Gene Expression Profiling Confirms the Dosage-Dependent Additive Neuroprotective Effects of Jasminoidin in a Mouse Model of Ischemia-Reperfusion Injury

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1. Introduction

Cerebral ischemia is a fatal disease, in which there is insufficient blood flow to the brain to meet metabolic demand; therefore, it leads to cerebral hypoxia and subsequent brain tissue death. The biochemical and molecular mechanisms triggered by ischemia, including reperfusion injuries and alterations in multiple genes, can cumulatively lead to progressive neurological damage and cell death [1]. Jasminoidin (JA) is a herbal extract that has shown to have protective effects in cases of ischemia, in a dosage-dependent manner [2]. Previous studies in rats with cerebral ischemia have shown that JA's pharmacological mechanisms involve multiple pathways [3], but its dosage effect remains poorly understood. It is therefore essential to elucidate the relationship between the complex pharmacological mechanisms and...
different JA dosages in cerebral ischemia, that is, assessing whether and how altered dosages affect the protective drug mechanisms.

JA is commonly used in traditional Chinese and clinical medicine to treat stroke, especially in Qingkailing [4]. The pharmacological properties of JA include neuroprotective, anti-inflammatory, cholangiara, anticoagulant, and anti-oxidative effects [2, 5]. Previous studies have demonstrated that JA affects multiple signaling cascades such as ATP-binding cassette (ABC) transporter [6], NF-κB [7], PI3K [8], TLR4 [9], and mitogen-activated protein kinase (MAPK) [6] signaling pathways.

In order to assess the similarities and differences of pharmacological mechanisms elicited by different JA dosages, we used the GeneGo MetaCore™ software [10], which was based on well manually curated database of protein-protein, signaling, and others, to analyze the data obtained from mice brain expression microarrays in our previous study [2]. This high-throughput data supported software was used to identify the differential pathway networks affected by JA in a rodent model of cerebral ischemia-reperfusion injury. The aim of this study was to determine the JA's dosage effect on its pharmacological mechanism in the treatment of cerebral ischemia, through analysis of signaling pathways and networks activated after JA and 2*JA administration.

2. Results

2.1. Pharmacodynamic Results. In our previous study, histological analysis observed that the Nissl bodies in hippocampus samples of Vehicle were considerably depleted, while the loss of Nissl bodies in JA group was significantly alleviated [11]. As shown in Figure 1, both 2*JA and JA were effective in reducing ischemic infarction volume when compared with the Vehicle group, with 2*JA showing the lowest level and thus the best protective result.

2.2. Comparison of Enriched Pathways among Treatment and Vehicle Groups. The pathways enriched in the Vehicle group were first examined to elucidate the pharmacological meaning of each treatment. Those statistically significant were identified by GeneGo MetaCore. The top 10 enriched pathways in the Vehicle, JA, and 2*JA groups are shown in Figures 2(a)–2(c), with each item ranked by calculated $p$ values. As shown in the Venn diagram of Figure 2(d), 3 top pathways overlapped between the JA and Vehicle groups, while 1 was found in both 2*JA and Vehicle groups. Of note, the $G$-protein signaling, $G$-protein alpha-i signaling cascades pathway found in the Vehicle and JA groups was not in 2*JA's top ten pathways. Besides, the apoptosis and survival, TNFRI signaling pathway was shared by all 3 groups (Figure 2(d)); importantly, JA and 2*JA treated animals shared only three top pathways, with the remaining seven exclusively enriched in the 2*JA group.

The pathways exclusively enriched in the JA and 2*JA groups might be involved in the pharmacological mechanisms of these regimens in the treatment of cerebral ischemia.

2.3. Comparison of Enriched Process Networks among Treatment and Vehicle Groups. The process networks enriched in various groups were also identified by GeneGo MetaCore. The top 10 enriched process networks in the Vehicle, JA, and 2*JA groups might be involved in the pharmacological mechanisms of respective regimens in the treatment of cerebral ischemia.

