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Common and unique mechanisms of Chinese herbal remedies on ischemic stroke

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Abstract

**Ethnopharmacological relevance:** Four traditional Chinese herbal remedies (CHR) including Buyang Huanwu decoction (BHD), Xuefu Zhuyu decoction (XZD), Tianma Gouteng decoction (TGD) and Shengyu decoction (SYD) are popular used in treating brain-related dysfunction clinically with different syndrome/pattern based on traditional Chinese medicine (TCM) principles, yet their neuroprotective mechanisms are still unclear. **Materials and methods:** Mice were subjected to an acute ischemic stroke to examine the efficacy and molecular mechanisms of action underlying these CHR. **Results:** CHR treatment significantly enhanced the survival rate of stroke mice, with BHD being the most effective CHR. All CHR were superior to recombinant tissue-type plasminogen activator (rt-PA) treatment in successfully ameliorating brain function, infarction, and neurological deficits in stroke mice that also paralleled to improvements in blood-brain barrier damage, inflammation, apoptosis, and neurogenesis. Transcriptome analyses reveals that a total of 774 ischemia-induced probe sets were significantly modulated by four CHR, including 52 commonly upregulated genes and 54 commonly downregulated ones. Among them, activation of neurogenesis-associated signaling pathways and down-regulating inflammation and apoptosis pathways are key common mechanisms in ischemic stroke protection by all CHR. Besides, levels of plasma CX3CL1 and S100a9 in patients could be used as biomarkers for therapeutic evaluation.
before functional recovery could be observed. **Conclusion:** Our results suggest that using CHR, a combinatory cocktail therapy, is a better way than rt-PA for treating cerebral ischemic-associated diseases through modulating a common as well as a specific group of genes/pathways that may partially explain the syndrome differentiation and treatment principle in TCM.

**Keywords:** Acute ischemic stroke (AIS); Buyang Huanwu decoction (BHD); Tianma Gouteng decoction (TGD); Shengyu decoction (SYD); Xuefu Zhuyu decoction (XZD); functional modules and genetic networks; genome-wide transcriptome analysis; neurogenesis; micro positron emission tomography (µPET); syndrome differentiation.

1. **Introduction**

Acute ischemic stroke (AIS) is a major cause of morbidity and mortality, and the leading cause of long-term disability (Roger et al., 2011). The grievous effects of AIS are the consequence of compromised blood circulation into brain, leading to inadequate supply of oxygen and nutrients and reduced clearance of metabolic toxin. Without proper medical treatments, more than millions of neurons in the brain die quickly as a result of excitotoxicity-mediated brain injury by over activation of ionotropic N-methyl-D-aspartate receptors (NMDAR) due to excessive extracellular glutamate accumulation (White et al., 2000; Lo et al., 2003; Syntichaki and Tavernarakis, 2003). Activation of NMDAR mediated sodium and calcium channel opening leads to depolarization and activation of cascades leading to neuronal death through inducing overproduction of reactive oxygen species (ROS) by impairing mitochondria. ROS damages tissue by inducing necrotic or apoptotic cell death through denaturing protein and lipid by inducing protein nitrosylation and malondialdehyde formation of the cell membrane and organelles and breaking DNA (Hou et al., 2010; Grupke et al., 2015). This phenomenon is further accompanied with activation of proinflammatory cytokines
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produced by recruited leukocytes, active microglial cells, damaged neurons and astrocytes that mediates early blood-brain barrier (BBB) dysfunction following stroke (Lo et al., 2003; Jin et al., 2010).

The thrombolytic recombinant tissue plasminogen activator (rt-PA) is the only FDA-approved drug for ischemic stroke but is limited by its serious side effects and very narrow therapeutic time window, and is applied only to a limited group of patients with acute ischemic stroke (Lapchak, 2011). Recent report evaluates all neurovascular protectants subject to clinical trial evaluation for the treatment of AIS that includes 241 studies conducted between 1978 and 2014; they propose that development of agents that reduce brain injury after AIS will require new and different approaches based on a deeper understanding of the pathophysiology of AIS. It is suggested that the future treatment for ischemic stroke is likely to lie in combination therapy rather than monotherapy (Kikuchi et al., 2014). Therefore, finding drugs or strategies from traditional or alternative medicine would be a very useful and time-saving approach for AIS therapy.

In traditional Chinese medicine (TCM), TCM practitioner treats patients based on pattern identification of the overall physiological and/or pathological pattern of the human body in response to a given internal and external condition by the state of qi and blood, the pathological changes of viscera and bowels, as well as eight basic principles (yin-yang, external-internal, cold-heat, and deficiency-excess). The ultimate goal of TCM treatment is to restore the qi (energy) and yin-yang (balance) of this complex system (Cheung, 2011). Numerous Chinese herbal remedies (CHR) have clinically been used for improving stroke-induced neurological-related disability and neuropsychiatric sequelae after stroke for centuries (Taiwan Herbal Pharmacopeia, 2nd ed., 2013). Among these CHR, four typical CHR based on different pattern identification were studied including Buyang Huanwu Decoction (BHD) (for pattern of qi-deficiency and blood-stasis), Xuefu
Zhuyu decoction (XZD) (for pattern of qi-depression and blood-stasis), Tianma Gouteng decoction (TGD) (for pattern of ascendant hyperactivity of liver-yang) and Sheng Yu decoction (SYD) (for pattern of both qi-deficiency and blood-deficiency) (Cai et al., 2007; Lee et al., 2011; Ho et al., 2008; Chen et al., 2015). However, how could these four CHR improve neurological functions in ischemic stroke animals and the potential common and unique molecular mechanisms of action based on a genome-wide view remains unclear.

