Danshen-Chuanxiongqin Injection attenuates cerebral ischemic stroke by inhibiting neuroinflammation via the TLR2/TLR4-MyD88-NF-κB Pathway in tMCAO mice

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[ABSTRACT] Danshen-Chuanxiongqin Injection (DCI) is a commonly used traditional Chinese medicine for the treatment of cerebral ischemic stroke in China. However, its underlying mechanisms remain completely understood. The current study was designed to explore the protective mechanisms of DCI against cerebral ischemic stroke through integrating whole-transcriptome sequencing coupled with network pharmacology analysis. First, using a mouse model of cerebral ischemic stroke by transient middle cerebral artery occlusion (tMCAO), we found that DCI (4.10 mL·kg⁻¹) significantly alleviated cerebral ischemic infarction, neurological deficits, and the pathological injury of hippocampal and cortical neurons in mice. Next, the whole-transcriptome sequencing was performed on brain tissues. The cerebral ischemia disease (CID) network was constructed by integrating transcriptome sequencing data and cerebrovascular disease-related genes. The results showed CID network was imbalanced due to tMCAO, but a recovery regulation was observed after DCI treatment. Pathway analysis of the key genes with recovery efficiency showed that the neuroinflammation signaling pathway was highly enriched, while the TLR2/TLR4-MyD88-NF-κB pathway was predicted to be affected. Consistently, the in vivo validation experiments confirmed that DCI exhibited protective effects against cerebral ischemic stroke by inhibiting neuroinflammation via the TLR2/TLR4-MyD88-NF-κB pathway. More interestingly, DCI markedly suppressed the neutrophils infiltrated into the brain parenchyma via the choroid plexus route and showed anti-neuroinflammation effects. In conclusion, our results provide dependable evidence that inhibiting neuroinflammation via the TLR2/TLR4-MyD88-NF-κB pathway is the main mechanism of DCI against cerebral ischemic stroke in mice.

[KEY WORDS] Danshen-Chuanxiongqin Injection; Cerebral ischemic stroke; Network pharmacology; Neuroinflammation; Neutrophil; Choroid plexus


Introduction

Stroke is the third most common cause of death worldwide [1]. It is also the leading cause of death in China with the growth of prevalence, incidence, and mortality in recent years [2]. Increasing evidence suggests that neuroinflammation plays a key role in the pathogenesis of stroke and acts as a major factor for the development of brain lesions and neurological deficits [3-4], marking it a promising target for therapeutic intervention. Notably, the progression of stroke and related neuroinflammation many are associated with multiple genes and components of several signaling pathways, rather than a single gene or target [5]. Thus, there is an urgent need to adopt drugs that act on multiple targets against this disease, especially those derived from plants used in traditional Chinese medicine (TCM).

Danshen-Chuanxiongqin Injection (DCI) is a commonly used TCM preparation, which has been recommended by many disease guidelines or consensus for the treatment of occlusive or ischemic cerebrovascular and cardiovascular disease (CCVD) [6]. It is composed of Salvia Miltiorrhiza Radix et Rhizoma (the root and rhizome of Salvia Miltiorrhiza Bunge) extract and ligustrazine hydrochloride. The
former, also named as Danshen in Chinese, has been widely used to treat CCVD in China and other Asia countries [8, 9], whereas ligustazine is the main constituent of a traditional Chinese herb Chuanxiong Rhizoma (the rhizome of Ligusticum Chuanxiong Hort) and also plays an important role in the treatment of CCVD [10]. Chemical analysis revealed that DCI consists of both hydrophilic and hydrophobic constituents, such as danshensu, salvianolic acid A, salvianolic acid B, ligustazine hydrochloride, protocatechuic aldehyde, and rosmarinic acid [11]. Our previous study demonstrated that salvianolic acid C, salvianolic acid A, danshensu, and ligustazine hydrochloride are the main effective constituents of DCI against ischemic brain injury [13]. Moreover, DCI can inhibit the production of malondialdehyde, scavenge oxygen free radicals, expand brain blood vessels, and improve cerebral blood flow both in animals and patients with stroke [13, 14], suggesting its role as an effective medicine for this disease. However, the systematic functions and molecular mechanisms of DCI on stroke, especially focusing on neuroinflammation, are still largely unclear.