2.4. Comparison of JA and 2*JA Induced Changes

2.4.1. Enriched Pathway Comparison between JA and 2*JA. The 3 overlapping pathways between the JA and 2*JA groups included Gamma-Secretase proteolytic targets, Apoptosis and survival, TNFRI signaling pathway, and regulation of lipid metabolism, Stimulation of arachidonic acid production by ACM receptors. A total of 5 and 7 top 10 ranked pathways were exclusively represented in the JA and 2*JA groups, respectively. For instance, G-protein signaling, EDG5 signaling was found in the JA group list, while Gamma-Secretase regulation of neuronal cell development and function, Development, Role of Activin A in cell differentiation and proliferation, and Cytoskeleton remodeling, TGF-β, WNT, and cytoskeletal remodeling were represented in the 2*JA group (Figure 2(d)).

Take two significant pathways in the 2*JA group as examples, that is, Gamma-Secretase proteolytic targets (Figure 4(a)) and Gamma-Secretase regulation of neuronal cell development and function (Figure 4(b)). In these two pathways, 5 out of 8 downregulated targets overlapped, including ADAM10, APP, Amyloid beta 40, AICD, and alpha APPs; other molecules such as CTFs were only found in the Gamma-Secretase
### Top 10 pathway of Vehicle group

1. Immune response_CD137 signaling in immune cell
2. Cell adhesion_Cell-matrix glycoconjugates
3. Cardiac Hypertrophy_NF-AT signaling in Cardiac Hypertrophy
4. Immune response_Th1 and Th2 cell differentiation
5. Apoptosis and survival_Lymphotoxin-beta receptor signaling
6. Cell adhesion_Cadherin-mediated cell adhesion
7. Regulation of degradation of deltaF508 CFTR in CF
8. Cell adhesion_Role of CDK5 in cell adhesion
9. apoptosis plus 090609
10. Apoptosis and survival_Role of IAP-proteins in apoptosis

Maps:

(a)

### Top 10 pathway of JA group

1. Development_PIP3 signaling in cardiac myocytes
2. Development_HGF signaling pathway
3. Immune response_IL-2 activation and signaling pathway
4. Immune response_Oncostatin M signaling via MAPK in mouse cells
5. G-protein signaling_EDG5 signaling
6. Immune response_Oncostatin M signaling via MAPK in human cells
7. Transcription_Role of AP-1 in regulation of cellular metabolism
8. Development_Growth hormone signaling via PI3K/AKT and MAPK cascades
9. Development_Leptin signaling via JAK/STAT and MAPK cascades
10. Development_Fh3 signaling

Maps:

(b)

### Top 10 pathway of 2·JA group

1. Gamma-secretase proteolytic targets
2. Gamma-Secretase regulation of neuronal cell development and function
3. Atherosclerosis_Role of ZNF202 in regulation of expression of genes involved in Atherosclerosis
4. Methionine metabolism
5. Regulation of metabolism_Bile acids regulation of glucose and lipid metabolism via FXR
6. Cell cycle_Regulation of G1/S transition (part 1)
7. Regulation of lipid metabolism_Stimulation of Arachidonic acid production by ACM receptors
8. Development_Role of Activin A in cell differentiation and proliferation
9. Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling
10. Neurophysiological process_HTR1A receptor signaling in neuronal cells

Maps:

(c)

### Top 10 GeneGo pathway comparison

1. Gamma-secretase regulation of neuronal cell development and function
2. Regulation of lipid metabolism_Stimulation of Arachidonic acid production by ACM receptors
3. Methionine metabolism
4. Regulation of metabolism_Bile acids regulation of glucose and lipid metabolism via FXR
5. Development_Role of Activin A in cell differentiation and proliferation
6. Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling
7. Neurophysiological process_HTR1A receptor signaling in neuronal cells

Maps:

(d)

**Figure 2:** Pathway profiles in different groups analyzed with GeneGo. (a–c) The top 10 GeneGo pathways of each group are shown; the overlapping cascades between Vehicle and JA (2, in green) and JA and 2·JA (2, in blue) and among the three groups (1, in red) are marked; no overlapping pathways were shared between Vehicle and 2·JA groups. (d) Overlapping and specific pathways of each group are presented in a Venn diagram.
proteolytic targets pathway, whereas beta APPs and NMDA receptor were only present in the other pathway.

