Unique Mechanisms of Sheng Yu Decoction (聖愈湯 Shèng Yù Tang) on Ischemic Stroke Mice Revealed by an Integrated Neurofunctional and Transcriptome Analysis

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**ABSTRACT**

Sheng Yu Decoction (聖愈湯 Shèng Yù Tang; SYD) is a popular traditional Chinese medicine (TCM) remedy used in treating cardiovascular and brain-related dysfunction clinically; yet, its neuroprotective mechanisms are still unclear. Here, mice were subjected to an acute ischemic stroke to examine the efficacy and mechanisms of action of SYD by an integrated neurofunctional and transcriptome analysis. More than 80% of the mice died within 2 days after ischemic stroke with vehicle treatment. Treatments with SYD (1.0 g/kg, twice daily, orally or p.o.) and recombinant thrombolytic tissue plasminogen activator (rt-PA; 10 mg/kg, once daily, intravenously or i.v.) both significantly extended the lifespan as compared to that of the vehicle-treated stroke group. SYD successfully restored brain function, ameliorated cerebral infarction and oxidative stress, and significantly improved neurological deficits in mice with stroke. Molecular impact of SYD by a genome-wide transcriptome analysis using brains from stroke mice showed a total of 162 out of 2081 ischemia-induced probe sets were significantly influenced by SYD. Mining the functional modules and genetic networks of these 162 genes revealed a significant upregulation of neuroprotective genes in Wnt receptor signaling pathway (3 genes) and regulation of cell communication (7 genes) and downregulation of destructive genes involved in stress, inflammation, angiogenesis, blood vessel formation, immune responses, and wound healing, as well as upregulating the genes mediating neurogenesis and cell communication, which make SYD beneficial for treating ischemic stroke.

**Key words:** Genome-wide transcriptome analysis, Ischemic stroke, Microarray, Positron emission tomography, Sheng Yu Decoction
INTRODUCTION

Ischemic stroke is ranked as the leading common cause of death in developed countries, even though some drop in death rates has been reported.[1] Excitotoxicity is the major pathophysiological mechanism mediating ischemic stroke–induced brain injury by excessive glutamate accumulation in the extracellular space through which ionotropic N-methyl-D-aspartate (NMDA) receptors in the brain are overactivated under stroke onset.[2,3] Excessive amount of glutamate excites neurons to death by inducing robust oxidative stress by overproduction of reactive oxygen and nitrogen species, such as hydroxyl radicals (OH·), superoxide anions (O₂⁻), hydrogen peroxide (H₂O₂), nitric oxide (NO), and peroxynitrite (OONO⁻).[4,5] The main free radical producing enzyme systems involved include mitochondria, cyclooxygenase (COX), xanthine oxidase, gp91phox (NOX2), and inducible nitric oxide synthase (iNOS) in response to the activation of proinflammatory mediators (e.g., cytokines) produced by recruited leukocytes (e.g., neutrophils, macrophages, and T-cells), active microglial cells, damaged neurons, and astrocytes in ischemic stroke–damaged tissues.[6,7] A key role for inflammatory cell activation in the development of ischemic stroke in humans has been highlighted.[8]

An inflammatory cascade is consequently activated in damaged tissue by ischemic stroke, leading to leukocyte infiltration and release of proinflammatory cytokines for amplification of inflammatory responses that mediate early blood–brain barrier (BBB) dysfunction following stroke.[9] Moreover, activation of inflammation-related transcriptional factor(s) plays a pivotal role in mediating oxidative stress–induced cell injury and in regulating post-ischemic inflammation, possibly through upregulation of inflammatory genes and proteins that contribute to cell death in cerebral ischemia.[10,11] Although thousands of studies provide important evidence in understanding the pathophysiology of ischemic stroke at the cellular, molecular, and animal levels,[10,11] the recombinant thrombolytic tissue plasminogen activator (rt-PA) has continued to be the only US Food and Drug Administration (FDA)-approved drug. According to clinical experience, rt-PA is limited by its serious side effects, narrow therapeutic time window, and is suitable only for a limited group of patients with acute ischemic stroke.[12] Therefore, searching for drugs with more efficacy and lower toxicity from alternative and complementary medicine would be a practical and important therapeutic strategy for the treatment of ischemic stroke.

