

# Puerarin accelerates neural regeneration after sciatic nerve injury

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## Abstract

Puerarin is a natural isoflavone isolated from plants of the genus *Pueraria* and functions as a protector against cerebral ischemia. We hypothesized that puerarin can be involved in the repair of peripheral nerve injuries. To test this hypothesis, doses of 10, 5, or 2.5 mg/kg per day puerarin (8-( $\beta$ -D-Glucopyranosyl-7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) were injected intraperitoneally into mouse models of sciatic nerve injury. Puerarin at the middle and high doses significantly up-regulated the expression of growth-associated protein 43 in the L<sub>4-6</sub> segments of the spinal cord from mice at 1, 2, and 4 weeks after modeling, and reduced the atrophy of the triceps surae on the affected side and promoted the regeneration of nerve fibers of the damaged spinal cord at 8 weeks after injury. We conclude that puerarin exerts an ongoing role to activate growth-associated protein 43 in the corresponding segment of the spinal cord after sciatic nerve injury, thus contributing to neural regeneration after sciatic nerve injuries.

**Key Words:** nerve regeneration; peripheral nerve injury; puerarin; growth-associated protein 43; sciatic nerve; repair; NSFC grant; neural regeneration

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## Introduction

Medication and surgery are the two most commonly used treatments for sciatic nerve damage, but neither is totally effective. In recent years, natural medicine and its extracts have been shown to stimulate nerve growth factor expression and Schwann cell proliferation after nerve injuries, contributing to neural regeneration and functional recovery (Qiu et al., 1999; Xue et al., 2007; Ye et al., 2007).

Puerarin, known as 8-( $\beta$ -D-Glucopyranosyl-7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, is extracted from the root of the kudzu plant, and has been widely used in traditional Chinese herbal medicine for the treatment of various diseases (including brain diseases). Dietary isoflavones have been shown to improve stroke outcome after focal cerebral ischemia/reperfusion injuries in such a way that a higher dietary isoflavone content results in a lower infarct volume and better neurological function (Burguete et al., 2006). Some scholars have found that puerarin can reduce focal cerebral ischemia/reperfusion injuries by scavenging oxygen free radicals, expanding cerebral blood vessels, and increasing cerebral perfusion volume (Han et al., 2007; Xu et al., 2005). The protective effects of puerarin against cerebral ischemia could act by: (1) an antioxidant and anti-free radical scavenger; (2) inhibition of p53 gene expression and anti-neuronal apoptosis; (3) inhibition of inflammatory response caused by focal cerebral ischemia and reperfusion; (4) improvement of cerebral microcirculation (Xu et al., 2005; He 2007; Saida et al., 1978;

Schwartz et al., 1982; Anselin et al., 1990; Pei et al., 1995; Yang, 1999). These findings suggest that puerarin is likely to reduce initial damage and facilitate nerve repair including neural regeneration.

In the present study, we developed a sciatic nerve injury model in BALB/c mice to observe nerve myelin recovery, muscular atrophy and expression of growth-associated protein 43 (GAP43) and its mRNA in the L<sub>4-6</sub> spinal segments of the spinal cord following administration with puerarin. This study was designed to explore the effects of puerarin on promoting neural regeneration following peripheral nerve injuries.

## Materials and Methods

### Animals

In total 160 BALB/c male mice, aged 4–6 weeks, weighing  $20 \pm 2$  g, were provided by the Experimental Animal Center of Jilin University, China, and housed at room temperature and allowed free access to food and water. All the mice were subjected to unilateral sciatic nerve complete transection and microscopic anastomosis. All experimental procedures were carried out in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals* (The Ministry of Science and Technology of the People's Republic of China, 2006).



**Figure 1** Corresponding spinal segments associated with the sciatic nerve during modeling.

A 1.5 cm longitudinal incision was made on the posterior femur of the unilateral lower limb. The sciatic nerve trunk at the lower edge of the piriformis muscle was bluntly separated. The lamina was bitten to expose the spinal segments associated with the sciatic nerve.