Network pharmacology breaks through the limitation of the traditional “one target, one drug” therapeutic mode and follows the holistic philosophy of TCM, which has been widely used to elucidate the pharmacological mechanisms of action at the molecular level from a systematic perspective [15-17]. Our laboratory and other researchers have successfully applied this method to explain the complex mechanisms of TCM [18-20]. Thus, in this study, we adopted network pharmacology analysis coupled with whole-transcriptome sequencing to investigate the systematic functions and molecular mechanisms of DCI on stroke, especially focusing on the neuroinflammation in stroke. In addition, in vivo experiments were also conducted to confirm the underlying mechanisms of DCI on stroke which were predicted by network pharmacology approaches.

Materials and Methods

Drug

danshen-Chuanxiongquin Injection (DCI) was obtained from Guizhou Baite Pharmaceutical Co., Ltd. (Guizhou, China; CFDA Med-drug permit No. H52020959). DCI is composed of Salvia Miltiorrhiza Radix et Rhizoma (Danshen, the dried roots and rhizomes of Salvia miltiorrhiza Bunge) and ligustazine hydrochloride (2, 3, 5, 6-tetramethylpyrazine hydrochloride, purity ≥ 98.5% by HPLC) at a ratio of 10 : 1 (dry weight) and manufactured in line with WS-10001-(HD-1138)-2002-2017 based on the Chinese Pharmacopoeia 2015 edition. In brief, per 4 g crude Danshen was extracted with water followed by alcohol-precipitation processes to make a transparent liquid of 1 mL, to which ligustazine hydrochloride, glycerol and water were added to reach 5 mL. HPLC-UV fingerprint analysis of DCI from 13 batches were conducted in our previous studies [13], and DCI (Batch No. 20180410) was applied and diluted by noraml saline for drug administration in the current study.

Animal model and drug treatment

Male C57BL/6 mice (22 ± 3 g, 8–10 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were housed in a temperature controlled room (25 ± 1 °C) for 12 h light/dark cycles with free access to food and water. All the animal experimental protocols were approved by the Animal Care and Use Committee of Zhejiang University School of Medicine.

After seven days of acclimatization, a cerebral ischemic stroke model was established through transient middle cerebral artery occlusion (tMCAO) in mice based on previous reports with modification [21, 22]. Briefly, the mice were anesthetized with 1% sodium pentobarbital by intraperitoneal injection. After midline skin incision, the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed, and then the L1800 silicone filament (Jialing Biotechnology Co., Ltd., Guangzhou, China) was inserted into the ICA about 10 mm to occlude the origin of the MCA. After 90 min, cerebral reperfusion was conducted by withdrawal of the silicone filament. Sham surgery was carried out without tMCAO. During the whole process, the rectal temperature of mice was maintained at 37.0 ± 0.5 °C using a heating lamp (NOMOY PET, Jiaxing, China).

The mice were randomly divided into five groups: (1) a sham operated group (Sham, intraperitoneally injected with normal saline at 0.01 mL·g⁻¹ body weight); (2) a model group (Model, intraperitoneally injected with normal saline at 0.01 mL·g⁻¹ body weight); (3) a low-dose Danshen-Chuanxiongquin treatment group (DCI-low, intraperitoneally injected with DCI at 2.05 mL·kg⁻¹, a clinically equivalent dose calculated based on the normalization of body surface area); (4) a high-dose Danshen-Chuanxiongquin treatment group (DCI-high, intraperitoneally injected with DCI at 4.10 mL·kg⁻¹); (5) an edaravone group, as a positive control (Edaravone, intraperitoneally injected with edaravone at 4 mg·kg⁻¹). The drugs were immediately administered after reperfusion.

