%–64% ( $P < 0.01$ ). Four tested compounds, including propyl gallate, calycosin-7-*O*- $\beta$ -D-glucoside, paeonol, and ferulic acid, clearly suppressed cytotoxicity ( $P < 0.01$ ,  $P < 0.05$ ) and elevated cell survival rates to 94%, 81%, 75%, and 73%, re-



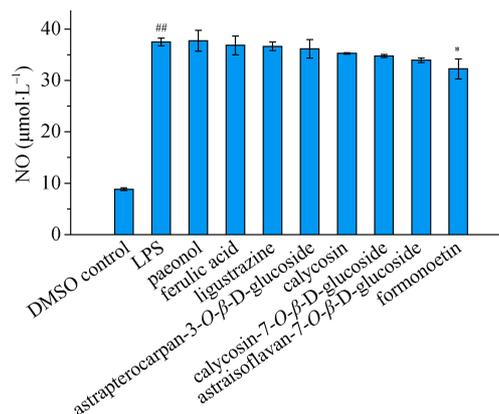
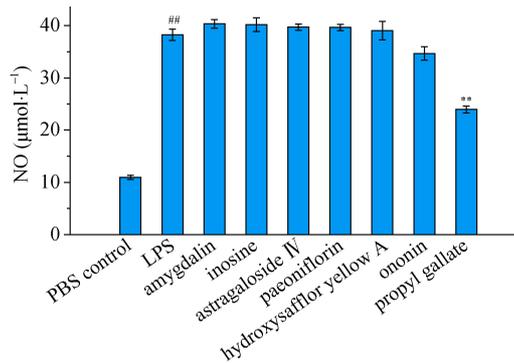
spectively.

*Effects of 15 compounds found in BYHWD on LPS-induced NO production in RAW264.7 cells*

As shown in Fig. 3, treatment with LPS increased NO production to  $38 \mu\text{mol}\cdot\text{L}^{-1}$  ( $P < 0.01$ ). Two tested compounds, propyl gallate and formononetin, inhibited the NO production ( $P < 0.01$ ,  $P < 0.05$ ); the inhibition rates were 37% and 13%, respectively.



**Fig. 2** Effects of 15 compounds found in BYHWD on LPS-induced cytotoxicity in RAW264.7 cells. Cells were pre-treated with BYHWD compounds ( $30 \mu\text{mol}\cdot\text{L}^{-1}$ ) for 12 h and subsequently treated with LPS ( $1 \mu\text{g}\cdot\text{mL}^{-1}$ ) for 24 h. Cell viability was determined via MTT assay. # $P < 0.05$  and ## $P < 0.01$  vs the control group, \* $P < 0.05$  and \*\* $P < 0.01$  vs the LPS-treated group. The data are presented as means  $\pm$  SD of three independent experiments



**Fig. 3** Effect of 15 compounds found in BYHWD on the LPS-induced NO production in RAW264.7 cells. Cells were pre-treated with BYHWD compounds ( $30 \mu\text{mol}\cdot\text{L}^{-1}$ ) for 12 h and subsequently treated with LPS ( $1 \mu\text{g}\cdot\text{mL}^{-1}$ ) for 24 h. The levels of secreted NO in the culture supernatants were determined by Griess assay. # $P < 0.05$  and ## $P < 0.01$  vs the control group, \* $P < 0.05$  and \*\* $P < 0.01$  vs the LPS-treated group. The data are presented as means  $\pm$  SD of three independent experiments

*Effects of 15 compounds found in BYHWD on glutamate-induced cytotoxicity in PC12 cells*

As shown in Fig. 4, the treatment of PC12 cells with glutamate induced cytotoxicity and reduced viability from 100% to 37% or 46% ( $P < 0.01$ ). When the cells were pre-treated with hydroxysafflor yellow A and inosine, cell viability significantly increased to 181% and 160% compared to the glutamate group, respectively ( $P < 0.01$ ).

