Convergent and Divergent Pathways Decoding Hierarchical Additive Mechanisms in Treating Cerebral Ischemia–Reperfusion Injury

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SUMMARY

Introduction: Cerebral ischemia is considered to be a highly complex disease resulting from the complicated interplay of multiple pathways. Disappointedly, most of the previous studies were limited to a single gene or a single pathway. The extent to which all involved pathways are translated into fusing mechanisms of a combination therapy is of fundamental importance. **Aims:** We report an integrative strategy to reveal the additive mechanism that a combination (BJ) of compound baicalin (BA) and jasminoidin (JA) fights against cerebral ischemia based on variation of pathways and functional communities. **Results:** We identified six pathways of BJ group that shared diverse additive index from 0.09 to 1, which assembled broad cross talks from seven pathways of BA and 16 pathways of JA both at horizontal and vertical levels. Besides a total of 60 overlapping functions as a robust integration background among the three groups based on significantly differential subnetworks, additive mechanism with strong confidence by networks altered functions. **Conclusions:** These results provide strong evidence that the additive mechanism is more complex than previously appreciated, and an integrative analysis of pathways may suggest an important paradigm for revealing pharmacological mechanisms underlying drug combinations.

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Introduction

Cerebral ischemia is one of the leading causes of death and disability in the world [1], which is considered to be a highly complex disease resulting from the complicated interplay of multiple pathways including excitotoxicity, acidotoxicity, and ionic imbalance induced by membrane depolarization, release of the pathological excitatory neurotransmitter glutamate, and intracellular calcium overload; peri-infarct depolarizations caused by cortical spreading depression; deleterious levels of reactive oxygen species produced by mitochondria due to high levels of intracellular Ca^{2+} , Na^{+} , and ADP; the responsibilities of cellular response, cytokine response, and response to Toll-like receptor (TLR) activation for inflammation; apoptosis triggered by oxygen free radicals, death receptor ligation, DNA damage, protease activation, and ionic imbalance [2]; and of course other unknown mechanisms of necroptosis [3]. These processes share overlapping and redundant features and cause injury to neurons, glia, and endothelial cells. Hence, it may be unrealistic to expect that a single drug will demonstrate sufficient benefit in cerebral ischemia. Two or more drugs in combination have the potential for improved efficacy through a variety of

synergistic or additive mechanisms [4–7]. Disappointedly, most of the previous studies were limited to a single gene or a single pathway. Encouragingly, it has been shown recently that compound/drug for certain complex diseases could act on multiple genes, pathways, and targets [8,9]. Furthermore, based on the theory of systems biology, network pharmacology, mapping the polypharmacology network onto the human disease-gene network, reveals that drugs often act on multiple targets and these drug targets are often involved with multiple diseases, which may provide us a new approach of drug discovery for complex diseases [10,11].

Scutellaria and Gardenia have been used to treat stroke patients in China for thousands of years. Baicalin (BA, effective component of Scutellaria) and jasminoidin (JA, effective component of Gardenia) were extracted from Qingkailing injection that could significantly reduce the ischemic infarct volume in our previous studies [8,9]. Additionally, a study found that in the view of pharmacodynamics, the combination of BA and JA based on a new model of compatibility was superior to BA or JA monotherapy in the treatment for cerebral ischemia in rats [12]. We have a great interest in investigating the pharmacological mechanism of combination therapies through the methodology of network pharma-

cology, so the Ingenuity Pathway Analysis (IPA) system was applied for further analyses. IPA can provide us a knowledge base, and a powerful data support about the omics was used to analyze the differentially expressed genes, which may help us identify statistically significant signaling pathways and network functions. The results of our study will present a network-based analysis of the additive mechanism of compound/drug combination therapy against cerebral ischemia.

Materials and methods

Animals

Animal use protocols were reviewed and approved by the Ethics Review Committee for Animal Experimentation, China Academy of Chinese Medical Sciences, and all animal experiments were conducted in accordance with the Prevention of Cruelty to Animals Act 1986 and the National Institute of Health guidelines on the care and use of laboratory animals for experimental procedures. The process of animal experiment and data analysis is showed in Figure 1. A total of 240 healthy, specific pathogen-free, adult male Kunming mice, 12 weeks of age, weighted 38–48 g, kept at 25°C with a 12 h light/dark cycle, were randomly divided into five groups, with 48 mice in each group.

