

New Phenomenon

Exploring the mechanism of *Panax notoginseng* saponin in inhibiting the inflammatory response of microglia in cerebral ischemia based on network pharmacology

Zhaoda Duan^{1,†}, Wenji Jia^{2,†}, Jianxiang Wang³, Dongyao Xu¹, Yujia Yang¹, Zhi Qi², Li Yang^{1,*}, and Chunyun Wu^{1,*}

¹Department of Anatomy and Histology/Embryology, Faculty of Basic Medical Sciences, Kunming Medical University, Kunming 650500, China,

²Department of Neurology, No.2 Affiliated Hospital, Kunming Medical University, Kunming 650101, China, and ³Ganmei Hospital Affiliated to Kunming Medical University, Kunming 650000, China

[†]These authors contributed equally to this work.

*Correspondence address. Tel: +86-13888797152; E-mail: wuchunyunkm@163.com (C.W.) / Tel: +86-13888566664; E-mail: yanglikm@163.com (L.Y.)

Received 11 March 2024 Accepted 11 April 2024

With the increasing global population and aging demographic, the incidence of stroke is rising. Among these, ischemic stroke (IS), also known as cerebral ischemia, constitutes over 80% of all stroke cases. This condition is characterized by an acute cerebrovascular disease caused by the blockage and interruption of the brain's blood supply, resulting in localized tissue ischemia, oxygen, and glucose deficiency, ultimately leading to the death of nerve cells and tissue necrosis [1,2]. "Vascular recanalization and the restoration of cerebral blood flow" are the primary clinical treatment objectives and are achieved through the intravenous administration of drugs such as tissue plasminogen activator or through surgical thrombectomy. These interventions not only restore the delivery of oxygen and glucose to the affected cerebral area but also help prevent the expansion of the infarcted region. However, the restoration of reperfusion cerebral blood flow similarly exposes the infarct area to peripheral immune cells, triggering the activation of the immune response and inflammation-induced injury [3]. Research indicates that IS elicits a robust inflammatory response, with neuroinflammation playing a crucial role in the secondary neurodegeneration process following stroke. Neuroinflammatory responses are initiated and perpetuated through injury cascades that include the release of inflammatory mediators, the migration and recruitment of white blood cells across the blood-brain barrier, and the impairment of endothelial nitric oxide synthase. These mechanisms collectively promote the activation of pro-inflammatory genes, which in turn activate microglia (MG) and exacerbate ischemic damage and neurological dysfunction [4]. MG are resident immune cells of the central nervous system (CNS). Its function is akin to that of macrophages, serving as the first line of defense against injuries within the central nervous system. Under typical conditions, brain microglia participate in immune surveillance and

defense against infectious agents. However, in the pathogenesis of neurodegenerative diseases such as IS, MG are activated by various stimuli. Once activated, MG are known to release numerous proinflammatory or cytotoxic factors, such as inducible nitric oxide synthase (iNOS), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and reactive oxygen species (ROS). These factors initiate the neuroinflammatory response, exacerbate inflammation, intensify damage to brain tissue and neurons, and significantly hinder the repair of brain injuries and neurogenesis [5,6]. Therefore, inhibiting the activation of microglia and reducing the inflammatory response in the central nervous system are crucial for minimizing brain damage caused by IS and are vital for developing effective prevention and treatment strategies.

In recent years, certain natural compounds extracted from traditional drug formulations have shown high therapeutic potential in protecting the brain from cerebral ischemic injury. These compounds reduce the neuroinflammatory response and apoptosis following stroke. Traditional Chinese herbal medicine (TCHM) and its constituent herbs feature a multiplicity of components, targets, and pathways owing to their complex formulations and therapeutic principles, making them promising sources for developing effective treatments for IS. *Panax notoginseng* saponin (PNS), as the principal bioactive component of *Panax notoginseng*, is extensively utilized in the prevention and treatment of cardiovascular and cerebrovascular diseases. Its pharmacological benefits include dissipating blood stasis, promoting hemostasis, alleviating swelling and pain, regulating energy metabolism disorders, balancing ion metabolism, and reducing and accelerating the clearance of free radicals [7]. Research indicates that PNS mitigates apoptosis by maintaining mitochondrial homeostasis, enhancing the integrity of the blood-brain barrier (BBB), augmenting cerebral blood supply,

