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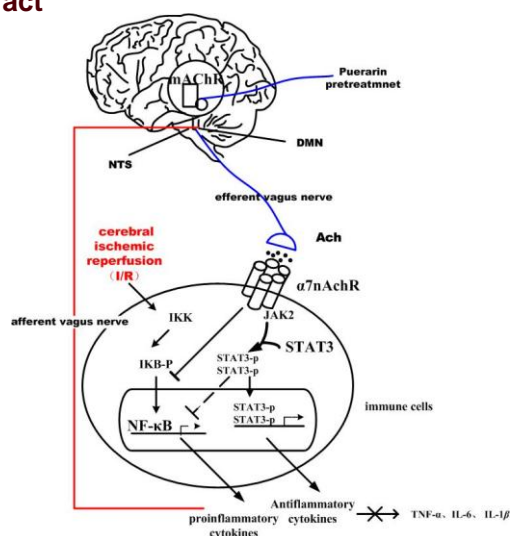
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Puerarin partly counteracts the inflammatory response after cerebral ischemia/reperfusion via activating the cholinergic anti-inflammatory pathway

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Graphical Abstract



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Abstract

Puerarin, a major isoflavonoid derived from the Chinese medical herb *radix puerariae* (Gegen), has been reported to inhibit neuronal apoptosis and play an anti-inflammatory role in focal cerebral ischemia model rats. Recent findings regarding stroke pathophysiology have recognized that anti-inflammation is an important target for the treatment of ischemic stroke. The cholinergic anti-inflammatory pathway is a highly robust neural-immune mechanism for inflammation control. This study was to investigate whether activating the cholinergic anti-inflammatory pathway can be involved in the mechanism of inhibiting the inflammatory response during puerarin-induced cerebral ischemia/reperfusion in rats. Results showed that puerarin pretreatment (intravenous injection) reduced the ischemic infarct volume, improved neurological deficit after cerebral ischemia/reperfusion and decreased the levels of interleukin-1 β , interleukin-6 and tumor necrosis factor- α in brain tissue. Pretreatment with puerarin (intravenous injection) attenuated the inflammatory response in rats, which was accompanied by janus-activated kinase 2 (JAK2) and signal transducers and activators of transcription 3 (STAT3) activation and nuclear factor kappa B (NF- κ B) inhibition. These observations were inhibited by the alpha7 nicotinic acetylcholine receptor (α 7nAChR) antagonist α -bungarotoxin (α -BGT). In addition, puerarin pretreatment increased the expression of α 7nAChR mRNA in ischemic cerebral tissue. These data demonstrate that puerarin pretreatment strongly protects the brain against cerebral ischemia/reperfusion injury and inhibits the inflammatory response. Our results also indicated that the anti-inflammatory effect of puerarin may partly be mediated through the activation of the cholinergic anti-inflammatory pathway.

Author contributions: Mei ZG was responsible for the study concept and design, validated the paper, was the head of funds, and the research director. Liu XJ was responsible for data acquisition and integration, and wrote the manuscript. Qian JP performed data analysis. Zeng YB and Wang MZ provided technical and information support. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

Ethical approval: All protocols were approved by the Experimental Animal Center of China Three Gorges University, China.

Key Words

neural regeneration; cerebral ischemia/reperfusion; inflammation; cholinergic anti-inflammatory pathway; alpha7 nicotinic acetylcholine receptors; nuclear factor kappa B; janus-activated kinase 2; signal transducers and activators of transcription 3; grants-supported paper; neuroregeneration

INTRODUCTION

Ischemic stroke involves death of brain tissue (cerebral infarction) resulting from an inadequate supply of blood and oxygen to the brain due to blockage of an artery. Although different mechanisms are involved in the pathogenesis of ischemic stroke, increasing evidence shows that inflammation plays an important role in the pathogenesis of ischemic stroke and other types of ischemic brain injury^[1-5]. Cerebral ischemia initiates a prominent inflammatory response, which includes the activation of brain microglia and macrophages and the upregulation of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF- α), interleukin (IL)-1 β and IL-6^[3, 6-7]. Cerebral ischemia/reperfusion (I/R) is critical for restoring normal function, and can paradoxically result in inflammation and secondary damage. Experimental data has shown that reperfusion represents an especially vulnerable period for the brain^[8-9], which is considered more amenable to treatment than acute neurotoxicity^[2, 10]. Thus, the anti-inflammatory approach may be a feasible potential therapeutic strategy of protecting against the I/R cerebral damage.

A neural signal transmitted through the vagus nerve that regulates cytokine production specifically *via* alpha7 nicotinic acetylcholine receptor (α 7nAChR)-dependent signaling termed the "cholinergic anti-inflammatory pathway" (CAP) is a highly robust mechanism for inflammation control^[11-12]. A number of preclinical studies have demonstrated that α 7nAChR may be a potential therapeutic target for inflammation as it has remarkable anti-inflammatory effects in many intractable diseases^[13-14]. Studies^[15-16] have demonstrated that selective α 7nAChR agonists might be useful for treating inflammation during traumatic brain injury or post-ischemic inflammation and neuronal damage.

Although the selective agonist of α 7AChR has not been used successfully in a clinical setting, several researchers have reported that nicotine can inhibit the inflammatory response through the activation of α 7AChR^[17-19]. We found that the selective agonist of nicotine α 7AChR significantly increased α 7nAChR mRNA expression, and inhibited levels of pro-inflammatory cytokines and nuclear

factor kappa B (NF- κ B) protein in ischemic cerebral tissue. Similar effects were observed with puerarin. Yoshikawa *et al*^[19] also demonstrated that the pharmacological concentration of nicotine inhibits the production of proinflammatory mediators in human monocytes through the α 7nAChR by inhibiting I- κ B phosphorylation and by suppressing NF- κ B DNA binding. Therefore, we also used nicotine as a control to compare with puerarin.

Many active ingredients of Chinese herbs have the same effect as cholinergic agonists. Physostigmine can suppress jugular venous O²⁻ generation, oxidative stress, early inflammation, and endothelial activation in the brain and plasma during the acute phase of cerebral I/R^[20]. Huperzine A can inhibit nuclear translocation of transcription factor NF- κ B, decrease overexpression of proinflammatory factors in both the ipsilateral cortex and striatum, and suppress activation of glial cells in the ischemic penumbra^[21]. The physiology of CAP can also be used to explain the mechanism of many traditional Chinese herbs and their active ingredients in modulating the inflammatory response^[12, 22-23]. Puerarin is a most abundant isoflavone-C-glucoside extracted from the root of the plant *pueraria lobata* (Gegen). It has been used for various medicinal purposes in traditional oriental medicine for thousands of years. Numerous studies have revealed that puerarin possesses many biological activities and medicinal properties, which include the treatment of various cardiovascular diseases, dilating the cardiac and cerebral vessels, and anti-inflammatory and antioxidative properties^[24-26]. In recent years, more attention has focused on the anti-inflammatory effect of puerarin. Several preclinical studies have demonstrated that puerarin protects the brain against inflammation through decreasing the expression of TNF- α and inhibiting the activity of NF- κ B following cerebral I/R^[27-29]. Clinical studies have shown that puerarin inhibits the increase of IL-6 after acute ischemic stroke and reduces lactate dehydrogenase levels following I/R^[30]. Similarly, puerarin in combination with aspirin treatment for acute cerebral infarction has protective effects on damaged vascular endothelial cells^[31]. These findings suggest that the anti-inflammatory mechanisms of puerarin are mediated by blocking NF- κ B signaling and inhibiting TNF- α levels. However, till now, the detailed mechanisms of its anti-inflammatory effect have not been clearly elucidated.