In traditional Chinese medicine (TCM), many formulas have clinically been used for treating stroke-induced disability for centuries. As a classic TCM formula, Sheng Yu Decoction (聖愈湯 Shèng Yù Tang; SYD) has been used for improving cardiovascular and neurological functional recovery in stroke-induced disability in China.[13] Recent clinical study reported that integrative medicine using SYD together with Western medicine showed promising neuroprotective effects against ischemic stroke in 42 patients,[14] and also the protective effect of SYD on the apoptosis of neural cells after traumatic brain injury in rats.[15] However, how SYD could protect and improve neurological functions in ischemic stroke animals and the potential molecular mechanisms of action based on a genome-wide view remain unclear.

In the present study, we investigated the protective effects and underlying molecular mechanisms of action of SYD, and compared it with a well-known drug for stroke (i.e., 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.

MATERIALS AND METHODS

Preparation of SYD

Sheng Yu Decoction (聖愈湯 Shèng Yù Tang; SYD) was prepared using the method as given in our previous report[16] (mentioned in supporting information) including microscopic examination of the property of the crude drug and decoction pieces, molecular sequence of internal transcribed spacer (ITS) region of the crude drug, and the high performance liquid chromatography (HPLC) fingerprint of each herb and whole remedy preparation. Briefly, the ingredients of SYD, i.e., Radix Rehmanniae (raw) (生地黃 Shèng Dì Huáng), Radix Rehmanniae Preparata (熟地黃 Shòu Dì Huáng), Rhizoma Chuanxiong (川芎 Chuān Xiōng), Radix Ginseng (人參 Rén Shēn), Radix Angelicae Sinensis (當歸 Dāng Guì), and Radix Astragali membranaceus (黃耆 Huáng Qí), were mixed in order in the ratio 20:20:20:20:15:15 (dry weight). SYD was prepared by 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 to a final concentration of 2.0 g/ml (equivalent to the dry weight of the raw materials). The chemical fingerprint [Figure 1] was determined using chromatographic separation carried out on a Thermo Synchrois C18 column (2.1 mm × 100 mm i.d., 1.7 μm) in Waters Acquity...
UPLC system equipped with a diode array detector (DAD), monitored at 230 nm. The mobile phase consisted of 0.1% phosphate water (A) and acetonitrile (B) using a gradient elution of 2% B at 0-1 min, 2-30% B at 1-10 min, and 30-70% B at 10-15 min. The flow rate was 0.4 ml/min, and the column temperature was maintained at 35°C.

**Animals and induction of ischemic stroke injury**

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. Ischemic stroke injury in mice was induced as reported previously with some modifications (NRIICM-IACUC-P-99-011). In brief, male imprinting control region (ICR) mice weighing 28-32 g (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan) were anesthetized with a mixture of isoflurane (1.5-2%), oxygen, and nitrogen. A fiberoptic 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, Millwely, Axminster, UK) for continuous monitoring of cerebral blood flow (CBF). For the induction of ischemic stroke, right middle cerebral artery (RMCA) occlusion in mice was performed using a heat-blunted monofilament surgical suture (6-0, around 100 μm in diameter) which was inserted into the exposed external carotid artery, further 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. This procedure leads to reproducible infarcts similar in size and distribution to those reported by others using transient RMCA occlusion of comparable duration. 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. Additional animals (as indicated in each result) from the groups as described were used for other assays including analysis of survival rates, positron emission tomography (PET), free radical production in living mice, and transcriptome analysis.

**Drug administration and animal grouping**

The mice were randomly divided into the following groups (n = 20 in each group) including sham control, stroke, stroke + SYD (1.0 g/kg, p.o., twice daily), and stroke + rt-PA (10 mg/kg, intravenously or i.v., once daily; Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany). Two hours after ischemic stroke induction, the mice were treated with SYD (p.o.) or rt-PA (i.v.), or vehicle control distilled water (sham and stroke groups) daily. All animals were free to move and take food.

**Assessment of neurological deficit scoring and analysis of survival rates**

The neurological deficit of mice was determined just before the sacrifice on day 1 after stroke by analyzing their tracking distance and appearance of the typical behavior pattern (circling clockwise) related to ischemic stroke within 3 min in an observation box (60 × 60 × 60 cm³) using a video-tracking system software (SMART v2.5.21 Barcelona; 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 immediately (day 0) and within 7 days after stroke induction.

**Evaluation of infarct volume**

Twenty-four hours after ischemic reperfusion, the 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, St Louis, MO, USA) 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 by Swanson et al. and corrected for edema by comparing the volume of ischemic and nonischemic hemispheres as described by Lin et al. The infarct volume was expressed as mm³ of the whole brain volume.

