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Danhong injection alleviates OGD-induced blood-brain barrier injury via VEGFR2/PI3K/AKT pathway based on network pharmacology and experimental evidence

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ABSTRACT

Objective: Previous studies have shown that Danhong injection (DHI) has a protective effect on the blood-brain barrier (BBB) function after ischemic stroke. However, the role of DHI in protecting the integrity of the BBB after ischemic stroke through endothelial cells is still unclear. The purpose of this study is to explore the role and mechanism of DHI in protecting BBB by interfering with endothelial cells.

Methods: We used network pharmacology technology to determine the potential pathways and mechanisms of DHI in treating ischemic stroke through endothelial cells. On account of the network pharmacology results, we assessed the effects of DHI in oxygen-glucose deprivation (OGD) model of mouse brain-derived endothelial (bEnd.3) cells via MTT, and validated the molecular mechanisms of DHI improving BBB injury through Western blot.

Results: Network pharmacology analysis suggested that DHI may regulate the PI3K/AKT signaling pathway of endothelial cells in the treatment of ischemic stroke. Cellular experiments showed that DHI stimulates endothelial cell migration and reduces OGD-induced BBB damage via VEGFR2/PI3K/AKT pathway.

Conclusion: Network pharmacology analysis and cellular experiments have shown that DHI alleviated the BBB damage by activating the VEGFR2/PI3K/AKT signaling pathway on endothelial cells.

1. Introduction

Stroke is one of the most common types of cerebrovascular disease, resulting in death or disability in aging people.[1,2] Although there are many new treatment strategies for ischemic stroke, its mortality and disability rates are still second in the world.[3] Thrombolytic therapy is the preferred treatment strategy for ischemic stroke and serves as an evaluation indicator of the effectiveness of stroke care outcomes.[4] However, thrombolytic therapy can damage the blood-brain barrier(BBB), exacerbating brain damage.[5] The BBB protects the brain from circulating blood by acting as a selective barrier.[6] Studies have shown that the BBB is disrupted in the early stages of ischemia,[7] and it exacerbates brain damage. Therefore, exploring drugs with the potential to protect the integrity of the BBB after ischemic stroke is of great significance.

Endothelial cells are an essential component of the blood-brain barrier.[8] Once cerebral ischemia occurs, endothelial cells are damaged by reactive oxygen species (ROS), oxidative stress and matrix metalloproteinase (MMPs).[9–12] More importantly, the injured endothelial cells

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will produce cell adhesion molecules and release inflammatory factors to aggravate brain injury.[9,13] In addition, angiogenesis is a critical function of endothelial cells that closely related to recovering BBB function after ischemic stroke.[14] After ischemic stroke, inducing angiogenesis around infarction can improve hemodynamics, promote vascular remodeling, and then restore vascular function.[15,16] In order to retain the blood-brain barrier's integrity after an ischemic stroke, it is crucial to maintain endothelial cells' normal activity. DHI is a frequent injection of traditional Chinese medicine for the treatment of ischemic stroke. DHI has a protective effect on the BBB, improving its permeability and upregulating tight junction proteins in MACO mice.[17,18] More significantly, DHI increases angiogenesis, which enhances the therapeutic effectiveness of mesenchymal stem cells in myocardial infarction.[19] In addition, the combination of DHI and t-PA can significantly improve the damage of BBB by activating the Notch-VEGF signaling pathway to promote angiogenesis.[20] However, it is still unclear whether DHI protects the integrity of the BBB after ischemic stroke by regulating the VEGFR2/-PI3K/AKT signal pathway of endothelial cells.

The holistic, multi-component, multi-target, and multi-pathway approach of traditional Chinese medicine is consistent with the network pharmacology principle. As a result, network pharmacology is acknowledged as a potential technique for figuring out how traditional Chinese medicine functions from the inside out. At present, studies have revealed the mechanism of DHI intervention in stroke using network pharmacology,[21] but have not focused on endothelial cells. In our research, we gathered the gene expression profiles of human umbilical vein endothelial cells (HUVEC) and concentrated on the function of DHI in controlling endothelial cells to protect the BBB following stroke. Network pharmacology can only make qualitative predictions about drug targets and components, and cell experiments are still required to confirm any definite pharmacological effects.

Compounds in DHI and their potential pathways for the treatment of cerebral ischemia stroke were screened using network pharmacology. Next, we further validated through cell experiments such as Western bolt and cell survival that DHI protects the integrity of the BBB after ischemic stroke by regulating the VEGFR2/PI3K/AKT signaling pathway in endothelial cells.

2. Methods and material

2.1. Identification of candidate active components in Danhong injection

A database of systemic pharmacological information on Chinese herbal medications is available at Traditional Chinese Medicine Systems Pharmacology (TCMSP).[22] To identify the chemical components present in DHI, a search was conducted in TCMSP using the keywords "Danshen (*Salvia miltiorrhiza*)" and "Honghua (*Flos carthami*)". The compounds were then screened based on their pharmacokinetic characteristics, specifically oral bioavailability (OB) and drug-likeness (DL). Only substances with OB $\geq$ 30% and DL $\geq$ 0.18 were considered potential active ingredients.[23,24]

2.2. Collection of targets of candidate active components

We used TCMSP and SwissTargetPrediction (http://www.swisstargetprediction.ch/) to obtain the related targets of candidate active components. The canonical SMILES information of the candidate active components was obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/). And the SMILES of candidate active components were imported into the SwissTargetPrediction to predict the potential targets of it. Only the targets with a probability >0.5 were selected.[25] And we used Uniprot Knowledgebase (http://www.uniprot.org/) to further normalize official symbols.

