PUERARIN PLAYS AN ANTI-INFLAMMATORY ROLE BY DOWN-REGULATE THE PHOSPHORYLATION OF MAPKS SIGNAL AND UP-REGULATE THE LEVEL OF O-GLCNAC GLYCOSYLATION PROTEIN

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ABSTRACT

Objective: *To investigate the mechanism by which puerarin inhibits inflammation damage in microglia and to further analyse the correlations of various signalling molecules.*

Methods: *Lipopolysaccharide (LPS) induced inflammatory damage in microglia. Microglia were untreated or treated with a low concentration (25 μM) or a high concentration (100 μM) of puerarin, respectively. The nitric oxide (NO) content of the microglia was detected by flow cytometry, and microglia-associated eggs were detected by Western blot. The expression of related genes was detected by PCR.*

Results: *The results of flow cytometry showed that the NO production of microglia stimulated by LPS was significantly higher than that of a control group (P<0.05). Compared with the untreated LPS group, the puerarin high concentration group could significantly inhibit NO production (P<0.05). The effect of the puerarin low concentration group on NO was not significantly different from that of the LPS group (P>0.05). Western blot analysis showed that the iNOS protein level increased significantly after LPS stimulation compared with the control group (P<0.05), decreased significantly after puerarin treatment compared with the LPS group, and was inhibited by puerarin in a dose-dependent manner (P<0.05). The results of real-time quantitative PCR showed that the change in the iNOS gene level was consistent with that of the protein level. Compared with the control group, the puerarin high concentration group significantly inhibited the activation of the NF-kB gene in microglia (P<0.05). Compared with the control group, LPS could significantly promote the phosphorylation of p38, ERK1/2 and JNK (P<0.05). Compared with the LPS group, puerarin at both high and low concentrations could inhibit the phosphorylation of ERK1/2 (P<0.05), while puerarin at the high concentration could significantly inhibit the phosphorylation of p38 and JNK (P<0.05). The level of O-GlcNAc glycosylated protein in microglia treated with LPS was significantly lower than that in the control group (P<0.05). The levels of O-GlcNAc glycosylated protein in microglia treated with different concentrations of puerarin were significantly higher than that in the LPS group (P<0.05).*

Conclusion: *Puerarin can inhibit the activation of the NF-kB gene by reducing the release of NO and the expression of iNOS, then down-regulating the phosphorylation of the MAPKs signal and up-regulating the level of O-GlcNAc glycosylation protein, thus playing an anti-inflammatory role.*

Keywords: *Puerarin, microglia, inflammation damage, mechanism of action, signal molecule regulation.*

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Introduction

Microglia are key cells involved in immune regulation in the brain, accounting for approximately 5~20% of the brain's glial cells. After activation, they can secrete harmful substances that play an important role in the inflammatory response in stroke[(1)]. Microglia in normal brain tissues can effectively and dynamically monitor the microenvironment of the brain, regulate the reconstruction of brain functions and nerve circuits, exert a phagocytosis effect, remove pathogens, and maintain the stability of the central nervous system. When the body is in a pathological state, microglia can be activated from the branching phenotype to the amoeboid phenotype. Activated microglia can secrete a large number of inflammatory cytokines, such as nitric oxide and reactive oxygen species clusters, and enhance the phagocytosis and

migration functions of cells, which further leads to irreversible damage to neurons[2]. In addition, studies have found that infection of pregnant women with bacteria and viruses during pregnancy can lead to brain damage in perinatal new-borns. It has been further found that the amniotic fluid of infected pregnant women is rich in lipopolysaccharide (LPS). LPS can damage the oligodendrocytes and white matter of the brain by activating the microglia[3]. Therefore, inhibiting the continuous activation of microglia and reducing the release of inflammatory cytokines has become a focus of clinical scholars. Puerarin is an isoflavone extracted from the root of Pueraria lobata that has the effects of anti-oxidation, anti-tumour, improving vascular circulation and anti-oxidative stress. In recent years, its anti-inflammatory effect, especially in improving cardiovascular and cerebrovascular diseases, has gradually been more widely recognized[4]. Therefore, this study mainly explores the effect of puerarin on inhibiting microgliitis. The mechanism of symptomatic injury and the correlation with the regulation of multiple signal molecules were further analysed.