**A PET evaluation of the brain function**

Cerebral glucose metabolism was measured to evaluate the brain function after stroke. Animals were injected with 100 μCi of 2-deoxy-2-(F-18) fluororo-D-glucose ([F-18] FDG) and imaged on a small animal PET scanner (μPET; Concorde Microsystems, Knoxville, TN). 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 the images according to the method published by Hsieh et al.

**Quantification of oxidative stress in living mice**

Dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA; 2.0 mg in 200 μl normal saline) was given i.v. to mice just before the onset of reperfusion for in vivo determination of free radical production in living mice. Reactive oxygen species (ROS) production was monitored at 24 h after reperfusion using the IVIS® Imaging System 50 Series (Xenogen Corp., Alameda, CA, USA) under 488-nm excitation light. Living Image software (Xenogen Corp.) was used to analyze the bioluminescence in the brain. Data were expressed as photon flux in a region of interest (ROI). All the animals were imaged under inhalation anesthesia (isoflurane 2%). Signal intensities from ROIs were defined manually and expressed as photon flux (photons/sec/cm²/sr), where steradian (sr) refers to the photons emitted from a unit solid angle of a sphere. Background photon flux was defined from an ROI of the same size drawn over the thorax of each animal, and these data were subtracted from the signal intensities measured at the sites of injury. Area under the curve (AUC) analyses were performed with AlphaEaseFC software (version 4.0; Alpha Innotech Corporation, San Leandro, CA, USA) and were expressed as the AUC of photon flux.
Array data sets, array probe preparation, and data processing

Twenty-four hours after induction of stroke, brains of sham-operated control mice, ischemic stroke mice, and SYD-treated mice were subjected to total RNA extraction and microarray hybridization. In each group, the RNAs from six different mice were hybridized onto two different chips to have biological replica. The Affymetrix™ Mouse Genome 430 2.0 chips were used. Robust multi-array analysis (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 the expression values. Significant difference between sample groups was identified using the “limma” package of the Bioconductor. To control the multiple testing errors, a false discovery rate (FDR) 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. Heatmap was created by the dChip software (http://biosun1.harvard.edu/complab/dchip/). Principal 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 were related. Gene annotation was performed by the ArrayFusion web tool (http://microarray.ym.edu.tw/tools/arrayfusion/). Gene Ontology (GO) database search were performed by the DAVID Bioinformatics Resources 6.7 interface (http://david.abcc.ncifcrf.gov/).

Statistical analysis

All values in the text and figures are presented as the mean ± SEM. 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 (SNK) t-test for multiple comparisons.

RESULTS

Effects of SYD on survival rate and cerebral infarction after ischemic stroke

Most of the mice (>80%) died within 2 days after stroke induction with vehicle (distilled water) treatment, but treatment with SYD (1.0 g/kg, twice daily, p.o.) and rt-PA (10 mg/kg, once daily, i.v.) both enhanced the survival rate as compared to vehicle (distilled water)-treated stroke group. This was especially true on day 1 after stroke [Figure 2] (P < 0.05). The infarct volume induced by ischemic stroke on day 1 after stroke (60 ± 11 mm³, around 40% of the whole brain) was comparable with previous reports. Treatment with SYD (1.0 g/kg) and rt-PA significantly decreased the stroke-induced cerebral infarction by 67% and 43%, respectively [Figure 3] (P < 0.05). 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 on day 1 after stroke by determining the tracking distance within 3 min in a box; the distance (cm) of mice with ischemic stroke injury (510 ± 180) was significantly lower than those in the sham-operated group (1510 ± 120) [Figure 4] (one-way ANOVA,

Figure 2. Protective effect of SYD on the survival rate in ischemic stroke-injured mice within 7 consecutive days. Animal groups include sham, vehicle treated (stroke), SYD treated (1.0 g/kg, twice daily, p.o.; +SYD), and rt-PA treated (10 mg/kg, daily, i.v.; +rt-PA). Survival curves were computed using the Kaplan–Meier method. Differences in survival rates were assessed using the log-rank test followed by Holm-Sidak method for all pairwise multiple comparisons. *P < 0.05 as compared to stroke group analyzed by one-way ANOVA followed by SNK t-test (n = 10 for each group).