2.3. Collection of targets related to ischemic stroke

Ischemic stroke-related genes were collected from GeneCards database,[26] OMIM (http://www.omim.org/) and DrugBank (http://go.drugbank.org/). The related targets were searched using "ischemic stroke" as keyword. Standardized protein names using the Uniprot Knowledgebase (http://www.uniprot.org/).

2.4. Collection of genes related to endothelial cells

We used the GEO database (https://www.ncbi.nlm.nih.gov/geo/) to collect genes from endothelial cells (HUVEC), and the GSEID is GSE223759.[27] Then, we used the DAVID database[28] to convert it to a Gene symbol.

2.5. Construction of active compound–potential targets network

We used the online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) to draw the venn diagram for analyzing the intersection of the targets related to DHI, cerebral ischemic stroke and endothelial cells. The remaining components and their corresponding potential targets were imported into the Cytoscape software (version 3.8.2) to construct the active compound-potential targets network after removing the candidate active components that could not act on the potential targets.

2.6. Gene Ontology (GO) and enrichment pathway analysis

Through GO analysis and KEGG analysis with the Database for Annotation, Visualization, and Integrated Discovery (DAVID),[28] biological processes, molecular function, cell composition, and pathways of the targets were examined to explore the roles of potential targets of DHI against cerebral ischemic stroke. Genes and gene products' biological activities are annotated using the GO database. The KEGG database is the knowledge base of gene function system analysis used to research the relationships between genes and biological pathways.

2.7. Molecular docking

In the first step, we used the RSCB PDB database (https://www.rcsb.org/) to download the "pdb" format file of the 3D structure of the VEGFR2 (ID number: 6GQP). And the Grid format file was used for docking. In the second step, we used Chem3D and Pymol software to preprocess the structural files of the active substance and the positive control that were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). To achieve greater accuracy docking, the grid file and the preprocessed ligand were loaded into the AutoDock Vina module in the third stage. Finally, the Pymol software was used for drawing.

2.8. Cell culture

The b.End3 cells (CL-0598, Procell, Wuhan, China) were cultured in DMEM basic medium (PM150210, Procell, Wuhan, China) supplemented with 10% FBS (164210-50, Procell, Wuhan, China), 100 μg/mL streptomycin, and 100 U/mL penicillin(all purchased from the Gibco Life Technologies Inc., Grand Island, NY, USA). An incubator (Thermo, USA) was used to maintain the cell culture conditions, which were 95% air and 5% CO_2 in a humid environment.

2.9. Preparation of drugs

First, the volume of Danhong injection and DMEM basic medium was calculated according to the concentration and dosage. Then, according to the calculated results, Danhong injection was added to DMEM basic

medium to prepare a series of concentrations. For example, add 60 μL of Danhong injection to 3940 μL of DMEM basic medium to prepare the dose of 15 μL/mL.

2.10. Oxygen-glucose deprivation (OGD) model and drug administration

The OGD model has been modified slightly in comparison to the previous study.[29] Briefly, bEnd.3 cells were plated at 5×10^3 or 12×10^4 cells per well in 96-well or 6-well plates. After that, the cells were given dose of 15 μL/mL and 20 μL/mL of DHI (Buchang Pharmaceutical, Shandong, China), and the culture medium was changed to sugar-free DMEM. The cells were grown in a Modular Incubator Chamber (Billups-Rothenberg) at 37 °C for 3 h. As the control group, bEnd.3 cells were grown in DMEM medium.

2.11. MTT assay

In 96-well culture plates, the bEnd.3 cells were planted at a density of 5000 cells per well. The MTT (BioFroxx, Guangzhou, China) test measured the cell viability after the cells were treated with OGD and DHI as previously mentioned, in accordance with the manufacturer's instructions. 10 μL of MTT solution was applied to each well of a 96-well plate, which was then incubated at 37 °C for 4 h. The absorbance (OD) at a wavelength of 490 nm was measured using a microplate reader (BIO-RAD, USA).

2.12. Ki8751 inhibits VEGFR2 expression of bEnd.3 cells in the OGD model

Ki8751 (Selleck, USA, S1363), a VEGFR2 inhibitor, significantly inhibits the expression level of VEGFR2.[30] Each well in the 6-well plate received 12×10^4 cells, which were then separated into the control group, model group, DHI group (20 μL/mL), model + Ki875 (10 μM) + DHI (20 μL/mL) group.

2.13. Wound healing test

The bEnd.3 cells were plated in 6-well plates at 12×10^4 cells per well. After the fusion degree of the cells reached 90%, a wound healing test was performed using a scratcher. After scratching, the control group's culture medium was replaced with the DMEM culture medium. In contrast, the DHI group's culture medium was replaced with DMEM medium with 15 μL/mL DHI, then photographed under a microscope at 0 h, 24 h and 48 h after scratching.