The neurological deficits were assessed when the animals completely recovered from anesthesia and 48 h after reperfusion in accordance with the modified Longa’s five-point test [23]. It was scored as follows: 0, no observable neurological deficits; 1, failure to extend left forepaw; 2, circling to the affected side; 3, falling to the affected side; and 4, no spontaneous walking. The observer was blind to the treatments.

Measurement of cerebral infarct volume

After 48 h, the mice were sacrificed and the brains were quickly removed and frozen at −20 °C for 20–30 min. The frozen brains were cut into 2 mm thick slices, stained with 2, 3, 5-triphenyltetrazolium chloride (TTC, 0.25% TTC in phosphate-buffered saline) at 37 °C for 30 min, immersed in 4% paraformaldehyde for 24 h, and photographed. The infarct (white) and normal (red) areas were analyzed by Image-Pro Plus 6.0, while the percentage of infarct volume for each mouse was calculated as below:
Infarct volume ratio (100%) = 
\[
\frac{\text{SUM of infarct area } \times 2 \text{ mm}^3}{\text{SUM of total brain area } \times 2 \text{ mm}^3} \times 100\%
\]

Hematoxylin-eosin (HE) staining and terminal deoxynucleotidyl transferase-mediated dNTP nick end labeling (TUNEL) assay

After 48 h of drug administration, three mice were randomly selected from each group, anesthetized with 1% pentobarbital sodium, and sacrificed by cardiac perfusion with 20 mL normal saline and 20 mL 4% paraformaldehyde successively. Then, the brains were carefully removed and fixed with 10% formalin for 48 h. The histopathological changes of brain tissues were measured using HE staining and observed under a microscope (E100, Nikon, Tokyo, Japan). The apoptosis of brain tissues was detected by TUNEL assay (Roche, Basel, Switzerland) and observed under a fluorescence microscope (ECLIPSE TI-SR, Nikon, Tokyo, Japan).

Whole-transcriptome sequencing and data analysis

Total RNA (n = 4 for the Sham and Model groups; n = 3 for the DCI group) from the junction of normal and infarction area of mouse brain tissues was extracted by Trizol reagent, following qualitative and quantitative detection by NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 1 μg RNA per sample was used as input material. Sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) according to the manufacturer’s instructions. Briefly, 250–300 bp double-stranded cDNA was synthesized, purified after ligation of adaptors, and sequenced on an Illumina Novaseq platform by Novogene (Beijing, China).

All the downstream analyses were performed based on the clean data after quality control of the fastq format raw data. Hisat2 v2.0.5 and featureCounts v1.5.0 were applied to build the index of the reference genome and quantify the gene expression of FPKM values, while DESeq2 R package (1.16.1) was performed for differential expression analysis of two groups. P value and Fold Change were calculated to screen out the differentially expressed genes (DEGs) between the Model and Sham or DCI and Model groups.

Construction and analysis of cerebral ischemia disease network

By integrating the sequencing data and the cerebrovascular disease-related genes collected in our previous study, an intersection of these two datasets was generated to construct a cerebral ischemia disease (CID) network. The interaction of these genes were analyzed in the String database (https://string-db.org/) and visualized by Cytoscape v3.7.1. The nodes in the biological network were the genes involved in CID, and the edges represented the protein-protein interactions (PPI) between these genes. Pathway analysis was conducted using Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/) and the results were used for selecting more critical pathways in CID.

Then, key nodes (i.e., core genes) in the CID network were determined by NTRA (the Network Topology and Transcriptomics based Approach) based on our previous report. As topology and expression level are the two main properties of nodes in the biological network, the most important genes in the disease network can be revealed based on NTRA. Briefly, the NTRA rank (Rank) is calculated by combining topological rank (RankT) and transcriptomics rank (RankR) (based on the Formula 1 displayed below). RankT consists of relative ranks of Degree (RankD) and Betweenness (RankB), calculated by Network Analyzer plugin in Cytoscape, while degree and betweenness indicate the importance of nodes in the entire biological network. Moreover, RankR is composed of Fold Change rank (RankF) and P value rank (RankP), where larger Fold Change and smaller P value represent more important genes. The higher the NTRA ranks, the more vital the genes are in the network.

