Integrating Network Pharmacological and Experimental Models to Investigate the Therapeutic Effects of Baicalein on Glaucoma

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Abstract

Background: Traditional Chinese medicine (TCM) has a long history of treating glaucoma with remarkable effects, but there is no clear conclusion on its mechanism.

Methods: Network pharmacology and molecular docking were used to analyze the mechanism and targets of TCM in the treatment of glaucoma, and use baicalein to intervene in chronic ocular hypertension rat animal models to verify.

Result: The results of animal experiments showed that baicalin could significantly reduce the intraocular pressure (IOP) in a rat model of chronic ocular hypertension and protect the structure of the retina and optic nerve, as shown by hematoxylin-eosin (H&E) staining and transmission electron microscope (TEM). Reduce the apoptosis of retinal ganglion cells (RGCs) by up-regulating the expression of anti-apoptotic protein BCL-2, and adjusted the expression of AKT1, HIF-1α up and down to a certain extent, this trend is basically consistent with the results of the molecular docking. In the network pharmacology analysis, many key proteins of biological pathways involved in the herbal therapeutic processes on glaucoma like AKT1 (core protein of PI3K/AKT signaling), TP53 (a tumor suppressor gene coding tumor protein P53), STAT3 (core protein of JAK/STAT signaling), IL6 and IL1B (pro-inflammatory factors). And their interactions built complicated chain reaction in the process of glaucoma.

Conclusion: By combining the analysis of network pharmacology and experimental verification revealed that baicalein can effectively improve the symptoms of glaucoma and reduce RGCs apoptosis, suggesting the potential mechanism of TCM in treating glaucoma is related to regulating inflammation and cellular immunity, and reducing apoptosis.

Introduction

Glaucoma, an optic neuropathy, has become a leading cause of irreversible blindness due to its unpredictable morbidity and atypical initial symptoms, resulting in worldwide and profound visual impairment. According to a report of the World Health Organization (WHO), there are approximate 76 million people (40 to 80 years old) are suffering from vision loss caused by glaucoma, and glaucoma has become the fifth cause of vision impairment in the world[1]. The etiology of glaucoma can be divided into the primary which is usually unknown and the secondary which can be attributed to trauma, drug, uveitis, and so on. But it can also be classified as the open-angle glaucoma (OAG) and angle-closure glaucoma (ACG) according to the pathological changes of anterior chamber angle (ACA). The ACG always exhibits rapid visual impairment and drastic eye pain or headache. But in contrast with ACG, the OAG have a chronic and nearly asymptomatic progression which allowed it to cause disastrous damage at retinal ganglion cells (RGCs) before visiting hospital[2].

The risks of glaucoma include family inheritance, black races, senior, use of corticosteroids and high IOP[2–4]. But the management of IOP, including drugs, surgery treatments and prognostic estimates, is the only verified way that which has been widely used in the clinic to slow down disease progression[5–7]. Loss of RGCs is the final pathological procession of glaucoma, and is directly responsible for the irreversible vision impairment. At the importance of RGCs in the pathology of glaucoma and limits of current treatments, ophthalmological practitioners and researchers put more attention on the neuroprotection and regeneration of RGCs[8,9].
TCM is widely used in the prevention and treatment of glaucoma in China, and some researches found out profound neuroprotection effects of TCMs\(^\text{10}\). Erigeron Breviscapus, Radix Salviae, Lycii Fructus, Croci Stigma and Ginkgo Folium are 5 herbs that are usually used in the treatment of glaucoma in China. But on account of multicomponent and complex effects of TCMs, it lacks an appropriate method to elucidate the treatment mechanism of TCM. The Network Pharmacology is a multiple and systemic research method on the treatment mechanism of drug based on system pharmacology and network biology, making it overcame the obstacles above\(^\text{11}\). In the present study, we constructed Glaucoma-Targets-Drug and Protein-Protein Interaction (PPI) Networks based on the herbal active ingredients and drug-disease common targets acquired from different databases, and performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways enrichment analysis. In the end, we processed the molecular docking verification on the herbal active ingredients and their targets proteins which play important roles in signaling pathways related to glaucoma, which allowed us to understand the possible connective patterns and binding energies of active ingredients with target proteins. We constructed an animal model of chronic ocular hypertension (COH) and gave the model group baicalein for treatment for animal experiment verification. We exhibited the work procedures of our research as a schematic illustration to help understanding in Fig. 1.