As mentioned above, puerarin and CAP stimulation can suppress the inflammatory response, and we hypothesize that the anti-inflammatory effect of puerarin is mediated through the stimulation of CAP. Therefore, we summarized the anti-inflammatory properties of puerarin in a middle cerebral artery occlusion-reperfusion rat model, and investigated a possible new mechanism for attenuating inflammation.

RESULTS

Quantitative analysis of experimental animals

A total of 162 adult rats were equally and randomly divided into six groups: sham, vehicle, puerarin 36 mg/kg, puerarin 54 mg/kg, nicotine and puerarin + α -bungarotoxin (α -BGT; α 7nAChR antagonist) groups. Middle cerebral artery occlusion (MCAO) was performed in all rats except those in the sham group. In the sham group, rats were pre-administered physiological saline *via* the tail vein for 5 days, but the origin of the middle cerebral artery occlusion was not occluded. Puerarin (36 mg/kg intravenous injection), puerarin (54 mg/kg intravenous injection), physiological saline, and nicotine were pre-administered for 5 days before introduction of MCAO in the puerarin 36 mg/kg, puerarin 54 mg/kg, vehicle and nicotine groups, respectively. Puerarin + α -BGT group underwent the same procedure as the puerarin 36 mg/kg group, and α -BGT (1.0 μ g/kg) was intravenously injected after MCAO. Rats excluded due to death or failed model establishment were supplemented. All 162 rats were included in the final analysis with 27 rats per group.

Puerarin improved neurological deficits induced by cerebral I/R

Forty-eight hours after cerebral I/R, rats in the sham group did not present with any symptoms of neurobehavioral dysfunction (Longa's score 0), while rats in the vehicle group showed significant neurobehavioral dysfunction ($P < 0.01$). Pretreatment with different concentrations of puerarin and nicotine produced significant improvements in neurological function ($P < 0.05$). The neurological function of rats in the nicotine group was significantly improved compared to that in the puerarin 54 mg/kg group ($P < 0.05$). However, puerarin + α -BGT treatment did not significantly improve neurological function of MCAO rats compared to puerarin 36 and 54 mg/kg treatment (Figure 1).

Puerarin reduced infarct volume after cerebral I/R

Twelve hours after cerebral I/R, the infarct volumes were measured by 2,3,5-triphenyltetrazolium chloride (TTC)

staining. Obvious cerebral infarct was detected in MCAO rats. Pretreatment with puerarin at 36 mg/kg and 54 mg/kg significantly reduced infarct volume of MCAO rats ($P < 0.01$, $P < 0.05$). Interestingly, the infarct volume was significantly reduced in MCAO rats after treatment with puerarin at 36mg/kg compared with puerarin at 54 mg/kg ($P < 0.05$). Furthermore, the protective effect of puerarin at 36 mg/kg was partly blocked by α -BGT. Nicotine showed a significant reduction effect on infarct volume compared with puerarin at 54 mg/kg ($P < 0.05$) (Figure 2).

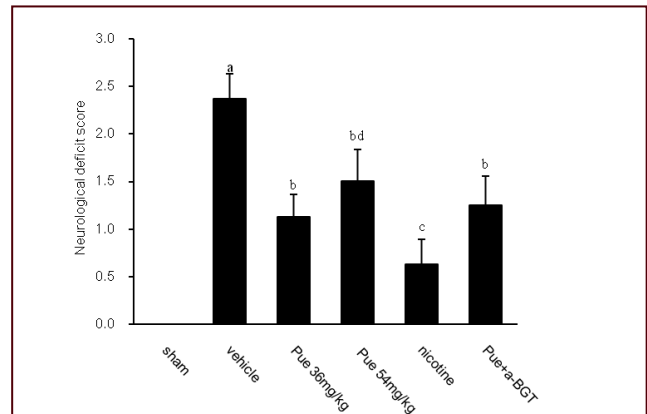


Figure 1 Effects of puerarin (Pue) on neurological deficits 48 hours after cerebral ischemia/reperfusion injury in rats.

Neurological function was assessed by Longa's score, with higher scores indicating more severe neurological deficits. Values are expressed as mean \pm SEM, $n = 6$ rats per group. ^a $P < 0.01$, vs. sham group; ^b $P < 0.05$, ^c $P < 0.01$, vs. vehicle group; ^d $P < 0.05$, vs. nicotine group. Differences between groups were compared by one-way analysis of variance followed by least significant difference *post-hoc* test.

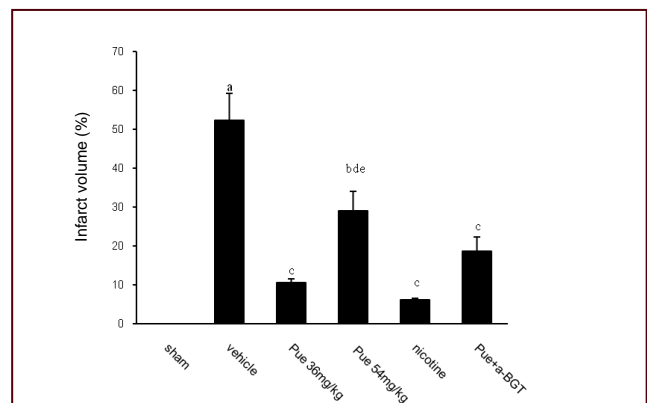


Figure 2 Effect of puerarin (Pue) on infarct volume 12 hours after cerebral ischemia/reperfusion injury in rats.

Infarct volume was assessed using 2,3,5-triphenyltetrazolium chloride (TTC) staining. $n = 5$ rats per group, values are expressed as mean \pm SEM. ^a $P < 0.01$, vs. sham group; ^b $P < 0.05$, ^c $P < 0.01$, vs. vehicle group; ^d $P < 0.05$, vs. Pue 36 mg/kg group; ^e $P < 0.05$, vs. nicotine group. Differences between groups were compared by one-way analysis of variance followed by least significant difference *post-hoc* test.