In the present study, we investigated the protective effects and underlying molecular mechanisms of action of these 4 CHR, and compared it with rt-PA on animal survival rate, neurological functions, infarction volume, biochemical and the genome-wide expression profiling in the transient focal cerebral ischemic mice brains. We try to address whether our results can provide partial explanation for the syndrome differentiation and the treatment principle in TCM by this model.

2. Materials and Methods

2.1. Preparation of Chinese Herbal Remedies

Chinese herbal remedies (CHR) including Buyang Huanwu decoction (BHD), Xuefu Zhuyu decoction (XZD), Tianma Gouteng decoction (TGD) and Shengyu decoction (SYD) were prepared in accordance with the official herbal pharmacopeia (Taiwan Herbal Pharmacopeia, 2nd ed., 2013) as in our previous report (Wang et al., 2011). BHD is composed of Astragalus membranaceus Bunge (Family Leguminosae), Angelica sinensis (Oliv.) Diels (Family Apiaceae), Paeonia lactiflora Pall. (Family Paeoniaceae), Ligusticum chuanxiong S. H. Qiu, Y. Q. Zeng, K. Y. Pan, Y. C. Tang & J. M. Xu (Family Apiaceae), Prunus persica (L.) Batsch (Family Rosaceae), Carthamus tinctorius L.
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**XZD** is composed of *Angelica sinensis* (Oliv.) Diels (Family Apiaceae), *Paeonia lactiflora* Pall. (Family Paeoniaceae), *Ligusticum chuanxiong* S. H. Qiu, Y. Q. Zeng, K. Y. Pan, Y. C. Tang & J. M. Xu (Family Apiaceae), *Prunus persica* (L.) Batsch (Family Rosaceae), *Carthamus tinctorius* L. (Family Asteraceae), *Rehmannia glutinosa* (Gaertn.) DC. (Family Plantaginaceae), *Citrus aurantium* L. (Family Rutaceae), *Achyranthes bidentata* Blume (Family Amaranthaceae), *Glycyrrhiza uralensis* Fisch. (Family Leguminosae), *Bupleurum marginatum* Wall. ex DC. (Family Apiaceae), and *Platycodon grandiflorus* (Jacq.) A.DC. (Family Campanulaceae);

**TGD** is composed of *Gastrodia elata* Blume (Family Orchidaceae), *Uncaria rhynchophylla* (Miq.) Miq. ex Havil. (Family Rubiaceae), *Achyranthes bidentata* Blume (Family Amaranthaceae), *Halotis diversicolor* Reeve (Family Haliotidae), *Eucommia ulmoides* Oliv (Family Eucommiaceae), *Scutellaria baicalensis* Georgi (Family Lamiaceae), *Leonurus sibiricus* L. (Family Lamiaceae), *Loranthus parasiticus* L. Merr. (Family Loranthaceae), *Gardenia jasminoides* J. Ellis (Family Rubiaceae), *Polygoni multiflori* Thunb. (Family Polygonaceae) and *Poria cocos* (Schw.) Wolff (Family Polyporaceae); **SYD** is composed of *Panax ginseng* C.A. Mey. (Family Araliaceae), *Astragalus membranaceus* Bunge (Family Leguminosae), *Angelica sinensis* (Oliv.) Diels (Family Apiaceae), *Paeonia lactiflora* Pall. (Family Paeoniaceae), *Angelica sinensis* (Oliv.) Diels (Family Apiaceae), *Paeonia lactiflora* Pall. (Family Paeoniaceae),
Ligusticum chuanxiong S. H. Qiu, Y. Q. Zeng, K. Y. Pan, Y. C. Tang & J. M. Xu (Family Apiaceae), and Rehmannia glutinosa (Gaertn.) DC. (Family Plantaginaceae). Briefly, these herbal remedies were mixed with the ratio according to TCM principles (Taiwan Herbal Pharmacopeia, 2nd ed., 2013), and were made through boiling with distilled water at 100 °C for 30 min twice and the drug solution was vacuum cool-dried and made into drug powder and dissolved with distilled water with the final concentration of 2.0 g/ml (equivalent to dry weight of raw materials). Their chemical fingerprints were determined (Fig. 1) by Dr. Lu CK and Prof Lin YL, two experts in nature products preparation and are also in charge of our chemical core lab (Wang et al., 2011). At least 4 active components in each CHR (Fig. 1) were identified and were compared to database, among them the chemical fingerprint of BHD was comparable as our previous report (Wang et al., 2011).

2.2. Animals and induction of acute ischemic stroke (AIS)

All animal procedures and protocols were performed in accordance with The Guide for the Care and Use of Laboratory Animals (NIH publication, 85-23, revised 1996) and were reviewed and approved by the Animal Research Committee at National Research Institute of Chinese Medicine. Acute ischemic stroke (AIS) in mice was setup by inducing cerebral ischemia/reperfusion (CI/R) injury as in our previous report (Wang et al., 2011). In brief, male ICR mice weighing 28–30 g (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan) were anesthetized with a mixture of isoflurane (1.5-2%), oxygen, and nitrogen. A fiber optic probe was attached to the parietal bone 2 mm posterior and 5 mm lateral to bregma, and connected to a laser-Doppler flowmeter (MBF3,
Moor Instruments Ltd., Millwey, Axminster, UK) for continuous monitoring of cerebral blood flow (CBF). For right middle cerebral artery (RMCA) occlusion in mice, a heat-blunted monofilament surgical suture (6-0, around 100 µm) was inserted into the exposed external carotid artery, advanced into the internal carotid artery, and wedged into the circle of Willis to obstruct the origin of the RMCA. The filament was left in place for 30 min and then withdrawn. Only animals that exhibited a reduction in CBF >85% during RMCA occlusion and a CBF recovery by >80% after 10 min of reperfusion were included in the study. The general successful induction rate is above 80%. This procedure leads to reproducible infarcts similar in size and distribution to those reported by others using transient RMCA occlusion of comparable duration (Kunz et al., 2008). Rectal temperature was monitored and kept constant (37.0±0.5°C) during the surgical procedure and in the recovery period until the animals regained full consciousness. The experimental grouping was designed as described below (2.3.). Additional animals (as indicated in each result) from the groups as described were used for other assays including analysis of survival rates, neuronal function (µPET) in living mice, and immunohistochemistry staining.