2.4.2. Comparison of Process Networks between JA and 2JA Groups. Four process networks were overlapped between the top 10 processes of the JA and 2JA groups, for example, signal transduction-Neuropeptides signaling pathways (Figure 3(d)). It is notable that 2JA group had 6 exclusive and representative processes, such as the Development_Neurogenesis: Synaptogenesis and cell adhesion_Amyloid proteins. Besides, JA group displayed 3 exclusive processes.

Next, we focused on the signal transduction-Neuropeptides signaling pathways, which are the most enriched processes in both the 2JA and JA groups. They contain 28 target genes, with 23 and 17 found in the JA and 2JA groups, respectively. Among them, 12 target molecules were activated by both JA and 2JA treatments (Figure 5, in black), including gamma-MSH, alpha-MSH, and ACTH; PACAP and PACAP receptor 1 were only activated after JA treatment (Figure 5, in blue); PKR1 and PKA-re (cAMP-dependent), among others, were exclusively found in the 2JA group (Figure 5, in red). Notably, 11 of the 12 target genes were downregulated in both groups. Further the Galpha(i)-specific peptide GPCRs was upregulated in the 2JA group but downregulated in JA group, indicating that 2JA could reverse target expression levels within the same process.
Figure 4: Two pathways of genes altered in the 2JA group. Gamma-Secretase proteolytic targets (a) and Gamma-Secretase regulation of neuronal cell development and function (b). The map legend can be viewed at http://lsresearch.thomsonreuters.com/static/uploads/files/2014-05/MetaCoreQuickReferenceGuide.pdf.

2.4.3. The Molecular Function Distribution of JA and 2JA Groups. The comparative analysis of the top 10 pathways and process networks of the 2 treatment groups found 3 common pathways and 4 common processes (Figures 2 and 3). Although common pathways and processes were identified, the differentially expressed genes in the 2JA group were significantly different from those obtained after JA treatment.

Seven of the top 10 pathways in the 2JA group were involved in neuroprotection, 40% in apoptosis and survival, 30% in the regulation of lipid metabolism, and 20% in anti-inflammatory reactions, all annotated by GeneGo. On the other hand, 40% of the top 10 pathways in the JA group were involved in neuroprotection and antiapoptosis, 10% in neuroprotection and anti-inflammation, and 10% in neuroprotection, antiapoptosis, and anti-inflammation.

2.5. Ischemia Pathways Observed in the Vehicle Group Were Not Activated in JA and 2JA. Seven of top 10 pathways in the Vehicle group were no longer activated by either JA or 2JA. For example, Immune response_CD137 signaling in immune cell (Figure 6), JNK, and NIK signaling pathways were activated in the Vehicle group. The activation of JNK contributes to cell death in brain ischemia through both necrosis and apoptosis pathways [12]; and NIK blocks both classical and alternative nuclear factor-κB (NF-κB) activation pathways and reduces the expression of several prosurvival and antiapoptotic factors in brain ischemia [13]. In cell adhesion_Role of CDK5 in cell adhesion, the upregulated activity of CDK5 was shown to contribute to neuronal death following ischemia [14]. These pathways were inhibited after treatment with either JA or 2JA, demonstrating that pathways contributing to brain injury after ischemia in the Vehicle group were altered.

3. Discussion

In this study, gene expression microarrays and GeneGo systematic pathway analysis elucidated the dose-effect mechanism of 2JA. Based on the enriched pathways, molecular processes, and key targets of JA and 2JA, it was consistently shown that the molecular mechanisms underlying the 2JA treatment of cerebral ischemia were significantly altered when compared to a single JA dose.

Among the top 10 enriched pathways, 2JA and JA both affected neuroprotection, anti-apoptosis, anti-inflammation, and lipid metabolism. However, 2JA treatment resulted in more pronounced effect on neuroprotection (70%) while JA was more anti-apoptotic (60%); 2JA and JA played nearly the same role in anti-inflammation and regulation of lipid metabolism (Figure 7). These findings demonstrated that the altered dosage changes the function distribution, which may constitute a mechanism for double JA dosage's additive therapeutic effect in the treatment of cerebral ischemia.