and fostering the differentiation of neural stem cells and proliferation of hippocampal neurons. In addition, PNS offers neuroprotection against focal cerebral I/R injury in rats by reducing brain edema, upregulating the expression of the heat shock protein HSP70, and downregulating the expression of transferrin [8,9]. Additionally, PNS has been reported to enhance the recovery of neurogenesis and neurological function in cerebral embolism induced by microspheres and to reduce sepsis-induced acute kidney injury by suppressing inflammation [10]. However, the mechanism by which PNS targets IS has not been fully elucidated. In this study, we investigated the anti-inflammatory effects of PNS on IS and identified potential target pathways that could inhibit microglia-mediated inflammatory responses, thereby providing experimental

evidence to support the development of new drugs derived from Chinese herbal medicine for the prevention and treatment of neurodegenerative diseases. A cerebral ischemia model was established through middle cerebral artery occlusion (MCAO). Western blot analysis and immunofluorescence double-labeling staining were employed to assess the changes in the expressions of the inflammatory factors iNOS, TNF- α , and IL-1 β in microglia. Western blot analysis revealed that, compared with those in the sham operation group, the protein expressions of iNOS, TNF- α , and IL-1 β in the MCAO group were significantly increased. Following PNS intervention (100 mg/kg), there was a notable reduction in the protein expressions of iNOS, TNF- α , and IL-1 β in the MCAO + PNS group compared to those in the MCAO group (Figure 1A,B).