Materials and methods

Main reagents and instruments

The N9 microglia cell line was purchased from the cell centre of the Institute of Basic Research, Union Medical University; puerarin was provided by the China Institute of Pharmaceutical and Biological Products Verification; LPS was purchased from the Gibco Company, USA; a BCA protein quantitative kit was purchased from the Beijing Bomeide Company; an RT kit was purchased from Biyuntian Biotechnology Research Institute, Jiangsu; anti-p38, ERK1/2, JNK, iNOS, iNOS. Beta-actin was purchased from the Cell Singaling Company in the United States.

The real-time quantitative PCR and gel imager were purchased from the RIO-RAD company of the United States. The electrophoretic and electrophoretic instruments were purchased in Beijing 61 factory; the ultra-fast refrigerated centrifuge was purchased from the HEMNLE company in Germany; the laser scanning confocal microscope was purchased from the Wetzlar company of Germany; and the flow cytometer was purchased from the Beijing BD company.

Cell culture

Microglia were cultured in IMDM complete medium containing 10% foetal bovine serum, 5 units/ml heparin, 1% glutamine and 100 units/ml streptomycin. The microglia were cultured in an incubator at 37°C and 5% CO_2. The cells were digested and passaged by trypsin.

Experimental methods

Flow cytometry was used to detect the NO content of the microglia. Cells in logarithmic growth phase were collected and inoculated into 6 well plates with $6x10^6$ cells. After 24 hours of cell culture, the supernatant was discarded and treated with 25 μM (low concentration group) or 100 μM puerarin (high concentration group) for 24 hours. The IMDM medium was used as a blank control group. The supernatant was then discarded, and the cells were washed twice with PBS. Approx. 1 ml of DAF-FM-DA diluted at a 1:1000 ratio was added to each pore. Cells reacted in an incubator at 37°C for 30 minutes. Cell suspensions were collected after PBS cleaning and analysed by flow cytometry.

Western blot was used to detect the expression of related proteins. The cell suspension was centrifuged at 1000 rpm for 4 minutes. After discarding the supernatant, 200 μl cell lysate was added to each group. The precipitate was dissolved completely and placed in a pre-cooled micro-centrifugal tube. After incubation for 25 minutes, the supernatant was centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was placed in a new centrifugal tube. Next, 6 μl was taken from each tube for protein quantification. The protein samples were mixed and centrifuged. According to the results of protein quantification, 40 μg protein was added into each pore. Marker localization was then carried out. After constant voltage electrophoresis, the strip was removed. According to the same size of PVDF film in the strip size monitoring area, the strip was placed in the order of filter paper, glue and film into the transfer clamp for membrane rotation. The PVDF membrane was soaked in 5% skim milk powder and sealed for 1 h. After being sealed, it was diluted with a single antibody and cultured for 12~16 h. After the PBS was cleaned 3 times, the membrane was immersed in diluted HRP-labelled secondary antibody, cultured 1 h at room temperature, and washed again with PBS 3 times. Then the ECL was detected using a chemiluminescence detector, and the optical density was analysed using Quant One software.

Polymerase chain reaction (PCR) was used to detect the expression of related genes. After the logarithmic growth cells were restarted by PBS, 0.9 ml pyrolysate was added to each pore. After repeated blowing of the pyrolysate without obvious floccu-

lent substance, it was transferred to an RNA-free EP tube. The chloroform accumulated in 1/5 pyrolysate was added to the audience. After standing at room temperature, the cells were centrifuged under 12,000 g centrifugal force for 15 minutes.