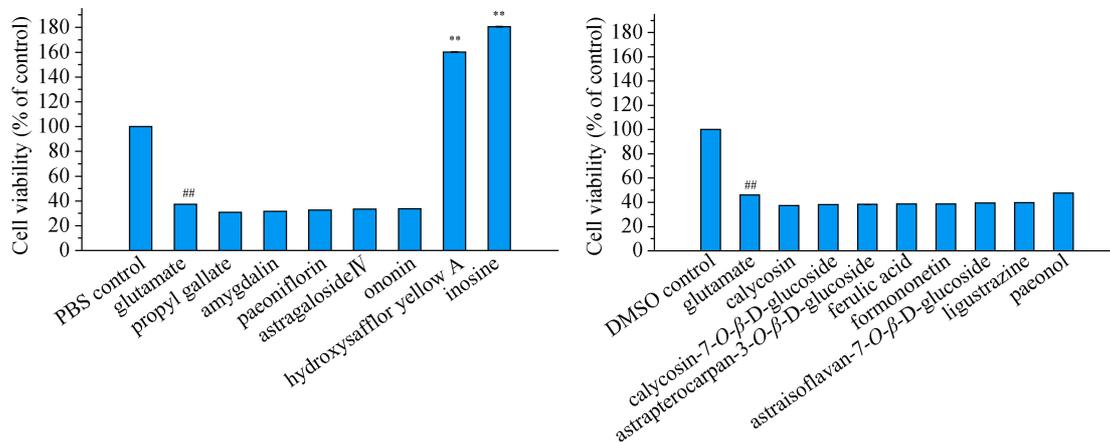
*Effects of 15 compounds found in BYHWD on KCl-induced contraction of aortic rings*

KCl-induced aortic contraction data are presented in Fig. 5.

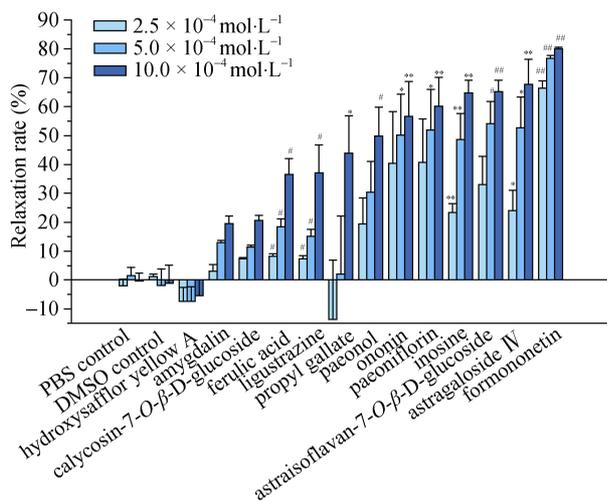
Ten tested compounds, i.e. formononetin, astragaloside IV, astraisoflavan-7-*O*- $\beta$ -D-glucoside, inosine, paeoniflorin, ononin, paeonol, propyl gallate, ligustrazine, and ferulic acid, produced marked relaxation responses in aortic rings that had been contracted by treatment with KCl ( $P < 0.01$ ,  $P < 0.05$ ); the maximal relaxation rates were 80%, 68%, 65%, 65%, 60%, 57%, 50%, 44%, 37%, and 37%, respectively.