2.3. Drug administration and animal grouping

The mice were randomly divided into following 7 groups (n=20 for each group) including sham control, stroke, stroke plus one of 4 CHR including BHD, XZD, TGD and SYD (1.0 g/kg, p.o., twice daily according to human daily dose), stroke plus recombinant rt-PA (10 mg/kg, i.v., once only; Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany). Two hours after stroke induction, the mice were treated with one of 4 CHR, rt-PA or vehicle control distilled water (sham and stroke only groups) daily. All animals were allowed to move and take food with freedom.

2.4. Assessment of neurological deficit and analysis of survival rates
The neurological deficit of mice was carried out just before the sacrifice at 24h after stroke by analyzing their tracking distance and appearance of the stroke-related behavior pattern (circling clockwisely) within 3 min in an observation box (60×60×60 cm³) using a video-tracking system software (SMART v2.5.21, Panlab, Spain). For survival rate analysis, mice were kept in isolators (individually ventilated cage systems) after stroke induction, given food and water ad libitum and kept at 22±2 °C with alternating 12 h periods of light and dark. Survival rates were calculated within 7 days after stroke induction.

2.5. Evaluation of infarct volume

Twenty-four hours after reperfusion, mice were sacrificed by rapid decapitation under deep anesthesia. The whole brain was rapidly removed. Immediately after being weighed, the brain was sliced into 2-mm-thick coronal sections and stained with 2% 2,3,5-triphenyltetrazoliumchloride (TTC, Sigma-Aldrich) for 30 min at 37°C in the dark, followed by fixation with 10% of formalin at room temperature (25°C) overnight. Brain slices lacking red staining defined the infarct area. The slices were photographed with a digital camera and analyzed by an image processing system (AlphaEaseFC 4.0, Alpha Innotech, San Leandro, CA, USA). Infarct volume was obtained according to the indirect method proposed (Swanson et al., 1990) and corrected for edema by comparing the volume of ischemic and nonischemic hemispheres as described (Lin et al., 1993). The infarct volume was expressed as mm³ of the whole brain volume.

2.6. A micro-positron emission tomography (µPET) evaluation of the brain function

Cerebral glucose metabolism was measured to evaluate the brain function after stroke. Animals were injected with 100 μ Curie of 2-deoxy-2-[F-18]fluoro-D-glucose ([F-18]FDG), and imaged on a small animal PET scanner (µPET; Concorde Microsystems). Images were acquired for 10 min under inhalation anesthesia (isoflurane
2%). The level of radioactivity in brain tissue (percentage dose per gram) was estimated from images according to the method published (Hsieh et al., 2009).

2.7. Immunohistochemical staining and quantification of the positively stained cells

Animals were anesthetized with sodium pentobarbital and then transcardially perfused with saline, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed, post-fixed overnight in a solution containing 4% paraformaldehyde and 4% sucrose in PBS, and then cryoprotected in solutions containing 10%, 15%, and 20% sucrose in PBS for 1 day each. The brains were then embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA) and frozen in liquid nitrogen. Coronal sections (16 μm) were taken 1.5-1.7 mm caudal to bregma by a cryostat (Microm HM560, Walldorf, Heidelberg, Germany). The sliced tissues were fixed in a solution containing 4% paraformaldehyde and 4% sucrose in PBS for 15 min, permeabilized with 0.3% Triton-X in PBS for 10 min, treated with 10% donkey serum in PBS containing 0.3% Triton X-100 to block non-specific binding, and then were randomly selected for incubation with appropriate first antibodies against calcium/calmodulin-dependent protein kinase II (CaMKII, 1:500, Abcam, Cambridge, UK), occludin (1:1000, Abcam, Cambridge, UK), caspase 3 (1:40, Calbiochem, CA, USA), doublecortin (1:1000, Millipore, CA, USA), and CD11b (1:100, Serotec, Oxford, UK) in PBST containing 3% albumin at 4 °C overnight. After washing twice with PBS containing Tween-20 (0.1%) for 30 min, sections were incubated with FITC- or Cy5-conjugated second antibodies (1:100 dilution for each, Jackson Lab, Bar Harbor, ME, USA) in PBS containing 3% albumin for 1 h, and then washed twice again for 30 min each. We also used proper neutralizing peptides or by omitting primary antibody during the staining procedure to check the specificity of the staining. All coverslips were mounted with Vectashield Mounting Medium (Vector Laboratories,
Burlingame, CA, USA) containing a proper dilution of 4',6-diamidino-2-phenylindole (DAPI) to counterstain DNA in the nuclei. The sliced tissues were examined using a laser-scanning confocal microscope (Zeiss LSM780; Carl Zeiss, Jena, Germany). The distribution and numbers of immuno-positively stained cells were determined and quantified using averaged florescence intensity (arbitrary units) by imaging software ZEEnet 2011 (black edition, Carl Zeiss MicroImaging GmbH, 1997-2011) in the entire field of the selected images or after sampling in the specific regions as indicated under high magnification (60×~100×) over 3~5 independent experiments.

2.8. Array data sets, array probe preparation and data processing

Twenty-four hours after stroke, brains of sham-operated control mice, stroke mice and CHR-treated stroked mice were subjected into total RNA extraction and microarray hybridization. In each group, RNAs from 6 different mice were hybridized onto 2 different chips to have biological replica. The Affymetrix™ Mouse Genome 430 2.0 chips were used. RMA log expression units were calculated from Affymetrix GeneChip array data using the ‘affy’ package of the Bioconductor (http://www.bioconductor.org/) suite of software for the R statistical programming language (http://www.r-project.org/).