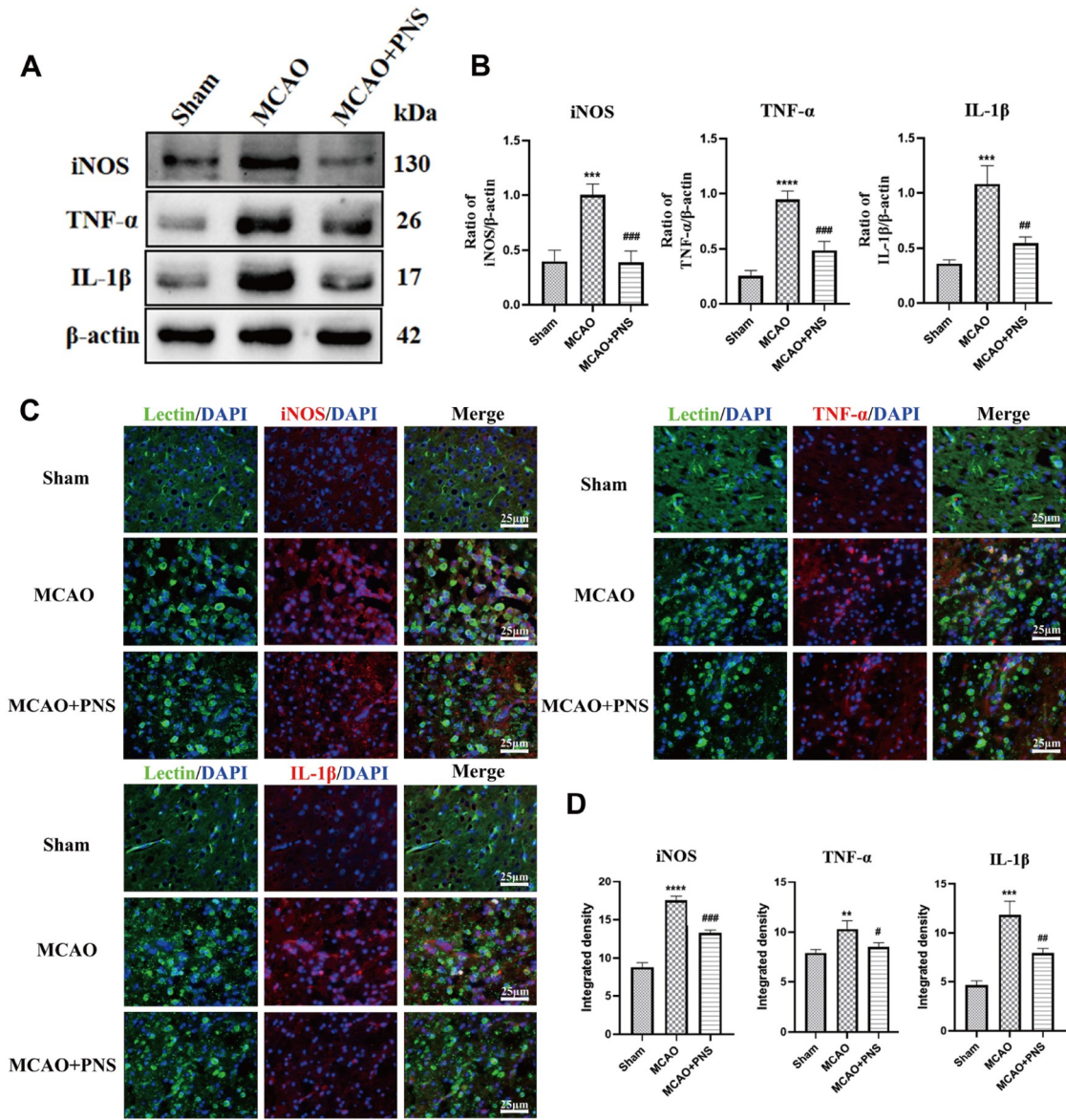


Figure 1. PNS inhibits the expressions of proinflammatory factors in microglia after MCAO in rats (A,B) Western blot analysis revealed that the protein expressions of iNOS, TNF- α , and IL-1 β were elevated in the MCAO group and subsequently decreased significantly following PNS treatment. (C,D) Immunofluorescence showed that the fluorescence intensity of iNOS, TNF- α , and IL-1 β increased, the number of positive cells increased in the MCAO group, the fluorescence intensity decreased, and the number of positive cells decreased after PNS intervention. Lectin-labeled microglia (green), DAPI-labeled nuclei (blue), and Cy3-labeled iNOS, TNF- α , and IL-1 β (red) are shown. Scale bar: 25 μ m. Data are presented as the mean $\pm$ SD ($n = 3$). ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared with the Sham group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with the MCAO group.

Immunofluorescence staining demonstrated that in the MCAO group, both the fluorescence intensity and the number of positive cells for iNOS, TNF- α , and IL-1 β were significantly increased. After PNS treatment, both the fluorescence intensity and the number of positive cells for iNOS, TNF- α , and IL-1 β were significantly reduced (Figure 1C,D). These results suggested that PNS (Figure 2A) significantly suppressed the protein expressions of iNOS, TNF- α , and IL-1 β in microglia following MCAO, thereby reducing the neuroinflammatory response.

To further explore the potential target pathways through which PNS inhibits microglial inflammatory responses, we utilized network pharmacology to screen for potential targets and pathways involved in the treatment of IS. Network pharmacology is a research field based on systems biology, genomics, proteomics, and other disciplines. It integrates computational analysis with *in vivo* and *in vitro* experiments to discover drug targets and molecular mechanisms through the integration of large amounts of data. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were employed to elucidate the roles of target genes in cellular functions and signaling pathways [11,12]. A total of 255 potential PNS targets were identified using the SwissTargetPrediction and PharmMapper databases. We retrieved 817 disease-related targets from the OMIM, GeneCards, and DrugBank databases. Intersecting targets between PNS targets and

IS-related disease targets were delineated using a Venn diagram on the WeChat online platform, which revealed 57 common targets (Figure 2B). These targets were visualized in Cytoscape 3.7.1, featuring 57 nodes and 710 edges (Figure 2C). Finally, the Metascape database was used to perform GO function and KEGG enrichment analyses of the core targets, and 667 biological process (BP) terms, such as “response to injury”, “response to hormones”, and “enzyme-linked receptor protein signaling pathway”, were identified. Twenty-seven cellular component (CC) terms, such as extracellular matrix, vesicle cavity, and membrane side, and 64 molecular function (MF) terms, such as endopeptidase activity, nuclear receptor activity, and chemoattractant activity, were identified. The top 10 items were selected to construct a bar chart (Figure 2D). KEGG enrichment analysis revealed 93 signaling pathways, and the top 10 pathways, including the cancer signaling pathway, relaxin signaling pathway, and MAPK signaling pathway, were selected to construct a pathway bubble map (Figure 2E). Notably, the MAPK signaling pathway comprises a series of protein kinases, such as extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK. It governs a variety of cellular biological processes and is crucial for cell proliferation, differentiation, apoptosis, and inflammation. Previous studies have shown that the MAPK signaling pathway, a significant regulator of proinflammatory cytokines in lipopolysaccharide (LPS)-induced