**Discussion**

Ischemic stroke is one of the leading causes of death and disability worldwide. Many risk factors contribute to the



**Fig. 4** Effects of 15 compounds found in BYHWD on glutamate-induced cytotoxicity in PC12 cells. Cells were pre-treated with BYHWD compounds ( $30 \mu\text{mol}\cdot\text{L}^{-1}$ ) for 4 h and subsequently treated with glutamate ( $12 \text{ mmol}\cdot\text{L}^{-1}$ ) for 20 h. Cell viability was determined by MTT assay. <sup>##</sup> $P < 0.01$  vs the control group, <sup>\*\*</sup> $P < 0.01$  vs the glutamate-treated group. The data are presented as means  $\pm$  SD of three independent experiments



**Fig. 5** Effects of 13 compounds found in BYHWD on KCl-induced constriction of rat thoracic aortas. The rat thoracic aortas were precontracted with KCl ( $60 \text{ mmol}\cdot\text{L}^{-1}$ ) to achieve maximum tension and different BYHWD compounds (final concentrations:  $2.5 \times 10^{-4}$ – $10 \times 10^{-4} \text{ mol}\cdot\text{L}^{-1}$ ) or the vehicle were subsequently cumulatively added to bath solution. Changes in the tensions of the aortic rings were recorded, and the relaxation ratios were calculated. <sup>#</sup> $P < 0.05$  and <sup>##</sup> $P < 0.01$  vs DMSO control group, <sup>\*</sup> $P < 0.05$  and <sup>\*\*</sup> $P < 0.01$  vs PBS control group. The data are presented as means  $\pm$  SD of three independent experiments

pathological development of ischemic stroke such as immune dysregulation, inflammation, neurological damage and vascular dysregulation [28]. Recently, an increasing number of agents have been studied in the context of the treatment of ischemic stroke. However, single-compound or single-target agents, such as neuroprotective agents, have shown limit effects on ischemic stroke [29]. Therefore, a promising treatment approach for ischemic stroke is identification and utilization

of multiple-component agents with multiple targets.

Buyang Huanwu Decoction (BYHWD) is a well-known, canonical and traditional Chinese medicine formula that has been widely used for the treatment of ischemic stroke and stroke-induced disability for about two hundred years. Traditional Chinese medicine suggests that BYHWD can promote blood circulation and dredge collaterals by invigorating the energy (Qi) of the body. Recent studies have shown that BYHWD exerts diverse effects that include protecting neurons from ischemic injury [8-9], reducing infarction volumes, and stimulating neural proliferation [10]. However, the effective constituents of BYHWD are unknown. The main chemical constituents of BYHWD include, but are not limited to, the following: astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, ononin, formononetin, astrapterocarpan-3-O-β-D-glucoside, and astraisoflavan-7-O-β-D-glucoside from Astragali Radix; ferulic acid and ligustilide from Angelicae Sinensis Radix; paeoniflorin, propyl gallate, paeonol and catechinic acid from Paeoniae Radix Rubra; ligustrazine and ligustilide from Chuanxiong Rhizoma; amygdalin and fatty acids from Persicae Semen; hydroxysafflor yellow A and safflomin-A from Carthami Flos; and inosine and hypoxanthine from Pheretima. In the present study, we investigated the anti-inflammatory, immune, neuroprotective and vasodilatory effects of 15 major, absorbable compounds of BYHWD with the same methods under the same conditions for the first time to identify the effective constituents of BYHWD and to explore the mechanisms by which BYHWD ameliorates the detrimental effects of ischemic stroke.

Extensive evidence indicates that activation of the immune system may increase the risk of stroke [30]. Mice engineered to lack selected T-cell subgroups are protected from ischemic damage [31]. Numerous immunosuppressive agents have shown promise in the treatment of ischemic stroke by providing neuroprotection during excitotoxic insults, and these agents may improve neurological functions [32-36]. In the

present study, we found that 9 compounds found in BYHWD, including ferulic acid, calycosin, formononetin, astrapterocarpan-3-*O*- $\beta$ -D-glucoside, paeonol, calycosin-7-*O*- $\beta$ -D-glucoside, astraisoflavan-7-*O*- $\beta$ -D-glucoside, ligustrazine, and propyl gallate, suppressed Con A-induced T cell proliferation. Furthermore, the effects of calycosin, astrapterocarpan-3-*O*- $\beta$ -D-glucoside, calycosin-7-*O*- $\beta$ -D-glucoside and astraisoflavan-7-*O*- $\beta$ -D-glucoside are reported here for the first time. Our results from the present study implied that the anti-ischemic stroke effects of 9 BYHWD compounds were mediated by the inhibition of T cell proliferation.