2.14. Western blotting

After being ultrasonically crushed, the bEnd.3 cells were lysed for 25

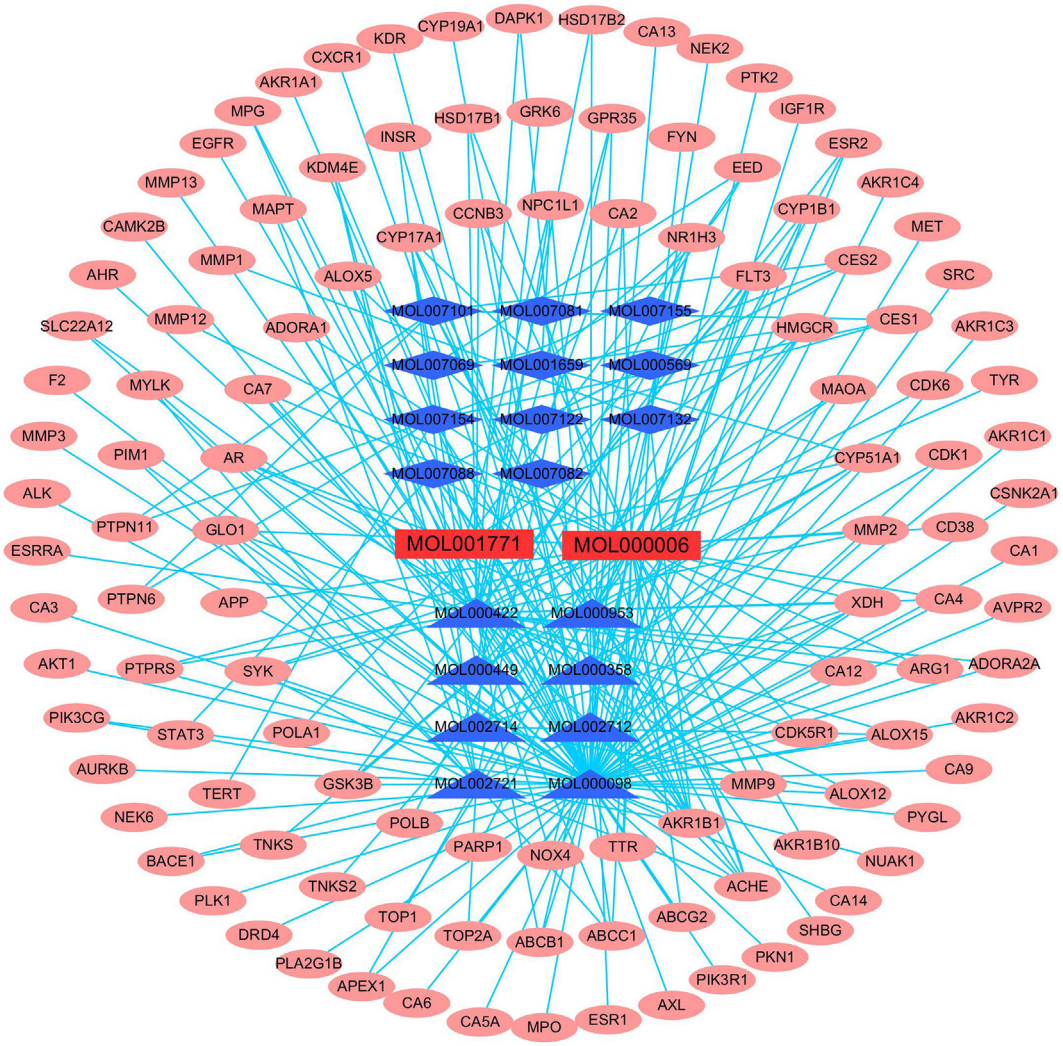

Fig. 1. Active compounds-targets network of DHI. "Diamond nodes" represent the compounds of Danshen, "triangle nodes" represent the compounds of Honghua, "rectangle nodes" represent the compounds of the common components of Danshen and Honghua and ellipse nodes represent potential targets.

Fig. 2. Venn diagrams. (A) Venn diagrams showing the targets overlap among GeneCards, OMIM and DrugBank database. (B) Venn diagrams showing the targets overlap among compound targets, ischemic stroke targets and endothelial cell gene symbols.