Middle Cerebral Artery Occlusion

After being anesthetized with 2% napental (4 mg/kg, i.p.), the mice were subjected to middle cerebral artery occlusion (MCAO), ligated with an intraluminal filament for 1.5 h, and then reperused for 24 h. In the sham-operated mice, the external carotid artery (ECA) was surgically prepared for the insertion of the fila-

ment, but the filament was not inserted. During the experimental process, blood pressure, blood gas, and glucose levels were monitored, rectal temperature was maintained at 37.0–37.5°C with a heating pad, body temperature was maintained at 37°C with a thermostatically controlled infrared lamp, and brain temperature (monitored with a 29-gauge thermocouple in the right corpus striatum) was maintained at 36–37°C with a temperature-regulating lamp. EEGs were monitored to ensure isoelectricity during ischemia. Based on the infarction volume or behaviors of these mice, we could determine whether the operations were successful.

Drug Administration

The experimental animals were randomly divided into five groups: vehicle group, sham-operated group, BA-treated group (5 mg/mL), JA-treated group (25 mg/mL), and BJ-treated group (combination of BA and JA, a mixture of equal volumes of BA and JA). The herbal preparations were chemically standardized products obtained from China Natural Institute for the Control of Pharmaceutical and Biological Product or Beijing University of Traditional Chinese Medicine, and their compositions had been validated using fingerprint chromatographic methodologies. All treatment drugs in the form of injection, dissolved in 0.9% NaCl just before use, were into the tail vein 1.5 h after the induction of focal cerebral ischemia. The animals in the vehicle group and the sham-operated group received an injection of saline (2 mL/kg body weight) from the tail vein at the same time point.

Combination Effect Analysis

One hundred and fifty mice were employed to analyze the combined effect of BA and JA (chemical structures shown in Figure 1A).

Figure 1 Validation of additive pharmacological effect between baicalin (BA) and jasminoidin (JA). Combination of BA and JA exerted an additive effect on the infarction volume according to the median-effect method. (A) The chemical structure of BA and JA. (B) BA, JA, and BJ all had dose-dependent effects in reducing the infarction volume. (C) The concentrations of JA and BA ranging from 1 to 16 mg/mL had CI values close to 1.0, suggesting an additive effect between JA and BA.

BA, JA, and their combination at five concentrations ranging among 1, 2, 4, 8, and 16 mg/mL were combined (1:1) to analyze the synergistic or additive effect using the CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). To determine the style of combination effect on infarction volume, the dose–effect curves of single or combined drug treatment were analyzed based on the median-effect method. In the median-effect method, a combination index (CI) value of less than, equal to, or more than 1 indicated synergy, additivity, or antagonism, respectively. The median-effect equation describes dose–effect relationships in the simplest possible term [13], which is given by the dose of a drug; F_a is the fraction affected by drug's dose. The effect (F_a) for any given dose (D) can be determined in this method.

RNA Isolation

The left hippocampus of nine mice from each group was homogenized in TRIzol Reagent, and the total RNA was isolated according to the manufacturer's instructions. RNA was further purified to remove the genomic DNA contamination and concentrated using an RNeasy micro kit (Qiagen, Valencia, CA, USA). The RNA quality was assessed by determining the 26 S/18 S ratio using Bioanalyzer microchip (Agilent, Palo Alto, CA, USA).

Microarray

The mouse brain array (Boao Capital, Beijing, China), a microarray chip containing 16,463 oligoclones (Incyte Genomics, Santa Clara, CA, USA), was used to conduct gene expression profiling. On a given chip, each clone was printed in duplicate spots, generating four technical replicates for each clone. A single intensity value for each clone was generated by averaging the quadruplet measurements after smoothing spline normalization. All clones had been verified by DNA sequencing. RNA from the vehicle group as a pool was labeled with Cy3, while RNA from the other groups was labeled with Cy5. The 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.