Inflammation is a key contributor to the pathophysiology of ischemic stroke [37]. LPS can directly stimulate inflammatory responses in macrophages by triggering the production of inflammatory mediators, such as NO, which can cause cytotoxicity [38]. In the present study, the effects of the compounds found in BYHWD on LPS-induced cytotoxicity and NO production were determined in the murine macrophage-like RAW264.7 cell line. Among the compounds studied, we found that propyl gallate, calycosin-7-*O*- $\beta$ -D-glucoside, paeonol, and ferulic acid inhibited the cytotoxicity induced by LPS; this effect of calycosin-7-*O*- $\beta$ -D-glucoside was reported for the first time. Furthermore, propyl gallate and formononetin reduced NO production. These results indicated that these compounds may attenuate the development of ischemic stroke by suppressing inflammation.

Stroke lesions are characterized by serious damages and devastating neuronal losses. Glutamate, the major excitatory neurotransmitter in the central nervous system, has been demonstrated to be capable of inducing excitotoxic neural injury during the ischemic cascade that results in cell death [39-41]. In the present study, we found that glutamate caused a significant decrease in the viability of PC12 cells; whereas pretreatment with hydroxysafflor yellow A or inosine significantly inhibited glutamate-induced cytotoxicity in PC12 cells.

Ischemic stroke occurs when one of the arteries in the brain becomes narrowed or is blocked by a blood clot. We measured the vasodilatory effects of the BYHWD compounds on KCl-induced thoracic aortic contraction. The results showed that KCl significantly increased vessel tension, and the contractile response was attenuated by formononetin, astragaloside IV, astraisoflavan-7-*O*- $\beta$ -D-glucoside, inosine, paeoniflorin, ononin, paeonol, propylgallate, ligustrazine, and ferulic acid. The effects of astraisoflavan-7-*O*- $\beta$ -D-glucoside and ononin were reported for the first time. Additionally, we found that amygdalin isolated from *Persicae Semen* did not have significant effects in these studies

Taken together, these results suggested that the components of BYHWD likely exerted anti-ischemic stroke effects via multiple targets and affect different pathological processes of stroke. The BYHWD components that affected the same targets were summarized as follows:

I. Con A-induced T lymphocyte proliferation: Nine components found in BYHWD, including ferulic acid, caly-

cosin, formononetin, astrapterocarpan-3-*O*- $\beta$ -D-glucoside, paeonol, calycosin-7-*O*- $\beta$ -D-glucoside, astraisoflavan-7-*O*- $\beta$ -D-glucoside, ligustrazine, and propyl gallate, suppressed T lymphocyte proliferation at the concentration of 30  $\mu\text{mol}\cdot\text{L}^{-1}$ .

II. LPS-induced cytotoxicity in RAW264.7 cells: Four components found in BYHWD, including propylgallate, calycosin-7-*O*- $\beta$ -D-glucoside, paeonol, and ferulic acid, inhibited cytotoxicity at the concentration of 30  $\mu\text{mol}\cdot\text{L}^{-1}$ .

III. LPS-induced NO release in RAW264.7 cells: Two components found in BYHWD, including propyl gallate and formononetin, inhibited NO release at the concentration of 30  $\mu\text{mol}\cdot\text{L}^{-1}$ .

IV. Glutamate-induced cytotoxicity in PC12 cells: Two components found in BYHWD, including hydroxysafflor yellow A and inosine, prevented cell cytotoxicity at the concentration of 30  $\mu\text{mol}\cdot\text{L}^{-1}$ .