Methods

Herbal data collection

The active ingredients of Erigeron Breviscapus, Radix Salviae, Lycii Fructus, Croci Stigma and Ginkgo Folium were acquired from Traditional Chinese Medicine Systems Pharmacology (TCMSP) Database (http://tcmspw.com) which focused on Traditional Chinese Medicine\(^\text{12}\), under the screening conditions of oral bioavailability (OB) $\geq 30\%$ and drug-likeness (DL) $\geq 0.18$. We also used the TCMSP to obtain the drug-related targets (DRTs), and then acquired their Gene Symbol from Uniprot Database (https://www.uniprot.org).

Acquirements of Glaucoma-Related Targets

The disease targets information were collected from 3 databases: Online Mendelian Inheritance in Man Database (OMIM, https://omim.org), GeneCards Database (https://www.genecards.org) and GEO Datasets of NCBI (https://www.ncbi.nlm.nih.gov/gds)\(^\text{13}\). We searched the key term “Glaucoma” in OMIM and GeneCards to obtain targets, and in GEO to obtain the gene chip data of GSE9944 and the platform files of GPL571 and GPL8300. The screening of differential genes expression was performed by limma package of R software (version 3.6.0) with the filters of $P > 0.05$ and $|\log_2$ fold change (FC)$| > 0.5$. Finally, we summarize the targets and differential genes above into the known glaucoma-related targets (GRTs).

Construction of Glaucoma-Targets-Drug and Protein-Protein Interaction (PPI) Networks

The Drug-Disease common targets were collected via the R software (version 3.6.0) and were exhibited as the Venn diagram. Then we used Cytoscape software (version 3.7.2) to construct and visualize the Glaucoma-Targets-Drug network. At the screening conditions of “Organism = Homo sapiens” and “High Confidence (> 0.9)”, the common targets were also used to build the PPI network through STRING Platform (version 11.0). The nodes
and edges between the nodes consisted of the network above. In the networks above, the degree of the node is the number of edges connected to the node, and a higher degree means more important the node it is.

**Gene Ontology (GO) Biological Processes Enrichment Analysis**

To explore the functional processes of Drug-Disease common targets, we perform the GO biological processes enrichment analysis on these targets in the R/Bioconductor environment (http://www.bioconductor.org/). Based on Gene Ratio, we has shown the top 20 processes ($P < 0.05$) as histogram and bubble chart.

**Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways Enrichment Analysis and Construction of Functional Pathways Network**

To clarify the potential molecular mechanisms, KEGG pathways enrichment analysis was performed by Metascape Platform (https://metascape.org/), and we also exhibited a heatmap of the top 20 KEGG pathways. Then 8 pathways highly related to glaucoma were selected to construct the Functional Pathways network which also involved related targets and active ingredients. The Functional Pathways network was constructed by Cytoscape software.

**Molecular Docking Study**

We performed molecular docking to verify the connective validity of active ingredients and core targets through Autodock Vina (version 1.1.2)[14]. The 3D structure of active ingredients and target proteins were obtained from the TCMSP database and RCSB PDB database (http://www.rcsb.org/) respectively. The proteins were dehydrated and hydrogenated and set grid box via AutoDockTools software (version 1.5.6). To find out exhaustive docking patterns, the docking of ingredients and targets were performed in a flexible and unrestrained manner which allowed the ligand to move through the entire volume of the grid box. After the steps above, the docking results were visualized by PyMOL software (https://www.pymol.org).

**Animal and Environmental Conditions**

Male Sprague-Dawley (SD) rats (200 ± 20)g from Laboratory Animal Centre, Chengdu University of Traditional Chinese Medicine were used, and the experiment was approved by the Committee of Scientific Research and the Committee of Animal Care of the Chengdu University of Traditional Chinese Medicine (permission number: 2020-12). Adaptively fed for one week at a cozy temperature of 23 ± 2 °C and temperate humidity of 40 to 60%. We abided by guidelines about animal use from the Association for Research in Vision and Ophthalmology (ARVO) throughout the study.

**Chemicals and antibodies**

Baicalein, sodium carboxymethyl cellulose (CMC-Na) and balanced salt solution were purchased from Shanghai yuanye Bio-Technology Co.,Ltd.(Shanghai, China).

**Animal Modeling, grouping and Treatment**

Total 20 SD rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium solution at the dose of 50mg/kg weight, and then received the episcleral veins cauterization (EVC) surgery in right eyes by same skillful operator. The EVC surgery were compliance with the procedures designed by Shareef[15]: three small incisions of conjunctiva in right eye were created to exposure episcleral veins, and then cauterized three vein stems using
cautery pen; and the intact left eye were taken as self-contrast. The sham-operated group rats’ left eyes received a sham procedure. Random grouping after the modeling: 10 rats performed sham operation on the left eyes as control group (CON) as with as right eyes-modeling as COH, and 10 rats in the BAL (right-modeling). The intragastric administration of drugs were begun one day after modeling surgery: the groups above received suspensions of baicalein (200mg/kg weight) and 0.5% CMC-Na solution respectively.

