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Dachengqi Decoction alleviate septic liver injury by regulating macrophage polarization through the JAK2/STAT3 pathway

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Abstract: The method applied in this paper were network pharmacology and animal experiment. Among them, network pharmacology was used to screen and predict the possible targets of Dachengqi Decoction and septic liver injury, and molecular docking and molecular dynamics were used to analyze the stability of the active components binding to JAK2. In animal experiment, 30 healthy male adult C57BL/6 mice were randomly divided into 6 groups: control group, model group, dexamethasone group (1.8 mg/kg), Dachengqi Decoction low (2.5 g/kg), medium (5 g/kg), and high (10 g/kg) concentration groups. The mice were given oral administration once a day. After 5 consecutive days of use, lipopolysaccharide was intraperitoneally injected (5mg/kg) was used to establish the model. The corresponding drugs were given orally 2 hours after model establishment, and the specimens were collected 6 hours later. HE staining was used to observe the pathological changes in the liver of mice; ELISA was used to detect IL-6, TNF-α, and IL-10 from serum and liver; liver function kit was used to detect ALT and AST from serum; Western blot was used to detect the protein expression of JAK2, p-JAK2, STAT3, p-STAT3, Arg-1, and iNOS in liver tissue; immunofluorescence was used to detect the protein expression of JAK2, STAT3, Arg-1, and iNOS; flow cytometry was used to detect the polarization of macrophages M1 and M2. The results of the above experiments show that, compared with the control group, the model group showed severe inflammatory reaction, liver tissue degeneration and inflammatory cell infiltration; Liver function ALT and AST were significantly increased ($P<0.05$); Pro-inflammatory factors IL-6, TNF-α were significantly increased, and anti- inflammatory factors IL-10 were increased ($P<0.05$); The protein expression of P-JAK2, P-STAT3 and iNOS in mouse liver tissue was significantly increased, and the protein expression of Arg-1 was significantly decreased ($P<0.05$); The proportion of M1 and M2 macrophages increased significantly ($P<0.05$). Compared with the model group, the liver injury of each drug intervention group was improved and the

inflammatory response was reduced; Liver function ALT and AST were significantly reduced ($P<0.05$); Pro-inflammatory factors IL-6 and TNF-α decreased, and anti-inflammatory factors IL-10 increased ($P<0.05$); The protein expression of P-JAK2, P-STAT3, and iNOS decreased, and the protein expression of Arg-1 increased ($P<0.05$); The proportion of M1 macrophages decreased and the proportion of M2 macrophages increased ($P<0.05$). Moreover, the change trend of dexamethasone group and Dachengqi Decoction high concentration group was more obvious. Therefore, we can draw the conclusion that Dachengqi Decoction can alleviate liver injury caused by sepsis, and its mechanism is related to the regulation of JAK2/STAT3 pathway and the regulation of macrophage polarization.

1. Background

Sepsis is a systemic inflammatory response syndrome (SIRS) caused by infection. Common causes include severe infections, trauma, and other factors. Clinically, sepsis often leads to multiple organ failure with a high mortality rate, representing an acute and critical condition[1]. During the course of sepsis, liver damage frequently occurs as a complication due to pathological mechanisms such as hepatocyte apoptosis induced directly by the inflammatory response, insufficient blood supply to the liver caused by microcirculatory disorders, coagulation dysfunction, and the unique physiological functions of the liver[2].

In the early stages of sepsis, macrophages play a crucial role in phagocytosing pathogens, cell fragments, and apoptotic cells, making them an important component of the innate immune system[3]. Macrophages, along with other antigen-presenting cells, can also initiate adaptive immunity by presenting antigens and secreting various cytokines through exocrine action to modulate immune responses. Macrophages exhibit extreme plasticity. Influenced by various specialized microenvironments, mature macrophages produce different phenotypes and perform distinct functions, a process known as macrophage polarization[4]. M1 macrophages, induced and activated by IFN-γ, LPS, and others, play a pro-inflammatory role in the early stages of sepsis. They possess strong anti-infection capabilities, eliminate apoptotic cells and necrotic tissues, and kill pathogens through antigen presentation-mediated Th1 immune responses. Conversely, M2 macrophages, induced and activated by IL-4, IL-13, and others, mediate anti-inflammatory responses and Th2 immune responses by secreting numerous anti-inflammatory factors, helping regulate immune responses in sepsis to control inflammation and minimize tissue damage[5]. Under normal circumstances, the proportions of M1 and M2 macrophages are balanced. When the tissue microenvironment changes, macrophages tend to differentiate into either M1 or M2 types, subsequently performing specific functions. In acute liver injury caused by sepsis, the severity of inflammation and tissue injury largely depends on the polarization state of macrophages[3, 4, 6].

Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signal pathway is the main pathway of signal transduction of various cytokines, mainly consisting of 4 JAK isoforms and 7 STAT isoforms. Typically, cytokine signals bind to receptors on the cell membrane, inducing the phosphorylation of JAK, which in turn activates STAT and forms a STAT dimer. This dimer then enters the nucleus as a transcription factor to regulate the expression of target genes[7]. Throughout

the course of sepsis, the body's tissue microenvironment changes dramatically from early hyperimmunity to later immunosuppression[8], the polarization state of macrophages also changes[9], with the JAK/STAT pathway continuously mediating this process. When LPS binds to TLR4 on the surface of macrophages, JAK2 and TYK2 within the cell are activated, subsequently phosphorylating STAT1, STAT3, and STAT4. This leads to an increase in the expression of iNOS, a marker gene for M1 macrophages, promoting M1 polarization, mediating the inflammatory response, and inducing apoptosis, which causes multiple organ damage, including liver injury. During M2 polarization, when cytokines such as IL-4 and IL-10 bind to their corresponding receptors on the macrophage membrane, JAK1/STAT6 plays a more significant role, increasing the expression of Arg-1, a marker gene for M2 macrophages. This promotes M2 polarization and reduces inflammatory damage[5, 7, 10].

Traditional Chinese medicine posits that the etiology and pathogenesis of "sepsis" are primarily due to infection by exogenous heat and toxins, internal heat and obstruction of the internal organs, and deficiency of the body's vital energy. Treatment methods primarily focus on clearing heat and detoxifying, alleviating internal obstructions, and strengthening the body's vital energy. Dachengqi Decoction is a renowned prescription attributed to Zhang Zhongjing, the esteemed physician of the Eastern Han Dynasty. This decoction originates from his book, Treatise on Febrile Diseases. It consists of four Chinese herbs: Rhei Radix Et Rhizoma, Natrii Sulfas, Aurantii Fructus Immaturus, and Magnoliae Officinalis Cortex. This decoction is a representative formula for clearing heat, alleviating congestion, and preserving Yin[11]. The four medicinal ingredients work together to detoxify the body, alleviate inflammation, and protect tissue cells. It is commonly used clinically to treat Yangming fu-viscera excess, internal heat syndrome, etc. In clinical practice, it is primarily used to treat acute pancreatitis, intestinal obstruction, postoperative gastrointestinal dysfunction, and other diseases, with its efficacy confirmed over many years of practice[12, 13]. The complexity of the ingredients and targets of Chinese medicinal compounds makes it challenging to conduct a comprehensive study on their mechanisms. Chinese medicine network pharmacology integrates multidisciplinary knowledge systems, such as biology and pharmacology, using computational methods to predict and screen the effective ingredients and potential targets of Chinese medicinal compounds, providing a new strategy for researching their mechanisms[14, 15].

Based on network pharmacology, this experiment screened the active ingredients of Dachengqi Decoction and the potential targets for the treatment of septic liver injury, and predicted the possibility of Dachengqi Decoction to treat septic liver injury through JAK2/STAT3 signal pathway. A model of acute liver injury due to sepsis was established in mice, and the mechanism of Dachengqi Decoction in alleviating this injury was explored through assessments of liver injury severity, inflammatory factors, and macrophage polarization trends. The potential relationship between the JAK2/STAT3 signaling pathway and macrophage polarization trends was established during the treatment of septic liver injury with Dachengqi Decoction, providing new insights into understanding its potential mechanisms in treating septic liver injury.

2. Materials and Methods

2.1. Network pharmacology-based analysis

2.1.1. Collection of active chemical components and potential targets of Dachengqi Decoction

The traditional Chinese medicine system pharmacology database analysis platform (TCMSP,

https://tcmsp-e.com/) was used to search for all active ingredients of Rhei Radix Et Rhizoma, Aurantii Fructus Immaturus, and Magnoliae Officinalis Cortex with oral bioavailability (OB) ≥ 30% and drug-likeness (DL) ≥ 0.18, allowing for the screening of active ingredients. All targets of the components were predicted by SwissTargetprediction (http://www.swisstargetprediction.ch/). After the sorted targets were calibrated by Uniprot (https://www.uniprot.org/) data, human genes were eliminated, invalid duplicate targets were deleted, and standardized gene names were obtained.

2.1.2. Acquisition of septic liver injury target genes and intersection targets

The keyword "Septic liver injury" was entered into the GeneCards (https://www.genecards.org/) and OMIM (https://www.omim.org/) databases to obtain disease-related targets. All targets in the database were integrated into excel, duplicate genes were removed, and the disease target gene information was obtained after correction by Uniprot database. The obtained drug component targets were mapped to the disease targets, and then a Venn diagram was drawn to obtain the intersection genes.