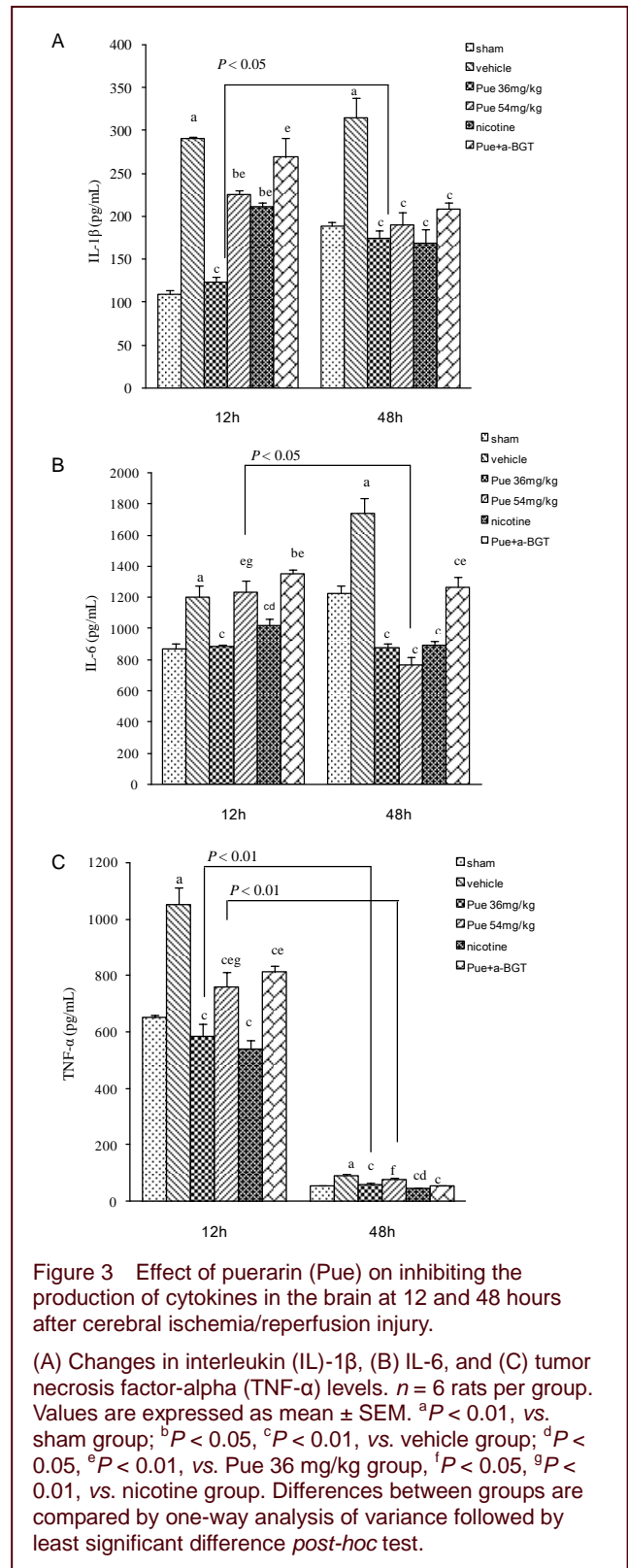
Puerarin reduced the expression of inflammatory cytokines in ischemic brain after cerebral I/R

To assess the effects of puerarin on anti-inflammatory response, we tested the expression of IL-1 β , IL-6 and TNF- α in the cortex, hippocampus and striatum in ischemic cerebral tissue by enzyme linked immunosorbent assay (ELISA) at 12 and 48 hours after cerebral I/R. As shown in Figure 3, the expression levels IL-1 β , IL-6 and TNF- α protein were significantly increased after cerebral I/R ($P < 0.01$). Pretreatment with puerarin at 36 mg/kg remarkably attenuated the increasing levels of IL-1 β , IL-6 and TNF- α ($P < 0.01$) and these decreases were significantly suppressed by α -BGT treatment ($P < 0.05$). In addition, pretreatment with puerarin at 54 mg/kg also significantly decreased the levels of IL-1 β , IL-6 and TNF- α ($P < 0.05$), but the difference was not statistically significant between puerarin 54 mg/kg and vehicle groups in the level of IL-6 at 12 hours after cerebral I/R. Two concentrations of puerarin treatment both attenuated the levels of IL-1 β , IL-6 and TNF- α at 12 and 48 hours after cerebral I/R, and statistical differences in the inflammatory factors between the two treatments were detected at 12 hours after cerebral I/R ($P < 0.01$), but no significant differences were seen at 48 hours after cerebral I/R. Compared to nicotine group, remarkably higher levels of IL-6 and TNF- α were observed in the puerarin 54 mg/kg group at 12 hours after cerebral I/R ($P < 0.01$), while there were no significant differences in these two indices between nicotine and puerarin 36 mg/kg groups. As shown in Figure 3, significant differences in the level of IL-1 β and TNF- α in the puerarin 36 mg/kg group, in the level of IL-6 and TNF- α in the puerarin 54 mg/kg group were detected when compared 48 hours after cerebral I/R with 12 hours after cerebral I/R.

Effects of puerarin on the expression of phosphorylated janus-activated kinase 2 (p-JAK2) and phosphorylated signal transducers and activators of transcription 3 (p-STAT3) in ischemic brain after cerebral I/R

The JAK2/STAT3 signaling pathway is one of the major mechanisms for cytokine signal transduction in CAP^[12]. To determine the possible anti-inflammatory pathways in which puerarin may participate, the present study used immunohistochemistry staining to evaluate p-JAK2 and p-STAT3 expression 12 and 48 hours after cerebral I/R. The sham and vehicle groups presented minimal p-JAK2 and p-STAT3 immunoreactivity. Pretreatment with puerarin significantly increased p-JAK2 immunoreactivity compared with the vehicle group ($P < 0.01$) and this increase was significantly higher at 12 hours after cerebral I/R compared to 48 hours after cerebral I/R ($P < 0.05$).

As shown in Figure 4B, puerarin at 36 mg/kg showed significantly higher immunoreactivity compared with puerarin 54 mg/kg ($P < 0.01$) at 48 hours after cerebral I/R, and nicotine pretreatment showed significantly higher immunoreactivity compared with puerarin at 54 mg/kg ($P < 0.01$).