After 28 days, all rats were euthanized by overdose anesthesia after experiment period. Eyeballs, retina and optic nerves were collected carefully for the subsequent assays.

**IOP Measurement**

The assay of IOP was performed at pre-operation (day surgery), the first day after surgery (before drug-administration), the third day of surgery, the seventh day after surgery, fourteenth day after surgery, and twenty-first day after surgery by TONOLAB tonometer TV02 (icare, Finland). The measurements of IOP were performed at least three times on each eye, and the nearest integer to the mean value of measurement results above were recorded as the final IOP of the eye. According to the IOP results of the first day after surgery (before drug-administration), we excepted rats whose IOP (operated eye) < 22 mmHg from the subsequent experiment.

**Retinal Thickness and Apoptosis Assays**

H&E stain and TdT-mediated dUTP Nick-End Labeling (TUNEL) assay were performed to quantitate the thickness of ganglion cell layer (GCL) and apoptosis of RGCs of the retina. Ocular tissues were fixed by formaldehyde, acetic acid, and saline (FAS) eyeball fixative and embedded in paraffin after dehydration by gradient concentration ethanol, and then sliced into 7µm thick slides by Rotary Slicer- RM2016 (Leica, Germany). The slides of H&E stain were stained by hematoxylin and eosin and sealed by neutral balsam. TUNEL stain were processed by In Situ Cell Death Detection Kit, Fluorescein (Roche Group, Switzerland). After dewaxing, the slides were incubated for 1 hour at the temperature of 37° in a dark room, and DAPI stain was used to visualize nuclei. The slides of the TUNEL stain were sealed by gelatin glycerin. The images of the slides above were collected and analyzed by Pannoramic 250 Flash (Danjier, Jinan, China).

**Immunofluorescence Stain for Retinal**

Ocular tissues were fixed by form 4% paraformaldehyde fixative and phosphate buffer saline (PBS) and dehydrated in sucrose, and then sliced into 7µm thick slides by Rotary Slicer- RM2016 (Leica, Germany). The BCL-2 antibody, FITC conjugated Goat Anti-Rabbit IgG were purchased from Bioss Inc and Servicebio Inc. After dewaxing, washed three times with PBS, blocking room temperature for 30 minutes in serum. Added primary antibody (BCL-2 1:100), secondary antibody and DAPI separately, and sealed by anti-fluorescence attenuation reagent. The images of the slides above were collected and analyzed by Pannoramic 250 Flash (Danjier, Jinan, China).

**Transmission Electron Microscopy for Optic Nerve**

The optic nerve samples were fixed in 3% glutaraldehyde and postfixed in 1% osmium tetroxide (Leica Company, German). After dehydration by gradient acetone dehydration, the optic nerve samples were permeated in Epon812 epoxy resin (Beijing Keyi, Beijing, China). Then the permeated samples were embedded in flat molds and heating polymerized for embedded blocks. The ultrathin sections (50 nm) were made with an ultramicrotome and double-
stained with Reynolds’s lead citrate and 0.5% aqueous uranyl acetate (Beijing Keyi, Beijing, China). The microscopic examination was performed by JEM-1400PLUS TEM (JEOL, Tokyo, Japan), and the image proceeded with OlyVIA software (version 2.8).

**Western Blot**

Retinal tissues were lysed, homogenized and then centrifuged at 12000 rpm for 10 minutes at 10 °C. The AKT1, HIF-1α and β-actin antibodies were purchased from Bioss Inc and Abclonal Inc. Sample protein extracts were attenuated into 25mg/ml as the standard solution and were mixed with BCA working solution on ferment plate for 30 minutes at 37°C. Concentrations of standard solution were assayed by multiscan spectrum. After the mixing with loading buffer, the protein lysate was electrophoresed for 15 minutes, and then transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were incubated with primary antibodies (AKT1 1:5000, HIF-1α 1:1000, β-actin 1:100000). And exposure scanning of the strips was carried out with Tianeng GIS Chassis Control Software (version 2.0) and quantified by ImageJ software (version 1.49).