\[
\begin{align*}
\text{RankT} & = R (\text{RankD} + \text{RankB}) \\
\text{RankR} & = R (\text{RankF} + \text{RankP}) \\
\text{Rank} & = R (\text{RankT} + \text{RankR})
\end{align*}
\]

Further measurement of the recovery function of DCI was conducted by EoR (Efficiency of Recovery regulation) based on our previous study, which is used to describe the recovery effect of drug on the network disturbed by disease. Moreover, the recovery level (RL) was calculated by comparing gene expression changes between DCI/Model and Sham/Model (displayed in Formula 2 as below). The maximum value of EoR is 100%, which means complete recovery from the gene disorder caused by tMCAO modeling, while EoR < 0 represents no recovery effect.

\[
\begin{align*}
\text{Fold Change (Sham/Model)} & = \log_2 E_{\text{Sham}} - \log_2 E_{\text{Model}} \\
\text{Fold Change (DCI/Model)} & = \log_2 E_{\text{DCI}} - \log_2 E_{\text{Model}} \\
\text{RL} & = \frac{\text{Fold Change (DCI/Model)}}{\text{Fold Change (Sham/Model)}} \\
\text{EoR} & = 100\% - |100\% - \text{RL}|
\end{align*}
\]

Pathway enrichment analysis

Genes ranked top 300 of NTRA with EoR > 0 were chosen as the significant recovery genes. Pathway enrichment analysis was performed on these genes by Ingenuity Pathway Analysis (IPA). P < 0.05 was considered to be significant.

Real-time quantitative PCR

Real-time quantitative PCR was performed on a Bio-Rad CFX96 Touch™ Real-time PCR Detection System (Hercules, CA, USA) to validate the results of whole-transcriptome sequencing analysis. Two micrograms RNA samples of mice from the same group were mixed as a sample to represent this group. Reverse transcription was performed using the Quant iT Nova Reverse Transcription Kit (QIAGEN, Hilden, Germany). RNA expression was detected by SYBR Green PCR Kit (biosharp, Hefei, China) with specific primers (Sangon, Shanghai, China) according to the manufacturer’s in-
structions. β-actin was used as the internal reference and the relative expression of genes was calculated by the 2^−ΔΔCt method. The experiment was conducted in duplicate and repeated three times.

### Western blot analysis

Total proteins were extracted from the ipsilateral ischemic hemisphere of the mouse brain tissues by RIPA with 1% PMSF. A nuclear-cytosol extraction kit (Beyotime Biotechnology, Shanghai, China) was used to isolate nuclear and cytosol proteins. The protein concentrations were determined by a BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein samples of four mice from the same group were mixed as a sample to represent this group. Equivalent amounts of 15 or 20 μg proteins were electrophoresed by 12% SDS-polyacrylamide gel, and then transferred to polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Darmstadt, Germany). The membrane was blocked in 5% nonfat dry milk at room temperature for 1 h before incubation at 4 °C overnight with corresponding primary antibodies against TLR2 (1 : 500, 13744S), TLR4 (1 : 500, 14358S), MyD88 (1 : 500, 4283S), p-NF-κB p65 (1 : 500, 3033T), p-IκBα (1 : 100, 2859S), β-actin (1 : 1000, 4970S), and Histone H3 (1 : 500, 9717S) (all purchased from Cell Signaling Technology, Beverly, MA, USA). The membrane was washed by TBST (Tris-buffered saline, Tween 20) for three times, and then incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG, 1 : 2000, Beyotime Biotechnology, Shanghai, China) at room temperature for 1 h. After washed by TBST, the blots were detected using an enhanced chemiluminescent substrate reagent (Bio-Rad, Hercules, CA, USA) and imaged by a Bio-Rad ChemiDoc™ Imaging System (Hercules, CA, USA). β-Actin and Histone H3 were used as the internal standards for nuclear and cytosol proteins, respectively. Data were analyzed by Image Lab software 6.0. The experiments were repeated in three times.