V. KCl-induced thoracic aortic constriction: Ten components found in BYHWD, including formononetin, astragaloside IV, astraisoflavan-7-*O*- $\beta$ -D-glucoside, inosine, paeoniflorin, ononin, paeonol, propyl gallate, ligustrazine and ferulic acid, inhibited thoracic aortic constriction at the concentrations of  $2.5 \times 10^{-4}$ ,  $5.0 \times 10^{-4}$  or  $10 \times 10^{-4} \text{mol}\cdot\text{L}^{-1}$ .

Based on the above results, we confirmed that several components found in BYHWD (as many as 10 compounds) could affect a same target. And we also found that one component of BYHWD affected multiple targets simultaneously. For instance, propyl gallate acted on four targets (I, II, III, V); ferulic acid, formononetin, and paeonol each affected three targets (I, II, V; I, III, V; I, II, V, respectively); and the other components acted on two targets each (calycosin: I and II; astraisoflavan-7-*O*- $\beta$ -D-glucoside: I and V; ligustrazine: I and V; and inosine: IV and V).

## Conclusion

In conclusion, multiple components (14 components) of Buyang Huanwu Decoction (BYHWD) might ameliorate the detrimental effects of ischemic stroke via modulations of multiple targets (at least five targets). This was the first study to report that effective BYHWD components, including calycosin, astrapterocarpan-3-*O*- $\beta$ -D-glucoside, calycosin-7-*O*- $\beta$ -D-glucoside, and astraisoflavan-7-*O*- $\beta$ -D-glucoside, suppressed the T lymphocyte proliferation induced by Con A. This study was also the first to report that calycosin-7-*O*- $\beta$ -D-glucoside markedly inhibited the apoptosis of RAW264.7 cells and that astraisoflavan-7-*O*- $\beta$ -D-glucoside and ononin suppressed the constriction of the thoracic aorta induced by KCl in rats. Whether a drug consisting of the same 14 effective compounds found in this study would exhibit the same pharmacological effects to those of BYHWD is an interesting question that deserves future study. The findings of the present study provided a potential pharmacological basis for the actions of BYHWD in the treatment of ischemic stroke and new pathways for the development of anti-ischemic

stroke drugs.