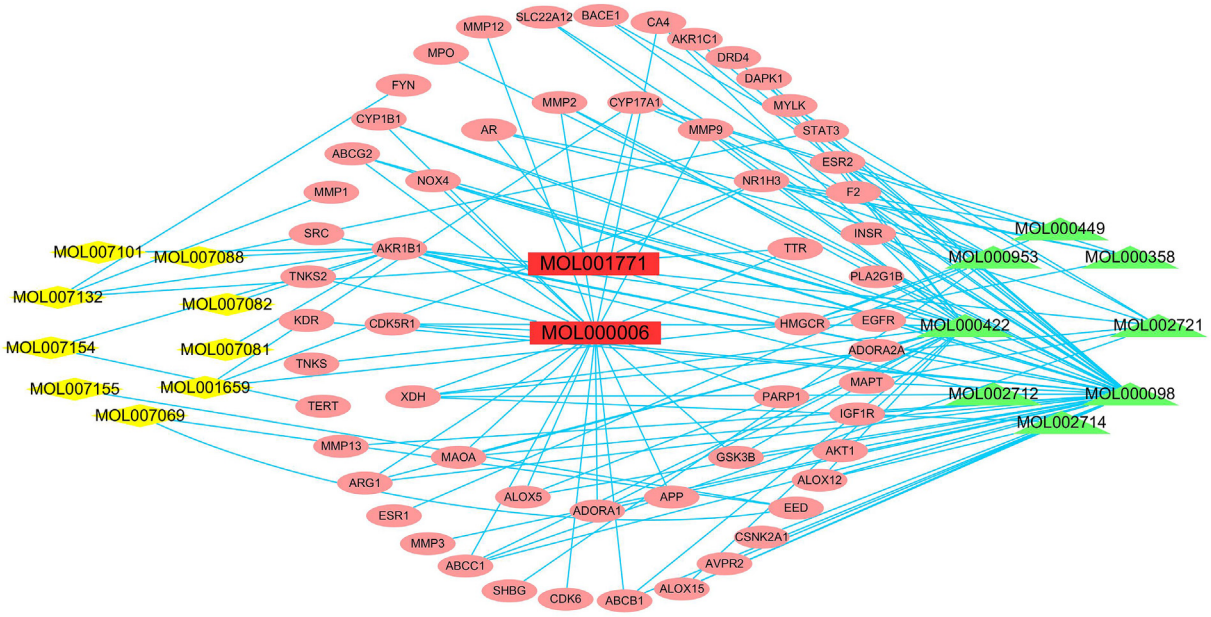

Fig. 3. Compounds-targets network of DHI against ischemic stroke. "Diamond nodes" represent the compounds of Danshen, "triangle nodes" represent the compounds of Honghua, "rectangle nodes" represent the compounds of the common components of Danshen and Honghua, and "ellipse nodes" represent ischemic stroke potential targets.

min on ice before being centrifuged for 10 min at 4 °C at 12,000 rpm. A BCA reagent kit (Thermo Fisher, 23227) was used to assess the protein level of the supernatant. The protein samples were separated on the 10% SDS-PAGE gel and then transferred to PVDF membranes. After blocking the membranes with TBST containing 5% nonfat dry milk, we treated the sample with primary antibodies and left it at 4 °C overnight. Rabbit-anti-ZO-1 (1:1500), rabbit-anti-VEGFR2 (1:1500), mouse-anti-PI3K p85 (1:1500), rabbit-anti-AKT (1:1500), mouse-anti-GAPDH (1:1500) and mouse-anti-β-actin (1:1500) were purchased from Proteintech Group (Rosemont, IL, USA). Goat anti-rabbit IgG secondary antibodies (1:8000, Proteintech Group, USA) or goat anti-mouse IgG secondary antibodies (1:8000, Proteintech Group, USA) were applied to the membranes and incubated with them for 1 h at room temperature before the membranes were exposed to the MiniChem610 (SAGECREATION, Beijing, China). Image J software was used to quantify the protein bands.

2.15. Statistical analysis

Data were analyzed using GraphPad Prism 8. Differences were assessed by t-tests and one-way ANOVA followed by Tukey''s post hoc test. Data were presented as mean ± SEM and $p < 0.05$ was considered statistically significant.

3. Results

3.1. DHI active compounds-targets network

The 84 compounds were filtered from the TCMSP database, and 62 of them came from Danshen (*Salvia miltiorrhiza* Bge.), 19 from Honghua (*Carthamus tinctorius* L.), and three from both. Among them, in the predicted results, only 21 compounds had potential targets with a possibility