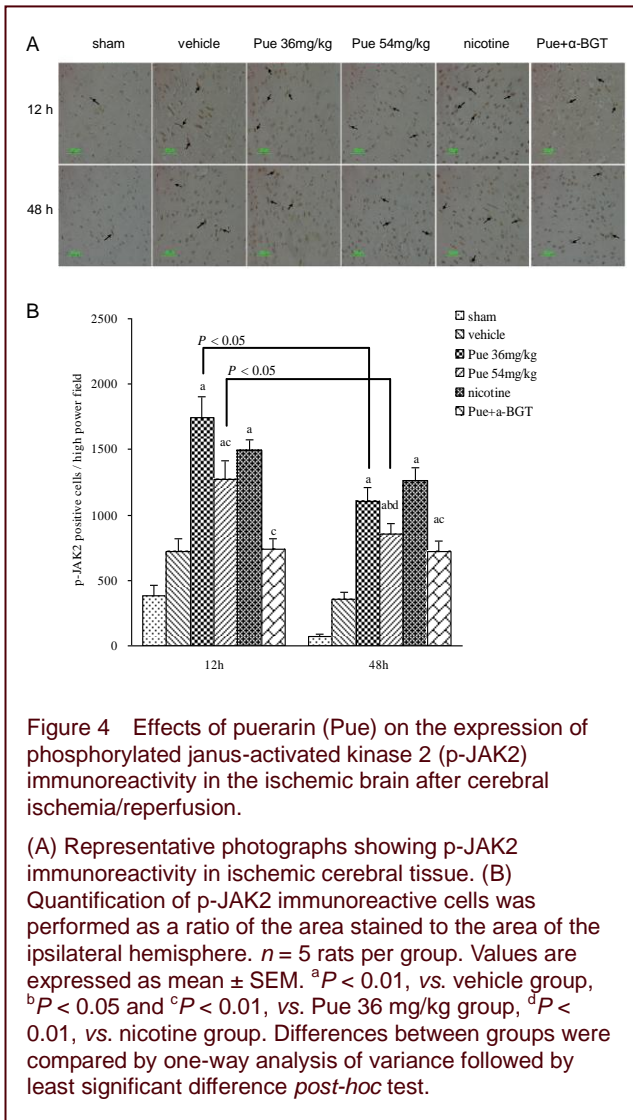


Figure 4 Effects of puerarin (Pue) on the expression of phosphorylated janus-activated kinase 2 (p-JAK2) immunoreactivity in the ischemic brain after cerebral ischemia/reperfusion.

(A) Representative photographs showing p-JAK2 immunoreactivity in ischemic cerebral tissue. (B) Quantification of p-JAK2 immunoreactive cells was performed as a ratio of the area stained to the area of the ipsilateral hemisphere. $n = 5$ rats per group. Values are expressed as mean \pm SEM. ^a $P < 0.01$, vs. vehicle group, ^b $P < 0.05$ and ^c $P < 0.01$, vs. Pue 36 mg/kg group, ^d $P < 0.01$, vs. nicotine group. Differences between groups were compared by one-way analysis of variance followed by least significant difference *post-hoc* test.

Furthermore, α -BGT significantly inhibited p-JAK2-immunoreactivity in the puerarin 36 mg/kg group ($P < 0.01$). As shown in Figure 5B, puerarin at 36 mg/kg markedly increased p-STAT3 immunoreactivity when compared with the vehicle group ($P < 0.01$). Rats in the puerarin + α -BGT group showed significantly lower p-STAT3 immunoreactivity when compared with the puerarin 36 mg/kg group at 12 hours after cerebral I/R ($P < 0.01$). Moreover, there was a significant difference in p-STAT3 immunoreactivity between puerarin 36 mg/kg and puerarin 54 mg/kg groups ($P < 0.01$) at 12 hours after cerebral I/R. A marked decrease in p-STAT3 immunoreactivity were observed at 48 hours after cerebral I/R when compared with 12 hours after cerebral I/R in the puerarin 36 mg/kg group.

Effect of puerarin on the generation of $\alpha 7nAChR$, JAK2 and STAT3 as well as NF- κ B mRNA expression in the ischemic brain after cerebral I/R

In this study, we used RT-PCR analysis to assess the

effect of puerarin on $\alpha 7nAChR$ mRNA expression 12 and 48 hours following MCAO-induced injury. As shown in Figure 6A–C, pretreatment with puerarin at 36 mg/kg resulted in significantly high expression of $\alpha 7nAChR$ mRNA compared to the vehicle group ($P < 0.05$) at both time points, but expression at 48 hours after cerebral I/R significantly decreased in comparison with 12 hours after cerebral I/R ($P < 0.01$).

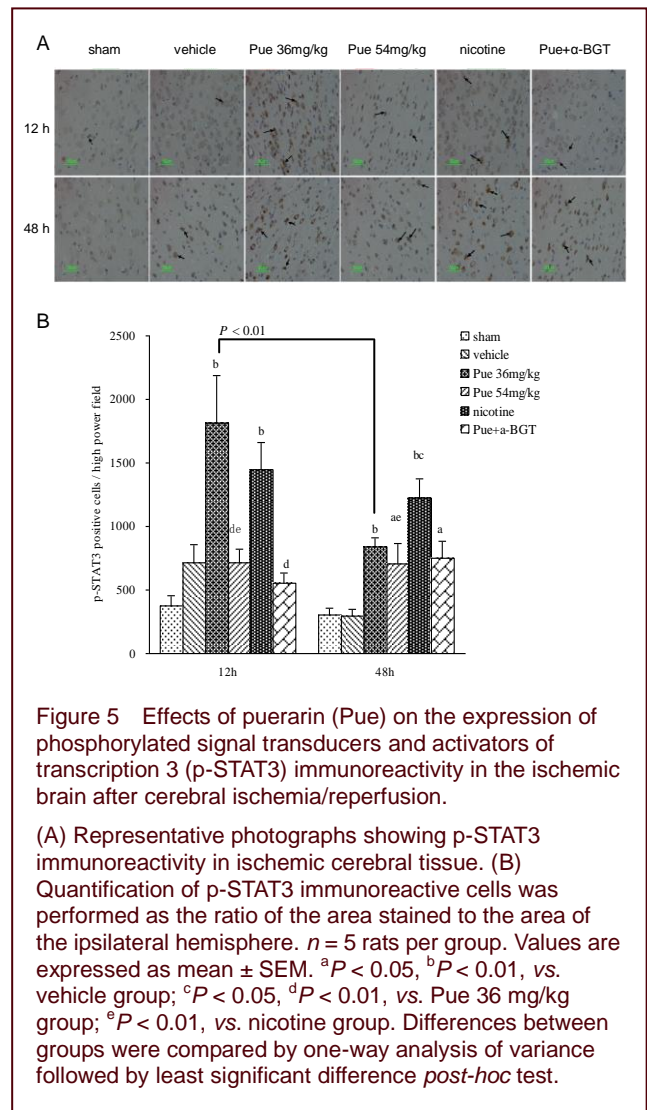
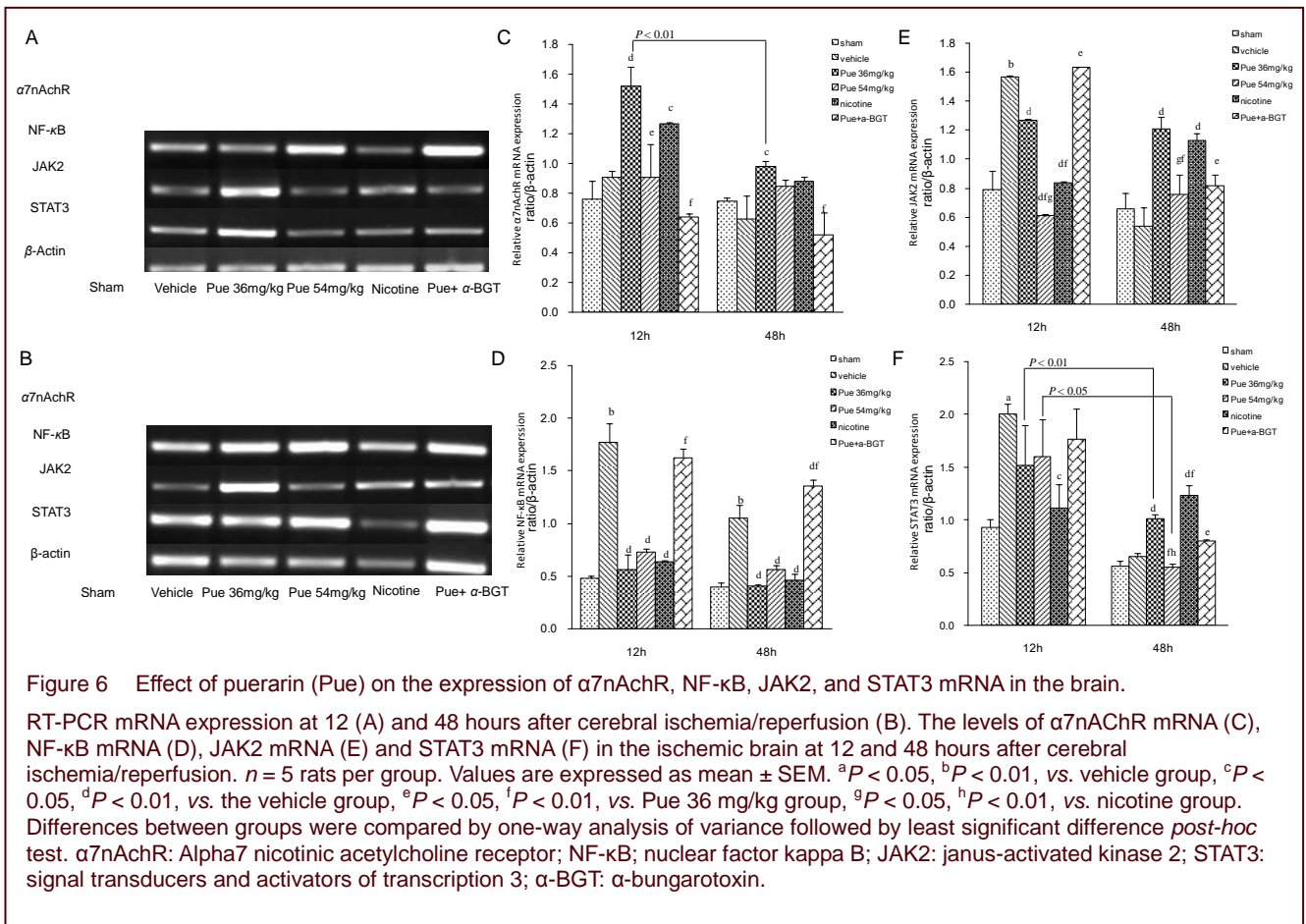