### Immunofluorescence staining

After 48 h, the mice were anesthetized and sacrificed by cardiac perfusion with 20 mL normal saline and 20 mL 4% paraformaldehyde, respectively. Then, the brains were carefully removed, placed in 10% formalin for 48 h, embedded with paraffin, and then sliced into 4 μm sections. After blocked with 10% normal serum for 30 min, the sections were incubated at 4 °C overnight with primary antibody of myeloperoxidase (MPO) (1 : 100, ab9535, Abcam, Cambridge, MA, USA) which is the marker of neutrophils.28,29 Then, the sections were incubated with secondary antibody and counterstained with DAPI to visualize the nuclei. Finally, the section images were collected under a fluorescence microscope (OLYMPUS, BX63, Tokyo, Japan) equipped with an OLYMPUS DP80 camera.

### Statistical analysis

All experimental data are expressed as mean ± standard error of mean (SEM). Statistical analysis was carried out by GraphPad Prism 7. Accordin to normality by Shapiro-Wilk test, all the data were normally distributed. The significant differences were determined by Student’s two-tailed t-test, or one-way ANOVA followed by Dunnett’s posterior analysis. P < 0.05 was defined to be significant.

## Results

### DCI relieved tMCAO-induced cerebral infarction and neurological deficits in mice

First, the therapeutic effects of DCI on tMCAO mice were assessed. The results of TTC staining showed that there was a significant change in the infarct volume ratio among the Sham, Model, DCI-low, DCI-high, and Edaravone groups (P < 0.0001). There was no infarction or edema formation in the brain of the Sham group, while those in the Model group appeared obvious brain damage with an average infarct volume ratio of 34.7% (significantly increased, P = 0.0001; Figs. 1A and 1B). Compared with the Model group, exposure to high-dose DCI dramatically decreased the brain infarct volume by 48.1%, with an average infarct volume ratio of 18.0% (P = 0.0104; Figs. 1A and 1B). Similarly, the positive control edaravone (P = 0.0014) or low-dose DCI (P = 0.1218) treated mice also presented a lower infarct volume ratio compared with the Model group, although there was no statistical difference with low-dose DCI treatment. Moreover, the results of neurological deficit score showed a similar trend (Fig. 1C), whereas DCI-low, DCI-high, and edaravone (P < 0.05) exposure markedly decreased neurological scores compared with the Model group. Taken together, it suggested that DCI effectively alleviates cerebral ischemic stroke. To further explore the protective effects of DCI against cerebral ischemic stroke in mice, high-dose DCI treatment (4.10 mL·kg⁻¹) was chosen.

### DCI ameliorated brain damage and neuronal apoptosis in tMCAO mice

Histopathological examination by HE staining was performed using the brain tissues in each group. As shown in Fig. 2A, mice in the Sham group displayed normal morphology, whereas those in the Model group showed obvious histopathological changes with the presence of vacuoles, nuclear shrinkage, and edema in the hippocampus and cortex regions. In contrast, these pathological damages were alleviated after administration of high-dose DCI. Furthermore, neuronal apoptosis was detected by TUNEL assay (Fig. 2B). The number of apoptotic cells obviously increased in the Model group compared with the Sham group, while DCI-high treatment reduced neuronal apoptosis in the mouse brain with cerebral ischemic stroke. These findings demonstrated that DCI protected brain tissues against pathological injury in tMCAO mice. Additionally, HE staining showed no obvious changes in mouse heart, liver, spleen, lung, and kidney after high-dose DCI treatment, which indicated that there was no apparent toxicity caused by high-dose DCI in tMCAO mice.