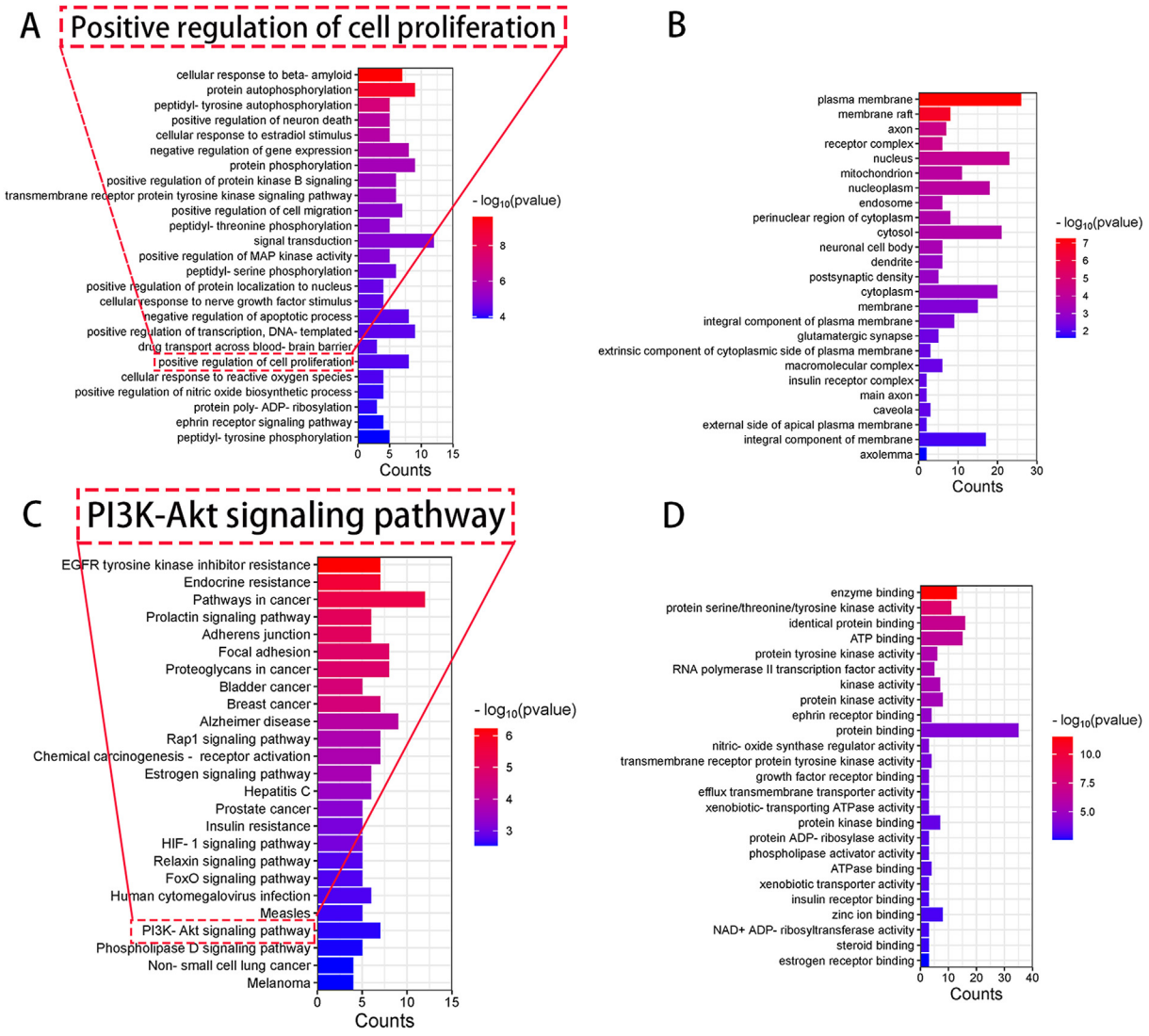

Fig. 4. GO and KEGG enrichment analysis. (A, B, D) The top 25 biological processes, Cellular components, and Molecular function, (C) The top 25 signal pathways of KEGG enrichment.

greater than 0.5. The active compounds-targets network (Fig. 1), showed that MOL000006 and MOL001771 were the common compounds from Danshen and Honghua.

3.2. Compounds-targets network of DHI against cerebral ischemic stroke

A total of 4114 potential targets associated with ischemic stroke were initially identified from the GeneCards database, from which 2283 targets were ultimately chosen for further analysis. Moreover, a comprehensive search across the GeneCards, OMIM, and DrugBank databases revealed a total of 2307 ischemic stroke-related targets (Fig. 2A). Following the intersection, 58 targets were linked to 19 substances and were considered prospective DHI targets for the prevention of ischemic stroke. Notably, 37 of these targets were particularly linked to endothelial cells, as shown in the Venn diagram (Fig. 2B). Additionally, an overlap of 58 targets was observed between the compound and ischemic stroke target categories. In the compounds-targets network of DHI against ischemic stroke (Fig. 3), there were 77 nodes (including 19 compound nodes, and 58 target nodes) and 131 edges.

3.3. GO and KEGG enrichment analysis

In order to further investigate the mechanism of DHI against ischemic stroke in endothelial cells, we performed GO and KEGG enrichment analyses on the 37 potential intersection targets. The GO analysis revealed a significant enrichment in biological processes related to positive regulation of cell migration (GO: 0030335). In respect of molecular function, the enriched targets were associated with enzyme binding (GO: 0019899), ATPase binding (GO: 0051117), phospholipase activator activity (GO: 0016004) and protein kinase binding (GO: 0019901). The enriched cellular components included membrane raft (GO: 0045121), plasma membrane (GO: 0005886) and macromolecular complex (GO:

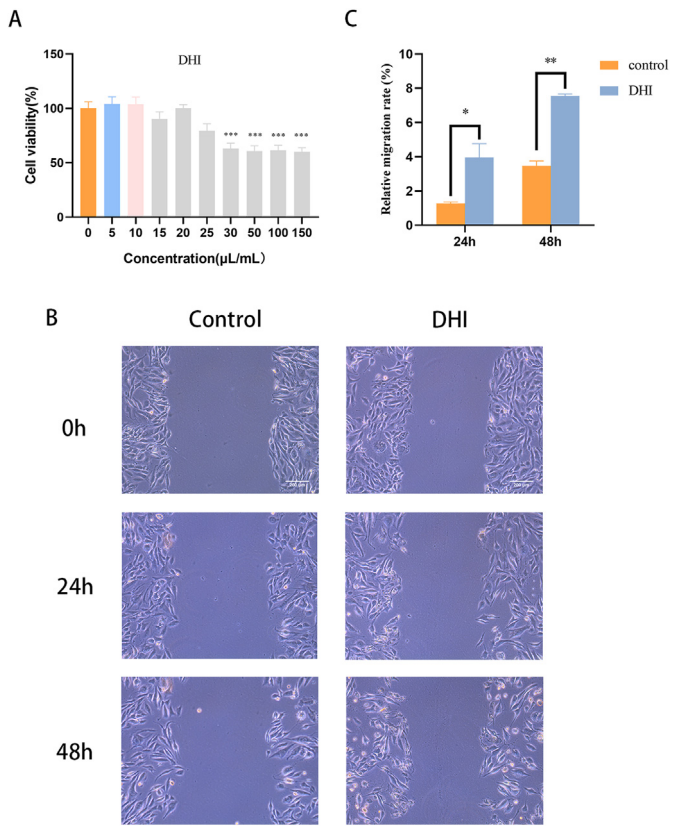

Fig. 5. DHI stimulates bEnd.3 cell migration. (A) Investigation of 24-h toxicity concentration of DHI, (B) Wound healing test indicates that DHI promotes bEnd.3 cell migration, (C) Relative migration rate. Results are expressed as means ± SEM (n ≥ 3). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ in comparison to the control group.