**Results**

*Screening of Active Ingredients and Drug Related Targets*

At the screening condition of OB ≥ 30% and DL ≥ 0.18, we selected 143 active ingredients from total ingredients of 5 Traditional Chinese Medicines (Erigeron Breviscapus, Radix Salviae, Lycii Fructus, Croci Stigma and Ginkgo Folium) and obtained 226 DRTs from TCMSP database. We listed some active ingredients in Table 1.

<table>
<thead>
<tr>
<th>MolID</th>
<th>Name</th>
<th>MolID</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOL000098</td>
<td>quercetin</td>
<td>MOL000093</td>
<td>dan-shexinkum d 1-hydroxy-2,3,5-trimethoxy-xanthone</td>
</tr>
<tr>
<td>MOL000006</td>
<td>luteolin</td>
<td>MOL007963</td>
<td>1-milionone I</td>
</tr>
<tr>
<td>MOL002714</td>
<td>kaempférol</td>
<td>MOL000119</td>
<td>Isotanshinone II</td>
</tr>
<tr>
<td>MOL007154</td>
<td>baicalein</td>
<td>MOL007111</td>
<td>beta-sitosterol</td>
</tr>
<tr>
<td>MOL003929</td>
<td>tanshinone iia</td>
<td>MOL00358</td>
<td>4-methylenemilitrone</td>
</tr>
<tr>
<td>MOL000439</td>
<td>formononetin</td>
<td>MOL007049</td>
<td>neocryptotanshinone ii</td>
</tr>
<tr>
<td>MOL000354</td>
<td>isorhamnetin</td>
<td>MOL007124</td>
<td>isocryptotanshi-none</td>
</tr>
<tr>
<td>MOL000449</td>
<td>Stigmasterol</td>
<td>MOL007108</td>
<td>cryptotanshinone</td>
</tr>
<tr>
<td>MOL008400</td>
<td>glycitein</td>
<td>MOL007088</td>
<td>2-isopropyl-8-methylphenanthrene-3,4-dione</td>
</tr>
<tr>
<td>MOL007100</td>
<td>dihydrotanshinlactone</td>
<td>MOL007041</td>
<td></td>
</tr>
</tbody>
</table>

*Glaucoma-Related Targets Acquirements and Drug-Glaucoma Common Targets Filtering*

We acquired 53 and 4130 glaucoma related targets from OMIM and GeneCards database respectively. We analyzed the gene chip GSE9944 and related platform files GPL571 and GPL8300 obtained from GEO datasets by searching key term “Glaucoma”, and the results provided 940 differential genes which has been exhibited as
Heatmap and Volcano plot through R software (Figure 2-a, b). Summarizing the targets and differential genes above, we obtained 4821 GRTs totally. 146 Drug-Disease common targets (Figure 2-c) were collected by the intersection of GRTs and DRTs performed by R.

_Glaucoma-Targets-Drug Network Analysis_

At the help of Cytoscape, we constructed the Glaucoma-Targets-Drug network based on 146 common targets and 99 glaucoma-related active ingredients of 5 herbs (Figure3-a). The active ingredients were shown as the MolIDs acquired from TCMSP, and were stained by different color to distinguish the herbal origins. In these glaucoma-related active ingredients, 2, 6, 10, 20, 54 of them only belong to Croci Stigma, Erigeron Breviscapus, Ginkgo Folium, Lycii Fructus and Radix Salviae respectively, but there are 7 ingredients which are contained by more than 2 herbs. The ingredients and targets were laid out as 2 and 3 concentric circles respectively according to their degrees (inner > outer). The top 10 active ingredients according to degree were listed in Figure 3-b.

_Drug-Glaucoma Common Targets Protein-Protein Interaction (PPI) Network Analysis_

As shown in Figure3-c, we constructed the PPI network based on 146 common targets at the work of String Platform. The nodes in PPI network represent different target proteins, and they are connected by edges. We excepted disconnected nodes and nodes with low interaction score (<0.9) from PPI network, and finally acquired 146 nodes and 626 edges. According to the degree (number of the node-connected edges) of nodes, we exhibited top 20 targets as histogram (Figure3-d). The protein was shown as the gene symbol, and some of them such as STAT3, AKT1, TP53, TNF and so on are involved in the pathogenesis and development of glaucoma.

_GO Enrichment Analysis of Drug-Glaucoma Common Targets_

After the PPI analysis, we performed GO enrichment analysis at the help of R software to clarify what biological processes were involved in and how they acted in the etiology of glaucoma and herbal therapeutic effects. There are 148 biological processes meet the \( P < 0.05 \), and the Gene Count decides the importance of the process. We built the histogram and bubble chart (Figure4-a, b) of biological processes (top 20) according to gene counts (x axis), and the gradient-colored bars/bubbles changed with the \( P \) value of related processes. The biological processes above are involved in multiple functions of cellular metabolism, signaling communications, gene expressions and so on, such as cytokine receptor binding, nuclear receptor activity, transcription activator and RNA polymerase activity and so on.