2.1.3. Construction of medicine-active ingredient-target network diagram

The network structure data of traditional Chinese medicines, active ingredients, and intersection targets were imported into Cytoscape 3.7.2 for topological calculations. Screening was performed with the condition that the median value was greater than two times, followed by visual analysis to illustrate their relationships. The setting node area and font size are positively correlated with the degree value, which is the number of connections between nodes. The higher the degree value, the more important the node is in the network.

2.1.4. Construction of PPI network targets of Dachengqi Decoction and septic liver injury

Drug and intersection genes were uploaded to the interaction database String (https://string-db.org/) for protein interaction network construction database; The species was set to "Homo sapiens," and the lowest interaction score was set to 0.7 to ensure credibility. The rest of the parameters were kept at the default settings, and the results were stored in TSV format. The TSV file was imported into Cytoscape 3.7.2, and the network was analyzed. The network analysis results were saved. The color and area of the nodes were positively correlated with the degree value, and the color and thickness of the edges were positively correlated with the relationship value score between the two connected target genes. The core target was selected to make a protein interaction network diagram.

2.1.5. GO analysis and KEGG pathway enrichment analysis

The drug-disease intersection gene was uploaded to the DAVID database (https://david.ncifcrf.gov/summary.jsp), the gene identifier was selected as OFFICIAL _ GENE _ SYMBOL, and the species was set as HomoSapiens. The function of DAVID6.8GO gene was used to annotate the effect of Dachengqi Decoction on septic liver injury from three aspects: biological process (BP), cellular component (CC), and molecular function (MF), as well as the role of target proteins in gene function. In order to clarify the target of Dachengqi Decoction in the treatment of septic liver injury, KEGG pathway enrichment analysis was carried out in the signaling pathway. The top 10 pathways of BP, CC and MF in GO function, and 20 pathways related to KEGG pathway entry ($P<0.01$) were selected as the main gene function enrichment process and signal

pathway of Dachengqi Decoction in the treatment of septic liver injury, and the mechanism in the treatment of septic liver injury was predicted.

2.1.6. Molecular docking analysis

The JAK2 protein was selected as the receptor protein among the core targets. Three effective active ingredients with the highest degree values related to JAK2 in the "drug-component-target" network were screened as ligands. The JAK2 protein structure was obtained from the Alphafold database (https://alphafold.com/), and the molecular structures of the effective active ingredients were obtained from TCMSP. Molecular docking was performed using the Autodock Vina software. The small molecules and proteins were preprocessed, and the active site was the entire protein. The docking center coordinates were set as center_x = -1.5, center_y = -5.2, and center_z = 8.0. The box size was set as a cube with an edge length of 90 A, and the Spacing step size was set to 0.375. Genetic algorithm was used for conformational sampling and scoring. The conformations were sorted based on the docking score, and the optimal conformation was selected for binding mode analysis. Finally, the results were analyzed and plotted using PyMOL software (Version 3.0.3).

2.1.7. Molecular dynamics analysis

Molecular dynamics simulations were conducted using the Gromacs2022.5 program. The Amber99sb force field was selected for the protein. The system was modeled with the TIP3P water model, and the protein and small molecule were placed in a cubic water box. The cutoff for both electrostatic and van der Waals interactions was set at 1.0 nm, and the time step was set at 2 fs. The Particle Mesh Ewald (PME) method was used for long-range electrostatic corrections. The simulation was carried out at a temperature of 30 ℃ (303.15 K) and a pressure of 1 bar. The established system was first subjected to energy minimization, followed by NVT equilibration dynamics for 100 ps and NPT equilibration dynamics for 100 ps. The V-rescale method was used for thermal coupling of the system temperature, and the Parrinello-Rahman method was used to control the pressure. Molecular dynamics sampling was performed for 100 ns. Calculations of root mean square deviation (RMSD), root mean square fluctuation (RMSF), solvent accessible surface area (SASA), radius of gyration (Rg), hydrogen bond number, and binding free energy were conducted using the gmx_MMPBSA module and Python provided by the Gromacs program.

2.2. Animal experimental materials

2.2.1. Experimental animals

Thirty C57BL/6 healthy male mice were purchased from SPF (Beijing) Biotechnology Co., Ltd., 8 weeks old, weighing approximately 18-22 grams. Animal License Number: [2022]IACUC Number: 3702.The experimental process was strictly carried out in accordance with the Ethical Review Guidelines. The mice were placed in a SPF environment and raised routinely, with a temperature of 18-23 ℃, natural circadian lighting, and a humidity of 55%-65%, with free access to food and water.

2.2.2. Preparation of Dachengqi Decoction

12g of Rhei Radix Et Rhizoma (back lower), 6g of Natrii Sulfas (Heat, stir and melt), 12g of Aurantii Fructus Immaturus and 24g of Magnoliae Officinalis Cortex were prepared from

Dachengqi Decoction, which were purchased from the Chinese Medicine Pharmacy of Union Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology. 96g of Magnoliae Officinalis Cortex and 48g of Fructus Aurantii were soaked in 1800mL of distilled water for 30 min, decocted together for 25 min and the filtrate was collected. Then, 48g of Rhei Radix Et Rhizoma was mixed with the previous filtrate and decocted for 10 min. Finally, 36g of Natrii Sulfas was added to the filtrate and stirred until it dissolved. A 0.22 μm microporous membrane was used for filtration on an ultra-clean bench. The above Chinese medicines were prepared into a medicinal liquid with a mass concentration of 1.0 g/ml and stored in a refrigerator at 4 ℃.

2.2.3. Reagents

TNF-α, IL-6, IL-10 enzyme-linked immunosorbent (ELISA) kits were purchased from Wuhan Pinofei Biotechnology Co., Ltd.; Chemiluminescent agent BCA and ECL kits were purchased from China Biyuntian Biological Company; Rabbit anti-mouse STAT3, Arg-1 and iNOS antibodies were purchased from Proteintech Group, rabbit anti-mouse JAK2 antibodies were purchased from Affinity, and goat anti-rabbit IgG antibodies were purchased from Proteintech Group; Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) detection kit were purchased from Nanjing Jiancheng Bioengineering Institute; Antibody CD80,CD206 were purchased from Becton, Dickinson and Company.

2.3. Animal experimental methods

2.3.1. Grouping and modeling administration

Thirty C57BL/6 healthy male mice were randomly divided into 6 groups: control group, model group, dexamethasone group (1.8 mg/kg), Dachengqi Decoction low (2.5 g/kg), medium (5 g/kg), and high (10 g/kg) concentration groups, with 5 mice in each group. Except for the control group, the remaining five groups received lipopolysaccharide intraperitoneal injections to establish septic acute liver injury mouse models. These groups were administered Dachengqi Decoction by gavage for five consecutive days, once daily, followed by a 5 mg/kg intraperitoneal injection of lipopolysaccharide to finalize the model. Different concentrations of Dachengqi Decoction were administered by gavage 2h after modeling, and samples were collected 6h later.

2.3.2. Pathological sections of liver tissue and HE staining

The liver tissue was fixed in 10% formaldehyde, dehydrated with ethanol, and cleared with xylene. Paraffin sections were made, 2μm sections were stained with HE, and the pathological changes of liver tissue were observed under light microscope, including the structural integrity of liver tissue cells, the degree of inflammatory cell infiltration, etc.

2.3.3. ELISA detection of TNF-α, IL-6, and IL-10 levels in serum and liver tissue

Six hours after modeling, the mice were sacrificed, and blood was collected from the eye socket. The blood was centrifuged at 3000 r/min for 15 minutes to collect the serum. The TNF-α, IL-6, and IL-10 ELISA kits were used according to the manufacturer’s instructions for detection.

2.3.4. Mouse liver function test

The above serum was tested in strict accordance with the instructions of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) detection kit.

2.3.5. Western blot (WB) analysis

Western blot analysis was conducted to detect the protein expression levels of JAK2, p-JAK2, STAT3, p-STAT3, Arg-1, and iNOS in mouse liver tissue. Pre-cooled RIPA cell lysate was added to the collected liver tissues of each group, and protein samples were prepared by centrifugation at 4 ℃, and the protein concentration was determined by BCA kit. According to the molecular weight of the protein, the concentration of SDS-PAGE separation gel, the voltage and time of membrane transfer were determined. The membrane was transferred to PVDF membrane, blocked at room temperature for 2h, washed three times, and incubated at 4 ℃ overnight. TBST was washed three times, and the secondary antibody was incubated at room temperature for one hour. After washing three times, the secondary antibody was exposed and developed. ImageJ was used to calculate the gray value, and the software was used to analyze the image, and the gray value of protein band was determined, and β-Actin was used as the internal reference.

2.3.6. Immunofluorescence (IF) analysis

The paraffin sections were dewaxed for immunofluorescence (IF) detection, permeabilized with PBS containing 0.5% Triton X-100 for 10 minutes, and incubated with the corresponding antibody at 4 ℃ overnight. Rinse 3 times with PBS for 5 minutes each. The IF slides were subsequently incubated with secondary antibody for 1 hour at room temperature in the dark and imaged with a light microscope.

2.3.7. Flow cytometry

Mice in each group were detected by flow cytometry, and the liver was grinded and filtered with a 200-mesh filter to obtain single cell suspension. Each group was divided into 2 tubes, centrifuged at 1000 r/min for 5 minutes, and the supernatant was discarded. The suspension was pipetted in PBS at 4 ℃ to prepare single cell suspension, then, transferred to 1.5 mL centrifuge tube and centrifuged at 1000 r/min for 5 minutes, and the supernatant was discarded. Then, The suspension was washed repeatedly for 3 times, and finally resuspended in 100μL PBS solution. 5μL CD80 antibody and 5μL CD206 antibody were added to the two centrifuge tubes in each group, and then 5uL F4/80 antibody was added at the same time. After 1 hour of incubation at 4℃ in the dark, then, the percentage of cells was measured.