#### Establishment of a mouse model of sciatic nerve injury

Mice were anesthetized with an intraperitoneal injection of 1% sodium thiopental (100 mg/kg) and fixed in the prone position. After the surgical region was disinfected, a 1.5 cm longitudinal incision was made on the posterior femur of the unilateral lower limb. The sciatic nerve trunk at the lower edge of the piriformis muscle was bluntly stripped from the surrounding tissues (Figure 1). Then, the sciatic nerve was completely transected 0.5 cm below the ischial tuberosity and anastomosed, viewed under a 120-fold microscope. After that, the fascia, subcutaneous tissue and skin were sutured layer by layer.

#### Specimen collection and preparation

Ten mice from each group were taken at the corresponding time points and intraperitoneally anesthetized with 1% sodium thiopental (100 mg/kg). The spinal canal was nipped to expose and dissociate the L<sub>4-6</sub> spinal cord segment connected with the sciatic nerve. The L<sub>4-6</sub> segment on the injury side was harvested, and immersed in liquid nitrogen quickly after being labeled.

#### Western blot assay of GAP43 protein expression in the injured spinal cord segment

The tissue specimens were taken from liquid nitrogen, and immediately placed in a mortar and pestle to be boiled in loading buffer for 15 minutes followed by centrifugation. The supernatant was taken and treated with radioimmuno-precipitation assay for cell lysis to separate and extract the proteins. The protein samples were electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with rabbit anti-mouse GAP43 antibodies (dissolved at a ratio of 1:1,000 with PBS containing 1% bovine serum albumin; Sigma, St. Louis, MO, USA) at 4°C overnight, and rinsed with 0.01 mol/L PBS 5 minutes × 4. After additional incubation with goat anti-rabbit IgG (1:10,000; Sigma) at room temperature for 1 hour, the membrane was rinsed with 0.01 mol/L PBS 5 minutes × 4, developed by 3,3'-diaminobenzidine (Sigma) according to instructions, and exposed by X-ray films followed by band scanning and analysis. GAPDH served as an internal

reference, and the result was expressed as an absorbance value of GAP43/GAPDH.

#### Real time-PCR detection of GAP43 mRNA expression in the injured spinal cord segment

Primer sequence for GAP43 was designed by Beacon Designer 9 software, and GAPDH served as an internal reference. The specificity of the primers was determined using BLAST. The total RNA was extracted from the L<sub>4-6</sub> segments on the injury side for real time-PCR detection of GAP43 mRNA. ΔCt value was detected, and the experimental results were described as  $2^{-\Delta\Delta Ct}$ .

#### Primer sequences:

Gene	Sequence (5'-3')
GAP43	Sense: GCC TAA ACA AGC CGA TGT GC
GAP43	Anti-sense: TTC GTC TAC AGC GTC TTT CTC C
GAP43-Probe	TGC TGC TGT CAC TGA TGC TGC TGC
GAPDH	Sense: AAT GTG TCC GTC GTG GAT CTG
GAPDH	Anti-sense: CAA CCT GGT CCT CAG TGT AGC
GAPDH-Probe	CGT GCC GCC TGG AGA AAC CTG CC