Microarray Data Analysis

All experimental data were uploaded to the ArrayTrack system. Experimental analysis was based on the Minimum Information about a Microarray Experiment guidelines and Microarray Quality Control project. The results were submitted to the Array Express database. All microarray data were normalized by locally weighted linear regression (Lowess) so as to reduce the experimental variability (smoothing factor, 0.2; robustness iterations, 3). A one-way ANOVA model and significance analysis of microarrays (SAM) were used to compare the means of the altered genes between sham and vehicle, BA and vehicle, JA and vehicle, and BJ and vehicle groups. Genes with a P value <0.05 and a fold change >1.5 were picked out for further analysis. In addition, an increase >1.5 -fold or a decrease <0.5 -fold of expression levels indicated up-regulation or down-regulation, respectively. And then, all of the significantly differentially expressed genes were

uploaded to the IPA system (<http://www.ingenuity.com/>). A cutoff was set to identify molecules whose expression was significantly differentially regulated. These molecules were called network-eligible molecules. Networks of network-eligible molecules were then algorithmically generated based on their connectivity. Right-tailed Fisher's exact test was used to calculate a P value determining the probability that each biological function assigned to that network was due to chance alone. The significance of association between these genes and the canonical pathway was measured in two ways: (1) a ratio of the number of genes from the data set that mapped to the pathway divided by the total number of genes that mapped to the canonical pathway was displayed, and (2) Fischer's exact test was used to calculate a P value determining the probability that the association between the genes and the canonical pathway was explained by chance alone. The level of statistical significance was set at $P < 0.05$. Finally, canonical pathways with a P value <0.05 and a fold change >1.5 were screened out and analyzed.

Defining Additive Index

Multiple pathways coordinate or interact with one another to achieve a complex biological process that can contribute to the additive effect. On the basis of pathway-components-related variation, we defined the additive exponent additive index (AI) of node j by

$$AI(j) = 1 - \frac{k}{\sum_{i=1}^T P_i} \quad (1)$$

where $AI(j)$ refers to the additive index of any pathway j in drug combination group, P represents the number of statistically significant pathway, which is induced by every compound from i , and k is defined as the total number of pathways stimulated by all the compounds connected with j .

Real-Time RT-PCR Analysis

Three animals from each group were anesthetized with chloral hydrate (400 mg/kg) and decapitated. Real-time polymerase chain reaction (PCR) (50 μ L) was performed using Thermo-fast 96 PCR plates (Bio-Rad Laboratories, Hercules, CA, USA), which were sealed with iCycler IQ optical-quality tapes (Bio-Rad Laboratories) on an iCycler IQ thermocycler (Bio-Rad Laboratories). Each measurement was taken in three replicates. A dilution series of positive control DNA was used in the same plates as the calibration standards. Positive control DNA was generated by the amplification of the gene from samples. The amplicon was purified, cloned into TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA), and reamplified. After purification, the concentrations of PCR products were determined using a fluorometer as described above and were serially diluted to generate calibration standards. Data analysis was carried out with iCycler software (Bio-Rad Laboratories). Based on the standard curve, a threshold cycle measured in a sample was converted to the copy number of the gene in a sample. Forward and reverse primers of regulators of

G-protein signaling 6 (Rgs6) (NM_015812) and SH2-B mRNA (AF036355) were as follows: -f ATGCCAGGAGCACATCTACA, -rGCGACTTTCCTTCTTCTTGG; and -fTGGCTCCCATGGTGTCTTCTT, -rTTAGTGACAAGCGCAGGTGCT, respectively.

Western Blot Analysis

The hippocampus was removed from the brains of the remaining six mice from each group. The proteins (40 µg per lane) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Hybond-C, Amersham, Buckinghamshire, UK) by electroblotting. Blots were stained with the mice anticyclic adenosine monophosphate cAMP-response-element-binding protein (CREB) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was developed by enhanced chemiluminescence (Amersham). Band density was determined with a GS-700 densitometer (Bio-Rad). Each measurement was taken in three replicates.

Results

Effects on Ischemia-Induced Infarction

It was found that BJ was more effective than BA or JA monotherapy in reducing the ischemic infarction volume ($P < 0.05$) [14,15]. The dose-effect curves of BA, JA, and combination therapy (Figure 1B) at concentrations of 1–16 mg/mL had CI values close to 1.0 (Figure 1C), indicating additive effects between the agents.