2.4. Statistical analysis

The data were statistically analyzed using the statistical software GraphPad Prism 9.5, and the counting data were expressed in $\bar{x} \pm s$. One-way variance test was used for comparison between multiple groups, and t test was used for comparison of differences between groups. $P<0.05$ indicated that the difference was statistically significant.

3. Results

3.1. Active chemical compositions and target prediction of Dachengqi Decoction

The active ingredients and targets of Dachengqi Decoction were predicted using TCMSP. After applying the criteria of OB ≥ 30% and DL ≥ 0.18, invalid components were eliminated. A total of 18 components from Aurantii Fructus Immaturus, 3 from Rhei Radix Et Rhizoma, and 2 from Magnoliae Officinalis Cortex were identified. A probability greater than 0 was used as the inclusion criterion for targets, yielding 473 targets after deduplication.

3.2. Prediction of Septic liver injury targets

A total of 1378 and 104 targets related to septic liver injury were identified from the GeneCards and OMIM databases, respectively. After deduplication, 1477 unique targets were obtained. The genes were then corrected using the Uniprot database. By intersecting the drug targets with the septic liver injury target genes, 140 intersection target genes were identified, which represent the interactive targets for drug treatment. A Venn diagram was created to illustrate the drug-disease-target network (Fig. 1).

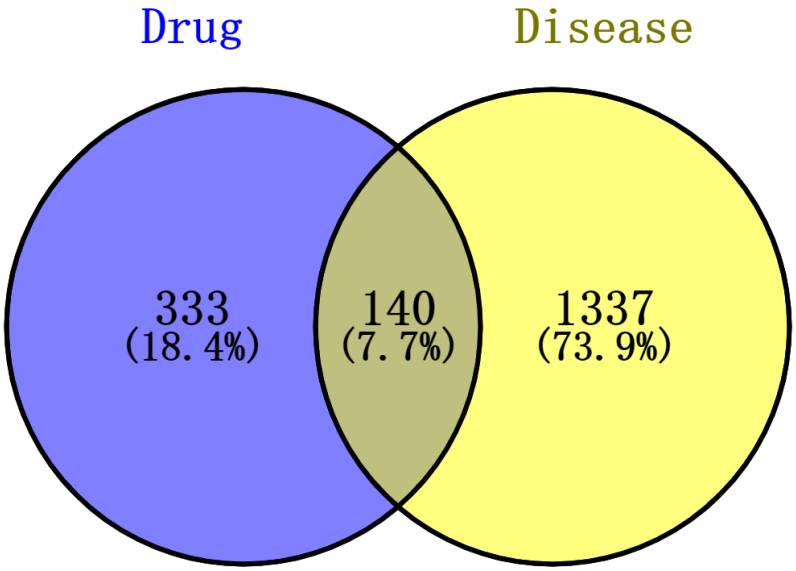


Fig.1 Venn diagram of drug-disease-target.

3.3. The PPI network of Dachengqi Decoction and septic liver injury intersection targets

After intersecting all drug targets with the septic liver injury target genes, 140 intersection target genes were identified as the interactive targets for drug treatment. The 140 intersection target genes were imported into the String (https://string-db.org/) database for protein-protein interaction prediction, with the species set to HomoSapiens and the confidence level set to 0.7. The network file was saved in TSV format, and the TSV file was imported into Cytoscape3.7. 2 to draw the protein interaction network. The graph included 125 nodes and 1068 edges. Topological analysis was conducted, using the degree value to indicate the size and color of the targets, while the

combined score reflected the edge thickness, thereby constructing a protein-protein interaction network (Fig. 2). Wherein AKT1, SRC, EGFR, MAPK3, HSP90AA1, BCL2, MAPK1, TLR4, CASP3, MMP9, JUN, STAT1, HSP90AB1, PTGS2, and JAK2 were core targets.

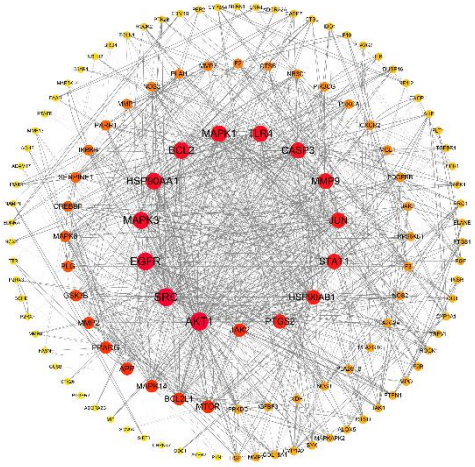


Fig.2 PPI network of Dachengqi Decoction and septic liver injury.

3.4. GO enrichment analysis

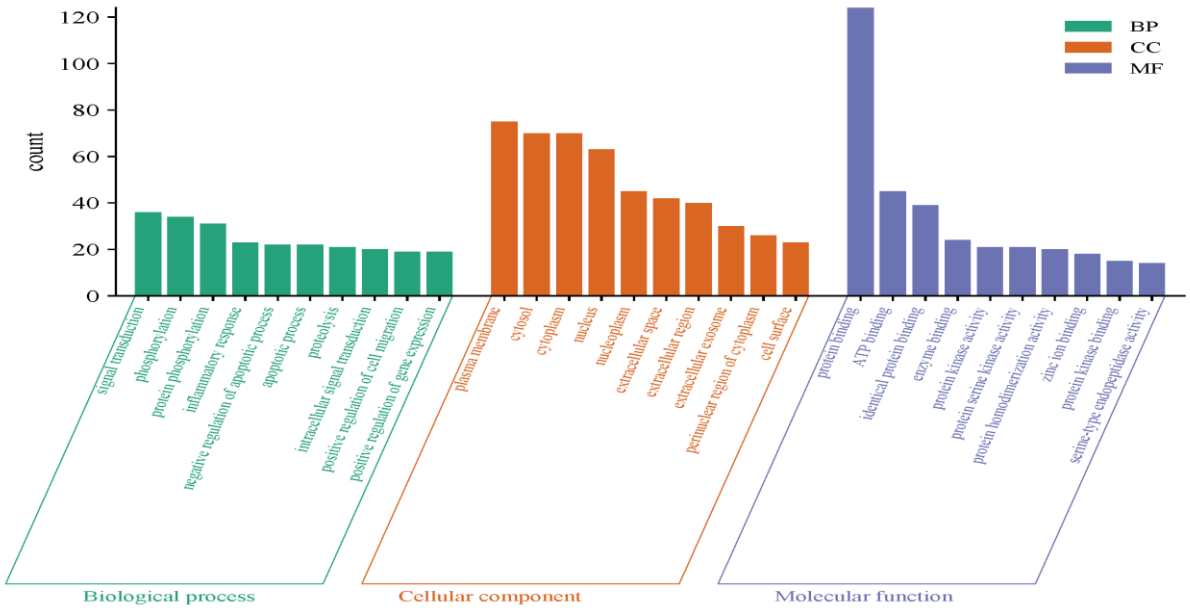


Fig.3 GO enrichment analysis.

The drug-disease intersection genes were analyzed using the DAVID database for GO gene functional enrichment. A total of 373 GO entries were screened, with 270 main entries significantly enriched by Dachengqi Decoction in the treatment of septic liver injury ($P < 0.01$). These included signal transduction, phosphorylation, inflammatory response, negative and positive regulation of apoptosis, proteolysis, intracellular signal transduction, positive regulation of cell migration, and positive regulation of gene expression. There are 46 related cellular components, involving plasma membrane, cytoplasm, cytoplasm, nucleus, karyoplasm, nucleoplasm, extracellular space, extracellular zone, extracellular exosome, cytoplasmic perinuclear zone, and cell surface; Among

them, there are 57 molecular functions related, involving protein binding, ATP binding, identical protein binding, enzyme binding, protein kinase activity, protein serine kinase activity, protein isomerism activity, zinc ion binding, protein kinase binding, serine-type endopeptidase activity (Fig.3).

3.5. KEGG enrichment analysis

Using DAVID database for pathway enrichment analysis, a total of 156 pathways related to Dachengqi Decoction in the treatment of septic liver injury were enriched. According to P<0.01, 123 pathways related to Dachengqi Decoction in the treatment of septic liver injury were screened, and the pathways related to septic liver injury were screened, respectively: hepatitis B, relaxin signal pathway, HIF-1 signal pathway, PI3K-Akt signal pathway, IL-17 signal pathway, apoptosis, Th17 cell differentiation, TNF signal pathway, C-type lectin receptor signal pathway, MAPK signal pathway, ErbB signal pathway, Ras signal pathway, Rap1 signal pathway, MAPK signal pathway, ErbB signal pathway, MAPK signal pathway, ErbB signal pathway, MAPK signal pathway (Fig.4).