The default RMA settings were used to background correct, normalize and summarize all expression values. Significant difference between sample groups was identified using the ‘limma’ package of the Bioconductor. To control the multiple testing error, a positive false discovery rate (pFDR) algorithm was then applied to these p-values to calculate a set of q-values: thresholds of the expected proportion of false positives, or false rejections of the null hypothesis. Heatmaps were created by the dChip software (http://biosun1.harvard.edu/complab/dchip/). Principle component analysis (PCA) was performed by the Partek Genomics Suite (http://www.partek.com/) to provide a visual impression of how the various sample groups are related. Gene annotation, Gene
Ontology database search, and KEGG pathway database search were performed by the DAVID Bioinformatics Resources 6.7 interface (http://david.abcc.ncifcrf.gov/).

2.9. Statistical analysis

All values in the text and figures are presented as the mean ± S.E.M. Data, except indicated, were analyzed by one-way or two-way analysis of variance (ANOVA) depending on the number of parameters for comparison, followed by post-hoc Student-Newman-Keuls (S-N-K) t-test for multiple comparisons. Values of $p<0.05$ were considered significant.

Results

3.1. Effects of 4 CHR on survival rate and cerebral infarction after stroke induction

Most of the mice (>80%) died within 2 days after stroke induction with vehicle (distilled water) treatment, but treatment of CHR (BHD, XZD, TGD and SYD) (1.0 g/kg, twice daily, p.o.), and rt-PA (10 mg/kg, once only at 2h after stroke, i.v.) all enhanced the survival rate as compared to vehicle (distilled water)-treated stroke group, with BHD being the most effective one (Fig. 2A, $p<0.05$). The infarct volume induced by stroke injury at 24h after stroke (65±6 mm$^3$, around 32% of the whole brain) was comparable with our previous reports (Wang et al., 2011). Treatments of these 4 CHR all significantly decreased the stroke-induced cerebral infarction by 38%~61% (Fig. 2B, $p<0.05$). Treatment with rt-PA ameliorated the infarct volume by around 23%. The hemodynamic and arterial blood-gas measurements showed no significant differences before, during, or after the experiments among these groups (data not shown). Neurological deficit scoring was measured at 24h after stroke by determining the tracking distance within 3 min in a box, the distance (cm) in mice with stroke injury (500±280) was significantly lower than those with the sham-operation (1500±210) (one-way ANOVA, $p<0.05$). Treatment with BHD, XZD, TGD, and SYD significantly enhanced the tracking distance (cm) to
1680±150, 1450±140, 1100±220 and 1030±180, respectively, and were all more potent than rt-PA (820±250) (Fig. 2C, one-way ANOVA, p<0.05). Besides, the typical neurological deficit behavior (circling clockwisely) induced by stroke was significantly ameliorated by 4 CHR, but still clearly observed in rt-PA treated group (Fig. 2C).

3.2. Effects of CHR on brain function of living mice after stroke induction

Neurofunctional study of the brain after stroke could be examined by determining the glucose metabolism in the brain (as assayed by µPET imaging). In this study, stroke injury dramatically impaired glucose metabolism (totally absence of red-colored image on the right brain hemisphere in untreated stroke mice) (Fig. 1D). Treatment with these 4 CHR (1.0 g/kg) all considerably ameliorated brain function at 24h after stroke injury (Fig. 2D).

3.3. Effects of CHR on BBB integrity and apoptotic brain injury after stroke induction

Stroke also induced remarkable BBB leakage and brain injury (loss of occludin (orange) and CaMKII (green) staining, Fig 3A) within the peri-infarct area of the ischemic brain and triggered apoptotic DNA damage as evidenced by increment in the immunoreactivity of caspase 3 (red) staining (Fig. 3A). Treatment of CHR including BHD, XZD, TGD, or SYD (1.0 g/kg), or rt-PA (10 mg/kg) in mice with ischemic stroke significantly reduced BBB leakage and brain injury (Fig. 3A) and the immunoreactivity for caspase 3 (apoptosis) staining (Fig. 3A) at 24h after stroke.

3.4. Effects of CHR on neurogenesis after stroke near the subgranular zone (SGZ) and the subventricular zone (SVZ)

In this study, the mice survived after stroke induction with vehicle treatment did not show significant neurogenesis at 24h after stroke (Fig. 3B, stroke) but showed strong evidence of apoptosis (caspase 3 staining) near subgranular zone (SGZ) and the subventricular zone (SVZ). Treatment of CHR (1.0 g/kg) in mice with ischemic stroke
significantly enhanced neurogenesis and reduced apoptosis near both SGZ and SVZ as determined by increment of doublecortin staining (a neuronal stem cell marker) and reduction of caspase 3 staining with BHD, XZD and TGD being more potent than that of rt-PA after stroke (Fig. 3B).

3.5. Molecular impacts of CHR on stroke mice brain

To provide more insights into the in vivo influences of these 4 CHR on ischemic stroked-mice brain, a genome-wide transcriptome analysis was performed. A total of 774 ischemia-induced probe sets were found significantly influenced by the 4 remedies tested, and a principle component analysis (PCA) plot based on these 774 probe sets was drawn to illustrate the differential gene expression patterns between different mice groups. The gene expression pattern of BHD-treated mice was closest to that of sham mice (Fig. 4A), reflecting the best survival situation of BHD-treating stroke mice in Figure 2A. Mice receiving XZD, SYD and TGD all expressed unique gene expression patterns and similarities to that of sham mice (Fig. 4A). Genes unique in or common between different CHR are illustrated in Figure 4B-C, and gene details are in Suppl. Table 1 online.