Canonical Pathways between Different Groups

Compared with the sham group, six statistically significant canonical pathways involving the differentially expressed genes were identified in the vehicle group, this result has been reported in our previous study [16]. In addition, a total of 7, 16, and 6 statistically significant canonical pathways involving the differentially expressed genes were found in BA, JA, and BJ groups (vs. vehicle), respectively ([16] and Figure 2). No more overlapping path-

Figure 2 Significant canonical pathways in all treatment groups. A total of 7, 16, and 6 significant canonical pathways were identified between baicalin (BA) versus vehicle, jasminoidin (JA) versus vehicle, and BJ versus vehicle groups, respectively. The orange vertical line indicates a threshold of $P < 0.05$.

ways were found between two or three groups except Huntington's Disease Signaling existing between JA and BJ groups. All canonical pathways in the three treatment groups presented a certain relationship with cerebral ischemia in previous research (Table S1).

Reconstructing Pathway Network

A network containing 29 nodes and 176 edges was reconstructed based on IPA knowledge (Figure 3A). Diverse relationships were found between these pathways except arachidonic acid metabo-

Figure 3 Construction of pathway network and additive index distribution. **(A)** The network contains 29 nodes and 176 edges. The pathway in the three treatment groups is regarded as a node, and common components as pathways relationship that confirms an edge between the pathway of monotherapy and combination therapy groups (pink and blue lines). The yellow lines represent relationships between pathways of BJ and BJ group, and the gray lines represent relationships between pathways of baicalin (BA) and jasminoidin (JA) group. Network reconstruction is based on pathway correlation in Ingenuity Pathway Analysis (IPA) knowledge base. **(B)** Histogram illustrates the additive index of the pathway in BJ group based on pathway correlation. **(C)** Pathway integration from both compounds; this micronetwork is divided into three regions, that is, immune-related cluster, neuron-related cluster, and ERK/MAPK signaling cascade-related cluster (gray shaded area).

lism in BA group and sulfur metabolism in BJ group. When sharing a component between two pathways, a line linked the two pathways. More lines indicated more relations to other pathways.

Distribution of AI

The histogram presenting the AIs of BJ pathways illustrated a hierarchical distribution (Figure 3B). The AI score of sulfur metabolism was 1, representing soundly additive change from the original pathways in BA and JA groups. However, the AI score of neuropathic pain signaling in dorsal horn neurons was 0.09, indicating a lower additive degree in which many of the original compounds in BA and JA groups could be found in BJ group.

Horizontal and Vertical Pathway Convergence or Divergence

Besides the analysis of additive degrees, additive direction of pathway convergence or divergence was also explored in this study. Those significant pathways of the BA and JA treatment groups could be divided into three regions, that is, immune-related cluster, neuron-related cluster, and ERK/MAPK signaling cascade-related cluster (Figure 3C). When different drugs targeted different clusters, a horizontal additive action might emerge; while a vertical pathway convergence took place between the upper-stream and downstream in a same cluster, broad horizontal and vertical pathway convergences or divergences were identified among those pathways in BJ group. Moreover, both pathway convergence and divergence might appear even in a same cluster. Some additive pathways followed a similar pattern in horizontal or vertical pathway convergence; for example, four pathways of Huntington's disease signaling, CDK5 signaling, cAMP-mediated signaling, and neuropathic pain signaling in dorsal horn neurons in BJ group similarly targeted T Cell receptor signaling in BA group and B Cell receptor signaling in JA group in the immune-related cluster.

Evidence of Pathways Convergence and Divergence

Pathways not only showed convergence or divergence through overlapping nodes, but also acted on sandy targets to produce novel pathways, such as fusing T Cell receptor signaling in BA group and ERK/MAPK signaling in JA group to form Huntington's disease signaling in BJ group. As AI was only 0.13, many original genes in monotherapy groups were still found in this pathway of BJ group. BA and JA affected different pathways and fused those differentially expressed genes to contribute to the additive effects of BJ (Figure 4).

The fold change in gene expression was demonstrated to be consistent in all treatment groups, determined by real-time reverse transcriptase (RT)-PCR and by microarray. For example, Rgs6 had the highest decreasing activity according to both analyses. However, SH2-B expression did not show any significant variation between these groups (Figure 5A). Results of Western blot analysis indicated that the CREB was increased significantly in the pathway of JA and BJ groups; and the p-ERK1/2 level was increased significantly in the pathway of BA, JA, and BJ groups

(Figure 5B), both of which were consistent with the findings displayed in Figure 4.