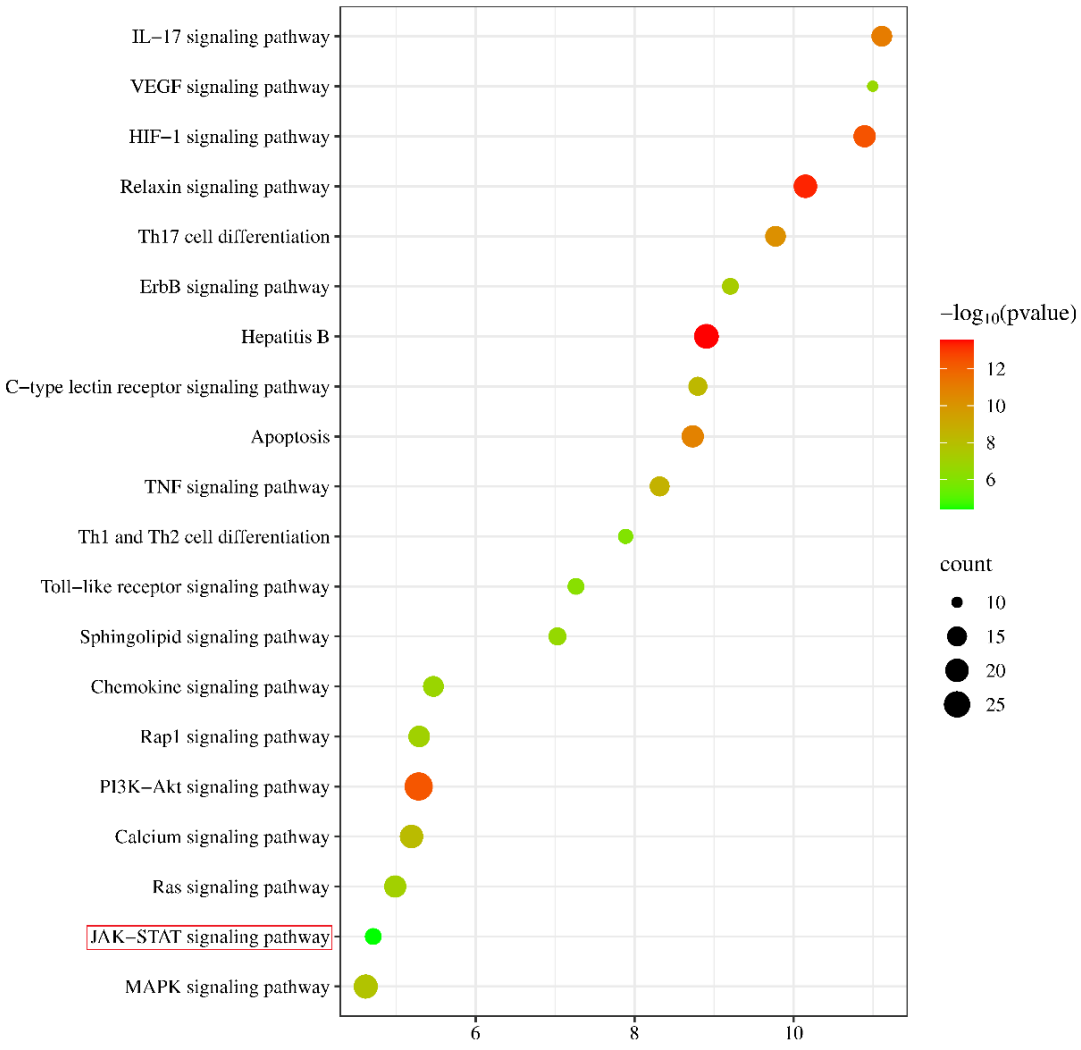


Fig.4 KEGG enrichment analysis.

3.6. Molecular docking analysis

JAK2 was selected as the receptor protein, and three effective active ingredients with the top degree values related to JAK2 in the "drug-component-target" network were screened as ligands, namely poncimarin, isoponcimarin, and Ammidin.

Molecular docking simulation was conducted using Autodock Vina, and the results were analyzed and visualized with PyMOL to obtain the best binding mode and interaction between poncimarin and the JAK2 receptor protein (Fig.5). The binding energy of the ligand-protein docking was -7.4 kcal/mol, and the binding was mainly achieved through hydrogen bonding and hydrophobic interactions. The amino acid forming hydrogen bonding interaction with the small molecule poncimarin was ASN395, with hydrogen bond lengths of 3.3 and 3.5 A. The hydrophobic amino acids around the small molecule poncimarin (5 A) were GLU65, GLU66, ILE69, and PHE432.

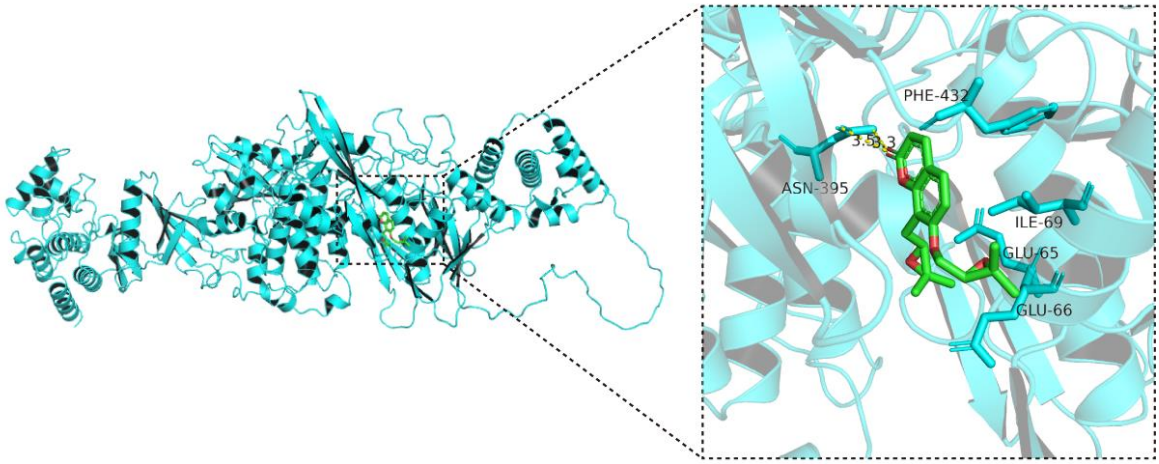


Fig.5 Molecular docking analysis of poncimarin with JAK2.

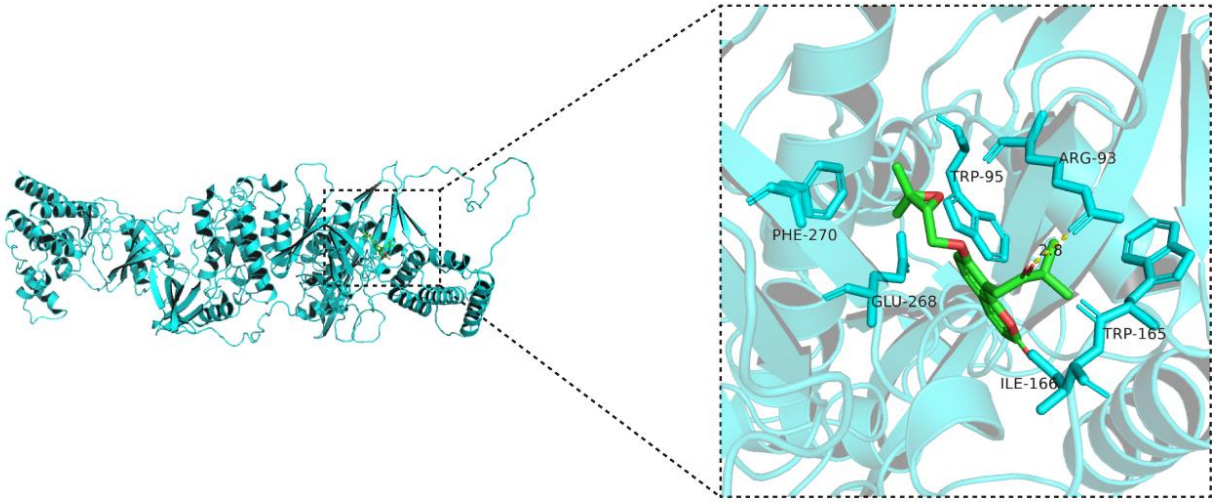


Fig.6 Molecular docking analysis of isoponcimarin with JAK2.

Molecular docking simulation was conducted using Autodock Vina, and the results were analyzed and visualized with PyMOL to obtain the optimal binding mode and interactions between isoponcimarin and the JAK2 receptor protein (Fig.6). The binding energy of the ligand-protein docking was -7.4 kcal/mol, and the binding was mainly achieved through hydrogen bond interactions and hydrophobic interactions. The amino acid that formed a hydrogen bond interaction with the small molecule isoponcimarin was ARG93, with a hydrogen bond length of 2.8 A. The hydrophobic amino acids within 5 A of the small molecule isoponcimarin were TRP95, TRP165, ILE166, GLU268, and PHE270.

Molecular docking simulation was conducted using Autodock Vina, and the results were analyzed and visualized with PyMOL to obtain the optimal binding mode and interactions between Ammidin and the JAK2 receptor protein (Fig.7). The binding energy of the ligand-protein docking was -7.6 kcal/mol, and the binding was mainly achieved through hydrogen bonding and hydrophobic interactions. The amino acid forming a hydrogen bond interaction with the small molecule Ammidin was GLU898, with a hydrogen bond length of 3.6 Å. The hydrophobic amino acids within 5 Å of the small molecule Ammidin were PHE860, ASP894, PHE895, and LEU997.