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## References

- [1] Lopez AD, Mathers CD, Ezzati M, et al. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data [J]. *Lancet*, 2006, **367**(9524): 1747-1757.
- [2] Tsuchiya M, Sako K, Yura S, et al. Cerebral blood flow and histopathological changes following permanent bilateral carotid artery ligation in Wistar rats [J]. *Exp Brain Res*, 1992, **89**(1): 87-92.
- [3] Deb P, Sharma S, Hassan KM. Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis [J]. *Pathophysiology*, 2010, **17**(3): 197.
- [4] Jauch EC, Saver JL, Adams HP Jr, et al. Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association [J]. *Stroke*, 2013, **44**(3): 870.
- [5] Hao CZ, Wu F, Shen J, et al. Clinical efficacy and safety of buyang huanwu decoction for acute ischemic stroke: a systematic review and meta-analysis of 19 randomized controlled trials [J]. *Evid Based Complement Alternat Med*, 2012, **2012**: 630124.
- [6] Li JH, Liu AJ, Li HQ, et al. Buyang huanwu decoction for healthcare: evidence-based theoretical interpretations of treating different diseases with the same method and target of vascularity [J]. *Evid Based Complement Alternat Med*, 2014, **2014**: 506783.
- [7] Wei RL, Teng HJ, Yin B, et al. A systematic review and meta-analysis of buyang huanwu decoction in animal model of focal cerebral ischemia [J]. *Evid Based Complement Alternat Med*, 2013, **2013**: 138484.
- [8] Li XM, Bai XC, Qin LN, et al. Neuroprotective effects of Buyang Huanwu Decoction on neuronal injury in hippocampus after transient forebrain ischemia in rats [J]. *Neuroscience letters*, 2003, **346**(1-2): 29-32.
- [9] Wang HW, Liou KT, Wang YH, et al. Deciphering the neuroprotective mechanisms of Bu-yang Huan-wu decoction by an integrative neurofunctional and genomic approach in ischemic stroke mice [J]. *J Ethnopharmacol*, 2011, **138**(1): 22-33.
- [10] Cai G, Liu B, Liu W, et al. Buyang Huanwu Decoction can improve recovery of neurological function, reduce infarction volume, stimulate neural proliferation and modulate VEGF and Flk1 expressions in transient focal cerebral ischaemic rat brains [J]. *J Ethnopharmacol*, 2007, **113**(2): 292-299.
- [11] Zhang YK, Han XY, Che ZY. Effects of buyang huanwu tang combined with bone marrow mesenchymal stem cell transplantation on the expression of VEGF and Ki-67 in the brain tissue of the cerebral ischemia-reperfusion model rat [J]. *J Tradit Chin Med*, 2010, **30**(4): 278-282.
- [12] Xu F. *Study on the effective substances of Buyang Huanwu Decoction-Absorption of seven crude drugs* [D]. Beijing: Peking University, 2005.
- [13] Li CY, Qi LW, Li P. Correlative analysis of metabolite profiling of Danggui Buxue Tang in rat biological fluids by rapid resolution LC-TOF/MS [J]. *J Pharm Biomed Anal*, 2011, **55**(1): 146-160.
- [14] Liang J, Xu F, Shang MY, et al. Metabolite profiling of propyl gallate in rat plasma and urine by HPLC-DAD-ESI-IT-TOF-MS(n) technique [J]. *China J Chin Mater Med*, 2013, **38**(22): 3970-3976.
- [15] Liang J, Xu F, Zhang YZ, et al. The profiling and identification of the absorbed constituents and metabolites of Paeoniae Radix Rubra decoction in rat plasma and urine by the HPLC-DAD-ESI-IT-TOF-MS(n) technique: a novel strategy for the systematic screening and identification of absorbed constituents and metabolites from traditional Chinese medicines [J]. *J Pharm Biomed Anal*, 2013, **83**: 108-121.
- [16] Liang J, Xu F, Zhang YZ, et al. The profiling and identification of the metabolites of (+)-catechin and study on their distribution in rats by HPLC-DAD-ESI-IT-TOF-MS(n) technique [J]. *Biomed Chromatogr*, 2014, **28**(3): 401-411.
- [17] Shaw LH, Lin LC, Tsai TH. HPLC-MS/MS analysis of a traditional Chinese medical formulation of Bu-Yang-Huan-Wu-Tang and its pharmacokinetics after oral administration to rats [J]. *PLoS One*, 2012, **7**(8): e43848.
- [18] Wen XD, Liu EH, Yang J, et al. Identification of metabolites of Buyang Huanwu decoction in rat urine using liquid chromatography-quadrupole time-of-flight mass spectrometry [J]. *J Pharm Biomed Anal*, 2012, **67-68**: 114-122.
- [19] Xu F, Zhang Y, Xiao S, et al. Absorption and metabolism of Astragali radix decoction: in silico, in vitro, and a case study in vivo [J]. *Drug Metab Dispos*, 2006, **34**(6): 913-924.
- [20] Yang D, Cai S, Liu H, et al. On-line identification of the constituents of Buyang Huanwu decoction in pig serum using combined HPLC-DAD-MS techniques [J]. *J Chromatogr B*, 2006, **831**(1-2): 288-302.
- [21] Yang DH, Ren XL, Xu F, et al. Absorptive constituents and their metabolites in drug-containing urine samples from Wuzhishan miniature pigs orally administered with Buyang Huanwu decoction [J]. *J Nat Med*, 2014, **68**(1): 11-21.
- [22] Zhang YZ, Xu F, Yi T, et al. Chemical profile analysis and comparison of two versions of the classic TCM formula Danggui Buxue Tang by HPLC-DAD-ESI-IT-TOF-MS<sup>n</sup> [J]. *Molecules*, 2014, **19**(5): 5650-5673.
- [23] He FY, Liu WL, He QP, et al. Reverse phase-high performance liquid chromatography to determine plasma levels of buyang huanwu decoction and its ligustrazine and a pharmacokinetic study [J]. *Chin Rem Clin*, 2005, **5**(12): 920-922.
- [24] Zhang YZ, Xu F, Liang J, et al. Isoflavonoids from roots of Astragalus membranaceus var. mongholicus [J]. *China J Chin Mater Med*, 2012, **37**(21): 3243-3248.
- [25] Chen GP, Ye Y, Li L, et al. Endothelium-independent vasorelaxant effect of sodium ferulate on rat thoracic aorta [J]. *Life Sci*, 2009, **84**(3-4): 81-88.
- [26] Kong X, Tian H, Fan H. Effects of ligustrazine on myocardium and coronary artery [J]. *China J Chin Mater Med*, 1998, **23**(8): 491-493, 512-inside back cover.