0032991). PI3K-Akt signaling pathway (hsa04151) was the major signaling pathway involved in the enrichment processes. The results of the top 25 GO and KEGG enrichment analyses are depicted in Fig. 4.

3.4. DHI stimulates bEnd.3 cell migration

We performed a wound healing assay on bEnd.3 cells to confirm the findings of network pharmacology that DHI positively influences cell migration. Firstly, we investigated the 24-h toxicity concentration of DHI on bEnd.3 cells by MTT assay and found that DHI was greater than 30 μL/mL has toxic effects on bEnd.3 cells (Fig. 5A). As described in previous studies,[31] wound healing tests can be used to assess cell migration ability. Then, we selected a concentration (15 μL/mL) non-toxic to bEnd.3 cells for wound healing test and found that DHI significantly stimulates cell migration in 24 h and 48 h after scratching(Fig. 5B and C), which is in accordance with the results of network pharmacology.

3.5. DHI mitigates OGD-induced BBB damage via the VEGFR2/PI3K/AKT pathway

Then, we developed an OGD for ischemic stroke in vitro cell model. OGD induces oxidative stress and cell apoptosis, and inhibits cell proliferation and angiogenesis.[32] Using MTT to detect cell survival, we found that DHI reverses OGD-induced brain microvascular endothelial cell death (Fig. 6A). Studies have pointed out that the disruption of the blood-brain barrier is not conducive to the prognosis of ischemic stroke.[33] A vital part of the blood-brain barrier is endothelial cells. ZO-1is an endothelial tight junction protein that are frequently used to assess the blood-brain barrier's integrity. We found that in the bEnd.3 cells OGD model, DHI significantly upregulated the expression level of ZO-1 (Fig. 6B). DHI can also considerably increase the expression of the VEGFR2/PI3K/AKT pathway (Fig. 6C, D, and E).

3.6. DHI showed BBB protective effects in a VEGFR2-dependent manner

In order to verify whether the protection of Danhong injection on BBB after ischemic stroke depends on VEGFR2, we selected MOL000006 (Luteolin) and MOL001771 (Poriferast-5-en-3β-ol), common components of *Salviae miltiorrhizae* and *Flos carthami*, to conduct molecular docking with VEGFR2 (ID number: 6GQP). The binding energy between MOL000006 and 6GQP is −9.6 kcal/mol (Fig. 7A), and the box center coordinates and size set are as follow (center_x = 16.933; center_y = −1.288; center_z = 10.343; size_x = 17.25; size_y = 21.75; size_z = 21.75). The binding energy between MOL001771 and 6GQP is −7.0 kcal/mol (Fig. 7B), and the box center coordinates and size set are as follow (center_x = 21.83; center_y = −0.604; center_z = 12.671; size_x = 64.05; size_y = 64.05; size_z = 64.05). Then, we used Ki8751 to inhibit the expression of VEGFR2 in bEnd.3 cells.[30] We found that Ki8751 downregulates the expression of VEGFR2 in bEnd.3 cells (Fig. 7C). Ki8751 significantly reversed the upregulation of VEGFR2 by DHI (Fig. 7D). In addition, Ki8751 significantly reversed the upregulation of ZO-1 by DHI (Fig. 7E). The protection of the BBB after ischemic stroke by DHI depends on VEGFR2.

4. Discussion

The blood-brain barrier (BBB) comprises endothelial cells, pericytes, astrocytes, tight junctions and the basement membrane.[34] Accumulating evidence suggests that apoptosis of endothelial cells in the BBB is the primary cause leading to cerebral hemorrhage associated with ischemia-reperfusion events.[35] Therefore, it is essential to explore the mechanism of drugs protecting the integrity of the BBB from the perspective of endothelial cells.

Our investigation focused on the impact of DHI on bEnd.3 cells using network pharmacology methodologies, indicating a potential association with the PI3K-Akt pathway and cell migration. Currently, it has been reported that DHI has a protective effect on the integrity of the BBB after ischemic stroke and is closely related to the VEGF pathway.[17,20] The results of network pharmacology are consistent with previous research findings. Previous studies have used network pharmacology and experiments to verify that endothelial inflammation is the mechanism of DHI in treating stroke.[21] However, our study focuses on the mechanism by which DHI regulates endothelial cells' signal pathways to protect the integrity of the BBB after ischemic stroke. Cell experiment verified that DHI stimulated endothelial cell migration to activate angiogenesis by regulating the VEGFR2/PI3K/AKT signaling pathway, thereby alleviating the damage of BBB after ischemic stroke (Fig. 8). Previous studies have shown that the VEGFR2/PI3K/AKT signaling pathway is closely related to the angiogenesis function of endothelial cells,[36–38] and promoting angiogenesis protects the integrity of BBB after ischemic stroke.[14] Our previous research[39] found that PI3K was closely related to BBB integrity. More importantly, we found that inhibiting the expression of VEGFR2 eliminated the protective effect of DHI on the integrity of the BBB. Therefore, VEGFR2 may be a key target for DHI to protect the integrity of the BBB after ischemic stroke. Further research on the relationship between VEGFR2 and the BBB will help us develop drugs