_KEGG Enrichment Analysis of Drug-Glaucoma Common Targets_

We acquired 178 signaling pathways (\( P < 0.05 \)) from the KEGG signaling pathways enrichment analysis performed by Metascape platform. Top 20 biological signaling pathways were shown in the Figure 5-b according to the \(-\log_{10}(P)\). Then we chose some signaling highly related to glaucoma, the common targets involved in these pathways and active ingredients of herbs to build the Functional Pathways network (Figure 5-a), which contained 8 pathways, 71 common targets and 82 active ingredients (shown as MolID). The 8 signaling pathways of the network include AGE-RAGE signaling pathway, IL-17 signaling pathway, HIF-1 signaling pathway, FoxO signaling pathway, NF-κB signaling pathway, Jak-STAT signaling pathway, Calcium signaling pathway and Wnt signaling pathway (Figure 5-c).

_Molecular Docking Verification of Core Targets and Active Ingredients_
Based on the results of KEGG signaling pathways enrichment analysis, we performed the molecular docking on the Core targets protein of the important signaling pathways, including AKT1(1unq), BCL2(2o22), HIF1A(4h6j), IL6(4cni), MDM2(5laz), STAT1(3wwt) and STAT3(6njs). And we obtained the active ingredients related to these core targets from the analysis of Glaucoma-Targets-Drug Network, including quercetin(MOL000098), baicalein(MOL002714), luteolin(MOL000006), kaempferol(MOL000422), beta-sitosterol(MOL000358), tanshinone iia(MOL007154) and cryptotanshinone(MOL007088). The 3D structures of ingredient molecules and core target proteins were obtained from TCMS and RCSB PDB databases respectively. After dehydration and hydrogenation, we performed docking work at the help of AutoDockTools and AutoDock Vina software. The absolute value of binding energy is positive correlation with connective stability of the ingredient molecule and target protein, and we visualized the most stable connective patterns in Figure6-a. Details of binding energies were listed in Figure 6-b.

IOP change

IOP was continuously monitored in each group before modeling and 1, 3, 7, 14, 21, 28 days after operation. Table 2 and Figure 7 shows IOP changes in Control group (CON), Baicalein group (BAL) and COH group (COH). BAL and COH were in high IOP after operation. BAL was significantly lower than COH group from 14 days after operation.

Table 2: IOP after operation (mmHg).

<table>
<thead>
<tr>
<th>DAO</th>
<th>Pre-operation</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>10.67±1.12</td>
<td>12.44±1.2</td>
<td>13.11±1.36</td>
<td>11.89±1.27</td>
<td>12.44±1.33</td>
<td>12.78±1.39</td>
<td>12.11±1.17</td>
</tr>
<tr>
<td>COH</td>
<td>11.33±0.71</td>
<td>26.56±3.3▲</td>
<td>26.44±4.85</td>
<td>24.89±3.26</td>
<td>23.78±2.39</td>
<td>23.00±2.92</td>
<td>23.00±2.8*#</td>
</tr>
<tr>
<td>BAL</td>
<td>10.67±2.24</td>
<td>28.33±2.2▲</td>
<td>23.44±1.01</td>
<td>23.44±3.05</td>
<td>23.56±0.88</td>
<td>16.78±2.28</td>
<td>17.33±2.6*#△</td>
</tr>
</tbody>
</table>

Note: Comparisons before and after modeling at different times ▲ P < 0.05, comparisons at 1 days after operation and 28 days after operation in the same group * P < 0.05, comparisons at 28 days after operation with CON # P < 0.05, comparisons between CON and BAL △ P < 0.05 at 28 days after operation.

GCL thickness change for retinal

Next, we observed morphology and structure changes in the ganglion cells and nerve fiber using H&E staining of ganglion cells and nerve fiber tissues (Figure 8-a). In CON, normal morphology and structure of ganglion cells and nerve fiber layer (NFL) were observed with H&E staining. In the COH, H&E staining revealed the structure of ganglion cells and NFL was disordered, and the thickness of GCL was decreased obviously (P < 0.0001). However, these destructive changes mediated by chronic ocular hypertension induction were markedly ameliorated by BAL (P = 0.0064). These results indicate that BAL has a protective effect against visual damage in COH rats.