#### Hematoxylin-eosin and Luxol fast blue staining

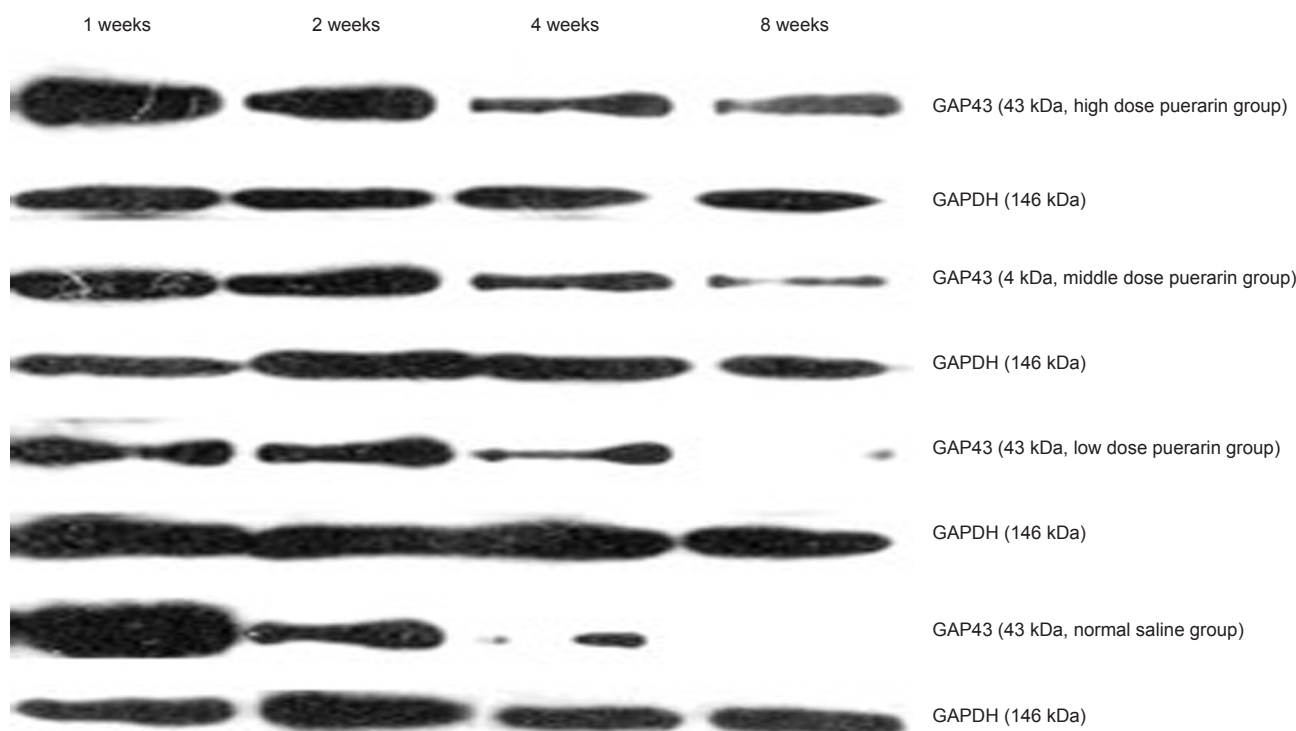
Ten mice from each group were selected at the corresponding time points. The neural stem was cut 0.5 cm distal to the sciatic nerve anastomosis (the anastomosis was also resected), and fixed in formalin for over 72 hours. Following dehydration in alcohol and paraffin embedding, the samples were cut into sections for hematoxylin-eosin staining. After initial screening, the damaged site was confirmed and stained with Luxol fast blue staining. Interactive image analysis system (IBAS2000; Kontron, Germany) was used to automatically correct image magnification, and the measured values were the actual values. Under high magnification, four square images per region were randomly selected to measure the diameter of sciatic nerve fibers and count the number of nerve fibers. Measurement by the operator abided by the following rules: (1) the sciatic nerve fibers adjacent to the contact boundaries were not counted; (2) the diameter of the nerve fiber was measured without considering the myelin sheath membrane, and the smallest diameter was selected as the nerve fiber diameter, depending on which, the diameter and area of myelinated nerve fibers of the spinal cord were determined.

#### Determination of muscular mass index in the soleus muscle

Eight weeks after the sciatic nerve transection, the triceps surae muscle was excised and weighed on an electronic analytical balance. The muscular mass index in the soleus muscle was calculated by the Cuadros method (Cuadros et al., 1987) using the following formula: muscle mass of the soleus muscle/body mass × 100.

#### Statistical analysis

Measurement data were expressed as mean ± SD, and statistically processed using SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). Multi-group comparison was performed using analysis of variance at each time point, and intergroup comparison was done using the least significant difference test. A value of  $P < 0.05$  was considered significant.



**Figure 2 Protein expression of growth-associated protein 43 (GAP43) in the L<sub>4-6</sub> segment of the injured side.**

After 1, 2, 4, 8 weeks of modeling, the protein expressions of GAP43 were upregulated in the high and middle dose groups as compared with the low dose and normal saline groups.