The transparent liquid containing DNA in the upper layer was added to another EP tube. Isopropanol of equal volume was added. After standing at room temperature, it was centrifuged at 12,000 g for 10 minutes. After discarding the supernatant, 1 ml ethanol was added to each tube. After centrifugation, the supernatant was absorbed. The purity and concentration of RNA were measured by ultraviolet spectrophotometer.

Statistical methods

The measurement data in this study are expressed as ($\bar{x}\pm s$) and data are compared between groups using the t-test, with $P<0.05$ regarded as representing a statistical difference.

The data in this study were analysed using the SPSS 21.0 software package.

Results

Effect of puerarin on NO production in microglia

The results of flow cytometry showed that the NO production of microglia stimulated by LPS was significantly higher than that of the control group ($P<0.05$). Compared with the LPS group, the puerarin high concentration group could significantly inhibit NO production ($P<0.05$). The effect of the puerarin low concentration group on NO was not significantly different from that of the LPS group ($P>0.05$). See Figure 1.

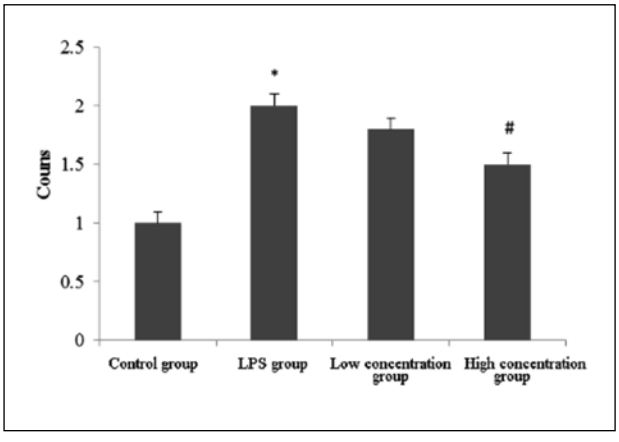

Figure 1: Puerarin inhibits LPS-induced NO production in microglia.
*Note: *Compared with control group P<0.05, #compared with LPS group P<0.05.*

Effect of puerarin on iNOS production in microglia

Western blot analysis showed that the iNOS protein level increased significantly after LPS stimulation compared with the control group ($P<0.05$), decreased significantly after puerarin treatment compared with the LPS group, and was inhibited by puerarin in a dose-dependent manner ($P<0.05$). The results of real-time quantitative PCR showed that the change in iNOS gene level was consistent with that of the protein level (see Figure 2, Table 1).

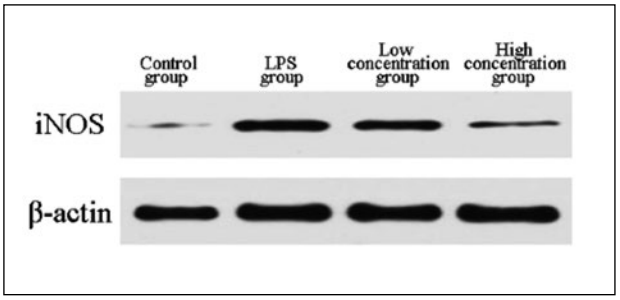

Figure 2: Effect of puerarin on iNOS protein level in microglia.

Groups	Cases	iNOS protein	iNOS mRNA
Control group	5	0.52±0.26	1.02±0.25
LPS group	5	1.61±0.39*	17.53±4.69*
Puerarin low concentration group	5	1.12±0.21#	10.78±3.12#
Puerarin high concentration group	5	0.56±0.19#	7.39±2.36#

Table 1: Effects of puerarin on iNOS protein and gene levels in microglia ($\bar{x}\pm s$).
*Note: *Compared with control group P<0.05, #compared with LPS group P<0.05.*

Effect of puerarin on activation of NF-kB in microglia

Compared with the control group, the puerarin high concentration group significantly inhibited the activation of the NF-kB gene in microglia ($P<0.05$, see Figure 3).