Subnetworks between Different Groups and Biofunctions Distribution

A total of 726, 361,414, and 465 significantly differentially expressed genes were found between vehicle versus sham, BA versus vehicle, JA versus vehicle, and BJ versus vehicle, respectively. Based on the IPA analysis of those network-eligible identifiers, 5, 8, 9, and 7 high score subnetworks were identified in vehicle, BA, JA, and BJ groups, respectively ([16] and Table S2). The top five networks in the BJ group were shown in Figure 6A–E. According to the molecular functions, IPA analysis indicated that compared with the vehicle group, 70, 74, and 76 statistically significant functions were identified in BA, JA, and BJ groups, respectively, and the percentage of overlapping functions (60) among these three groups was 71.43%. Each function represented a cluster, including the same functional molecules, and these biofunctions were divided into three categories, that is diseases and disorders, molecular and cellular functions, and physiological system development and function (Table S3). Those biofunctions indicated not only the same regulation process in each group but also their own unique tendencies (Figure 6F). Three functions of protein synthesis, RNA posttranscriptional modification, and psychological disorders contributed special actions to the additive mechanism in networks analysis. All of these functions were also demonstrated to have certain relationships with cerebral ischemia in previous literatures (Table S3).

Discussion

We displayed a vivid example that each of the monotherapy drugs and the combination therapy not only shared common targets, but also had their unique targets that may play an important role in the emergence of additive effect. Obviously, as a network, the wiring diagram is determined by overlapping components between pathways, and such a complicated relationship reflects that pathways of BA and JA groups could work together through shared genes, where the additive effect of BJ is created. Evidence suggests that pathways convergence and divergence often intersect and regulate each another to trigger a response to a complex set of stimulation, whether it is positive or negative [17–19]. In our study, multiple pathways coordinate or interact with one another to achieve a complex biological process that may contribute to the additive effect of the combination therapy. A high AI represents a strong variation, even though merely for the pathways of sulfur metabolism and cell cycle regulation by BTG family proteins in this reconstructed network. Beyond that, the construction of another network different from the component correlation-based network reveals pathway integration from both compounds (Figure 3C), and this micronetwork is divided into three regions, namely immune-related cluster, neuron-related cluster, and ERK/MAPK signaling cascade-related cluster. We find that the pathways in BA and JA groups present an interactive connection, including horizontal regulation such as TGF- β signaling and EGF signaling regulating ERK/MAPK signaling, and Amyotrophic Lateral Sclerosis signaling and Huntington's disease signal-

Figure 4 Additive mechanism of pathway convergence and divergence in Huntington's disease signaling pathway. On the basis of Ingenuity Pathway Analysis (IPA) knowledge base, the overlapping components between T Cell Receptor Signaling of baicalin (BA) group and Huntington's disease signaling of BJ group were Ca^{2+} , PIP3, JNK1, PI3K, GRB2, SOS, hRas, and ERK1/2; those between ERK/MAPK signaling of jasminoidin (JA) group and Huntington's disease signaling of BJ group were CREB, SHC, PIP2, IP3, DAG, PI3K, GRB2, SOS, hRas, and ERK1/2; and the overlapping ones among the three groups were PIP2, IP3, DAG, PI3K, GRB2, SOS, hRas, and ERK1/2. Differentially expressed genes in the three groups are marked with pink, blue, green, and yellow arrows, up- and down-regulated genes were labeled with the up and down arrow, respectively. The overlapping components between the different groups are marked with different color stars.

Figure 5 Validation of *Rgs6*, *SH2-B*, cAMP-response-element-binding protein (CREB), p-ERK1/2 expression levels using both real-time polymerase chain reaction (RT-PCR) and Western blot analysis. **(A)** No significant discrepancies were found between the findings of microarray and real-time RT-PCR analyses. **(B)** Western blot analysis was employed to identify CREB and p-ERK1/2 between different groups. * $P < 0.05$ versus vehicle.