We found that in total 52 probe sets were commonly induced by these 4 CHR in ischemia mice, while another 54 were down (Fig. 4D-E) at 24h after stroke. According to the Gene Ontology (GO) database, genes involved in Ras/Rho signaling pathways (including Arhgef9, Klrn, Pafah1b1, Itsn2, and Psd3) were significantly enriched (Table 1, upper part) among CHR-induced ones. Five neurogenesis genes (including Cdk5r1, Cx3cl1, Elmo1, Mycbp2, and Pafah1b1) and 3 angiogenesis genes (including Cx3cl1, Rtn4, and Wars) were also specifically induced in brains of stroke mice by these 4 CHR at 24h after stroke (Table 1).

Among CHR-downregulated genes, genes response to wounding, inflammation, or leukocyte mediated cytotoxicity (including Ncf1, Cxcl1, Plaur, Cd163, and Fcgr3;
Genes involved in apoptosis (Srgn, Ncf1, Id1, 18100110Rik, and Rps6; P=0.037) and chemotaxis (Creb3, Fcgr3, and S100a9; P=0.035) were also significantly inhibited by tested CHR (table 1). Vasculature development (Notch4, Emcn, Nos3, and Id1) and cell proliferation (Met, Pim1, Ncf1, and Rps6) genes were less active in CHR-treated mice at 24h after stroke (Table 1).

3.6. Unique mechanisms of BHD in ischemic stroke mice

We next examined the unique gene expression patterns in mice treated with each CHR. CHR-affected genes were organized into functional groups according to the GO Biological Processes definition or KEGG pathways for having better insights into the biological consequences of gene expression changes. We started from the BHD-affected mice genes since comparing with another 3 CHR, BHD had the best influence on ischemia mice, and the gene expression pattern of BHD-treated stroked mice showed nice reversion to that of sham controls (Fig. 5A). BHD-treated mice and sham mice were clustered together (highlighted by a red box, Fig. 5A).

According to the GO database, BHD treatment significantly enriched genes involved in promoting neurogenesis and cell morphogenesis (P=1.40e-6 and 3.00e-4, respective; Fig. 5B). Twelve neurogenesis genes and 7 neuron differentiation genes were specifically induced in brains of stroked mice by BHD (Fig. 5B). Genes involved in neuron function, such as synapse transmission (10 genes, P=6.10e-4) and cell migration (8 genes, P=0.041), were also induced by BHD in mice (Fig. 5B). Among BHD-downregulated genes, genes response to wounding (9 genes, enrichment P value=0.004) or inflammation (7 genes, P=0.006) were reverted by BHD (Fig. 5C). Genes involved in promoting cell death (9 genes, P=0.03) or inhibiting cell differentiation (6 genes, P=0.011) were also significantly inhibited by BHD.
Increasing evidence shows that genes do not act as individuals but collaborate in signaling pathways or genetic networks. To better understand how genes affected by BHD are related to each other, we further performed gene pathway analysis based on the KEGG pathway database. Pathways involved in neuronal function, such as neuroactive ligand-receptor interaction and calcium signaling pathway, were significantly up-regulated by BHD (Fig. 5D). Six genes (Cacng3, Cacng7, Fgfr3, Map3k5, Ntrk2, and Nr4a1) involved in mitogen-activated protein (MAP) kinase pathway were also enriched in BHD-treated ischemia mice (Fig. 5D).

3.7. **Unique mechanisms of other 3 CHR in ischemic stroked mice**

As to other 3 CHR, TGD-affected genes only partially reverted stroke-induced gene abnormality (Fig. 6A). The unique GO biological processes induced by TGD include those participated in neuron differentiation, visual learning, learning or memory, adult walking behavior, chromatin modification, mRNA splice site selection, and nerve-nerve synaptic transmission (detail lists in Fig. 6B). XZD-affected genes mainly repressed stroke-induced genes (Fig. 6C). As a result, XZD repressed many biological processes (according to the GO categories) rather than inducing them. Having said that, XZD still induced nerve function-related genes, including those involved in nerve-nerve synaptic transmission, ion transport, and synaptic transmission (indicated by arrows, Fig 6D), suggestion BHD, TGD and XZD could all rescue stroked mice by inducing neuron function.

Interestingly, biological functions induced by SYD do not include those related to neuronal function; SYD activated Wnt receptor signaling pathway (Fig. 6E). SYD repressed biological processes rather than inducing them. Stimulus or stress response genes were repressed/reverted by SYD (P=4.36e-05 and 1.30e-04, respectively; Fig. 6E). Chemotaxis, inflammatory response, and lymphocyte activation were reverted by SYD.
(indicated by arrows, Fig. 6E). Finally, angiogenesis and vasculature development were less active in SYD-treated mice (Fig. 6E).

4. Discussion

Although Chinese herbal remedies like BHD, XZD, TGD and SYD have been reported to be neuroprotective in many animal models and even in TCM clinic (Cai et al., 2007; Lee et al., 2011; Ho et al., 2008; Chen et al., 2015), the mechanisms of action based on a brain functional and a genome-wide transcriptome analysis, here referred as a TCM translational research, has not been elucidated before. Our results demonstrate for the first time that treatment with 4 different CHR exhibit protective effect against ischemic stroke in mice as compared with vehicle- and rt-PA-treated stroke mice. Among these 4 CHR, the neuroprotective effect of BHD is more potent than that of XZD, TGD, SYD and rt-PA, indicating that novel mechanism(s) or targets more than what XZD, TGD, SYD and rt-PA modulate could be involved in the neuroprotective effects of BHD on stroke-induced injury. Herein, we reveal the brain protective effect of these 4 CHR in living mice by modulating a common and unique group of genes that parallels with significant improvement in brain function and neurological deficits, as well as a reduction of BBB impairment and apoptosis without significant modulation of the hemodynamic, arterial blood-gas, or physiological conditions.