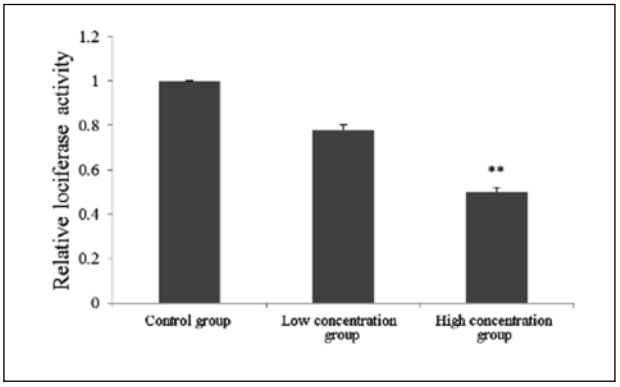

Figure 3: Effect of puerarin on activation of NF-kB in microglia.

Effect of puerarin on phosphorylation of the MAPK signal in microglia

Compared with the control group, LPS could significantly promote the phosphorylation of p38, ERK1/2 and JNK (P<0.05). Compared with the LPS group, puerarin at both high and low concentrations could inhibit the phosphorylation of ERK1/2 (P<0.05), while puerarin at high concentration could significantly inhibit the phosphorylation of p38 and JNK (P<0.05). See Table 2.

Groups	Cases	p38	ERK1/2	JNK
Control group	5	0.53±0.15	0.32±0.11	0.23±0.02
LPS group	5	1.45±0.38*	1.58±0.35*	1.02±0.23*
Puerarin low concentration group	5	1.62±0.40	0.62±0.16#	0.89±0.19
Puerarin high concentration group	5	0.75±0.22#	0.51±0.13#	0.37±0.06#

Table 2: Effect of puerarin on phosphorylation of MAPK signal in microglia ($\bar{x}\pm s$).
*Note: Compared with control group *P<0.05, compared with LPS group #P<0.05.*

Effect of puerarin on O-GlcNAc glycosylated protein in microglia cells

The level of O-GlcNAc glycosylated protein in microglia treated with LPS was significantly lower than that in the control group (P<0.05). The level of O-GlcNAc glycosylated protein in microglia treated with different concentrations of puerarin was significantly higher than that in the LPS group (P<0.05). See Figure 4.

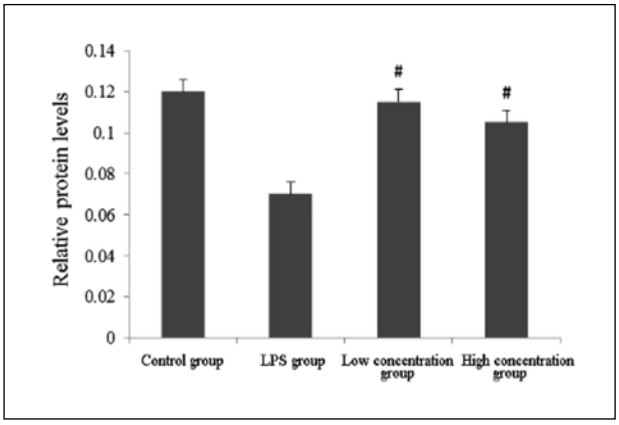

Figure 4: Effects of puerarin on O-GlcNAc glycosylated protein in microglia.

Discussion

Microglia are innate immune cells of the central nervous system, accounting for about 10% of the total number of adult central nervous system cells. They are widely distributed in the hippocampus, basal ganglia and substantia nigra and play an important role in maintaining the stability of the local microenvironment of brain tissue[5]. In a physiological state, microglia can monitor the entire brain within several hours and play a perceptual role, which is the main regulator of inflammatory response after ischemic stroke.