**Table 1 Changes in the protein expression of growth-associated protein 43 (GAP43) in the L<sub>4-6</sub> segment of the injured side after administration of puerarin**

Group	After modeling (week)			
	1	2	4	8
<b>Puerarin</b>				
High dose	1.172±0.022*	0.862±0.036*	0.423±0.029*	0.323±0.021
Middle dose	1.863±0.023*	1.009±0.024*	0.544±0.018*	0.254±0.026
Low dose	1.122±0.038	0.611±0.040	0.321±0.030	0.012±0.021
Normal saline	1.294±0.031	0.600±0.021	0.201±0.023	0.003±0.029

The experimental results were expressed as the absorbance value of GAP43/GAPDH. Data were expressed as mean ± SD ( $n = 10$ ), and analyzed by one-way analysis of variance and least significant difference test. \* $P < 0.05$ , vs. low dose group and normal saline group.

## Results

### Quantitative analysis of animals

After the initial surgery, 160 mice were equally and randomly divided into low dose, middle dose, high dose, and normal saline groups, and injected with 2.5, 5, 10 mg/kg per day puerarin and an equal volume of saline. Hereafter, 10 mice from each group were selected at 1, 2, 4, 8 weeks after injury, respectively. Finally, all 160 mice were included in result analysis with no dropout.

### Puerarin effects on mRNA and protein expressions of GAP43 in the L<sub>4-6</sub> segment of the injured side of mice

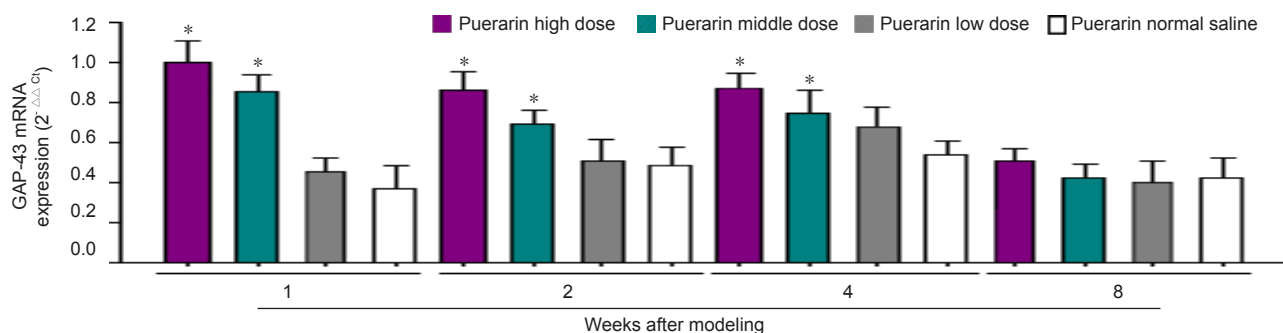
After 1, 2, and 4 weeks of modeling, mRNA and protein expressions of GAP43 were upregulated in the high and middle

dose groups as compared with the low dose and normal saline groups ( $P < 0.05$ ). After 8 weeks, there were no significant differences between the four groups (Figures 2, 3, Table 1).

### Puerarin effects on the morphology of the triceps surae and spinal cord tissues on the injured sciatic nerve side of mice