Compared with JA, pathways specific to 2JA were mainly involved in neuroprotection, apoptosis and survival, anti-inflammation, and regulation of metabolism. There is overwhelming evidence that these biological pathways are important therapeutic mechanism in ischemia therapy [15]. For example, Gamma-Secretase regulation of neuronal cell
development and function is related to Gamma-Secretase, an important pathway that may help combat cerebral ischemia and that is discussed further ahead. Its regulation by 2JA had antiapoptotic and neuroprotective effects on neuronal cells after ischemia by targeting apoptotic protease, cleaved caspase-3, and NF-κB, among others [16]. Development_Role of Activin A in cell differentiation and proliferation was another 2JA’s signature pathway. Activin A, a member of the transforming growth factor-beta (TGF-β) family, is an endogenous neuronal survival factor increased by 2JA; after acute brain injury, it may have proven beneficial therapeutic activity against cerebral ischemia [17]. Indeed, Activin A protects neurons against oxidative challenge and promotes tissue survival after focal cerebral ischemia/reperfusion, with an extended therapeutic window [18]. In Cytoskeleton remodeling, TGF-β, WNT, and cytoskeletal remodeling, 2JA regulated the TGF-β and WNT signaling pathways. Studies have indicated that increased TGF-β signaling after stroke is neuroprotective and likely to be an important target for future stroke therapies [19, 20]. WNT signaling can enhance neurogenesis and improve neurological function after ischemic injury [21, 22]; it also has anti-inflammatory effects [23] and is neuroprotective in stroke [24]. Besides, Atherosclerosis_Role of ZNF202, Regulation of metabolism_Bile acids, regulation of glucose, and lipid metabolism via FXR, Neurophysiological process_HTR1A receptor signaling in neuronal cells, are also pathways that play important roles in neuroprotection [25–28]. Taken together, the pathways exclusively identified in the 2JA group were mostly related to inflammatory response, neuroprotection, apoptosis, and survival, which may constitute the double dosage additive mechanism of JA in the treatment of cerebral ischemia.

As mentioned above, the significant nodes ADAM10, APP, Amyloid beta, AICD, CTFs, and NMDA receptor may be the key targets related to 2JA effect (Figure 4), since their expression level changing activated 2 of the top 10 pathways, including Gamma-Secretase proteolytic targets and Gamma-Secretase regulation of neuronal cell development and function. ADAM10 has been shown to mediate repair in response to neuronal damage in the brain [29]; it is an important enzyme for proteolytic processing of amyloid precursor protein (APP) [30]. Cleavage products of APP include Amyloid beta, Alpha APPs, beta APPs, AICD, and CTFs. Several studies have demonstrated the neuroinflammatory effects of Amyloid beta...
Figure 6: JNK1 and NIK pathways were upregulated and contributed to cell death in brain ischemia in the Vehicle group.

[31]; *alpha APPs* have been reported to have neurotrophic and neuroprotective properties; *beta APPs* seemed to have a proapoptotic function [31]. *AICD* (amyloid precursor protein intracellular cytoplasmic/C-terminal domain) regulates *Amyloid beta* degradation [32]; meanwhile, degradation of *CTFs* (APP C-terminal fragments) can suppress *Amyloid beta* generation [32, 33]. The common variants of *Amyloid beta* are *amyloids beta 40 and 42*, of which only the monomeric forms exhibit neuroprotective and antioxidant effects [34]. It has also been shown that *NMDA receptor* activation leads to *ADAM10* upregulation and induces *APP* breakdown [30]. These important nodes may be the potential targets of 2JA, providing neuroprotection and antiapoptotic effects in the treatment of cerebral ischemia.