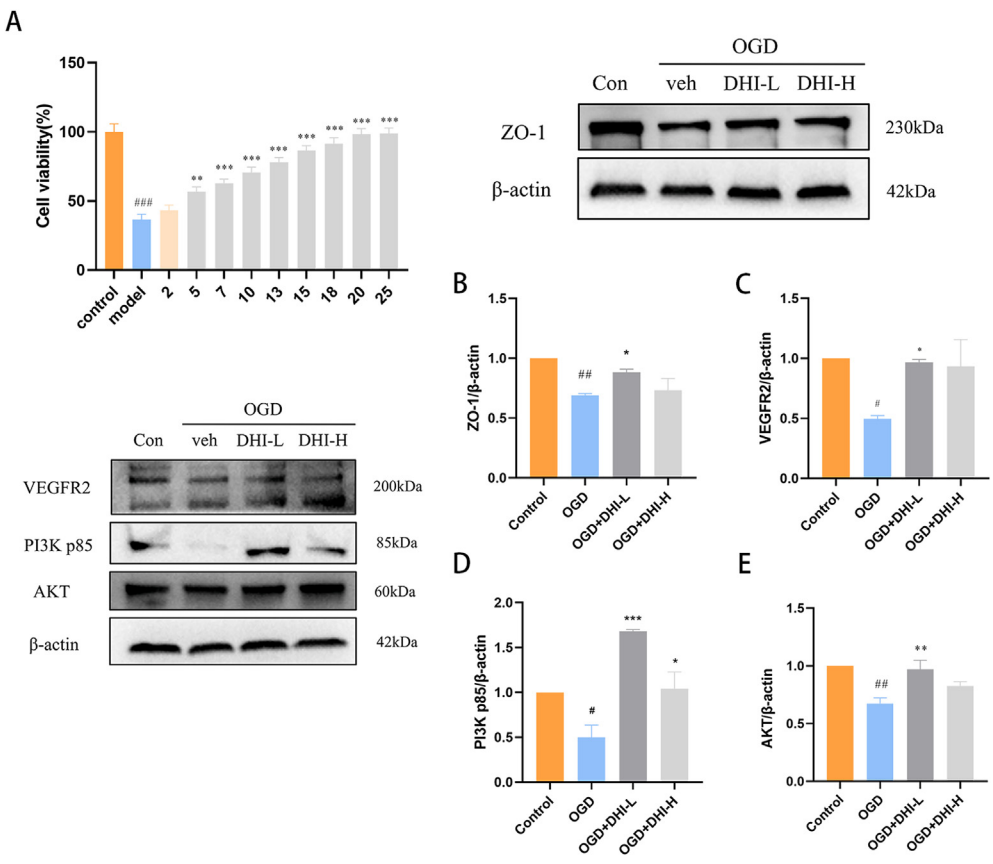

Fig. 6. DHI mitigates OGD-induced BBB damage via the VEGFR2/PI3K/AKT pathway. (A) DHI improves the cell survival rate of bEnd.3 cells after OGD, 2, 5, 7, 10, 13, 15, 18, 20, 25 is the concentration of DHI (μL/mL). The cells were collected and detected for (B) ZO-1, (C) VEGFR2, (D) PI3K p85, and (E) AKT protein content in the cells after lysis. Results are expressed as means ± SEM ($n \geq 3$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ in comparison to the sham group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ in comparison to the model group.

targeting VEGFR2. The destruction of BBB is the pathogenesis of many nervous system diseases, including Alzheimer's disease and Parkinson's disease.[40,41] This may play an essential role in the treatment of ischemic stroke and other nervous system disease, attracting more researchers' attention to the role of endothelial cells in the BBB.

Overall, there are several components and multiple targets involved in the synergistic pharmacological effects of DHI in the treatment of ischemic stroke. The limitation of our study is the lack of in-depth research on the role of luteolin and Poriferast-5-en-3β-ol, the key substances of DHI. So, in our subsequent works, we should apply network pharmacology to deeply explore the mechanism of monomer components in DHI. We want to provide this portion of the research findings in the future.

5. Conclusion

We found that DHI can upregulate the levels of VEGFR2/PI3K/AKT signaling pathway in bEnd.3 cells and its protective effects of the integrity of the BBB depends on VEGFR2 via network pharmacology and cellular experiments, which may be a potential mechanism of DHI in alleviating BBB damage after ischemic stroke.

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Availability of data and materials

All data generated or analyzed during this study are available. It shall be provided by the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