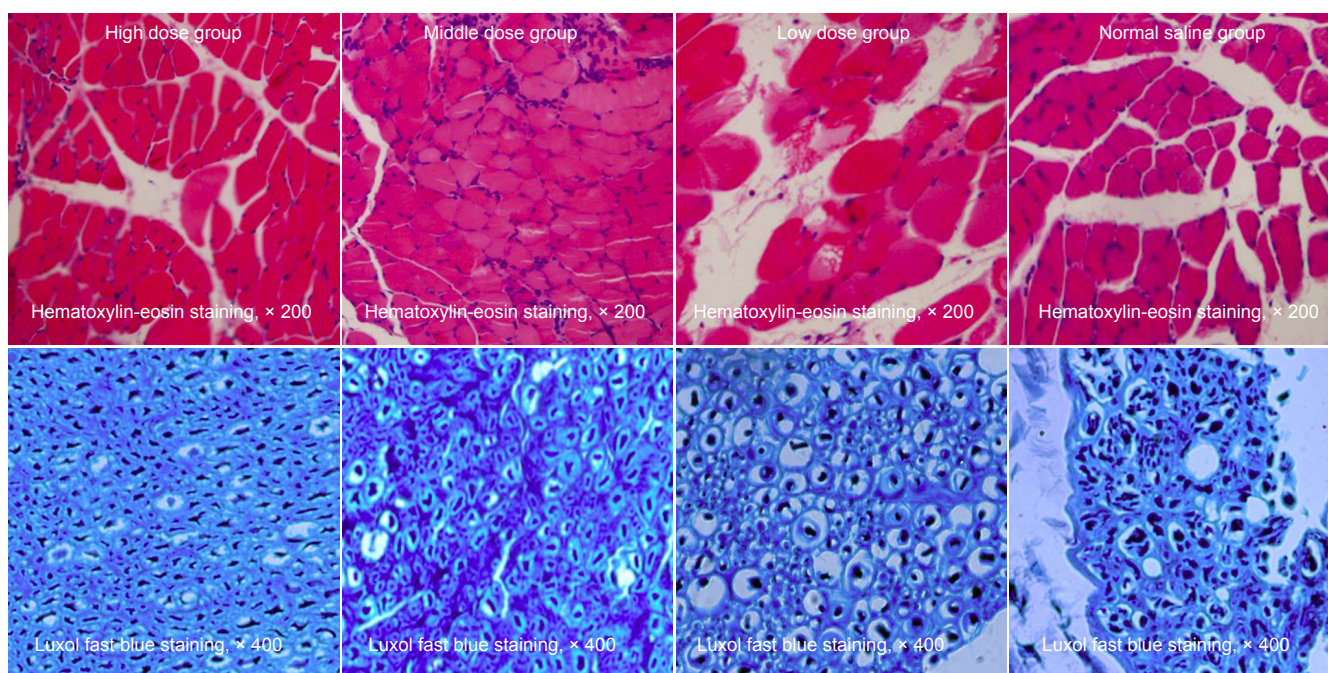
At 8 weeks after injury, the muscle cells in the high and middle dose groups were arranged regularly and tightly, and there was a large cross-sectional area of muscle fibers with small gaps between them. While in the low dose and normal saline groups, varying degrees of muscle atrophy were visible and the muscle fibers arranged loosely with smaller cross-sectional areas (Figure 4).

Under the Luxol fast blue staining, the myelin fibers appeared to be blue, and the background was gray in color. At 8 weeks after injury, the high and middle dose groups showed regular arrangement of the myelin sheaths with uniform thickness and clear outline, but there were no proliferated fibrous connective tissues. While in the low dose group, the shape and thickness of the myelin sheaths were both irregular but still with clear boundaries, and the fibrous connective tissues had proliferated significantly; the normal saline group exhibited disordered myelin sheaths and significantly proliferated fibrous connective tissues (Figure 4). Based on randomized fields of view, the number of myelinated nerve fibers and their average diameter were calculated and analyzed statistically. The number and average diameters of myelinated nerve fibers in the high and middle dose groups were significantly higher than in the low dose and normal saline groups. Moreover, the low dose group exhibited less damage than the normal saline group ( $P < 0.05$ ; Table 2).



**Figure 3** Real time-PCR detection of growth-associated protein 43 (GAP43) mRNA expression ( $2^{-\Delta\Delta C_t}$ ).

Data were expressed as mean  $\pm$  SD ( $n = 10$ ), and analyzed by one-way analysis of variance and least significant difference test. \* $P < 0.05$ , vs. low dose group and normal saline group.



**Figure 4** Puerarin effects on the morphology of the triceps surae and nerve fibers of the spinal cord on the injured side at 8 weeks after sciatic nerve surgery.

The hematoxylin-eosin staining showed that muscle cells in the high and middle dose puerarin groups arranged regularly and tightly with a larger cross-sectional area of muscle fibers and smaller gaps. Luxol fast blue staining showed that the myelin sheaths arranged regularly with uniform thickness and clear outline and no proliferated fibrous connective tissues were found in the high and middle dose groups. While in the low dose group, the shape and thickness of the myelin sheaths were both irregular but still with a clear boundary, and fibrous connective tissues had proliferated significantly; the normal saline group displayed disordered myelin sheaths and proliferated fibrous connective tissues.