ing regulating Apoptosis signaling, as well as vertical regulation such as VDR/RXR activation regulating PDGF signaling and IGF-1 signaling, primary immunodeficiency signaling regulating T Cell receptor signaling and B Cell receptor signaling. Cross talk between pathways in this network displays cross-inhibition, cross-activation, and pathway convergence [20]. In fact, the additive effect of drug combination does not mean the sum of pathways simply, but transmitting information each other, triggering different space-time effect by drug interactions, and generating fire-new phenotypic correlation information set. Specifically, to illustrate the therapeutic mechanism of the combination group, we base on the published literatures to further explain the differentially expressed genes of the pathways in BJ group. Neuropathic pain signaling in dorsal horn neurons arises from an injury discharge originating at the site of nerve injury which may result from stroke and other causes [21]. Molecules such as BDNF and CAMK1G in this pathway in our experimental data showed down-regulated expressions, which can directly or indirectly activate N-methyl-D-aspartate-(NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepro-pionic acid (AMPA) receptor, the dependent receptor of calcium ions, leading to calcium influx restrictedly. NMDA and AMPA receptor antagonists resulted in almost complete neuroprotection [22]. FOSB, BDNF, and EGR1 can cause down-regulation of MAPK cascade, which were down-regulated in CDK5 signaling. A very early expression of MAPKs suggested that MAPKs might be closely involved in signal transduction during cerebral ischemia [23]. Activation of MAPK cascades was involved in neuroprotection against gerbil global ischemia/reperfusion injury [24]. Dnajb1-Hsp70 as a complex structure had a down-regulated expression in Huntington's disease signaling, and this would weaken the expression of HD, whose reduction can effectively control the influx of calcium by

calcium channel and GRIN2B, against the excitotoxic death and release of cytochrome c, cut down the action of calpain, a type of calcium-dependent protease that can activate caspase 3/7 to induce apoptosis. Moreover, a low expression of BDNF can block cell death that exists in the path of suppression of gene transcription from Hdac-Rcor-Rest-Sin3a. Interestingly, recent research shows similar molecular pathological mechanisms between Huntington's disease and cerebral ischemia, such as endoplasmic reticulum (ER) dysfunction, mitochondrial disturbances, excitotoxicity, and neuroinflammation [25–28]. As a second messenger central to a number of biological processes, cAMP-mediated signaling has four down-regulated genes in our findings, that is, GRM3, CAMK1G, DUSP6, and DUSP4. Low expressions of CAMK1G and GRM3 would contribute to the activation from cAMP to ERK; meanwhile, DUSP6 and DUSP4, members of MKP, would weaken the inhibition of ERK. This complex process can help CREB, the cAMP-response-element-binding protein, to perform transcriptional regulation, which plays a critical role in synaptic plasticity and neuronal survival [29]. Consistently, another study revealed that the number of new neurons was increased in cerebral ischemic stroke, which was correlated with the activation of CREB [30]. A down-regulation of BTG2 would reduce the level of activation of the complex structure CDC2-CDK4-BTG1-CNOT7, together with the PPP2R1B lower expression, and resist antiproliferation effectively. Although research on the relationship between BTG family and cerebral ischemia is very limited, BTG protein is considered to be a modulator of cell survival and differentiation and could help to protect against cell death by inhibition of necrosis and/or apoptotic signaling pathways [31]. In the treatment of cerebral ischemia, sulfur metabolism is a very novel factor in the additive mechanism of combination therapies, which may play an important neuroprotective role in the brain during cerebral hypoxia [32]. Previous studies indicated that inorganic sulfur significantly inhibited MMP-2 and MMP-9 expressions in the cells [33], and MMP inhibitors might have an important significance in the treatment for cerebral ischemia, especially antineuroinflammation and reestablishment of cerebral blood flow [34,35].

As a functional community [36], genes with a same molecular function are listed together, which present a kind of biofunction. A high frequency of overlapping biofunctions indicates that a similar regulatory process between BA and JA has been inherited after drug combination, constituting a fusion background. Amino acid metabolism, lipid metabolism, cell cycle, cell death, and immune response from the overlapping functions were thought to have important relevance to cerebral ischemia. It has been shown that branched chain amino acids are reduced in ischemic stroke, and the degree of reduction correlates with worse neurological outcome [37]. Moreover, glutamate as a nonessential amino acid is an excitatory neurotransmitter that plays a major role in the pathogenesis of ischemia brain injury [2]. Excitatory amino acid transporters may provide neuroprotection during cerebral ischemic insult [38]. A relation between lipid alterations and dysfunction of energy metabolism has also been confirmed after brain infarction in rats [39]. Phospholipid degradation with subsequent free fatty acid release and alteration in membrane-bound enzymes may have a direct effect on metabolic machinery and may slow cerebral metabolic rate. Brain lipid metabolism was studied by bilateral common carotid artery ligation, a model for chronic cere-