TUNEL apoptosis and Immunofluorescence Stain assay for Retinal

To investigate the protective effects of BAL on visual damage, in particular RGCs apoptosis (a main mechanism of glaucoma), we evaluated the protein (Figure 9-a) expression of cells apoptosis regulating factors by TUNEL
and Immunofluorescent staining (Figure 9-b).

No significant TUNEL staining positive cells were found in each field of CON. Significant TUNEL staining positive cells were observed in the sections of both the COH ($P<0.0001$) and BAL ($P<0.0001$) (Figure 9-a,b).

Only a small amount of BCL-2 was expressed in retina in CON, which was increased in BAL, the protein expression was significantly higher than in CON ($P<0.01$) (Figure 9-c,d).

**Ultrastructural change of optic nerve**

Compared with the CON, the COH had obvious neurofibropathy and apoptosis-related features, myelin lamellar release and partial demyelination, but complete demyelination and axonal dissolution have not yet occurred. The BAL also had myelin lamina changes, but to a lesser extent than COH. When the nerve Myelin was significantly released, the Myelin thickness was increased by lamellar separation, and the diameter ratio of Axon/Axon plus Myelin (A /APM) was significantly reduced, which is considered to be one of the manifestations of chronic degeneration of the optic nerve. According to the ratio of $A/AM$ in the COH, it was significantly lower than that in the CON and BAL, and the difference was statistically significant($P<0.05$). However, there was no significant difference between CON and BAL, with no statistical significance, as shown in Figure10.

**Western blot**

To investigate the effects of AKT1, HIF-1α in the neuroprotective function of BAL, on the survival of RGCs, we evaluated the regulatory factor AKT1, HIF-1α by Western blot analysis(Figure 11-a). There was no difference in the protein expression of AKT1 and HIF-1α among the groups ($P > 0.05$) (Figure 11-b).

**Discussion**

Traditionally, glaucoma is caused by increased IOP, which changes the structure of the optic nerve sieve plate, resulting in mechanical damage of nerve fibers$^{[16]}$. Elevated IOP can lead to characteristic optic neuropathy, but it is not the only cause. Several studies have shown that the occurrence of glaucomatous optic neuropathy is associated with ocular hemodynamics, oxidative stress and inflammatory response, excitatory glutamate toxicity, and nutrient factor deficiency$^{[17, 18, 19, 20, 21]}$. However, recent studies have found that some patients' disease continues to develop, even though the IOP is under stable control$^{[22]}$. Instead, researchers turned to the damage and loss of retinal ganglion cells and their axons, leading to the idea of a pathological mechanism for glaucoma characterized by optic neuropathy. With the irretrievable damage of RGCs, the loss of vision seems to be the destiny of chronic glaucoma patients due to the lack of effective treatments. A meta-analysis performed by Li suggested that the TCM interference can actually improve the visual acuity of glaucoma patients$^{[23]}$.

Traditional Chinese medicine (TCM) has a history of thousands of years of clinical practice and are widely used to treat glaucoma in China$^{[24]}$. However, due to its complex composition and many targets, we cannot fully understand its mechanism of action. However, network pharmacology is a discipline that can predict the target of drug action and improve the efficiency of drug discovery, which can help analyze and understand the targets and mechanisms of Chinese herbs$^{[25]}$. *Erigeron Breviscapus, Radix Salviae, Lycii Fructus, Croci Stigma and Ginkgo Folium* are usually used by practitioners of TCM in the treatment of chronic glaucoma$^{[26, 27, 28, 29, 30]}$. Based on network pharmacology, we obtained 99 active ingredients and 146 target proteins related to the pathological
progression of chronic glaucoma. But most of the current studies about TCM were limited in specific ingredient reaction with special targets, which ignored the multiple ingredients and their cooperation with TCM. There are more herbal ingredients filtered by us that need further researches to verify their therapeutic mechanism on glaucoma.