A pattern recognition analysis illustrated that CHR treatment reversed stroke-induced brain damage at a molecular level. Commonly upregulated functional groups by the 4 tested CHR including Ras/Rho signal transduction pathways, neurogenesis and angiogenesis. Two important downstream signal transduction pathways of Ras protein are mitogen-activated protein kinases (MAPK) kinase-extracellular signal-regulated kinase (MEK)/Erk pathway and phosphatidylinositol 3-kinase (PI3K) pathway (Malumbres and Pellicer, 1998). Activation of MEK/Erk and PI3K pathways promotes the
phosphorylation/ inactivation of glycogen synthase kinase 3 (GSK3), a pivotal molecule mediating neurodevelopment (Hur and Zhou, 2010), in turn, enhances angiogenesis and neurogenesis after ischemic stroke (Chuang et al., 2011). Besides, activation of Rho related signaling pathways have been confirmed in mammalian hematopoietic stem cells to regulate mammalian stem cell self-renewal, adhesion, and migration (Nayak et al., 2013; Ito et al., 2014). In this study, three angiogenesis genes including Rtn4, Wars, Cx3cl1 were expressed in all CHR-treated mice. Cx3cl1 is also involved in neurogenesis, indicating its critical roles in CHR-mediated stroke recovery. CX3CL1 (fractalkine) is a unique chemokine that is constitutively expressed on neurons where it serves as an adhesion molecule for lymphocytes and monocytes. CX3CL1 can also be cleaved from the surface of these cells and enter the circulation to act as a traditional chemokine for serving as an immune modulator and neuroprotector in a variety of neurodegenerative diseases (Jones et al., 2010). Higher plasma fractalkine is associated with better 6-month outcome from ischemic stroke patients (Donohue et al., 2012). These CHR may achieve their neuroprotection function by way of, at least in part, inducing CX3CL1/fractalkine expression in vivo.

Here we further found that S100a9 and CXCL1 (GRO-alpha, a potent neutrophil chemoattractant) are two common gene repressed by all CHR, indicating that inflammation could be compromised by all CHR here. Similar observation using DNA microarray chips containing 512 cDNA probe also identified S100a9 as one of the 6 potential targets down-regulated by BHD (Li et al., 2004). In a gene expression microarray study using the core, peri-infarct and contralateral cortex parts of adult Sprague-Dawley rats, chemokines like CXCL1 and CXCL12 were found overexpressed after both 24 hours (acute phase) and 3 days (delayed stage) of permanent middle cerebral artery (MCA) occlusion (Ramos-Cejudo et al., 2012). Nevertheless, Serum CXCL1 levels
in stroke patients did not differ from controls (Losy et al., 2005).

Nevertheless, only the Toll-like receptor (TLR) signaling pathway were found specifically repressed in stroked-mice treated with BHD, but not with another 3 CHR. Among these genes, Tlr7, Nfkbia and Jun are unique to BHD-treated mice. Toll-like receptor (TLR) 7 and TLR8 expression was shown to associate with poor outcome and greater inflammatory response in acute ischemic stroke (Brea et al., 2011). TLR7 is known to induce \( IL6 \) gene expression via TRAF6 (Loniewski et al., 2007), and the Il6 cytokine was particularly down-regulated in BHD- and XZD-treated mice. The Tlr7-Il6 signaling pathway is therefore activated in stroke mice and BHD treatment is effective in suppressing this inflammation circuit. We weighted in this field by showing that BHD significantly reverts genes involved in promoting neurogenesis while repressing inflammation (Wang et al., 2011). BHD also significantly potentiated the expression of a protective factor in the damage area, for example, Frzb (frizzled-related protein, alias Sfrp3), a secreted protein activating the Wnt survival and proliferating pathway, was induced by BHD (Wang et al., 2011). Furthermore, signaling pathway analysis revealed that BHD significantly upregulated 6 genes involved in MAPK pathway. BHD also upregulated 6 genes, including camk2a, involved in the calcium signaling pathway. CaMKII, is one member of \( Ca^{2+}/\text{calmodulin} \)-dependent protein kinase (CaMK) cascade which is well-established for its effects on modulating synaptic plasticity and learning and memory (Wayman et al., 2008).

The unique GO biological processes induced by TGD include those participated in neuron differentiation, visual learning, learning or memory, and adult walking behavior. This could be further evidenced as previous studies reported that TGD is beneficial for memory enhancement (Ho et al., 2005; 2008). Besides, according to the GO categories, XZD repressed many biological processes including angiogenesis. This observation is
different from those reported by Song’s and Gao’s groups who demonstrated that XZD could enhance angiogenesis in endothelial cell (Song et al., 2012) and animal model (Gao et al., 2012). The discrepancy between others and ours report could be attributed to different cellular or animal model used. Conversely, for the XZD-induced upregulation in nerve function-related genes, ours results is in agreement with Gao’s repot in which XZD promoting regeneration of bone marrow hematopoietic stem cells through improving hematopoietic function by means of increasing the number and enhancing the function of premature hematopoietic stem cells (HSC) in mice (Gao et al., 2007). On the contrary, SYD did not interfere biological functions related to neuronal function, but activated Wnt/β-catenin pathway that regulates stem cell pluripotency and cell fate decisions during development (Angers and Moon, 2009) and suppressed inflammation associated pathways, contrasting that SYD is the less effective CHR in this study.