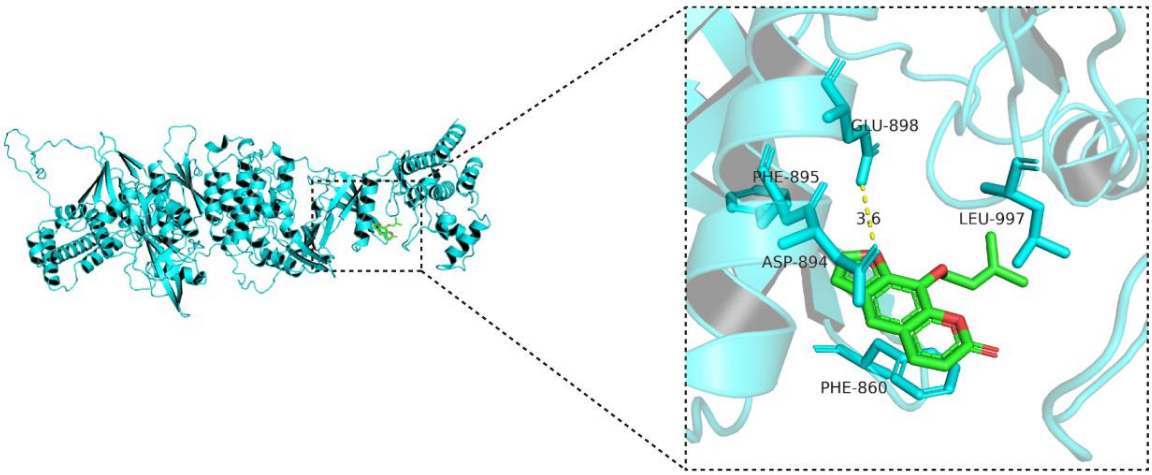


Fig.7 Molecular docking analysis of Ammidin with JAK2.

3.7. Molecular dynamics analysis

The Ammidin with the lowest binding energy in the molecular docking was selected for further molecular dynamics simulation. Gromacs was used for the molecular dynamics simulation, and Gromacs and Python were used to calculate the root mean square deviation (RMSD), root mean square fluctuation (RMSF), solvent accessible surface area (SASA), radius of gyration (Rg), number of hydrogen bonds, and binding free energy, etc.

Among them, the RMSD curve is an indicator for judging the stability of the protein-ligand complex. A more stable complex is represented by a more stable RMSD curve. The RMSF curve represents the degree of fluctuation of amino acid residues during the dynamic simulation. A lower RMSF value indicates that the amino acid residues have less fluctuation and the structure is more stable. The Rg curve is used to characterize the compactness and stability of the structure. A smaller radius of gyration indicates that the system remains compact and stable during the dynamic

simulation. The SASA curve represents the size of the solvent-accessible surface area of the protein, which characterizes the folding degree and stability of the protein. The curve of the number of hydrogen bonds represents the fluctuation of the number of hydrogen bonds formed between the protein and the ligand during the simulation. The more hydrogen bonds formed, the more stable the binding. The free energy landscape is used to describe the conformation with the lowest energy throughout the dynamic simulation process of the complex structure. If the interaction between the protein and the ligand is weak or unstable, multiple small energy clusters will appear in the free energy landscape; a strong and stable interaction can form a nearly single energy cluster in the free energy landscape.

As shown in the figure (Fig.8), the RMSD curves of the JAK2 protein and the Ammidin molecule system fluctuate to some extent in the early stage of the dynamics and then tend to equilibrium. The Ammidin molecule stabilizes at around 0.1 nm, while the JAK2 protein fluctuates in the early stage and then stabilizes at around 0.7 nm. The RMSF curve shows that the RMSF of the N-terminal amino acids is relatively large, while that of the remaining amino acids is relatively small, and the overall variation is small. The Rg curve indicates that the compactness of the protein system varies little and stabilizes at around 4.4 nm. The SASA curve shows that the solvent-accessible surface area of the protein fluctuates slightly and stabilizes at around 570 nm ². The curve of the number of hydrogen bonds shows that in the 100 ns molecular dynamics simulation, the ligand and the protein can form hydrogen bond interactions, and the number of hydrogen bonds formed between the protein and the ligand is 0-3. The hydrogen bond interaction contributes to the binding of the protein and the small molecule. The free energy landscape shows that the complex conformation is dominant in the range of RMSD of 0.6-0.8 nm and Rg of 4.35-4.50 nm, and the structure is more stable.

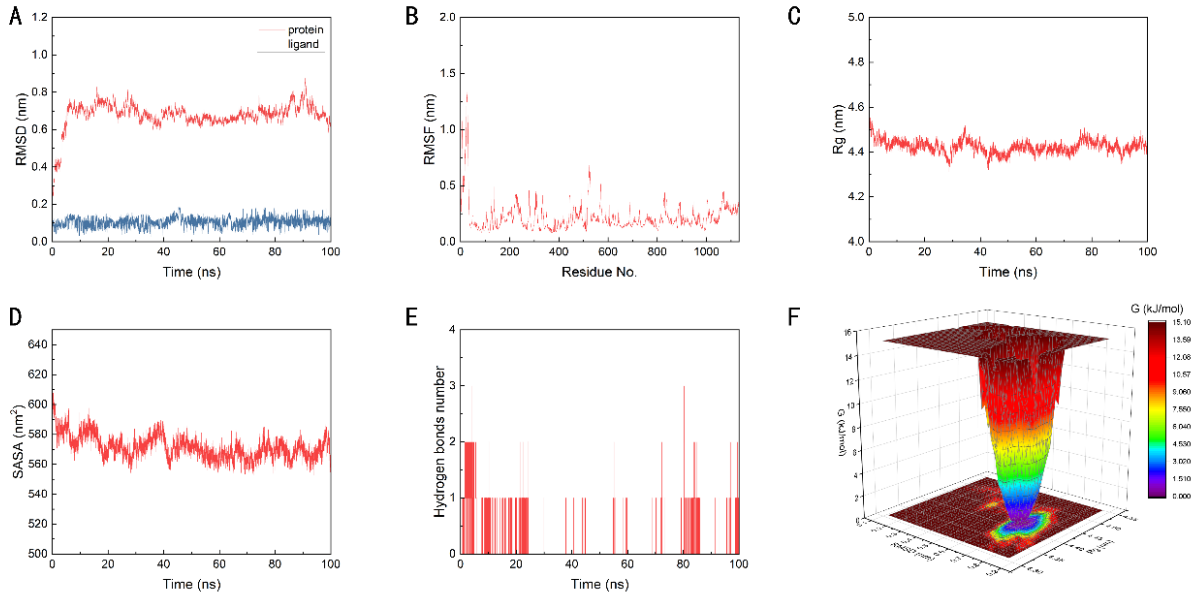


Fig.8 Molecular dynamics analysis. A. RMSD; B. RMSF; C. Rg; D. SASA; E. Hydrogen bonds numbers; F. Free energy landscape.

3.8. Histopathology of liver tissue

In the control group, vacuolar degeneration of hepatocytes was observed, but no obvious hepatic

sinusoidal congestion and inflammatory cell infiltration were observed. Except for the low concentration group of Dachengqi Decoction, liver tissue degeneration, inflammatory cell infiltration, hepatocyte hemorrhage and necrosis were improved in each intervention group compared with the model group, among which the improvement in the high concentration group of Dachengqi Decoction and the dexamethasone group was the most obvious, and the degree of remission was higher than that in the middle and low concentration groups (Fig.9).

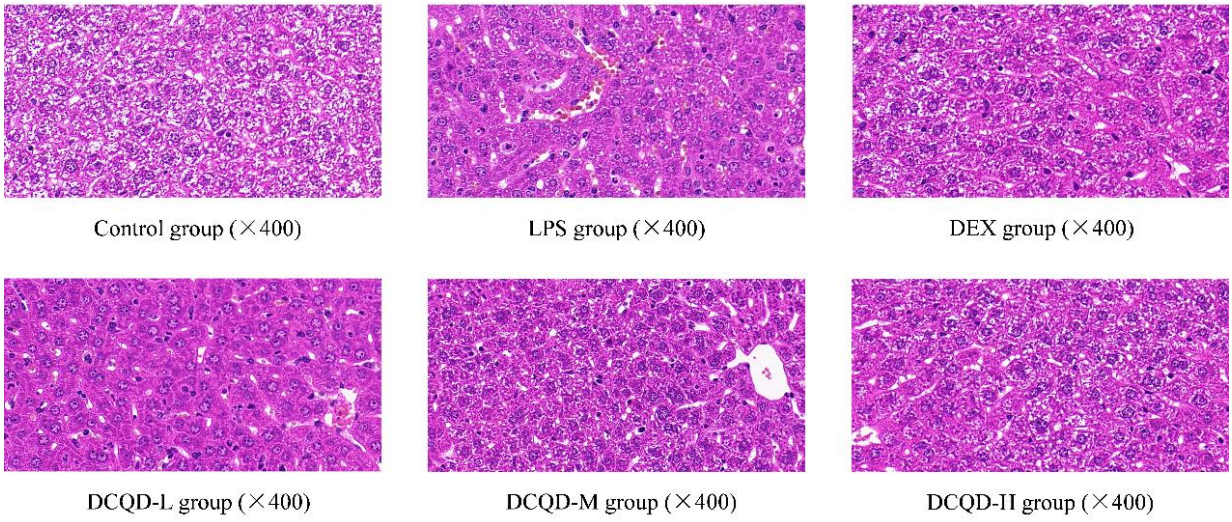


Fig.9 HE staining to observe the pathological changes of liver tissue of mice in each group (x400).