Figure 3. Protective effect of SYD on cerebral infarction at 24 h after ischemic stroke. Animal groups include sham, vehicle treated (stroke), SYD treated (1.0 g/kg, twice daily, p.o.; +SYD), and rt-PA treated (10 mg/kg, daily, i.v.; +rt-PA). *P < 0.05 as compared to stroke group analyzed by one-way ANOVA followed by SNK t-test (n = 10 for each group). NA, data not available.
P < 0.05). Treatment with SYD and rt-PA both significantly enhanced the tracking distance (cm) to 1050 ± 160 and 800 ± 150, respectively [Figure 4] (one-way ANOVA, P < 0.05). Besides, the typical neurological deficit behavior (circling clockwise) induced by ischemic stroke was significantly ameliorated by SYD and rt-PA [Figure 4].

**Effects of SYD on brain function and oxidative stress of living mice after stroke**

To determine the neurofunctional effect of SYD, the brain image after stroke was examined by measuring the glucose metabolism in the brain of living mice using μPET imaging system. To determine the brain oxidative stress generated from free radical production, oxidative stress was monitored by DHE fluorescence in the brain of living mice using IVIS imaging system. In this study, ischemic stroke injury dramatically impaired glucose metabolism [Figure 5] and produced a large amount of free radical (as assayed by IVIS imaging) [Figure 6] in living mice, which may create enormous oxidative stress to damage brain tissues after stroke. Treatment with SYD and rt-PA both significantly ameliorated brain function compromised by ischemic stroke [Figure 5]. SYD and rt-PA both significantly reduced free radical production on day 1 after stroke [Figure 6].

**Molecular impacts of SYD on ischemic mice brain**

To provide more insight into the in vivo influences of SYD on ischemic mice brain, a genome-wide transcriptome analysis was performed. A total of 162 out of 2081 ischemia-influenced probe sets were found significantly reversed by the SYD using transcriptome analysis [Figure 7a]. A PCA plot using these 162 probe sets illustrated the differential gene expression patterns between different mice groups. The gene expression pattern of SYD-treated mice was closer to that of sham mice [Figure 7b]. On measuring the average linkage distances between mice transcriptomes and comparing with ischemic mice, SYD-treated ones were found to be closer to sham controls [Figure 7c]. A heat map shows 64 probe sets were commonly more abundant in SYD-treated and sham mice, while another 98 ischemia-induced probe sets were suppressed by SYD [Figure 7d]. The top 50 up- and downregulated genes are listed in Tables 1 and 2, respectively.
The top 50 up-regulated known genes in ischemic stroke mice treated with remedy SYD