Figure 6 The top five statistically significant signaling pathway networks between BJ and vehicle groups and Wayne figures of network functions among baicalin (BA), jasminoidin (JA), and BJ groups. The top five statistically significant signaling pathway networks involving the differentially expressed genes between BJ and vehicle groups, with functions in organ morphology, reproductive system development and function, cellular compromise (A); cell-to-cell signaling and interaction, nervous system development and function, cellular movement (B); skeletal and muscular system development and function, tissue morphology, connective tissue disorders (C); cell-to-cell signaling and interaction, cell-mediated immune response, organ morphology (D); and cell signaling, molecular transport, vitamin, and mineral metabolism (E). The red node denotes an up-regulated gene, and the green node denotes a down-regulated gene. The intensity of colors indicates the degree of up-regulation or down-regulation. (F) Only significant functions are shown in this figure based on a P value <0.05 and a fold change >1.5 between groups.

bral hypoperfusion, which showed that dysbolism might be caused by compensatory secretory phospholipase A (2)-mediated vasodilation [40]. Another experiment used compound to prevent membrane lipid loss and suggested that lipid metabolism was important for the development of ischemic damage [41]. A recent study suggested that cell cycle modulation resulted in neuroprotection in ischemia, which was mediated at least in part through inhibition of microglia proliferation and production of inflammatory cytokines [42]. Besides that, researchers also confirmed that the application of natural medicine induced cell cycle arrest and cell survival to resist cerebral ischemia injury [43]. It is essential to understand the different cell death mechanisms after cerebral ischemia [44]. Furthermore, autophagy-like injury may contribute to ischemia-induced cell death in the brain, which could be regulated by the NF- κ B pathway, suggesting potential treatments for ischemic stroke [45]. Confidently, T cells were required for stroke-induced reductions in non-T immune cells, and they were the most crucial lymphocytes for stroke-induced immunodepression [46]. A study analyzing the temporal dynamics of immune cell accumulation in a rodent stroke model showed that peculiar activation pattern and massive increase of antigen-presenting cells in temporal conjunction with regulatory cells might provide additional insight into poststroke immune regulation [47]. A better understanding of the roles of the different T-lymphocyte subpopulations and their temporal profile of damage versus repair will help to clarify whether T-lymphocyte targeting may be a viable post-stroke therapy for clinical use [48].

A novel nonoverlapping function, RNA posttranscriptional modification, was identified in BJ group, which might turn out to be a factor of more general importance in gene expression, and was identified as a participant in precise decoding of the genetic code that had been demonstrated to be associated with the treat-

ment for cerebral ischemia [49]. An investigation focusing on the role of miRNAs in ischemia indicated that miRNAs played a role in the regulation of gene expression at the posttranscriptional level, which may serve as biomarkers and therapeutic targets in cerebral ischemia [50]. Another review examined the implicated RNA-based networks in the molecular pathogenesis of stroke and revealed therapeutic strategies that more effective neuroprotective and neural regenerative responses may be developed through the modulation of RNA transcription, posttranscriptional RNA processing, and RNA translation [51]. Although found to be associated with ischemia in previous studies, other special functions of psychological disorders and protein synthesis in BJ group may need to be studied further in order to get a more complete understanding of the underlying additive mechanism of the combination therapy.

Conclusions

In conclusion, system-pathways hierarchical analysis may need a kind of integrative and quantitative method, and analysis of known pathways convergence and divergence may suggest an interdependent paradigm of revealing pharmacological additive mechanisms of drug combinations, such as BA and JA.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Table S1. Relationships between canonical pathways and cerebral ischemia which were supported by published literatures.

Table S2. Statistically significant signaling pathway networks between BJ and vehicle groups.

Table S3. Cerebral ischemia/stroke-related overlapping functions among BA, JA and BJ groups which were supported by published literatures.