The PPI network constructed by common targets showed that many key proteins of biological pathways involved in the herbal therapeutic processes on glaucoma like AKT1 (core protein of PI3K/AKT signaling), TP53 (a tumor suppressor gene coding tumor protein P53), STAT3 (core protein of JAK/STAT signaling), IL6 and IL1B (pro-inflammatory factors). And their interactions built complicated chain reaction in the process of glaucoma. Li's research showed that PI3K/Akt1 signaling activation was a positive correlation with the apoptosis of RGCs both in vitro and in vivo in the simulated ocular hypertension environment[31]. An in vitro experimental study showed that IL6 activation regulates fibrosis of trabecular meshwork induced by TGF-β to attenuate glaucoma progression[32]. Astrocytes, the most typical within the glia of the central nervous system (CNS), have been also confirmed involving in the pathology of glaucoma. STAT3 plays a core role in the activation of astrocytes in glaucoma, which can exhibit neuroprotective or neurotoxic effects on RGC in different locations and conditions. As the structural cells in the lamina, excessive proliferation of astrocytes can result in glial scar and become the obstacle for regeneration of damaged axons of RGCs[33]. But Danniel found that knockdown of STAT3 resulted in lower activated astrocytes and higher apoptosis rate of RGCs in the rat glaucoma model[34]. Such a complicated PPI doesn't only mean the complex of glaucoma, but also suggest that therapeutic effects of TCM involved multiple molecular mechanism and network interactions of target proteins.

In the present GO biological processes enrichment analysis, most of important processes have been contained in our results such as activation of transcription factors and activators, cytokine and related receptors activation, ubiquitylation and protease activity, messenger molecules activity and phosphatase activity. Those processes managed cell DNA replication and transcription, functional proteins synthesis and metabolism, signaling transmissions and so on.

As shown in KEGG enrichment analysis, signaling pathways main involved in the treatment effects are AGE-RAGE signaling pathway, IL-17 signaling pathway, HIF-1 signaling pathway, FoxO signaling pathway, NF-κB signaling pathway, Jak-STAT signaling pathway, Calcium signaling pathway and Wnt signaling pathway, and they have many mutual proteins to sustain the complicated cross-talk among them[35,36,37,38,39]. We performed the molecular docking on the Core targets protein of the important signaling pathways, including AKT1(1unq), BCL2(2o22), HIF1α(4h6j), IL6(4cni), MDM2(5laz), STAT1(3wwt) and STAT3(6njs).

In this study, we established the chronic ocular hypertension model and found that baicalein has a protective effect on RGCs, which was also consistent with the previous results of Zhang's study of acute ocular hypertension in mice[40]. These results suggest that baicalein may protect astrocytes from oxidative stress-induced apoptosis and promote synaptic regeneration of ganglion cells by promoting the high expression of BCL-2. Bcl-2 family are the key regulators in apoptosis, and are highly-related to the loss of RGCs in glaucoma[41]. Bax, the downstream executor of the Bcl-2 family, eliminating the Bax gene is considered to be the only effective strategy to protect RGCs in glaucoma models[42]. Our network pharmacology research was based on the current status of treatment and the experiment on the mechanism of baicalein was not in-depth. Suggesting future can increase in vitro experiments, screen drug concentration, and by blockers more comprehensive verification of theoretical predictions.
Conclusion

In this study, we explored the protective effects and potential molecular mechanisms of TCM in the treatment of glaucoma through network pharmacology, and used baicalein to intervene in an animal model of COH to verify. Network pharmacology analysis demonstrated that the mechanisms of herbal therapeutic against glaucoma were multi-component, multi-target, and multi-pathway. And their interactions built complicated chain reaction in the process of glaucoma. Moreover, AKT1, TP53, STAT3, IL6, IL1B and several pathways, which were closely related to inflammation and oxidative stress, were thought to play a significant role in the mechanism of action of herbal therapeutic against glaucoma. Molecular docking results showed that the core active ingredients showed good affinity with BCL-2, IL-6, STAT3, HIF-1α, AKT1 and so on, as with as the binding sites had stable hydrogen bonds. Further experimental evidences revealed that baicalein therapeutic notably ameliorated the visual injury possibly through inhibiting oxidative stress and inflammation in COH rats model, which at least partially verified the predicted consequences of network pharmacology. The therapeutic effect of TCM on glaucoma may be related to regulation PI3K/Akt pathway, and up-regulation of the expression of anti-apoptotic protein BCL-2. These findings provide direct evidence for TCM therapeutic in the prevention and treatment of glaucoma.