Figure 5 Effects of puerarin (Pue) on the expression of phosphorylated signal transducers and activators of transcription 3 (p-STAT3) immunoreactivity in the ischemic brain after cerebral ischemia/reperfusion.

(A) Representative photographs showing p-STAT3 immunoreactivity in ischemic cerebral tissue. (B) Quantification of p-STAT3 immunoreactive cells was performed as the ratio of the area stained to the area of the ipsilateral hemisphere. $n = 5$ rats per group. Values are expressed as mean \pm SEM. ^a $P < 0.05$, ^b $P < 0.01$, vs. vehicle group; ^c $P < 0.05$, ^d $P < 0.01$, vs. Pue 36 mg/kg group; ^e $P < 0.01$, vs. nicotine group. Differences between groups were compared by one-way analysis of variance followed by least significant difference *post-hoc* test.

Nicotine pretreatment had a similar effect to puerarin at 36 mg/kg. In addition, the high expression of $\alpha 7nAChR$ mRNA was remarkably attenuated by α -BGT *versus* puerarin at 36 mg/kg group ($P < 0.01$). Puerarin at 54 mg/kg also increased $\alpha 7nAChR$ mRNA expression, but it was not concentration-dependent. NF- κ B is known to play an important role in regulating the expression of a variety of cytokine genes during the inflammatory response, so we investigated NF- κ B mRNA expression in the ischemic brain as shown in Figure 6D. NF- κ B mRNA expression at both time points was significantly increased after cerebral I/R (both $P < 0.01$).



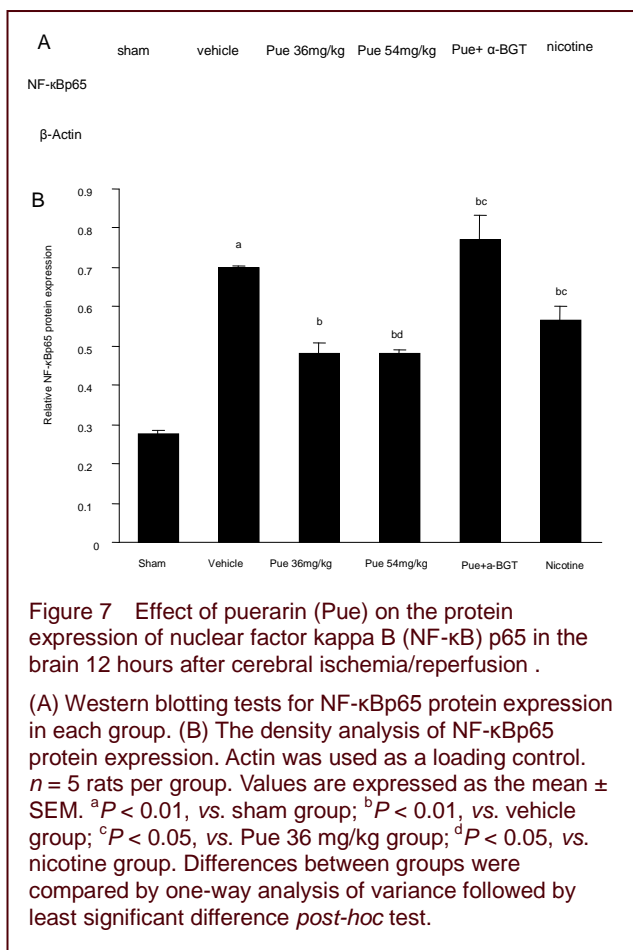
Pretreatment with different concentrations of puerarin and nicotine significantly inhibited the high mRNA expression of NF- κ B (all $P < 0.01$). However, α -BGT treatment markedly suppressed the efficacy of puerarin at 36 mg/kg ($P < 0.01$). Twelve hours after cerebral I/R, the mRNA expression of JAK2 and STAT3 were significantly increased in the vehicle group when compared with the sham group ($P < 0.05$), but both indices were significantly decreased in the nicotine group ($P < 0.05$) (Figure 6E, F).

As shown in Figure 6E, pretreatment with the two concentrations of puerarin significantly reduced the mRNA expression of JAK2 (both $P < 0.01$) at 12 hours after cerebral I/R. Compared with the puerarin 36 mg/kg group, low mRNA expression of JAK2 was significantly blocked in the puerarin + α -BGT group ($P < 0.01$). As shown in Figure 6F, STAT3 mRNA expression was significantly increased at 12 hours after cerebral I/R ($P < 0.05$). Nicotine pretreatment significantly attenuated the expression of STAT3 mRNA at 12 hours after cerebral I/R when compared with the vehicle group ($P < 0.05$), while puerarin did not. In comparison with 12 hours after cerebral I/R, both puerarin at 36 and 54 mg/kg significantly decreased the expression of STAT3 mRNA at 48 hours

after cerebral I/R ($P < 0.05$). Furthermore, RT-PCR data showed similar results in mRNA expression of JAK2 and STAT3 at 48 hours after cerebral I/R to immunohistochemistry staining. At 48 hours after cerebral I/R, pretreatment with puerarin at 36 mg/kg and nicotine significantly increased mRNA expression when compared with the vehicle group ($P < 0.01$), while the high expression was markedly attenuated in the puerarin + α -BGT group ($P < 0.05$).