With reference to the process networks, both 2JA and JA acted on *signal transduction-neuropeptides signaling pathways* and the differential expressed genes, such as *alpha-MSH, ACTH, GPCRs, beta-LPH, and POMC*, which play important roles in ischemia injury. The family of *GPCRs* involved in the *c-AMP/PKA* pathway is known to provide neuroprotection against ischemic injury [35] and activate *CREB* for neuronal cell-survival after ischemia injury [36]. *Alpha-MSH* confers anti-inflammatory [37], antioxidative, and antiapoptotic [38] effects after ischemia. In addition, the neuroprotective, anti-inflammatory, and antiapoptotic effects of *ACTH* have been demonstrated [39, 40]. In the JA group, *PACAP*, an exclusive target, is involved in antiapoptosis [41] and neuroprotection [42]; its receptor *PACAP receptor 1* is neuroprotective [43]. *Neuromedin U, PKR 1, PKA-reg (cAMP-dependent), APOE, and TBRI* may constitute key nodes related to 2JA’s effect. *PKR 1* is involved in angiogenesis and inflammation [44, 45]. The neuron-specific transcription factor *TBRI (T-box brain 1)* is involved in the second process *Development_Neurogenesis: Synaptogenesis* and is known to regulate brain development [46] and to play a role in adaptive cytoskeletal remodeling [47]. Targets with altered expression in *cell adhesion_Amyloid proteins*, the forth process, were also noteworthy: *Amyloid beta, ADAM10, APP, alpha APPs, and WNT* are related
Figure 7: Altered genes representing 2-JA (in blue), JA (in green), and Vehicle (in red) that target pathways involved in cerebral ischemia. Lipid metabolism, apoptosis and survival, inflammation pathways, and others were affected.

to neuroprotection, anti-inflammation, and antiapoptosis. These important nodes are potential targets involved in the pharmacological mechanism of 2-JA in the treatment of cerebral ischemia.

In future investigations, the differential expression of some of these target genes should be tested quantitatively. Ideally, the expression of main molecules (ligands, receptors, and effectors such as transcription factors) of the more relevant pathways and networks associated with each therapeutic group (JA and 2-JA) will be quantitatively monitored. Such data would enable further understanding of the pharmacological mechanism and potential targets of JA’s dosage effect.

4. Conclusion

System-based pathway and network analysis of transcriptomic microarrays revealed that 7 pathways and 3 process networks of the 10 most significantly regulated after 2-JA treatment mainly affect neuroprotection (70%), apoptosis and survival (40%), and anti-inflammation (20%). These pathways, which include Gamma-Secretase regulation of neuronal cell development and function, alpha-MSH, ACTH, PKRI, and WNT, and the overall altered functional profiles reveal the molecular mechanism of 2-JA dosage’s effect in treatment of cerebral ischemia.

5. Methods

5.1. Animals. A total of 48 healthy specific pathogen-free adult male Kunming mice (12 weeks old, 38 to 48 g) were housed at 25 °C in a 12-hour light/dark cycle environment. The exploratory assessment of dose-effect relationship focused on individual- and double-JA dosages. Therefore, mice were randomly divided into 4 groups (each consisting of 12 samples): Sham, Vehicle, JA, and 2-JA. Protocols for animal use were reviewed and approved by the Ethics Review Committee for Animal Experimentation of the China Academy of Chinese Medical Sciences; all animal experiments were conducted in accordance with the Prevention of Cruelty to Animals Act of 1986 and the National Institute of Health guidelines for care and use of experimental laboratory animals.

5.2. Middle Cerebral Artery Occlusion. For the Vehicle, JA, and 2-JA groups, mice were intraperitoneally anaesthetized with 2% pentobarbital (40 mg/Kg) and surgery was carried out to induce middle cerebral artery occlusion (MCAO). The focal cerebral ischemia-reperfusion model was induced by occluding the left middle cerebral artery for 1.5 h with an intraluminal filament followed by reperfusion for 24 h. For sham treated animals, the external carotid artery was surgically prepared, but no filament was inserted. During the experimental procedures, blood pressure, blood gas levels, and glucose amounts were monitored; in each animal, rectal temperature was maintained at 37.0–37.5 °C with a heating pad; body temperature was kept at 37 °C with a thermostatically controlled infrared lamp; brain temperature (monitored with a 29-gauge thermocouple in the right corpus striatum) was maintained at 36–37 °C with a temperature-regulating lamp. An electroencephalogram was acquired to ensure isoelectricity during the ischemic period. Operational success was determined based on infarct volume. The infarct volume of each group was calculated using a Pathology Image
5.3. Drug Administration. The animals were treated as follows: sham group: 0.9% NaCl; Vehicle group: 0.9% NaCl; JA group: 25 mg/ml Jasminoidin; 2 JA group: 50 mg/ml Jasminoidin. The drug preparation used was a chemically standardized product obtained from the National Institutes for Food and Drug Control, which was validated by fingerprint chromatographic methodologies. The herbal preparations were dissolved in 0.9% NaCl immediately before use. All preparations were administered by tail vein injection at the same time point, 1.5 h after focal cerebral ischemia induction at 2 ml/kg. The preparations were chemically standardized products obtained from the China Natural Institute for the Control of Pharmaceutical and Biological Products or the Beijing University of Traditional Chinese Medicine.