- [27] Zhang JY, Cao YX, Weng WL, *et al.* Paeonol induces vasodilatation in rat mesenteric artery *via* inhibiting extracellular Ca(2)(+) influx and intracellular Ca(2)(+) release [J]. *Chin J Integr Med*, 2013, **19**(7): 510-516.
- [28] Pandya RS, Mao L, Zhou H, *et al.* Central nervous system agents for ischemic stroke: neuroprotection mechanisms [J]. *Cent Nerv Syst Agents Med Chem*, 2011, **11**(2): 81-97.
- [29] MENG WT, LI DX, TONG L. Progress in research of the treatment of ischemic stroke [J]. *Chin J New Drugs*, 2016, **25**(10): 1114-1120.
- [30] Kaptoge S, Di Angelantonio E, Lowe G, *et al.* C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis [J]. *Lancet*, 2010, **375**(9709): 132-140.
- [31] Yilmaz G, Arumugam TV, Stokes KY, *et al.* Role of T lymphocytes and interferon-gamma in ischemic stroke [J]. *Circulation*, 2006, **113**(17): 2105-2112.
- [32] Capano M, Virji S, Crompton M. Cyclophilin-A is involved in excitotoxin-induced caspase activation in rat neuronal B50 cells [J]. *Biochem J*, 2002, **363**(Pt 1): 29-36.
- [33] Chen J, Adachi N, Tsubota S, *et al.* Dexamethasone augments ischemia-induced extracellular accumulation of glutamate in gerbil hippocampus [J]. *Eur J Pharmacol*, 1998, **347**(1): 67-70.
- [34] Sharkey J, Butcher SP. Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischaemia [J]. *Nature*, 1994, **371**(6495): 336-339.
- [35] Yrjanheikki J, Keinanen R, Pellikka M, *et al.* Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia [J]. *Proc Natl Acad Sci USA*, 1998, **95**(26): 15769-15774.
- [36] Yrjanheikki J, Tikka T, Keinanen R, *et al.* A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window [J]. *Proc Natl Acad Sci USA*, 1999, **96**(23): 13496-13500.
- [37] Moskowitz MA, Lo EH, Iadecola C. The science of stroke: mechanisms in search of treatments [J]. *Neuron*, 2010, **67**(2): 181-198.
- [38] Guha M, Mackman N. LPS induction of gene expression in human monocytes [J]. *Cell Signal*, 2001, **13**(2): 85-94.
- [39] Arundine M, Tymianski M. Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury [J]. *Cell Mol Life Sci*, 2004, **61**(6): 657-668.
- [40] Choi DW, Rothman SM. The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death [J]. *Annu Rev Neurosci*, 1990, **13**: 171-182.
- [41] Rothman SM, Olney JW. Glutamate and the pathophysiology of hypoxic-ischemic brain damage [J]. *Ann Neurol*, 1986, **19**(2): 105-111.

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