Ischemia, infection, trauma and other pathological factors can change the activation status of microglia, and once activated, their deficient physiological function and structural abnormality aggravate the inflammatory damage of brain tissue[6]. Therefore, inhibiting the over-activation of microglia and reducing the inflammatory damage to the central nervous system has become a focus of clinical scholars. Puerarin is commonly used in traditional Chinese medicine. Its main components include isoflavones, alkaloids, and triterpenoid saponins. Chinese medicine utilises puerarin for many functions, such as "eliminating yin-qi", "dispelling depression and fire" and "clearing blood".

Modern pharmacology considers that it has many pharmacological effects, including antioxidant, anti-tumour, and hypoglycaemic properties. It has been widely used in the treatment of ischemic cardiovascular and cerebrovascular diseases[7-8]. Relevant data show that puerarin can inhibit the expression of inflammatory factors in monocytes and macrophages and alleviate the inflammatory response of the body by blocking the activation of the NF-kB signalling pathway. In addition, puerarin can also play a neuroprotective role by inhibiting the activation of neutrophils[9].

NO is a double-acting messenger and toxic molecule that is present mainly in the immune, cardiovascular and nervous systems. Its physiological effects on the body depend primarily on the nature of stimulating factors. Relevant data show that NO is not only a neurotoxin released after tissue and cell injury, but also has the function of regulating immune signals. NO structural iNOS can induce the release of a large amount of NO, cause cell damage and play a cytotoxic role. Because iNOS itself has a neurotoxic effect, its transcription is regulated by NF-kB[10]. Some scholars have found that activated microglia can express a large amount of iNOS, thereby releasing a large amount of NO and aggravating the damage to nerve cells[11].

The results of this study showed that the NO production of microglia stimulated by LPS was significantly higher than that of a control group

($P<0.05$). Compared with the LPS group, the group subsequently treated with a high concentration of puerarin could significantly inhibit NO production ($P<0.05$). The iNOS protein level after puerarin treatment was significantly lower than that of the LPS group and was dose-dependent. The expression of iNOS protein ($P<0.05$) suggested that puerarin could significantly inhibit NO production and iNOS expression in microglia and alleviate inflammation. NF-kB is a nuclear transcription factor that is present in many kinds of cells and plays a regulatory role in inflammation and immune response.

It participates in the expression of iNOS, gene expression and cell apoptosis, and other signal transduction processes can be regulated by binding of NF-kB with gene promoter and enhancer. It is widely used in head and neck tumours, cervical cancer, breast cancer, etc.[12]. Overexpression was found in several malignant tumours.

The results showed that puerarin could significantly activate the NF-kB gene in microglia, suggesting that puerarin could inhibit the production of NO and the expression of iNOS in microglia, which may be related to the reduction of the expression of NF-kB. The MAPKs signalling pathway is located upstream of NF-kB, including p38, ERK1/2 and JNK, which can mediate the whole process of cell growth, development and differentiation.

The MAPKs pathway can transmit extracellular stimulus signals to the nucleus, which is closely related to the permeability of vascular endothelial cells[13]. In recent years, it has been found that macrophages in the ischemic core of patients with cerebral ischemic stroke contain abundant p38MAPK, suggesting that the inflammatory response of ischemic stroke is closely related to the MAPKs signalling pathway[14].

The results showed that puerarin could significantly inhibit the phosphorylation of the MAPKs signalling pathway. O-GlcNAc is abundant in the brain and plays an important role in gene regulation, protein expression and signal transduction.

It has been proved that O-GlcNAc glycosylation can inhibit inflammatory response and reduce the activation of NF-kB in a cerebral ischemia-reperfusion injury model[15].

The results showed that the levels of O-GlcNAc glycosylated protein increased significantly after puerarin treatment at different concentrations, suggesting that the inhibition by puerarin of the activation of NF-kB may be related to the increase in the O-GlcNAc glycosylated protein level. In conclusion, puerarin can play an anti-inflammatory role by reducing NO release and iNOS expression, inhibiting the activation of the NF-kB gene, then down-regulating the phosphorylation of the MAPKs signal and up-regulating the level of O-GlcNAc glycosylation protein.

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