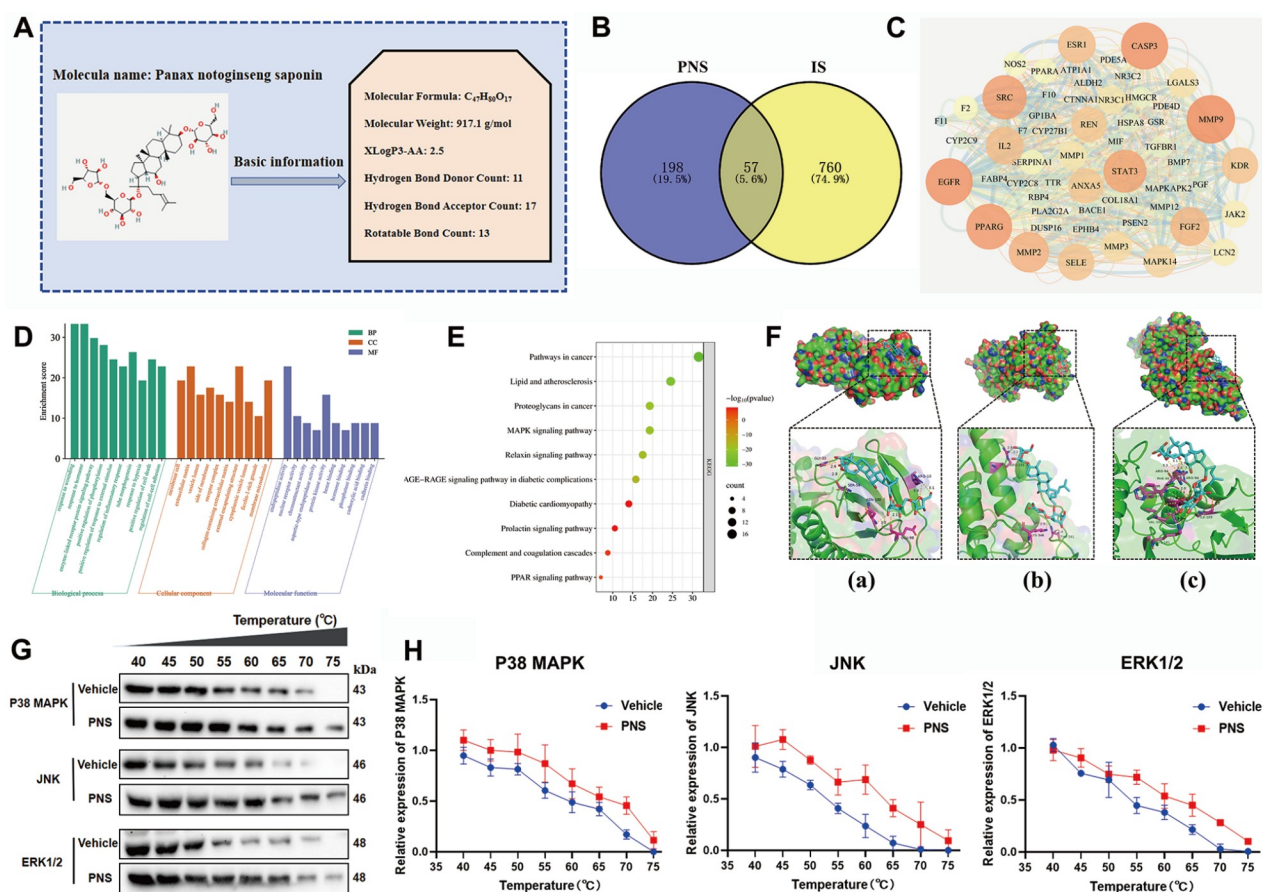


Figure 2. Network pharmacology screening of potential target pathways for the PNS treatment of IS. (A) PNS chemical information [15]. (B) Venn diagram of intersection targets between the PNS and IS. (C) Visualization of the PPI protein network interaction. (D) Gene Ontology (GO) functional enrichment analysis. (E) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. (F) Visualization of molecular docking between PNS and P38 (a), JNK (b), and ERK1/2 (c) in the MAPK signaling pathway, with binding energies of -7.8 kcal mol $^{-1}$, -8 kcal mol $^{-1}$, and -9.5 kcal mol $^{-1}$, respectively. (G,H) CETSA protein expression bands and apparent melting curves for P38, JNK, and ERK1/2 ($n = 3$).

MG, influences the production and release of proinflammatory factors by activated MG [13,14]. Based on these findings, we hypothesized that the inhibition of microglia-mediated neuroinflammation by PNS after MCAO in rats may be linked to the modulation of the MAPK signaling pathway.

To test this hypothesis, we conducted molecular docking and visualization of PNS with three major components of the MAPK pathway—P38, JNK, and ERK1/2 (Figure 2F). Binding energies below 0 kcal mol⁻¹ indicate spontaneous binding between the drug molecule and receptor protein. Values below -5.0 kcal mol⁻¹ suggest good binding activity, while those below -7.0 kcal mol⁻¹ indicate strong binding activity. Molecular docking results demon-

strated that PNS could bind strongly and spontaneously to P38 (-7.8 kcal mol⁻¹), JNK (-8 kcal mol⁻¹), and ERK1/2 (-9.5 kcal mol⁻¹). Western blot analysis results showed that after post-PNS treatment, with the increase of temperature, the protein expression levels of P38, JNK, and ERK1/2 were higher than those of the vehicle group at the same temperature. And the apparent melting curves were significantly shifted to the right compared to those in the vehicle group, suggesting that these proteins are more stable after PNS treatment (Figure 2G,H). Subsequent western blot analysis (Figure 3A,B) and immunofluorescence double staining (Figure 3C,D) were employed to monitor and assess changes in the expressions of P38, JNK, and ERK1/2 in microglia. The results indicated that, compared