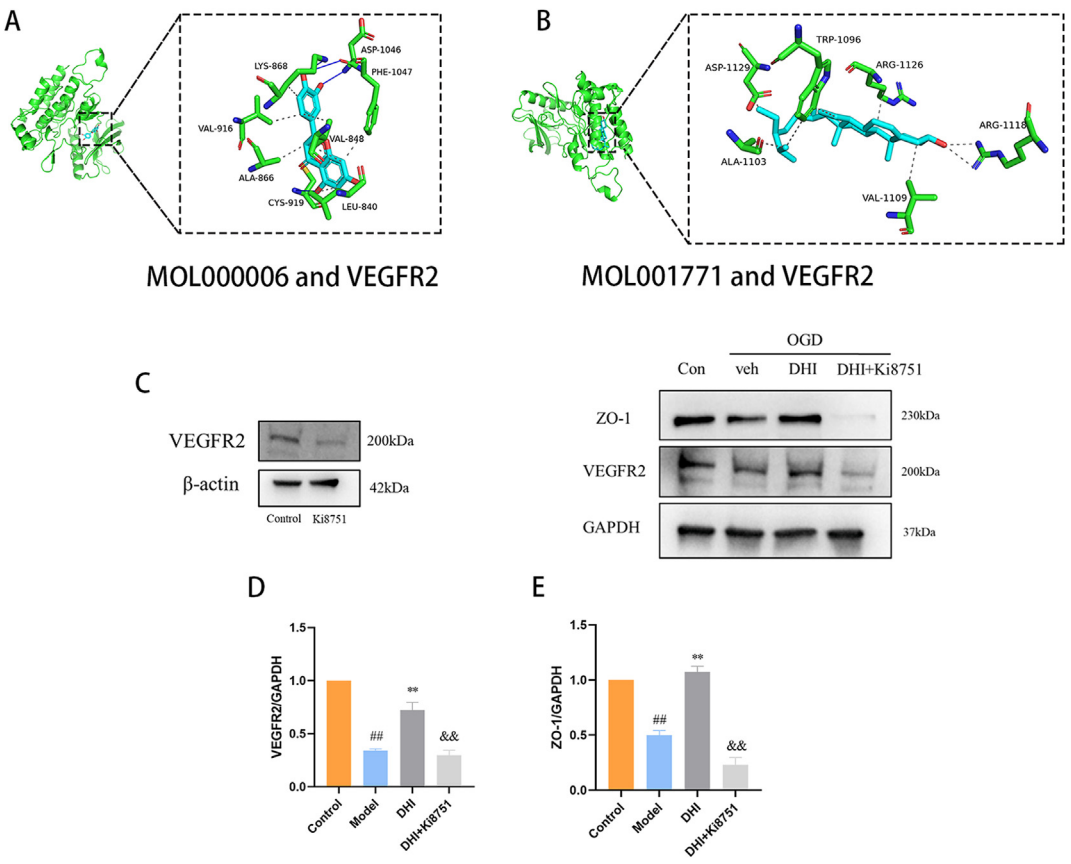

Fig. 7. DHI showed BBB protective effects in a VEGFR2-dependent manner. (A) Molecular docking pattern of MOL000006 and VEGFR2. (B) Molecular docking pattern of MOL001771 and VEGFR2 (C) The inhibition efficiency of Ki8751. The cells were collected and detected for (D) VEGFR2, and (E) ZO-1 protein content in the cells after lysis. Results are expressed as means ± SEM (n ≥ 3). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ in comparison to the sham group. $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison to the model group. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ in comparison to the DHI group.

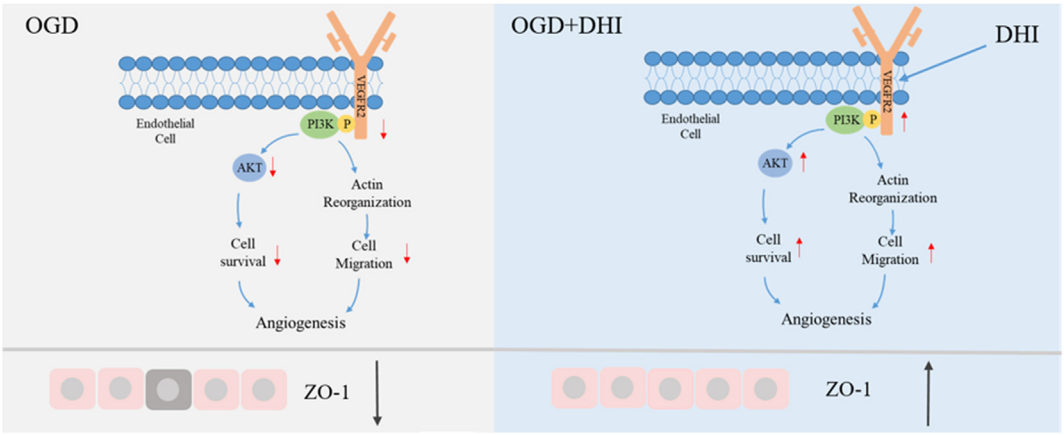

Fig. 8. Integrating network pharmacology and experimental validation reveals the mechanism of Danhong injection alleviating OGD-induced BBB injury via VEGFR2/PI3K/AKT Pathway.

Patient consent for publication

Not applicable.

CRediT authorship contribution statement

Yutong Zhang: Writing – original draft, Validation, Methodology, Investigation, Data curation. **Meixia Xie:** Investigation, Data curation. **Jiayin Liang:** Investigation, Data curation. **Li Li:** Writing – review & editing. **Shumei Wang:** Writing – review & editing, Supervision. **Minghua Xian:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare the following personal relationships which may be considered as potential competing interests: Li Li is currently employed by Celularity.Inc.

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Abbreviations

VEGFR2	Vascular Endothelial Growth Factor Receptor 2
PI3K	Phosphatidylinositol 3-kinase
BCA	kit Bicinchoninic acid kit
TCMSP	Traditional Chinese Medicine System Pharmacology Database
GO	Gene ontology
KEGG	Kyoto encyclopedia of genes and genome

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