| Probe Set ID | UniGene ID | Gene title | Gene symbol | Location |
|--------------|------------|------------|-------------|----------|
| 1427371_at   | Mm.439858  | ATP-binding cassette, sub-family A (ABCA1), member 8a | Abca8a | chr11 E1 |
| 1421024_at   | Mm.8684   | 1-acylglycerol-3-phosphate O-acyltransferase 1 expressed | Agpat1 | chr17 B1|17 18.7 cM |
| 1435417_at   | Mm.27054  | sequence AI464131 | AI464131 | chr4 A5 |
| 1436258_at   | Mm.44841  | Cdc42 guanine nucleotide exchange factor (GEF) 9 | Arhgef9 | chrX C3 |
| 1429190_at   | Mm.300178 | arylsulfatase B | Arsb | chr13 C3-D1|13 50.0 |
| 1426336_at   | Mm.214994 | calcium channel, voltage-dependent, gamma subunit 7 | Cacng7 | cM |
| 1442707_at   | Mm.131530 | calcium/calmodulin-dependent protein kinase II alpha | Camk2a | chr7 A1|7 2.0 cM |
| 1452453_a_at | Mm.131530 | calcium/calmodulin-dependent protein kinase II alpha | Camk2a | chr18 E1|18 33.0 cM |
| 1441572_at   | Mm.167882 | deleted in colorectal carcinoma | Dcc | chr18 E1|18 33.0 cM |
| 1451751_at   | Mm.250841 | DNA-damage-inducible transcript 4-like | Ddit4l | chr18 E2|18 45.0 cM |
| 1419581_at   | Mm.27256  | discs, large homolog 4 (Drosophila) distal-less homeobox 1, | Dll4 | chr3 G3 |
| 1419845_at   | Mm.4543   | antisenes deosmplakin | Dlx1as | chr11 B4 |
| 1435494_s_at | Mm.355327 | Enhancer of polycomb homolog 1 (Drosophila) Fibronectin | Dsp | chr2 C2|2 44.0 cM |
| 1442279_at   | Mm.312133 | leucine rich transmembrane protein 2 frizzled-related | Epc1 | chr13 A3.3 |
| 1447204_at   | Mm.341948 | protein | Flrt2 | chr18 A1 chr12 E |
| 1416658_at   | Mm.427436 | frizzled homolog 2 (Drosophila) | Frzb | chr2 C3|2 49.75 cM |
| 1418533_s_at | Mm.36416  | GC-rich promoter binding protein 1-like 1 | Fzd2 | chr11 E1 chr4 C7 |
| 1449113_at   | Mm.41715  | O protein-coupled receptor 34 | Gpbb11l | chrX |
| 1422542_at   | Mm.391323 | Glutaminase | Gpr34 | A1.3 |
| 1435708_at   | Mm.398608 | inhibitor of DNA binding 4 | Gls | chr1 C1.1|1 25.9 cM |
| 1432529_at   | Mm.458006 | imprinted gene in the Prader-Willi syndrome region | Id4 | chr13 B1|3 31.0 cM |
| 1431229_at   | Mm.335715 | kalirin, RhoGEF kinase | Ipw | chr7/7 28.0 cM |
| 1442341_at   | Mm.441119 | Kv channel-interacting protein 2 | Kalmn | chr16 B3 |
| 1425870_a_at | Mm.213204 | lin-7 homolog A (C. elegans) | Kcnip2 | chr19 D1|19 45.2 cM |
| 1438450_at   | Mm.268025 | membrane-associated ring finger (C3HC4) 6 | Lin7a | chr10 D1 |
| 1445928_at   | Mm.272185 | RAB3C, member RAS oncogene family | Mar6 | chr15 B2 chr13 D2.2 |
| 1432432_a_at | Mm.390643 | RAB3C, member RAS oncogene | Rab3c | chr13 D2.2 |
| 1449494_at   | Mm.390643 | poliovirus receptor-related 3 | Rab3c | chr16 B5 chr6 C3 |
| 1423351_a_at | Mm.328072 | leucine rich repeat transmembrane neuronal 4 | Prrt3 | chr6 |
| 1437214_at   | Mm.94135  | LUC7-like 2 (S. cerevisiae) | Lrtrim4 | B1 |
| 1436767_at   | Mm.276133 | mitogen-activated protein kinase kinase kinase 5 | Luc7l2 | chr10 A3 |
| 1439830_at   | Mm.6595   | Mitochondrial ribosomal protein S18A | Map3k5 | chr17 C |
| 1457813_at   | Mm.287443 | nuclear factor I/A | Mrps18a | chr4 C4-C6|4 45.8 |
| 1421163_a_at | Mm.31274  | netrin G1 | Nfia | cM |
| 1441634_at   | Mm.39262  | O-linked N-acetylglucosamine (GlcNAc) transferase | Ntng1 | chr3 G1 |
| 1436780_at   | Mm.259191 | oxysterol binding protein-like 3 | Ogt | chrX D |
| 1428484_at   | Mm.44153  | p53-associated parkin-like cytoplasmic protein | Osbp13 | chr6 B3 |
| 1427620_at   | Mm.329076 | protocadherin 20 | Parc | chr17 C |
| 1424701_at   | Mm.128861 | phosphatidylinositol transfer protein, cytoplasmic 1 | Pdcd20 | chr14 E1 |
| 1431074_a_at | Mm.439910 | poliovirus receptor-related 3 | Pitpap1 | chr11 E1 |
| 1448673_at   | Mm.328072 | R3H domain 1 (binds single-stranded nucleic acids) | Prcl3 | chr16 B5 |
| 1458539_at   | Mm.221041 | solute carrier organic anion transporter family, member 1c1 | R3hdm1 | chr1 E4 |
| 1423343_at   | Mm.284495 | transducin-like enhancer of split 1, homolog of Drosophila | Slocl1c | chr6 G1 |
| 1422751_at   | Mm.278444 | E(sp1) | Tle1 | chr4 C3 |
| 1432130_a_at | Mm.275710 | tetratricopeptide repeat domain 14 | Ttc14 | chr3 B |
| 1415694_at   | Mm.38433  | tryptophanyl-tRNA synthetase | Wars | chr12 F2 |
| 1436485_s_at | Mm.300397 | whirlin | Whrn | chr4 C1|4 31.4 cM |