3.9. The levels of liver function and inflammatory factors

Compared to the control group, serum levels of liver function markers ALT and AST were significantly increased in the model group ($P < 0.05$). Pro-inflammatory factors IL-6 and TNF-α in both serum and liver tissue were significantly elevated (Fig.10), while anti-inflammatory factor IL-10 was also increased ($P < 0.05$). Compared with the model group, the liver function ALT and AST of each drug intervention group were significantly reduced ($P<0.05$); IL-6 and TNF-α decreased, and IL-10 increased ($P<0.05$), and the improvement in the dexamethasone group and the high concentration group of Dachengqi Decoction was more obvious than that in the middle and low concentration groups (Fig.11).

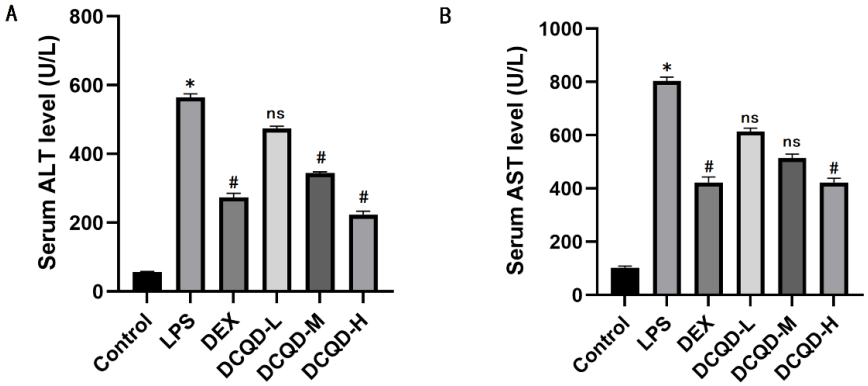


Fig.10 Changes of serum liver function in each group of mice. A. Changes of ALT in each group. B. Changes of AST in each group. *P<0.05 vs. control group. #P <0.05 vs. model group. ns indicates no statistical significance.

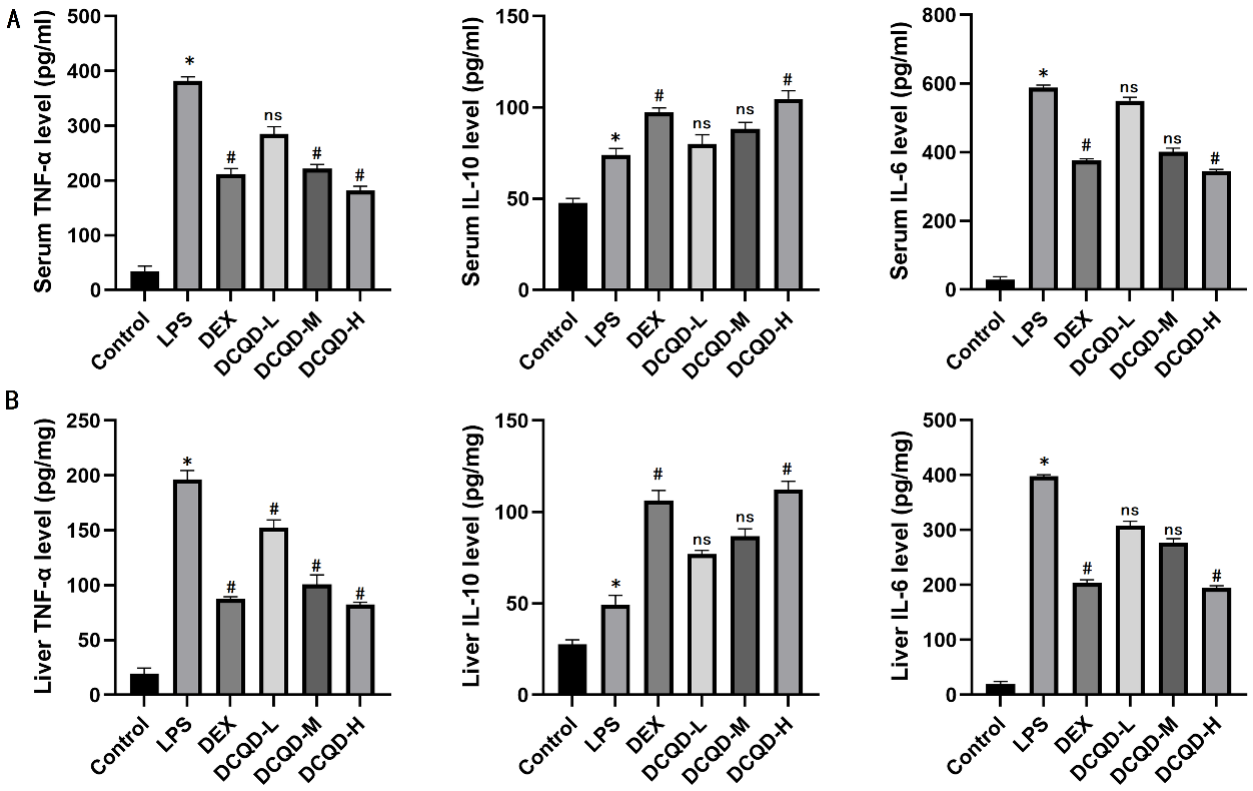


Fig.11 Changes of inflammatory factors in each group of mice. A. Changes of inflammatory factors in serum. B. Changes of inflammatory factors in liver tissue. *P<0.05 vs. control group. #P <0.05 vs. model group. ns indicates no statistical significance.

3.10. Protein expresstion of JAK2, P-JAK2, STAT3, P-STAT3, Arg-1, and iNOS.

The protein expressions of JAK2, P-JAK2, STAT3, P-STAT3, Arg-1, and iNOS in liver tissues were analyzed using Western blot (WB) and immunofluorescence. Compared to the control group, the expressions of P-JAK2, P-STAT3, and iNOS were significantly increased, while Arg-1 expression was significantly decreased ($P < 0.05$). Compared with the model group, the protein expression of P-JAK2, P-STAT3, and iNOS in each intervention group decreased, and the expression of Arg-1 increased ($P<0.05$). Moreover, the change trend of dexamethasone group and high concentration group was more obvious (Fig.12-14).

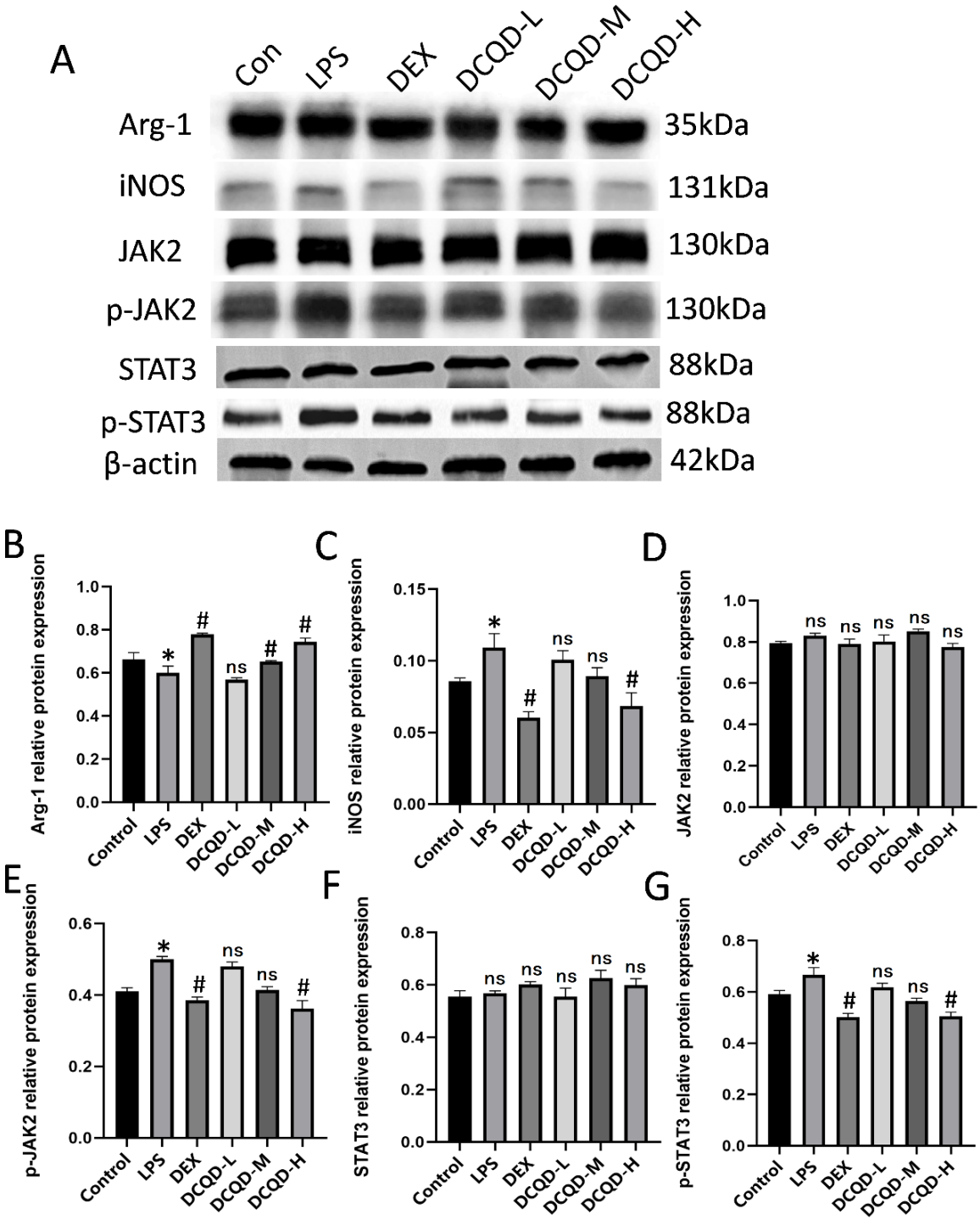


Fig.12 Protein expression of Arg-1, iNOS, JAK2, STAT3 in liver tissues of mice in each group (WB). A. Expression of Arg-1, iNOS, JAK2, p-JAK2, STAT3, p-STAT3 proteins. B-G. Relative protein expression of Arg-1, iNOS, JAK2, p-JAK2, STAT3, p-STAT3 proteins. *P<0.05 vs. control group. #P <0.05 vs. model group. ns indicates no statistical significance.