#### Results of muscular mass index in the soleus muscle

At 8 weeks after injury, the muscular mass index in the soleus muscle was significantly higher in the high and middle dose groups ( $2.32 \pm 0.01$ ,  $2.09 \pm 0.02$ ) than the low dose and normal saline groups ( $1.82 \pm 0.03$ ,  $1.36 \pm 0.03$ ;  $P < 0.05$ ). Moreover, there was also a significant difference between the low dose and normal saline groups ( $P < 0.05$ ).

#### Discussion

GAP43 is a membrane-associated phosphorylated protein that can be rapidly attached to the membrane. It is closely related to neuronal growth, neurite formation and plasticity (Chen et al., 2012; Zhang et al., 2013). GAP43 can potentially control G proteins, which act as a signal transduction factor

and amplifier in membrane signal transduction systems, through its amino terminus domain (Fishman, 1996). Purified GAP43 is a guanine nucleotide release protein that can enhance guanosine diphosphate release, increase the activity of guanosine-5'-triphosphate and stimulate guanine nucleotide binding to guanosine-5'-triphosphate (McIlvain et al., 2003; Mendonça et al., 2010; Zhang et al., 2012; Chen et al., 2013). In this study, we found that after intervention with high- and middle-dose puerarin, the expressions of GAP43 protein and mRNA in the L<sub>4-6</sub> segments of the injured side were dramatically increased. Our findings suggest that puerarin can trigger the overexpression of GAP43, thus promoting neural regeneration.

In the present study, Luxol fast blue staining further illus-

**Table 2** Puerarin effects on numbers and average diameters of myelinated nerve fibers at 8 weeks after sciatic nerve injury

Index	High dose group	Middle dose group	Low dose group	Normal saline group
Number of myelinated nerve fibers ( $n/mm^2$ )	1,213±286 <sup>*#</sup>	1,213±212 <sup>*#</sup>	1,028±225 <sup>#</sup>	964±199
Average diameter of myelinated nerve fibers ( $\mu m$ )	2.23±0.27 <sup>*#</sup>	2.29±0.12 <sup>*#</sup>	2.10±0.20 <sup>#</sup>	1.32±0.36

Fields of views were randomly selected using the interactive image analysis system (IBAS2000; Kontron, Germany) to calculate the number and diameter of myelinated nerve fibers. Data were expressed as mean  $\pm$  SD ( $n = 10$ ), and analyzed by one-way analysis of variance and least significant difference test. \* $P < 0.05$ , vs. low dose group; # $P < 0.05$ , vs. normal saline group.

trated that the number, integrity and thickness of the myelin sheaths were increased in the process of neural regeneration following sciatic nerve injury, suggesting that puerarin strongly promotes the growth of damaged nerve myelin. After nerve injury, local muscle atrophy certainly occurs, which can be eased following neural regeneration and functional recovery. Our findings from hematoxylin-eosin staining and muscular mass index in the soleus muscle indicate that puerarin promoted sciatic nerve regeneration better than untreated controls.

Interestingly, after intervention with puerarin for 8 weeks, GAP43 levels showed no differences at any dose, whereas the myelinated nerve and recovery of muscle atrophy were significantly different in the high, middle and low dose groups from the normal saline group. These results indicate that the nerve repair and regeneration were completed at 8 weeks in the high and middle dose groups, followed by decreased GAP43 that was close to the level of the low dose and normal saline groups. Meanwhile, nerves and muscles recovered well.

Taken together, puerarin has an ongoing role in the up-regulation of GAP43 in the corresponding segment after sciatic nerve injury, and thus promotes sciatic nerve repair. During the repair, puerarin could also have antioxidant, anti-apoptotic and anti-inflammatory roles.

**Author contributions:** All the authors participated in study design and concept, data collection, integration analysis, manuscript preparation and approved the final version of the paper.

**Conflicts of interest:** None declared.

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