### DCI recovered the CID network disturbed by tMCAO

To further understand the underlying pharmacological mechanisms of DCI against cerebral ischemic stroke, we ad-
opted the network pharmacology strategy. First, whole-transcriptome sequencing was conducted using mouse brain tissue samples. The accuracy of gene expression data was verified by qRT-PCR. The results confirmed the reliability of the data from sequencing due to the concordant trend. Then, the CID network was constructed and visualized with a total number of 864 nodes and 24076 edges. In this network, typically critical pathways were clustered in the middle surrounding by other genes (Fig. 3A). Based on NTRA, several genes, including Ccl2, Gfap, Hmox1, Cd44, Fn1, Tlr4, Tnf, Cxcl10, Il6, and Tlr2 were highly related to cerebral ischemia injury as ranking in the top 20. Then, the disturbance of tMCAO modeling was visualized by the Log$_2$(Fold Change) of the genes in the CID network. Specifically, genes with red color represented Log$_2$(Fold Change) > 0 and implied up-regulation by tMCAO modeling, whereas genes with green color indicated Log$_2$(Fold Change) < 0 and meant down-regulation by tMCAO (Fig. 3B). Thus, colored network in Fig. 3B indicated a severe damage of brain function after tMCAO, which mainly led to up-regulation of the NF-κB signaling pathway, Toll-like receptor signaling pathway, TNF signaling pathway, cytokine-cytokine receptor interaction, and chemokine signaling pathway accompanied by down-regulating the neurotrophin signaling pathway and neuroactive ligand-receptor interaction. However, when treated with DCI, 71.6% (619/864) genes presented recovery regulation based on EoR > 0 (labeled blue in Fig. 3C). Among them, 35.4% (219/619) nodes exhibited more than 50% efficiency of recovery regulation, implying the efficient recovery effect of DCI on cerebral I/R injury.

**DCI attenuated tMCAO-induced cerebral ischemic stroke in mice mainly by the neuroinflammatory signaling pathway**

Based on the topological and transcriptomics ranks of nodes in the network, 264 genes were selected for pathway enrichment as they were in the top 300 of NTRA rank with an effective recovery trend (i.e., EoR > 0). According to the results, 433 pathways in total were enriched by IPA and the top 10 canonical pathways were presented in Fig. 4A. Furthermore, the neuroinflammation signaling pathway was significantly enriched as the top 1 pathway ($P < 0.05$), suggesting that anti-neuroinflammation was the mainly mechanism of DCI against cerebral ischemic stroke. Meanwhile, several inflammation-related pathways, such as granulocyte adhesion and diapedesis, acute phase response signaling, and agranulocyte adhesion and diapedesis, were also significantly enriched ($P < 0.05$).

Furthermore, the neuroinflammation signaling pathway was further analyzed. The subnetwork of the 55 genes with the recovery efficiency in this pathway after DCI treatment was visualized in Fig. 4B. In particularly, the TLR2/TLR4-MyD88-NF-κB pathway was found to be activated during cerebral ischemic stroke, and all the key molecules in this pathway showed an obvious recovery trend based on EoR, indicating that TLR2/TLR4-MyD88-NF-κB may be one of the
main targets of DCI against tMCAO injury.

**DCI attenuated cerebral ischemic stroke in tMCAO mice by inhibiting neuroinflammation via the TLR2/TLR4-MyD88-NF-κB pathway**

As we found that the TLR2/TLR4-MyD88-NF-κB pathway was obviously affected in neuroinflammation cascade, the gene and protein expression of the key molecules in this pathway were further verified by qRT-PCR and Western blot. As shown in Fig. 5, tMCAO modeling up-regulated the levels of TLR4 and TLR2 mRNA in mouse brain, while significant increases in the protein levels of TLR4 ($P = 0.0129$) and TLR2 ($P = 0.0008$) were observed in brain tissues in the Model group compared with the Sham group. Downstream adaptor protein MyD88 was also activated by tMCAO model-
ing \( (P = 0.0241) \), which induced the translocation of p-NF-κB p65 \( (P = 0.0001) \) to the nucleus and increased the transcription of pro-inflammatory factor IL-6 (Fig. 5). However, when treated with DCI, the expression of TLR2, TLR4, MyD88, p-NF-κB p65, and p-IκBα were all significantly suppressed \( (P < 0.05) \), accompanied by decreasing the transcription of IL-6 (Fig. 5). These results indicated that DCI alleviated cerebral ischemic stroke via the TLR2/TLR4-MyD88-NF-κB pathway.