Abbreviations

ACG: Angle-closure glaucoma

ACA: Anterior chamber angle

A /APM: Axon/Axon plus Myelin

ARVO: Association for Research in Vision and Ophthalmology

AKT1: Proline-rich AKT1 substrate 1

BAX: Bcl-2-associated X protein

BCL-2: B-cell lymphoma 2

BAL: Baicalein group

CNS: Central nervous system

CON: Control group

COH: Chronic Ocular Hypertension group

CMC-Na: Sodium carboxymethyl cellulose

DL: Drug likeness

DRTs: Drug-related targets

EVC: Episcleral veins cauterization

GO: Gene Ontology
GRTs: Glaucoma-related targets

GCL: Ganglion cell layer

H&E: Hematoxylin-eosin

HIF-1α: Hypoxia-inducible factor 1, alpha subunit

IOP: Intraocular pressure

IL-6: Interleukin 6

IL-17: Interleukin 17

Jak-STAT: Janus kinases-Signal transducer and activator of transcription

KEGG: Kyoto Encyclopedia of Genes and Genomes

MDM2: Mouse double minute 2 homolog

NF-κB: Nuclear factor kappa-B

NFL: Nerve fiber layer

OAG: Open-angle glaucoma

OB: Oral bioavailability

PPI: Protein-Protein Interaction

RGCs: Retinal ganglion cells

SD: Sprague-Dawley

STAT3: Signal transducer and activator of transcription 3

STAT1: Signal transducer and activator of transcription 1

TCM: Traditional Chinese Medicine

TCMSP: Traditional Chinese Medicine Systems Pharmacology

TUNEL: TdT-mediated dUTP Nick-End Labeling

TEM: Transmission Electron Microscopy

TP53: Tumor protein p53

TNF: Tumor necrosis factor

TGF-β: Transforming Growth Factor Beta

Wnt: Wingless and Int-1
WHO: World Health Organization

**Declarations**

**Availability of data and material**

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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**Contributions**

JY, MZ and SZ conceived this study. JY, MZ, QS, XZ and LK performed the experiments. JY, MZ, QS and SL conducted the network pharmacology analysis and molecular docking verification. JY and MZ wrote the manuscript. MZ and QS edited pictures. SZ revised the manuscript. All authors read and approved the final manuscript.

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**Ethics approval and consent to participate**

This study was approved by the Committee on Ethical Use of Animals of Chengdu University of Traditional Chinese Medicine.

**Consent for publication**

Not applicable.

**Competing interests**
Not applicable.

References


Figures
Figure 1

Schematic Illustration of The Network Pharmacological Analysis and Molecular Docking-Based Study.
Glaucoma-related differential genes analysis and Drug-Disease common targets collection. (a) Heatmap of differential genes analysis on GSE9944; (b) Volcano plot of differential genes analysis on GSE9944; (c) Drug-Disease common targets collection.
Figure 3

Glaucoma-Targets-Drug network and common targets PPI network analysis. (a) Glaucoma-Targets-Drug network; (b) Degree of active ingredients of TCMs (top 10); (c) PPI network of common targets; (d) Degree of common targets (top 20) in PPI network.
Figure 4

GO-enrichment analysis of common targets. (a) Histogram of top 20 biological processes according to gene counts. (b) Bubble chart of top 20 biological processes according to gene counts.
Figure 5

KEGG enrichment analysis of common targets. (a) Functional Pathways network based on KEGG signaling enrichment analysis; (b) Histogram of top 20 signaling pathways according to -log10(P); (c) Important glaucoma-related signaling pathways and their target proteins.
Figure 6

Molecular docking analysis. (a) docking patterns of core proteins in glaucoma related signaling pathways; (b) binding energy of docking results.
Figure 7

IOP change during experiment (mmHg).
Figure 8

(a) Effects of BAL on the histological changes (a) and the GCL thickness (b) in retinal tissues in rats. All tissues were observed under a light microscope (x20), and representative pictures were shown. CON, control group; COH, chronic ocular hypertension group; BAL, baicalein group; **P < 0.01, ***P < 0.001, ****P < 0.0001. At the end of experiment, rats were sacrificed and the retinal was removed for histological analysis.
Figure 9

(a) The results of TUNEL apoptosis assay (× 400) for each group (n=3); (b) The proportion of apoptosis (apoptosis ratio = number of TUNEL/DAPI) in each group (*P<0.05, ****P<0.0001); (c) Immunofluorescent staining expression (× 400) of BCL-2 (n=3); (d) Quantitative analysis of BCL-2 fluorescent positive protein expression (**P<0.01).
Figure 10

(a) The ultrastructural changes of optic nerve tissue in different groups, CON showed a healthy optic nerve tissue. COH had myelin lamellar release and partial demyelination, which was more severe than BAL's damage (Bar=1μm; 2μm); (b) Axona(black, Bar=1μm) and axon plus myelin(red, Bar=1μm) diameter of each group were measured under high power(×20000); (c) Quantitative analysis of A/APM among the three groups(****P<0.0001).
Figure 11

(a) Relative levels of AKT1, HIF-1α proteins were normalized to β-actin expression and determined by western blotting; (b) The expressions of AKT1 and HIF-1α in retina of each group. Data are expressed as the mean ± S.D (n = 3).