**Table 1.** The top 50 up-regulated known genes in ischemic stroke mice treated with remedy SYD

**Figure 6.** IVIS imaging of free radical production in live mice at 24 h after stroke. Animal groups include sham, vehicle treated (stroke), SYD treated (1.0 g/kg, twice daily, p.o., +SYD), and rt-PA treated (10 mg/kg, daily, i.v., +rt-PA). Left panel: Representative IVIS imaging for free radical production; ROI, region of interest. Right panel: Statistical analysis. *P < 0.05 as compared to stroke group analyzed by one-way ANOVA followed by SNK t-test (n = 10 for each group). NA, data not available.
Coordinated changes of functional modules in SYD-treated mice

We next organized SYD-affected genes into functional groups for having better insight into the biological consequences of gene expression changes. According to the GO database, SYD treatment significantly enriched genes involved in the regulation of cell communication and Wnt receptor signaling pathway \( P = 0.01 \) and 0.04, respectively) [Table 3]. Seven genes related to the regulation of cell communication and three Wnt receptor signaling pathway genes were specifically induced in the brains of ischemic mice by SYD [Table 3]. Among the SYD-downregulated genes, genes’ response to stress (13 genes, enrichment \( P \) value = 0.0015), wounding (7 genes, enrichment \( P \) value = 0.0021), inflammation (5 genes, \( P = 0.0110 \), development of vasculature (6 genes, \( P = 0.0027 \)) and blood vessel (5 genes, \( P = 0.0145 \), angiogenesis (3 genes, \( P = 0.0130 \), defense response (7 genes, \( P = 0.0074 \), chemotaxis (4 genes, \( P = 0.0086 \)), immune response (7 genes, \( P = 0.0094 \), cytokine production (4 genes, \( P = 0.0165 \), antigen processing and presentation (3 genes, \( P = 0.0445 \), and leukocyte-mediated cytotoxicity (2 genes, \( P = 0.0545 \)) was reverted by SYD [Table 3]. The details of enriched GO categories, as well as the genes involved can be found in Tables 1-3.
Table 3. Functional module analysis for SYD-induced neuroprotection in ischemic stroke mice.

| SYD-induced GO terms                      | Count | %    | P value |
|-------------------------------------------|-------|------|---------|
| Regulation of cell communication          | 7     | 12.96296 | 0.010505 |
| Wnt receptor signaling pathway            | 3     | 5.55556 | 0.040555 |
| SYD-repressed GO terms                    |       |       |         |
| Response to stress                        | 13    | 16.83132 | 0.001596 |
| Response to wounding                      | 7     | 9.090909 | 0.002142 |
| Vasculature development                   | 6     | 7.792208 | 0.002744 |
| Defense response                          | 7     | 9.090909 | 0.007438 |
| Chemotaxis                                | 4     | 5.194805 | 0.00861 |
| Immune response                           | 7     | 9.090909 | 0.009402 |
| Inflammatory response                     | 5     | 6.493506 | 0.011088 |
| Regulation of angiogenesis                | 3     | 3.896104 | 0.013076 |
| Blood vessel development                  | 5     | 6.493506 | 0.014564 |
| Regulation of cytokine production         | 4     | 5.194805 | 0.016565 |
| Antigen processing and presentation       | 3     | 3.896104 | 0.044586 |
| Leukocyte mediated cytotoxicity           | 2     | 2.597403 | 0.045459 |

DISCUSSION

Although SYD has been reported to be neuroprotective against brain injury in clinical and animal studies, its mechanism of action based on a brain functional and a genome-wide transcriptome analysis has not been revealed. Our results demonstrate for the first time that treatment with SYD (1.0 g/kg, p.o., twice daily) shows a protective effect against ischemic stroke in mice and significantly extends their survival rate and lifespan, as compared with vehicle-treated ischemic stroke mice. The neuroprotective effect of SYD is effective up to at least 3 days after ischemic injury (around 40% survival), and is more potent than rt-PA in the improvement of neurological function, indicating that novel mechanism(s) or targets could be involved in the neuroprotective effects of SYD on ischemia-induced brain injury. Here, we reveal the brain protective effect of SYD in living mice that parallels with significant improvement in brain function (by PET imaging) and neurological deficits, as well as reduction of inflammation and oxidative stress (by IVIS imaging) without significant modulation of the hemodynamic, arterial blood gas, or physiological conditions.