5.4. RNA Isolation. The left hippocampus samples of 9 mice per group were homogenized in TRizol® Reagent (Invitrogen, USA). According to the manufacturer's instructions, total RNA was extracted using the RNeasy Micro Kit (Qiagen, Valencia, CA). The RNA integrity was determined by the 26S/18S ratio, using a Bioanalyzer microchip device (Agilent, Palo Alto, CA, USA).

5.5. Microarrays. Gene expression profile was assessed using a mouse brain array (BoaoCapital, Beijing, China), which consists of 374 stroke-related cDNAs, in-house prepared with a microarray chip for the whole mouse genome (Incyte Genomics, Santa Clara, CA, USA). Duplicate clones were imprinted on each chip, generating 4 technical replicates per clone. A single intensity value for each clone was generated by averaging quadruplet measurements after smoothing spline normalization. All clones were verified by DNA sequencing. RNA from the Vehicle group was pooled and labelled with Cy3, while Cy5 was used for the other groups. Microarrays were hybridized, washed, and scanned according to standard protocols. For each group, the above procedures were repeated in at least biological triplicates and technical quadruplets.

5.6. Microarray Data Analysis. All experimental data were uploaded into the ArrayTrack system. Experimental analysis was based on the Minimum Information about Microarray and the Microarray Quality Control Project, and the results were submitted to the Array Express database (Accession number: E-TABM-612). All microarray data were normalized by locally weighted linear regression to reduce experimental variability (smoothing factor: 0.2; robustness iterations: 3). One-way analysis of variance (ANOVA) and Significance Analysis of Microarrays (SAM) were used to compare the means of genes altered between JA or 2JA treated animals and the Vehicle group. The differentially expressed genes (DEGs, \( p < 0.05 \)) were selected for further analyzed; the DEGs of JA and 2JA were listed in Supplementary Table 1. This cutoff value was set to identify molecules significantly differentially regulated, which were labelled as Network Eligible Molecules (NEMs). Networks of NEMs were algorithmically generated based on connectivity. Right-tailed Fisher's exact test was used to calculate the probability (\( p \) value) that each biological function assigned to a network was due to chance alone. The significance of the association between these genes and the canonical pathways was measured by one of the following 2 ways:

1. A ratio of the number of genes from the dataset mapping the pathway by the total number of genes mapping to the canonical pathway
2. A \( p \) value, calculated by Fisher's exact test, determining the probability that the association between the genes and canonical pathway was explained by chance alone. The level of statistical significance was set at 0.05. Finally, canonical pathways with \( p < 0.05 \) and a log fold change > 1.5 screened and analyzed.

5.7. Network Calculation of Enrichment. Enrichment analysis is a computational method for identifying the functional distribution of genomic/proteomic expression profiles and significantly enriched functional categories [48]. Biological processes, subcellular locations, and molecular function distributions of differentially expressed genes were computed using MetaCore based on Gene Ontology annotations [49]; the network distribution of selected genes was computed using MetaCore based on GeneGo network ontologies.

5.8. Statistical Analysis. Significance of enrichment and pathways was evaluated with scores produced using the Expression Analysis Systematic Explorer software (National Institute of Health, USA) [50], which employs modified Fisher's exact test [51]. To assess pathways, the statistical significance of ontology matches was evaluated as the probability of a match to occur by chance, taking database size into account. Lower significance, which denoted higher ratings for matched terms, was expected as the number of genes/proteins belonging to a single process/pathway increased, with \( p < 0.05 \) considered statistically significant.

Data Availability
The microarray data used to support the findings of this study have been deposited in the ArrayExpress repository (E-TABM-612).

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Authors’ Contributions
Haixia Li and Jingtao Wang contributed equally to this work.

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Supplementary Materials

Supplementary Table 1: there are differentially expressed genes (DEGs) in IA and 2JA groups. (Supplementary Materials)

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