In conclusion, our results reveal for the first time at a side-by-side manner the neuroprotective effects of these 4 different CHR on stroke-induced brain injury in mice. The distinct therapeutic effects between different CHR may due to the fact that each CHR revert and modulate a specific alone with a common group of molecular targets (genes) and pathways. Our results provide a possible explanation for the necessary of pattern differentiation and treatment in TCM based on a genome-wide transcriptome analysis integrated with neurofunctional assay, and the opportunity for the prognostic evaluation of CHR efficacy. We suggest the levels of plasma CX3CL1 and S100a9 in patients for predicting neurogenesis and neural inflammation before functional recovery could be observed. Since each famous CHR has its own unique profile of mechanisms of action, using a combinatory cocktail therapy like CHR based on TCM syndrome identification, might be a better way for personalized treatment of cerebral ischemic-associated diseases.

**Author disclosure statement**
No conflicting financial interests exist.

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Fig. 1. The representative chemical fingerprints of the Chinese herbal remedies (CHR) examined in this study. HPLC chromatogram was carried out on a Cosmosil 5C\textsubscript{18} AR II column (4.6 x 250 mm) in Hitachi L-7100 HPLC system with a diode array detector (DAD), monitored at 280 nm for Bu-yang Huan-wu Decoction (BHD) product, at 230 nm for Xue-fu Zhu-yu Decoction (XZD) product and Sheng-yu Decoction (SYD) product, and at 203 nm for Tian-ma Gou-teng Decoction (TGD) product. The mobile phase consisted of 0.1% phosphate water (A) and acetonitrile (B) using a gradient elution of 2% B at 0-5min, 2-10% B at 5-10min, 10% B at 10-20min; 10-25%B at 20-50min; 25-45% at 50-70min; 45-100%B at 70-75min. The flow rate was 1.0 ml/min. PGG,
1,2,3,4,6-pentagalloylglucoside. At least 4 active components (arrows indicated) in each CHR were identified and compared to data base in our chemical core lab.

**Fig. 2. Protective effect of Chinese herbal remedies on ischemic stroke-injured mice.**
(A) The survival rate among sham-operated (sham) or vehicle-, BHD-, XZD-, TGD-, SYD-, and control drugs (rt-PA)-treated mice with an ischemic stroke (Stk); survival rates were calculated within 7 days (N=20 for each group). (B) Brain infarction analysis (TTC stains) at 24h after stroke. (C) Neurological deficit scoring by determining the tracking distance within 3 min in a box measured at 24h after ischemic stroke. (D) Micro-PET analysis of the brain function (glucose metabolism) in living mice at 24h after stroke, arrows indicate sites for ischemia induction in ischemic groups. † † † p<0.05 as compared to sham or vehicle-treated group (stroke only (Stk)), respectively, analyzed by one-way ANOVA followed by S-N-K t-test. N.D., data not detected.

**Fig. 3. Protective effect of Chinese herbal remedies (CHR) on ischemic stroke injured mice revealed by immunohistochemical staining.** (A) Examples of a brain slice taken from 1.5~1.7 mm caudal to bregma. BBB integrity near the peri-infarct area (cortex) was revealed at 24h after stroke by the staining of occludin (O, orange); apoptosis revealed by caspase 3 staining (R, red); preserved area revealed by calcium/calmodulin-dependent protein kinase II (CaMKII staining (G, green); DAPI (blue, a marker for nuclei); arrows indicate occludin staining; (B) Neurogenesis near the subgranular zone (SGZ) and the subventricular zone (SVZ) was examined at 24h after stroke; arrows indicate the staining of doublecortin (DCX) (G, green), a marker for neuronal stem cells; apoptosis revealed by caspase 3 staining (R, red); CD11b (O, orange), a marker for inflammatory cell. Sham, sham-operated mice without treatment; Stroke,
vehicle-treated mice with cerebral ischemia reperfusion; Stroke+Chinese herbal remedy (CHR: BHD, XZD, TGD, or SYD), stroke mice treated with CHR (1.0 g/kg, p.o., twice daily); Stroke+rt-PA, stroke mice treated with rt-PA (10 mg/kg, i.v., once-shot). At least 3 independent experiments were confirmed in this study. *p<0.05 as compared to vehicle-treated group (stroke only (Stk)), respectively, analyzed by one-way ANOVA followed by S-N-K t-test. N.D., data not detected.

**Fig. 4. Transcriptome analyses on sham and CHR-treated ischemic stroke injured mice.** (A) A PCA plot shows the transcriptome relationships between sham mice, stroked mice without treatment (stroke) and stroked-mice treated with different CHR (BHD, XZD, TGD and SYD). There were 774 probe sets deregulated in stroke mice but were then rescued by CHR treatment (indicated by a Venn diagram), and this PCA plot was drawn using these 774 probe sets. (B-C) Venn diagrams illustrate genes common or unique in each CHR. B: up genes; C: down ones. (D-E) Genes commonly up- (D, 52 genes) or down-regulated (E, 54 genes) in CHR-treated stroke mice.

**Fig. 5. Transcriptome analyses on BHD-treated ischemic stroke injured mice.** (A) A heat map shows the up (in red) or down-regulation (in blue) pattern of the 481 probe sets that were deregulated in stroked-mice but were then rescued by BHD treatment. B: BHD; S: SYD. Red transparent box: BHD-treated stroked-mice and sham mice were clustered together. (B-C) Altered biological processes that were up (B) or down (C) in BHD-treated stroked-mice according to the Gene Ontology (GO) database. The number of genes, their percentages in the whole 481 BHD-affected stroke genes, and p values for each category that was significantly (p<0.05) enriched are listed. (D) Up-regulated pathways in BHD-treated stroked-mice according to the KEGG database.
Fig. 6. Unique mechanisms of another 3 CHR (XZD, TGD, and SYD) on ischemic stroke injured mice.