Effect of puerarin on NF- κ Bp65 protein expression in the ischemic brain after cerebral I/R

As shown in Figure 7, low levels of NF- κ Bp65 protein expression was detected in the ischemic brain 12 hours after cerebral I/R by western blotting. In the vehicle group, NF- κ Bp65 protein was significantly up-regulated when compared to the sham group ($P < 0.01$), while puerarin (36 and 54 mg/kg) and nicotine treatment markedly decreased NF- κ Bp65 protein expression when compared with the vehicle group ($P < 0.01$). NF- κ Bp65 protein was significantly increased in the nicotine group when compared with the puerarin 36 mg/kg and 54 mg/kg groups ($P < 0.05$). Moreover, α -BGT treatment significantly suppressed the effect of puerarin 36 mg/kg on the protein expression of NF- κ Bp65 ($P < 0.01$).



DISCUSSION

Pueraria lobata root and its components are useful in the prevention and treatment of various inflammatory diseases and oxidative stress-related diseases^[24]. The protective mechanisms of puerarin, at least in part, are related to its action of dilating the coronary artery^[32] and cerebral vessels, increasing cerebral blood flow^[33], improving hemorheology^[34], blocking Na⁺ channels^[35] and its antioxidative properties^[25, 36]. In recent years, many studies have demonstrated that pueraria has protective effects against ischemia-induced neuronal injury, with pueraria flavonoid being widely used for the prevention and treatment of ischemic stroke^[37]. Studies have shown that inflammation as a protective response to microbial invasion or injury is an important pathophysiological mechanism during ischemic stroke, and that up-regulation of the inflammatory response rapidly leads to further cerebral injury^[2, 11]. In our prior research, we have found that pretreatment with puerarin can prevent cerebral I/R-induced inflammatory injury, which may be related to the stimulation of CAP^[38-39]. In the present study, we have provided further insights into the function

and potential mechanism of puerarin in inhibiting inflammation. Consistent with previous findings, results from this study showed that different concentrations of puerarin (36 mg/kg and 54 mg/kg) at different time points (12 hours and 48 hours) after cerebral I/R could reduce the cerebral inflammatory response *via* CAP. Furthermore, this study examined the effect of puerarin on the JAK2/ STAT3 and NF-κB signaling pathway.

We found that the expression of IL-6, IL-1β and TNF-α were increased after focal cerebral I/R in rats, and this result was also observed by other studies^[7, 40]. Among these inflammatory cytokines, TNF-α and IL-1β appear to exacerbate cerebral injury. The production of TNF-α is associated with neuronal death and inflammation in stroke, and it has a critical role in coordinating the inflammatory response and activating mediators distally in the cytokine cascade^[41]. IL-1β has been reported to activate the production of other cytokines and evoke metabolic and hemodynamic changes which are similar to TNF-α^[7, 42]. Moreover, high circulating levels of TNF-α and IL-1β may elevate the expression of IL-6, another well known cytokine following cerebral ischemia^[43]. Depending on the location and time course, IL-6 has both pro- and anti-inflammatory effects^[44]. In the present study, we demonstrated that puerarin significantly attenuated the production of IL-1β, IL-6 and TNF-α in brain tissue, and this observation is supported by many other studies^[28-30]. In addition, the α7AChR antagonist α-BGT suppressed the beneficial effects of puerarin, which indicates that puerarin attenuates inflammation possibly through α7AChR. However, the anti-inflammatory effect degraded as time passed from 12 hours to 48 hours after cerebral I/R. Evidences showed that the levels of IL-1β, IL-6 and TNF-α were lower at 48 hours compared to 12 hours after cerebral I/R in the puerarin 54 mg/kg group, but not in the puerarin 36 mg/kg group, which indicates that higher concentrations of puerarin have a much stronger effect on weakening inflammatory factors after acute cerebral I/R. However, the underlying mechanisms are complicated and CAP can not fully explain this phenomenon.

The cholinergic anti-inflammatory pathway, a link between the parasympathetic and innate immune system, is composed of the efferent vagus nerve, the neurotransmitter acetylcholine and α7nAChR^[45]. Stimulation of the efferent vagus nerve can release the important neurotransmitter acetylcholine, which acts through the α7nAChR expressed on macrophages. After combining with acetylcholine, this ligand binding receptor transmits

cholinergic anti-inflammatory signals into the cytoplasm to activate JAK2 with subsequent STAT3 phosphorylation and activation^[46]. Stimulating CAP can inhibit cytokine synthesis, protect against myocardial ischemia and prevent inflammation through activation of $\alpha 7$ nAChR, decreasing translocation of NF- κ B into the cell nucleus and activating the JAK2/STAT3 signaling pathway^[11, 47-49]. It has been demonstrated that $\alpha 7$ nAChR is expressed not only on the immunocytes, such as monocytes, neutrophils and macrophages, but also on coronary microvascular endothelial cells^[50]. It was also observed in our study that the expression of $\alpha 7$ nAChR mRNA existed in brain tissue, and this expression was decreased after cerebral I/R. Pharmacological activation of $\alpha 7$ nAChR may provide significant protection against cerebral ischemia-related cell death and inflammation.

NF- κ B regulates the expression of various genes, including TNF- α , IL-1 β , IL-6, and vascular adhesion molecule 1, and many of them also re-activate NF- κ B itself, resulting in a positive regulatory loop that helps amplify and perpetuate the inflammatory response^[51-52]. It has been shown that cerebral I/R injury promotes inflammation to activate the NF- κ B pathway, which stimulates the downstream-associated inflammatory response and ischemic neuronal apoptosis^[53]. Guarini *et al*^[54] demonstrated that vagus nerve stimulation protected against brain injury through reducing NF- κ B nuclear translocation. We and Chen *et al*^[30] found that puerarin can inhibit the activation of NF- κ B and prevent inflammation in rats with cerebral I/R, and our results also confirmed that the reduction of NF- κ B in the brain by nicotine pretreatment was coupled to a reduction in brain inflammation. In addition, puerarin and nicotine attenuated the transcriptional and translational levels of NF- κ B protein. Furthermore, the JAK/STAT signaling pathway is common to class I and II cytokine receptor families, and STAT3 is generally recognized as an anti-inflammatory transcription factor^[55-56]. STAT3 phosphorylation by JAK2 can translocate from the cytoplasm into the nucleus and compete with NF- κ B to bind DNA in CAP^[11]. Boersma *et al*^[57] reported that activation of the JAK2/STAT3 signaling pathway in mice can attenuate inflammation and postoperative ileus, but failed to do so in mice devoid of STAT3 in their macrophages. The data presented in this study also showed that the effect of puerarin increased the protein expression of p-JAK2 and p-STAT3, and low doses, but not high doses, of puerarin increased the expression of JAK2 and STAT3 mRNA at 48 hours after cerebral I/R, while NF- κ B mRNA decreased at 12 and 48 hours after cerebral I/R. This evidence demonstrated that the weakening of NF- κ B mRNA might attribute to the

activation of CAP by low doses of puerarin. However, α -BGT restricted some of these effects in our study, indicating that puerarin is likely to regulate the inflammatory response partly through inhibiting NF- κ B expression and the activating JAK2/STAT3 signaling pathway *via* stimulating the $\alpha 7$ AChR.