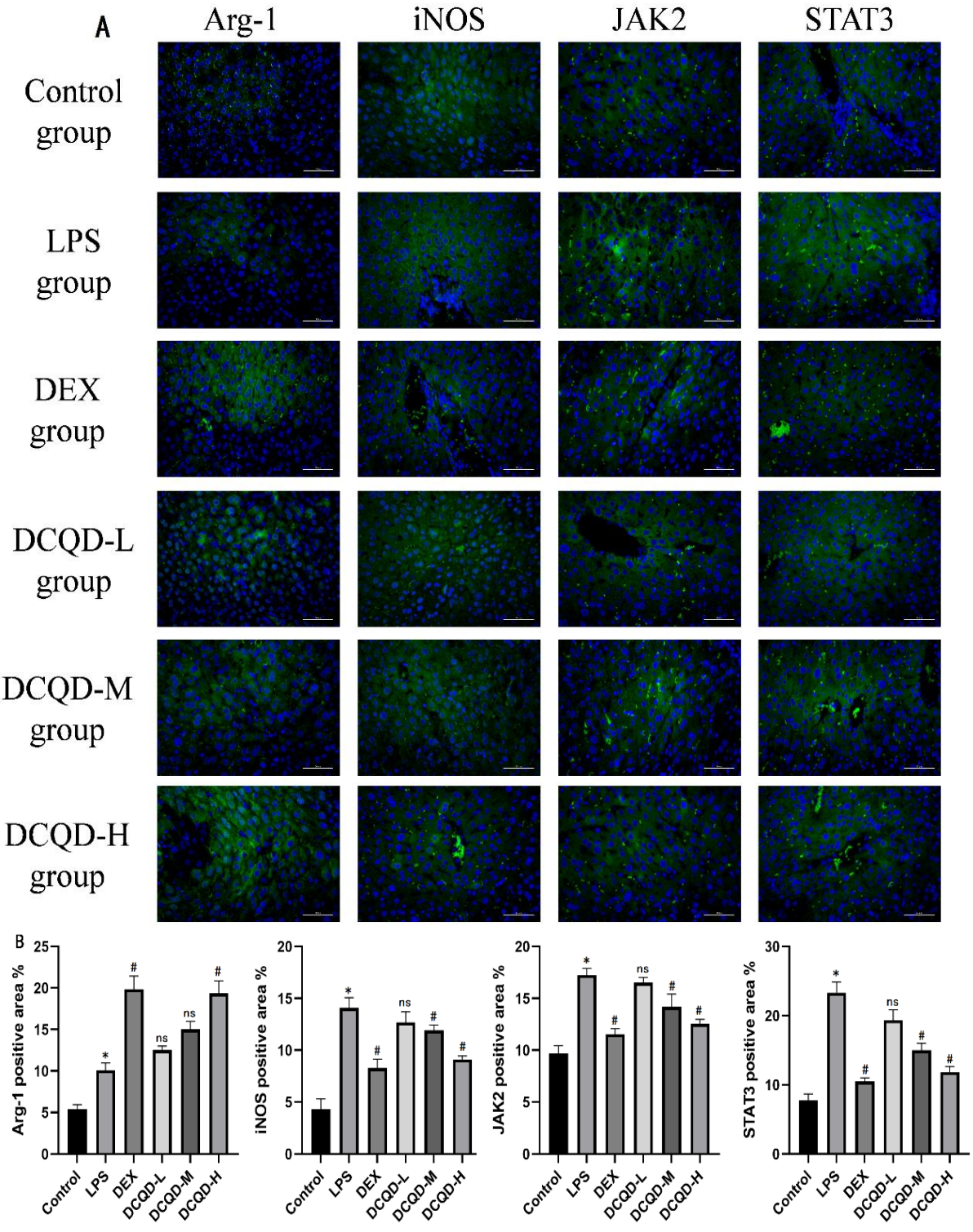


Fig.13-14 Protein expression of Arg-1, iNOS, JAK2, STAT3 in liver tissues of mice in each group (IF). A. Expression of Arg-1, iNOS, JAK2, STAT3 proteins. B. Positive area of each protein. *P<0.05 vs. control group. #P <0.05 vs. model group. ns indicates no statistical significance.

3.11. Polarization trend of liver macrophages

Compared with the control group, the proportion of CD80/F4/80 double-positive cells and CD206/F4/80 double-positive cells significantly increased in the model group, indicating a rise in both M1 and M2 macrophages, with a more pronounced M1 polarization. Compared with the model group, the proportion of CD80 and F4/80 double-positive cells in the Dachengqi Decoction group decreased significantly, while the proportion of CD206 and F4/80 double-positive cells increased significantly, that is, the proportion of M1 decreased while the proportion of M2 increased, Macrophages started M2 polarization (Fig.15).

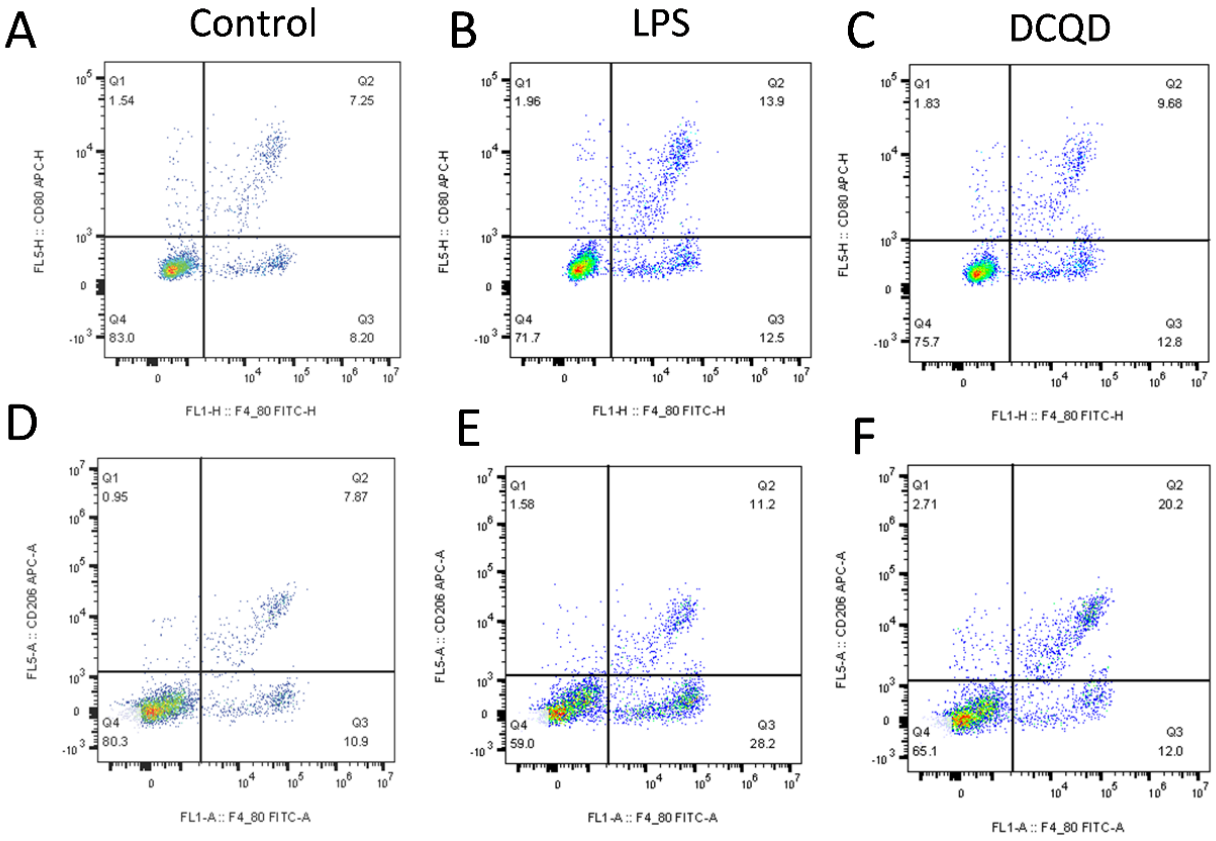


Fig.15 Changes of polarization trend of liver macrophages in each group of mice. A-C. M1 polarization trend of liver macrophages in each group. D-F. M2 polarization trend of liver macrophages in each group.