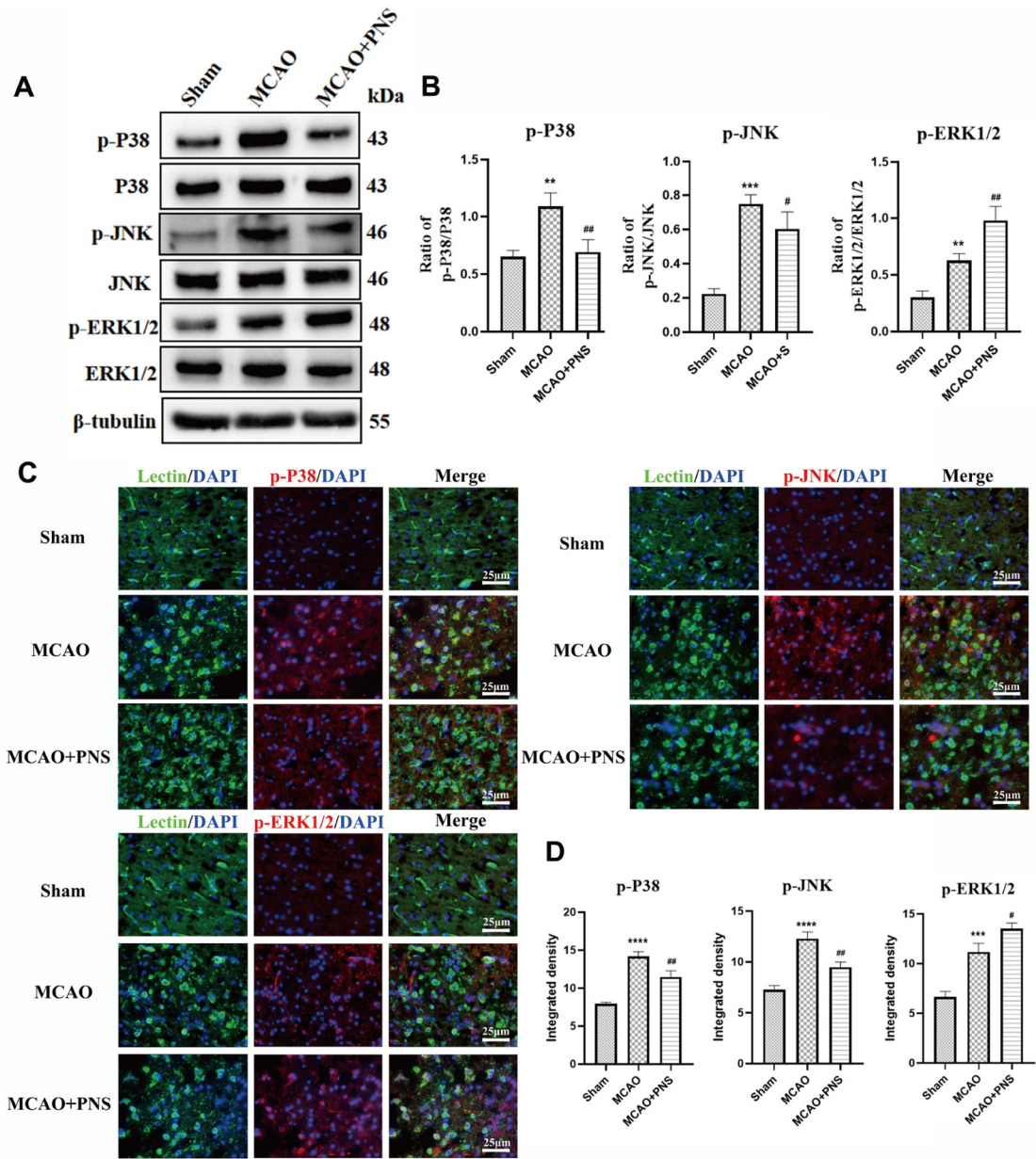


Figure 3. Effect of PNS on the MAPK pathway in microglial cells after MCAO in rats (A,B) Western blot analysis indicated that the phosphorylation levels of P38, JNK, and ERK were increased in the MCAO group. Following PNS treatment, the phosphorylation levels of P38 and JNK significantly decreased, while the phosphorylation level of ERK further increased. (C,D) Immunofluorescence staining results are consistent with the western blot analysis data. Lectin labeled microglia (green), DAPI labeled nuclei (blue), and Cy3 labeled phosphorylated P38, JNK, and ERK (red). Scale bar: 25 μm. Data are presented as the mean ± SD (n = 3). ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 compared with the Sham group, #*P* < 0.05, ##*P* < 0.01 compared with the MCAO group.

with those in the sham group, the phosphorylation levels and fluorescence intensity of P38, JNK, and ERK1/2 and the number of positive cells in the MCAO group increased significantly. After PNS intervention, compared with the MCAO group, the phosphorylation levels and fluorescence intensity of P38 and JNK were significantly reduced in the MCAO + PNS group, and the number of positive cells decreased. Conversely, the phosphorylation levels and fluorescence intensity of ERK1/2 further increased, and the number of positive cells also increased following PNS intervention.

In summary, we demonstrated that PNS significantly inhibited the expressions of neuroinflammatory factors in microglia after cerebral ischemia in rats. Through network pharmacology screening, the MAPK signaling pathway was found to be significantly enriched, suggesting that it is a potential target pathway by which PNS modulates the inflammatory response. Additionally, molecular docking revealed that PNS strongly binds to P38, JNK, and ERK1/2, the key components of the MAPK pathway. PNS could inhibit P38 and JNK while enhancing the activation of ERK1/2 phosphorylation. These findings suggest that PNS may alleviate microglia-mediated neuroinflammation after cerebral ischemia in rats by regulating the MAPK signaling pathway. To further elucidate the mechanism by which PNS regulates microglial inflammatory responses, additional experimental studies are required to refine our understanding of this mechanism.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (No. 81960223 to C.W.), the Applied Basic Research Projects of Yunnan Province (No. 202301AY070001-163 to L.Y.), and the Scientific Research Fund of Education Department of Yunnan province (Nos. 2023Y0793 and 2024Y206 to Z.D., Y.J.).

Conflict of Interest

The authors declare that they have no conflict of interest.

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