α -BGT, one of the bungarotoxins, has been shown to play an antagonistic role in the binding of the $\alpha 7$ nAChR in the brain, and as such has numerous applications in neuroscience research. As described in the present results, rats showed neurological function deficits and infarcts after cerebral I/R, which was consistent with the results from Wang *et al*^[58]. Pretreatment with puerarin and nicotine both significantly improved the neurological function of rats, and the $\alpha 7$ AChR antagonist α -BGT suppressed the beneficial effects of low dose puerarin. Meanwhile, α -BGT had a better efficacy in both neurobehavioral evaluation and brain infarct volume than high doses of puerarin. One possible explanation accounting for these results is the concentration of puerarin used in these experiments. High doses of puerarin may induce additional systemic effects. α -BGT maybe just partly block the protective effect of puerarin during cerebral I/R, and in future studies, more attention should be paid to the pharmacological effect of α -BGT pretreatment after cerebral I/R because of two distinct effects.

In conclusion, our study recapitulated three important issues. First, puerarin can protect against brain injury by inhibiting the cerebral I/R-induced inflammatory response, and no dose-dependent effects were observed in our study. A possible explanation for these different results from others may be the different administration routes and the concentration of puerarin used in these experiments. Our study found that puerarin 36 mg/kg had a better neuroprotective effect than puerarin 54 mg/kg. This may be due to the current commercially available preparation, which is poorly soluble in water, and its reverse physicochemical properties, which block it from penetrating the blood brain barrier^[59]. How to improve its solution in water and penetration into the brain is still a hurdle for clinical application, and is a key element in experimental research. Second, CAP played an important role in restraining the inflammatory response induced by cerebral I/R. In this study, tiny amounts of nicotine pretreatment protected rats from brain dysfunction, decreased the expression of proinflammatory cytokines and inhibited the expression of NF- κ B in ischemic cerebral tissue. However, due to the well-known side effects of nicotine when stroke occurs, we need to pay more attention to other $\alpha 7$ nAChR

agonists, particularly to Chinese medical herbs and their ingredient such as puerarin. Third, puerarin protects against the cerebral I/R-induced inflammatory response possibly *via* activation of CAP. Our data showed that puerarin and nicotine have similar effects in reducing cytokine synthesis, regulating the NF- κ B and JAK2/STAT3 signaling pathway, and protecting against MCAO-induced injury. Pretreatment with α -BGT weakened the inflammation-inhibiting effects of puerarin. However, understanding CAP and its role in mitigating inflammation during cerebral I/R continues to grow. Therefore, future studies aim to refine and enhance the development of puerarin and CAP for highly targeted therapeutic approaches.

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

The study was performed at the Medical College of China Three Gorges University, China from October 2011 to July 2012.

Materials

Animals

Male Sprague-Dawley rats, aged 8 weeks old, weighing 250–300 g were offered by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, China (License No. SCXK (E) 2010-0007) and housed in a light-controlled room with a 12-hour light/dark cycle and allowed free access to water and standard rat chow. Rats were acclimatized for 1 week before experimentation. Animal care in this study was in compliance with guidelines approved by the Provisions and General Recommendation of Chinese Experimental Animals Administration Legislation.

Drug

Puerarin (7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one-8-(β -D-glucopyranoside) was purchased from Shandong Reyoung Pharmaceutical Co., Ltd., (Zibo, Shandong, China). Freshly prepared puerarin was dissolved in physiological saline to reach a final concentration of 36 mg/mL and 54 mg/mL.

Methods

Preparation of MCAO models

MCAO was performed according to the intraluminal suture method described by Su *et al* [60]. Briefly, all rats

were anesthetized with chloral hydrate (350 mg/kg, intraperitoneal; Merck, Darmstadt, Germany). The right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were exposed *via* a midline incision in the neck. The ECA was then isolated and ligated. A 4-0 nylon suture was inserted into the ICA through the ECA stump and gently advanced approximately 18–22 mm to block the origin of the middle cerebral artery (MCA). At 2 hours after ischemia, the nylon suture was removed for reperfusion. After neurological status evaluation, rats were sacrificed and brain tissues were collected for further evaluation of inflammatory indices and cerebral infarct sizes.

Drug administration

Puerarin (36 mg/kg or 54 mg/kg) or nicotine (200 μ g/kg; Sigma-Aldrich, St. Louis, MO, USA) or physiological saline was injected through the caudal vein for 5 days (once daily) before MCAO. α -BGT (1.0 μ g/kg; Sigma-Aldrich) was intravenously injected after MCAO for 5 consecutive days (once daily). The dose of puerarin used in this study was based on our previous study [38] and estimated for the desired dose according to the pharmacokinetics and pharmacodynamics data provided by the manufacturer. The body surface area (BSA) normalization method was used to determine the animal dose, which was based on the extrapolation of the human equivalent dose (HED) [61]. All drugs were dissolved in physiological saline for use.

Evaluation of neurological status

All animal neurobehavioral tests were performed at 48 hours after cerebral I/R using an investigator blind to the experiment according to the standard of Longa's report [62]. Briefly, the neurological findings were scored on a 4-point scale: no neurological deficit (spontaneous activity) was scored as 0; a mild focal neurological deficit (failure to extend left forepaw fully) was scored as 1; a moderate focal neurological deficit (circling to the left) was scored as 2; and a severe focal deficit (circling without displacement, or spinning) was scored as 3; rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness. Higher scores indicate worse neurobehavioral dysfunction.

Determination of infarct volume

To determine the infarct volume, five rats per group were euthanized under deep anesthesia using 10% (v/v) chloral hydrate (300 mg/kg) 12 hours after cerebral I/R. The frozen brain sections were prepared and successively sliced into 2 mm-thick coronal sections. Brain slices were incubated in 2% solution of TTC (Sigma-

Aldrich) at 37°C for 10 minutes and then fixed by 4% (w/v) buffered paraformaldehyde solution. The normal area of brain was stained dark red while the infarct area remained white. Each brain slice was scanned by a color flatbed scanner. The infarct areas were determined with an image analysis program Beta 4.0.2 of Scion Image (Scion Corp., Frederick, Maryland, USA). Data were expressed as the percentage of infarct volume/ brain slice volume (%).