**DCI inhibited neuroinflammation by decreasing the infiltration of neutrophils into the brain via the choroid plexus route in tMCAO mice**

According to pathway enrichment results, granulocyte adhesion and diapedesis were significantly enriched and highly related to neuroinflammation. According to recent studies, the choroid plexus is a key cerebral invasion route for immune cells migrating into the brain parenchyma after stroke, indicating a new route for therapeutic intervention \([30, 31]\).

Since neutrophils are the first cells infiltrating into the injured areas after stroke \([32]\), we further detected the effects of DCI on the trafficking of neutrophils via the choroid plexus route. The results showed that 48 h after tMCAO modeling, the ipsilateral cortex and choroid plexus presented more MPO (the marker of neutrophils) positive neutrophils (Figs. 6A and 6B), indicating that tMCAO modeling may result in the invasion of neutrophils from the choroid plexus to the injured
Fig. 4 DCI attenuated cerebral ischemic stroke in tMCAO mice mainly by the neuroinflammatory signaling pathway. (A) The top 10 canonical pathways enriched by IPA based on NTRA and EoR methods. (B) The PPI network of the 55 genes affected in the neuroinflammatory signaling pathway. Each node represents a gene and each edge indicates the interaction between two genes which was mapped based on the protein-protein interaction. The size of nodes means the NTRA rank. The higher the ranking of the node is, the larger the size it has.

Discussion

DCI is widely used to treat ischemic stroke in clinical settings which can significantly improve neurological function and the quality of life of patients [36]. Although the curative effects of DCI on ischemic stroke have been investigated in many clinical studies [7], the detailed functions and underlying mechanisms are not completely understood. In the present study, we provided solid evidence that DCI exhibited significantly protective effects against cerebral ischemic stroke by ameliorating infarct volume, promoting neurologic function, attenuating pathological injuries, and inhibiting neuron apoptosis in tMCAO mice. More importantly, by integrating network pharmacology analysis and whole-transcriptome sequencing, we found that 31.6% CID-related genes which were imbalanced due to tMCAO presented a recovery trend after DCI treatment, and inhibiting neuroinflammation through suppression of the TLR2/TLR4-MyD88-NF-κB pathway is the main mechanism of DCI against cerebral ischemic stroke in mice.

Many inflammatory factors are involved in stroke-related neuroinflammation, such as cytokines, chemokines, and MMPs [37]. It is reported that IL-6 and IL-1β provoke and aggravate the inflammatory responses after ischemic stroke and positively correlate with the severity of stroke [38]. Similarly, our results indicated that these two major proinflammatory cytokines were up-regulated after stroke and recovered by DCI treatment. Moreover, chemokines (such as Cxcl1, Cxcl2, and Ccl2) and MMPs (such as Mmp-2, Mmp-3, Mmp-9, Mmp-13, and Mmp-14) in the granulocyte or agranulocyte adhesion and diapedesis pathways were similarly affected by DCI treatment. CXCL1 and CXCL2 serve as the chemoattractants for neutrophils, which mediate neutrophil infiltration and co-localize with the pathogenesis of stroke [39]. The obvious recovery trend of these two genes after DCI treatment indicates the inhibitory effects of DCI on neutrophil trafficking to the ischemic brain region. Furthermore, a previous study reported that disruption of CCL2 gene decreased infarct volume in cerebral ischemic mice [37], indicating the role of DCI to ameliorate the infarct volume of tMCAO mice. In addition, MMPs are crucial in the neuroinflammatory cascade and brain blood barrier disruption during ischemic stroke [37], and the recovery effects of DCI on MMPs indicated that DCI suppressed the stroke-related neuroinflammation. These data demonstrated that inhibiting neuroinflammation is the main function of DCI against ischemic stroke.