(A) TGD partially restore gene expression patterns in stroke mice. This heat map shows the up (in red) or down-regulation (in blue) pattern of the 292 probe sets that were deregulated in stroke mice but were then rescued by TGD treatment. B: BHD; X: XZD. 

(B) Functional module analyses as a framework for the interpretation of the TGD-induced neuroprotection. Arrows: discussed in the text. (C) A heat map shows the up- and down-regulation pattern of the 474 probe sets that were deregulated in stroke mice but were then rescued by XZD treatment. T: TGD; S: Sham. (D) Up-regulated biological processes in XZD-treated CI/R mice according to the Gene Ontology (GO) database. (E) Altered biological processes that were up (upper part) or down (lower part) in SYD-treated stroke mice according to the Gene Ontology (GO) database. Arrows: discussed in the text.

Fig. 7. Common and unique mechanisms of 4 CHR (BHD, XZD, TGD, and SYD) on ischemic stroke injured mice. Acute ischemic stroke (AIS) induces an excitotoxicity associated neuronal damage and depletion of endogenous neuronal stem cells by strong oxidative stress and inflammation. CHR modulates a common as well as a specific unique group of genes/pathways to rebalance the abnormal (imbalanced of yin-yang) gene expression profiles by AIS that may partially explain the syndrome differentiation and treatment principle in traditional Chinese.

Table

Table 1 Components and relative amount of the plants in the four CHR

<table>
<thead>
<tr>
<th>Name of CHR</th>
<th>Plant name (family) and the digital show relative amount (ratio)</th>
<th>Human maximum</th>
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<tr>
<th>Formula</th>
<th>Ingredients</th>
<th>Daily Dose (gm)</th>
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<td><strong>Buyang Huanwu decoction</strong></td>
<td><em>Astragalus membranaceus</em> Bunge (Family Leguminosae) 20; <em>Angelica sinensis</em> (Oliv.) Diels (Family Apiaceae) 1; <em>Paeonia lactiflora</em> Pall. (Family Paeoniaceae) 1; <em>Ligusticum chuanxiong</em> S. H. Qiu, Y. Q. Zeng, K. Y. Pan, Y. C. Tang &amp; J. M. Xu (Family Apiaceae) 0.5; <em>Prunus persica</em> (L.) Batsch (Family Rosaceae) 0.5; <em>Carthamus tinctorius</em> L. (Family Asteraceae) 0.5; <em>Pheretima aspergillum</em> (E. Perrier) (Family Megascolecidae) 0.5.</td>
<td>24.0</td>
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<td><strong>Xuefu Zhuyu decoction</strong></td>
<td><em>Angelica sinensis</em> (Oliv.) Diels (Family Apiaceae) 4.5; <em>Paeonia lactiflora</em> Pall. (Family Paeoniaceae) 3; <em>Ligusticum chuanxiong</em> S. H. Qiu, Y. Q. Zeng, K. Y. Pan, Y. C. Tang &amp; J. M. Xu (Family Apiaceae) 2.3; <em>Prunus persica</em> (L.) Batsch (Family Rosaceae) 6; <em>Carthamus tinctorius</em> L. (Family Asteraceae) 4.5; <em>Rehmannia glutinosa</em> (Gaertn.) DC. (Family <em>Plantaginaceae</em>), <em>Citrus aurantium</em> L. (Family Rutaceae) 3; <em>Achyranthes bidentata</em> Blume (Family <em>Amaranthaceae</em>) 4.5; <em>Glycyrrhiza uralensis</em> Fisch. (Family <em>Leguminosae</em>) 1.5; <em>Bupleurum marginatum</em> Wall. ex DC. (Family <em>Apiaceae</em>) 1.5; <em>Platycodon grandiflorus</em> (Jacq.) A.DC. (Family <em>Campanulaceae</em>) 2.3.</td>
<td>37.6</td>
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<tr>
<td><strong>Tianma Gouteng decoction</strong></td>
<td><em>Gastrodia elata</em> Blume (Family Orchidaceae) 2; <em>Uncaria rhynchophylla</em> (Miq.) Miq. ex Havi. (Family <em>Rubiaceae</em>) 3; <em>Achyranthes bidentata</em> Blume (Family <em>Amaranthaceae</em>) 4; <em>Halotis diversicolor</em> Reeve (Family <em>Haliotidae</em>) 5; <em>Eucommia ulmoides</em> Oliv (Family <em>Eucommiaceae</em>) 4; <em>Scutellaria baicalensis</em> Georgi (Family <em>Lamiaceae</em>) 2; <em>Leonurus sibiricus</em> L. (Family <em>Lamiaceae</em>) 3; <em>Loranthus parasiticus</em> L. Merr. (Family <em>Loranthaceae</em>) 3; <em>Gardenia jasminoides</em> J. Ellis (Family <em>Rubiaceae</em>) 2; <em>Polygonum multiflori</em> Thunb. (Family <em>Polygonaceae</em>) 3; <em>Portia cocos</em> (Schw.) Wolff (Family <em>Polyporaceae</em>) 3.</td>
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<td><strong>Shengyu decoction</strong></td>
<td><em>Panax ginseng</em> C.A. Mey. (Family <em>Araliaceae</em>) 5; <em>Astragalus membranaceus</em> Bunge (Family <em>Leguminosae</em>) 5; <em>Angelica sinensis</em> (Oliv.) Diels (Family <em>Apiaceae</em>) 2.5; <em>Paeonia lactiflora</em> Pall. (Family <em>Paeoniaceae</em>) 5; <em>Ligusticum chuanxiong</em> S. H. Qiu, Y. Q. Zeng, K. Y. Pan, Y. C. Tang &amp; J. M. Xu (Family <em>Apiaceae</em>) 2.5; <em>Rehmannia glutinosa</em> (Gaertn.) DC. (Family <em>Plantaginaceae</em>) 5.</td>
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