Measurement of cytokine levels by ELISA

Six rats in each group were deeply anesthetized by 10% (w/v) chloral hydrate (300 mg/kg) and decapitated at given times after cerebral I/R. The ischemic cerebral tissue (0.5 g) was quickly removed and grinded fully into brain tissue homogenates. Normal sodium (500 μ L) was added to samples and lysates were centrifuged at 13 400 \times g for 10 minutes. The supernatant was collected and stored at -20°C until analysis. All standards were prepared before starting the assay. The ELISA was performed according to the manufacturer's recommendations. Rat IL-1 β and IL-6 ELISA kits were purchased from Wuhan Boster Bioengineering Co., Ltd. (Wuhan, Hubei, China). The TNF- α ELISA kit was purchased from NeoBioscience Technology Co., Ltd., (Shenzhen, Guangdong Province, China).

The mean absorbance value A_{450nm} for each set of reference standards and samples was calculated. The standard density was X, the B/BO was Y, sitting to mark the paper in the logit-log up to draw a standard curve. According to the B/BO that need to be measured, the sample can from sit to mark the density value that the paper looks up the sample up.

Immunohistochemical staining

The antibody against polyclonal p-JAK2 (1:200 dilution) was purchased from Beijing Biosynthesis Biotechnology Co., Ltd., (Beijing, China). Antibodies against polyclonal p-STAT3 (1:100 dilution) (4°C overnight), streptavidin-biotin complex (SABC) the immunohistochemistry kit and diaminobenzidine (DAB) dye were purchased from Wuhan Boster Bioengineering Co., Ltd. (Wuhan, Hubei, China). The secondary antibody was horseradish peroxidase-labeled goat anti-rabbit IgG (KPL Company, Maryland, Washington, USA) and was incubated at 20–37°C for 20 minutes and washed with PBS three times for 2 minutes each. Rats were perfused transcardially with 200 mL of 0.9% (w/v) saline and 200 mL of 4% (w/v) paraformaldehyde (pH 7.4). Brains were removed quickly and postfixed in 4% (w/v) paraformaldehyde for 1 day and subsequently with 75% (v/v)

ethanol. These paraffin-embedded brain sections were cut into 4- μ m thick sections using a cryostat. These sections were deparaffinized in dimethylbenzene, and dehydrated successively in gradient ethanol. Subsequently, the sections were quenched by incubation with 3% (v/v) H₂O₂ for 15 minutes and then washed in PBS three times for 5 minutes each. Thereafter, the other procedures were performed strictly according to the manufacturer's specifications. Slides were counterstained with hematoxylin, dehydrated in gradient ethanol and mounted with neutral gum. Under a microscope (Olympus, Tokyo, Japan), cells with brown granules in the cytoplasm or nucleus were considered as positive cells. Ten fields of view per slice were randomly observed under a microscope. The total positive area and total ipsilateral hemisphere area were quantified using an image processing and analysis system (Leica, Cambridge, UK).

RT-PCR analysis

Five rats per group were used for RT-PCR analysis. Total RNA was extracted from 100 mg ischemic hippocampal tissue using Trizol reagent (Takara, Dalian, Liaoning, China) and was reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara). Subsequently, the PCR reaction was performed according to the manufacturer's instructions (Sangon Biotch Co., Ltd., Shanghai, China). The primers (Invitrogen, Carlsbad, CA, USA) used for the amplification are shown as follows: β -actin (207 bp): 5'-CAC CCG CGA GTA CAA CCT TC-3', 3'-CCC ATA CCC ACC ATC ACA CC-5', α 7nAChR (235 bp): 5'-ACA TTG ACG TTC GCT GGT TC-3', 3'-CTA CGG CGC ATG GGT TAC GT-5', NF- κ Bp65 (319 bp): 5'-CAC AGA TAC CAC TAA GAC GCA CC-3', 3'-GAC CGC ATT CAA GTC ATA GTC C-5', JAK2 (284 bp): 5'-GGT GCC CTA GGA TTT TCT GG-3', 3'-CGA CCA GCA CTG TAG CAC AC-5', STAT3 (284 bp): 5'-TAT CTT GGC CCT TTG GAA TG-3', 3'-GTG GGG ATA CCA GGA TGT TG-5'. The PCR condition for β -actin was as follows: denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 60.4°C for 45 seconds, 72°C for 10 seconds, and a final elongation step at 72°C for 5 minutes. The PCR condition for α 7nAChR was as follows: denaturation at 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 60.5°C for 30 seconds, 72°C for 30 seconds, and a final elongation step at 72°C for 5 minutes. The PCR condition for NF- κ Bp65, JAK2 and STAT3 was as follows: denaturation at 94°C for 5 minutes, followed by 36 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72 C for 10 seconds, and a final elongation step at 72°C for 5 minutes. The amplified products were electrophoresed on 1.5% (w/v) agarose

gel and analyzed using the SIM gel imaging analysis system (SIM, Charlotte, North Carolina, USA).

Determination of NF- κ Bp65 protein by western blotting

Five rats per group were used for western blotting analysis. The ischemic cerebral tissue (0.1 g) was cut into small pieces and mixed with lysate, and the suspension was homogenized and centrifuged at 12 000 \times g for 10 minutes to remove debris. The protein concentration was determined using the Bradford assay^[63]. Equal amounts of total protein were separated by 10% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes, and then blocked in 5% (w/v) non-fat milk in PBS containing 0/1% (v/v) triton X-100 (PBST) for 1 hour. The members were incubated overnight at 4°C with NF- κ Bp65 antibodies (KangChen Biotech, Shanghai, China) diluted at 1:1 000 in PBST. Members were washed twice in PBST (10 minutes each) and incubated with horseradish peroxidase-labeled secondary antibody at a dilution of 1:3 000 (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) at 4°C for 1 hour. Membranes were washed twice in PBST (10 minutes each) and then visualized with enhanced electrochemiluminescence reagent (KangChen Biotech, Shanghai, China) and exposed to X-ray film.

Statistical analysis

The experimental data are presented as mean \pm SEM. SPSS 11.7 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Intergroup comparison was made by one-way analysis of variance (ANOVA) followed by least significant difference *post-hoc* test. Differences were considered to be significant when $P < 0.05$.

Research background: Although puerarin has been confirmed to exhibit good anti-inflammatory and brain-protective effects, few studies have investigated the anti-inflammatory mechanism of puerarin and puerarin activation of the cholinergic anti-inflammatory pathway.

Research frontiers: This study reported that puerarin can inhibit NF- κ Bp65 and upregulate α 7nAChR, JAK2 and STAT3 expression from the prospective of molecular biology. In addition, nicotine and α 7nAChR antagonist α -BGT were used as controls, which fully indicated that puerarin exhibits anti-inflammatory effects by activating the cholinergic anti-inflammatory pathway.

Clinical significance: Results from this study provide evidence for the feasibility of puerarin use in the neuroprotective treatment of stroke.

Academic terminology: The cholinergic anti-inflammatory pathway includes a neural circuit that regulates the immune response to injury, invasion or tissue ischemia. This pathway regulates the activation of the efferent vagus nerve through interaction with peripheral α 7nAChR expressed on macrophages.

Peer review: The role of the cholinergic anti-inflammatory pathway in the prevention against cerebral ischemia is an increasing area of research. Researchers innovatively correlated the brain-protective effect of puerarin to the cholinergic anti-inflammatory pathway.

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