4. Discussion

Sepsis has acute onset, high mortality rate, poor prognosis, and easy to produce excessive economic burden[1]. Therefore, the gist of its treatment is to cut off the disease in time in the early stage of sepsis to prevent it from further developing in the direction of critical illness, which coincides with the theoretical thought of "preventing disease" in traditional Chinese medicine[16, 17]. According to TCM syndrome differentiation, sepsis can be mainly categorized into toxin-heat syndrome, internal organ and qi obstruction syndrome, blood stasis syndrome, and acute deficiency syndrome. The corresponding treatment strategies include heat-clearing and detoxifying methods, internal attack clearing methods, promoting blood circulation to remove blood stasis, and strengthening the body's resistance. Dachengqi Decoction is a fundamental prescription for treating

severe heat knots and preserving acute yin, making it suitable for addressing heat and qi obstruction in sepsis[18].

Network pharmacology was employed to investigate the material basis and molecular mechanisms of Dachengqi Decoction in treating septic liver injury by constructing a "drug-component-target" network. Among them, Glauber's salt is a mineral medicine. Although essential in the clinical application of Dachengqi Decoction, Glauber's salt is often used as a ministerial medicine to enhance the purgative and heat-clearing effects of the formula. However, its components are not included in the TCMSP database, and its mechanisms are beyond the scope of this study, thus it was excluded from the network pharmacology analysis[19]. The results from the network pharmacology part showed that some active components of Dachengqi decoction could bind closely to JAK2.

The model was established through intraperitoneal injection of LPS, and administration of Dachengqi Decoction via gavage improved liver function markers ALT and AST, reduced levels of pro-inflammatory factors IL-6 and TNF-α, and increased levels of the anti-inflammatory factor IL-10. Among them, IL-6 is a highly active pro-inflammatory factor secreted by lymphoid cells (e.g., T cells and B cells) and non-lymphoid cells (e.g., monocytes, macrophages, dendritic cells, and mast cells)[20]. It is primarily activated by TNF-α and IL-1β, making it a key marker of the inflammatory process. TNF-α itself is a classic pro-inflammatory factor.In sepsis, TNF-α levels are significantly elevated, binding to mTNF receptors on hepatocyte and hepatic sinusoidal endothelial cell membranes, leading to damage of these cells and promoting IL-6 secretion, resulting in a cascade amplification of inflammation[21]; As a negative regulator of cell-mediated immune responses, IL-10 inhibits the production of prostaglandin E2 and pro-inflammatory factors such as TNF-α, IL-1, IL-6, and IL-8, thereby mitigating the inflammatory response[22]. Therefore, this experiment believes that Dachengqi Decoction can improve the liver injury caused by sepsis.

The JAK2/STAT3 signaling pathway is involved in regulating immune responses, cell proliferation, and other important physiological processes. It has been found that it also plays an important role in regulating macrophage polarization[23]. In the early stage of sepsis, when LPS and inflammatory factors bind to receptors on the surface of macrophages, such as LPS binds to TLR4, IFN-γ binds to IFN-γR, etc., JAK is recruited, and then phosphorylated to activate STAT3. The phosphorylated STAT3 dimerizes via the SH2 domain and, upon entering the nucleus, initiates the expression of NF-κB, IL-1β, iNOS, IL-6, TNF-α, and others, creating a vicious cycle that polarizes macrophages to the M1 phenotype and exacerbates inflammatory damage[7, 24]. Arg-1 is an ideal phenotypic marker for identifying M2 macrophages. After gavage administration of Dachengqi Decoction, expressions of p-JAK2, p-STAT3, and iNOS in liver tissue were down-regulated, while Arg-1 expression was up-regulated. Flow cytometry indicated that macrophages were polarized to the M2 phenotype[23]; This suggests that Dachengqi Decoction may regulate macrophage polarization to the M2 phenotype by affecting the JAK2/STAT3 pathway, thereby protecting against septic liver injury.

In conclusion, this experiment shows that Dachengqi Decoction can regulate the polarization of macrophages to M2 by affecting the JAK2/STAT3 pathway, thereby protecting septic liver injury. Other studies have also indicated that intervening in the JAK2/STAT3 pathway through inhibition or gene knockout can enhance survival rates in mouse models of sepsis[25, 26]. This experiment proved that Dachengqi Decoction can improve septic liver injury in mice injected with LPS intraperitoneally, and the degree of improvement is related to JAK2/STAT3 signal pathway and macrophage polarization. However, as a traditional Chinese medicine decoction, the mechanisms of

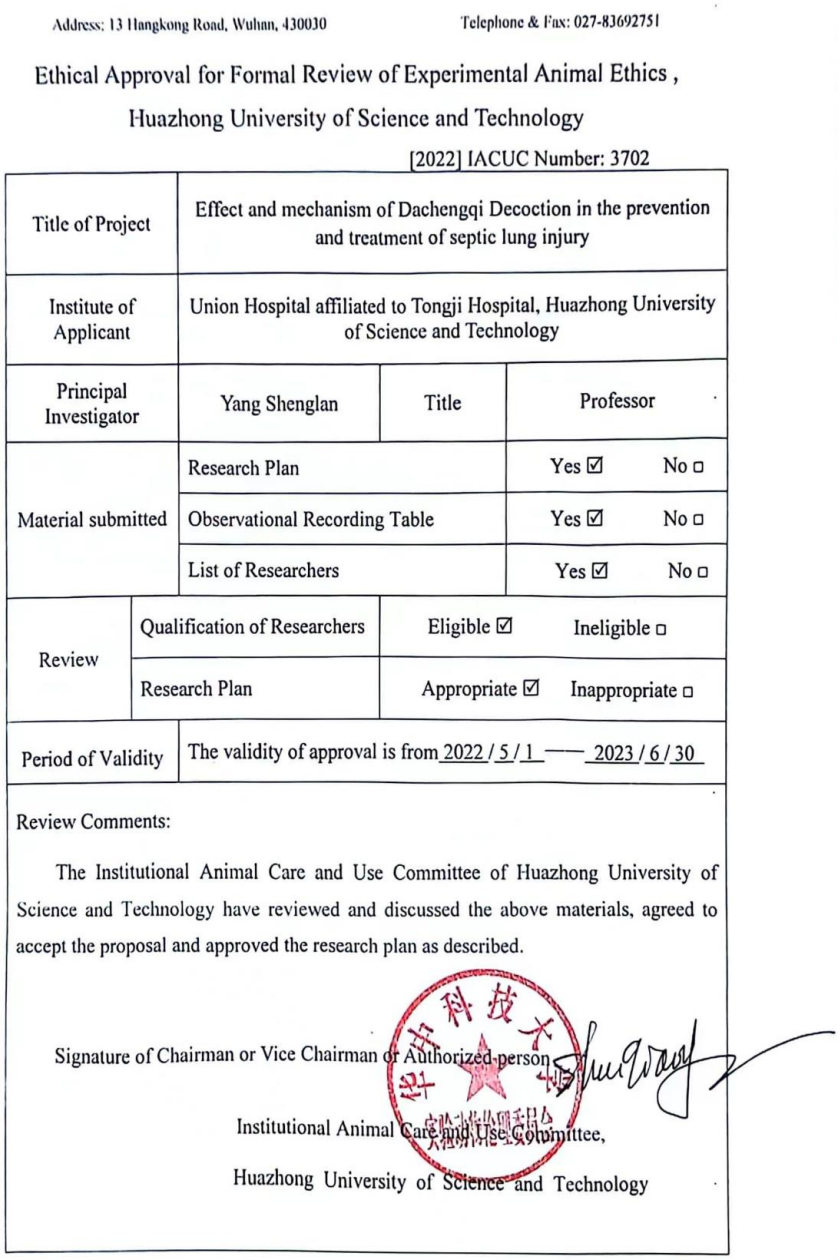
action of Dachengqi Decoction, with its multiple components and targets, are not fully understood, and further exploration of other possible pathways is needed.

Acknowledgements

We would like to express our gratitude to people and animals who helped.

Animal Ethics Statement

The experimental process was strictly carried out in accordance with the Ethical Review Guidelines. The animal ethics of this experiment was reviewed and approved by the Ethics Committee of Animal Center, Tongji Medical College, Huazhong University of Science and Technology. Animal License Number: [2022] IACUC Number: 3702.



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Ethical Approval for Formal Review of Experimental Animal Ethics ,
Huazhong University of Science and Technology

[2022] IACUC Number: 3702

Title of Project	Effect and mechanism of Dachengqi Decoction in the prevention and treatment of septic lung injury		
Institute of Applicant	Union Hospital affiliated to Tongji Hospital, Huazhong University of Science and Technology		
Principal Investigator	Yang Shenglan	Title	Professor
Material submitted	Research Plan	Yes ☑	No □
	Observational Recording Table	Yes ☑	No □
	List of Researchers	Yes ☑	No □
Review	Qualification of Researchers	Eligible ☑	Ineligible □
	Research Plan	Appropriate ☑	Inappropriate □
Period of Validity	The validity of approval is from 2022 / 5 / 1 —— 2023 / 6 / 30		

Review Comments:

The Institutional Animal Care and Use Committee of Huazhong University of Science and Technology have reviewed and discussed the above materials, agreed to accept the proposal and approved the research plan as described.

Signature of Chairman or Vice Chairman or Authorized person:

Institutional Animal Care and Use Committee,
Huazhong University of Science and Technology

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Conflicts of Interest

The authors declare no conflicts of interest.

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