The major pathological mechanisms leading to ischemic brain injury include ionic imbalance, oxidative/nitrosative stress, inflammatory responses, and apoptotic cell death, and all these mechanisms have been linked to excitotoxicity, an inappropriate activation of ionotropic NMDA receptors, in particular CaMKII. In this study, our results showed that ischemia induced a dramatic production of free radicals, which could be measured by oxidized DHE in living mice (by IVIS). Treatment with SYD dramatically reduced the oxidative stress and spared the tissue against ischemia-induced brain infarction. An inflammatory cascade is initiated in the tissue damaged by free radicals, which leads to complement activation (e.g., C5a), firm adhesion molecule upregulation, microglial activation, leukocyte infiltration, chemotaxis, and release of pro-inflammatory cytokines that may amplify inflammation through activation of inflammatory signals (e.g., transcription factors) for induction of brain infarction.

A pattern recognition analysis illustrated that SYD treatment reversed ischemia-induced brain damage at a molecular level (162 genes reversed) [Figure 7]. Among these genes modulated by SYD, proinflammatory cytokine/chemokine receptor, cytotoxic T lymphocyte–associated proteins and genes promoting inflammation, such as Cxcl1, Fcgr3, Ck8, Ck8b, Ifi202, Ifitm6, CD109, CD163, CD1d1, and CD3001f, were suppressed by SYD. We showed for the first time by GO database-based gene set enrichment analysis that SYD also upregulated a neuroprotective gene, camk2a, which is involved in the calcium signaling pathway. The gene product CaMKII is a member of calcium/calmodulin-dependent kinase (CaMK) cascade, which is well-established for its effects on modulating neuronal synaptic plasticity and learning and memory. SYD also significantly potentiated the expression of a protective factor in the damaged area [e.g., frizzled-related protein (Frzb), alias Sfrp3], a secreted protein activating the Wnt survival and proliferating pathway, was induced by SYD [Tables 1 and 3]. On the other hand, genes involved in wounding, stress, and acute inflammation were reverted by SYD [Table 3]. Leukocyte-mediated cytotoxicity, immune response, and chemotaxis genes were all reduced after SYD treatment, implying the prevention of leukocyte infiltration in damaged regions, as well as
the rescue of inflammation response and prevention of neuron cells from inflammation-associated cell death. Furthermore, S100 calcium-binding proteins (e.g., S100a9) have been reported to display many proinflammatory functions and act as damage-associated molecular pattern molecules. Here, we observed that S100a4, S100a6, S100a8, and S100a9 were all significantly downregulated Table 2, indicating that damaged BBB and inflammation could be compromised by SYD. Similar observation using DNA microarray chips containing 512 cDNA probe also identified S100a9 as one of the six potential targets downregulated by an effective TCM remedy.

Angiogenesis genes such as vascular endothelial growth factor (VEGF) and its receptor Flk1 have been reported to be upregulated by an effective TCM remedy for stroke protection in rats on days 7-14 after stroke. In our array analysis, however, blood vessel and vasculature development as well as angiogenesis genes were less active in SYD-treated mice Tables 1 and 2. This could be due to the upregulation of VEGF signaling, mediating increased BBB permeability, possibly by enhancing VEGF-mediated activation of Rac-1 caused free radical generation and anti-VEGF receptor antagonist (VGA1155) has been demonstrated to reduce infarction in rat permanent focal brain ischemia. According to these observations, we propose that suppression of angiogenesis-related pathways by SYD may be due to the fact that at the time point (day 1 after stroke) we harvested brain RNA for transcriptome analysis, ischemia-induced inflammatory responses, especially BBB leakage, may have been ameliorated by SYD in vivo, so blood vessel formation is no longer active in vivo.

In conclusion, our results reveal for the first time that the neuroprotective effect of SYD on ischemic stroke–induced brain injury in mice may depend on modulation of multiple molecular targets (162 genes) and pathways involved in the downregulation of stress, inflammation, immune response, and angiogenesis, as well as upregulation of neurogenesis-related signals (Frzb/Wnt) and regulation of cell communication Figure 8. Our results provide a possible explanation based on a genome-wide transcriptome analysis integrated with neurofunctional assay, and the opportunity for the evaluation of SYD, a typical traditional Chinese medicine formula, in the treatment or combination therapy of cerebral ischemia–associated diseases.

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