According to the current study, the TLR2/TLR4-MyD88-NF-κB pathway played a major role in the protective effect of DCI against stroke-related neuroinflammation (Fig. 7). The Toll-like receptor signaling is of great concern in cerebrovascular disorder, and related to immune responses [40]. MyD88...
is one of the adaptor proteins, and can be activated by almost all TLRs \[41\]. Once MyD88 is activated, it causes the activation of NF-κB, which leads to the transcription and translation of inflammatory factors and results in brain damage and neurological impairment \[41\]. It was reported that the levels of TLR2, TLR4, and MyD88 increased in MCAO rats \[42, 43\]. The similar trend was observed in this study as the gene and protein expression of TLR2, TLR4, and MyD88 were all up-regulated by tMCAO modeling, but down-regulated by DCI treatment (Fig. 5). Furthermore, our results revealed that DCI treatment decreased cytoplasmic p-IκBα and nuclear p-NF-κB which were up-regulated in the Model group. Additionally, two studies confirmed that TLR2 is involved in the process of leukocyte infiltration to the cerebrospinal fluid in the brain \[44, 45\], which indicated that DCI may suppress neutrophil infiltration by the TLR2 signaling in ischemic stroke. These results provided evidence that DCI attenuates cerebral ischemic stroke in vivo by inhibiting neuroinflammation via the TLR2/TLR4-MyD88-NF-κB pathway.

More interestingly, the function of DCI on neutrophil trafficking via the choroid plexus route in stroke-related neuroinflammation was investigated in the current study. In the past few years, special attention has been paid to the relationship between leukocytes and neuroinflammation in stroke as it is a major factor for the development of brain lesions and neurological deficits \[46\]. Invasion of circulating leukocytes is the key inflammatory process after stroke and neutrophils are the first leukocytes infiltrating into the injured areas \[32\], which rapidly infiltrate the ischemic brain from 30 min to a few hours, peak among days 1–3, and are positively related with the infarct volume and neurological function deficit \[32, 34\]. Recent studies from neonatal stroke and other brain diseases indicated that the choroid plexus is the alternative cerebral infiltration route for neutrophil trafficking to the ischemic brain region \[30, 45\]. Similarly, in this study, 48 h after tMCAO, substantial invasion of neutrophils from the choroid plexus to the injured brain tissues was observed in the tMCAO mice, whereas DCI treatment markedly suppressed neutrophil trafficking via this route, suggesting that DCI is a novel drug for treating stroke-related neuroinflammation. A
schematic diagram of the mechanisms of DCI on cerebral ischemic stroke is shown in Fig. 7.

**Conclusions**

In summary, the present study elucidated that DCI exerts protective function against cerebral ischemic stroke in vivo, which are characterized by reduced infarct volume, neurological deficits, pathological damages, and apoptosis. Moreover, this study first combined network pharmacology analysis and whole-transcriptome sequencing to reveal the mechanisms of DCI against cerebral ischemic stroke. DCI treatment induced 71.6% CID-related genes with a recovery trend and inhibition of neuroinflammation via the TLR2/TLR4-MyD88-NF-κB pathway. More importantly, we first discovered that DCI markedly suppressed the infiltration of neutrophils in the brain parenchyma via the choroid plexus route. Taken together, our study demonstrates that DCI can protect tMCAO mice against cerebral ischemic stroke mainly through anti-neuroinflammation.

**Supplementary Information**

All the Supplementary Tables and Figures can be acquired by contact with corresponding author. The RNA sequencing data have been uploaded to Sequence Read Archive at NCBI (https://www.ncbi.nlm.nih.gov/sra/, accession ID: PRJNA674888).
**Fig. 7** Schematic diagram of the mechanisms of DCI on cerebral ischemic stroke. DCI exhibited protective effects against cerebral ischemic stroke via the TLR2/TLR4-MyD88-NF-κB pathway and suppressing neutrophil infiltration via the choroid plexus route.

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