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Quercetin promotes motor and sensory function recovery following sciatic nerve-crush injury in C57BL/6J mice

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Abstract

Injuries and diseases that occur in the nervous system are common and have few effective treatments. Previous studies have shown that quercetin has a therapeutic effect on nervous system injuries, but its potential effects on and mechanisms of action related to behavioral recovery and axonal regrowth have not been investigated. Here, we showed that quercetin administration promotes behavioral recovery following sciatic nerve-crush injury in mice. Long-term evaluation showed that mice administered 20 $mg \cdot kg^{-1} \cdot day^{-1}$ quercetin for 35 days had a greater sensorimotor recovery compared with all other treatment groups. The mechanisms behind these effects were further investigated, and quercetin was found to regulate the expression of genes involved in regeneration and trophic support. Moreover, quercetin increased cyclic adenosine monophosphate expression and downstream pathway activation, which directly leads to neuronal growth activation in peripheral axon regeneration. In addition, quercetin enhanced axon remyelination, motor nerve conduction velocity and plantar muscle function, indicating that the degree of distal portion hypotrophy during the peripheral axon regeneration process was reduced. These results suggest that quercetin accelerates functional recovery by up-regulating neuronal intrinsic growth capacity and postponing distal atrophy. Overall, quercetin triggered multiple effects to promote behavioral recovery following sciatic nerve-crush injury in mice.
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Keywords: Quercetin; Sciatic nerve injury; Axonal regeneration; Intrinsic growth capacity; Cyclic adenosine monophosphate

1. Introduction

Damage to the nerves of the peripheral nervous system, or neuropathy, is a common, disabling and complex disorder. The prevalence of neuropathy is greater than 2% in the general population [1], accounting for as many as 3% of all trauma patients [2] and for 15% of people over the age of 40 years [3]. Despite decades of significant advances in human nerve repair, patients with peripheral nerve injuries often face poor clinical outcomes, even after surgical nerve repair. There are three surgical strategies: direct repair, nerve transfer and nerve grafting [4], but each method has its own drawbacks. For direct repair, the difficulties include reproducing the alignment of nerve fascicles, inflammation and a neuropathy-inducing tension [5]. For nerve transfer, the biggest challenges are the limited number and lengths of available donor nerves, the additional surgery associated with donor-site morbidity and the few effective nerve graft alternatives [6]. For nerve grafting, the disadvantage of this technique is that it completely depends on the formation of a fibrin clot, and when this clot does not form, no nerve regeneration occurs [7]. Despite the momentous developments in surgical techniques and extensive research, there are no current therapies aimed at the molecular mechanisms of nerve regeneration. In humans, nerves often have to regrow over long distances, and the distal portion of nerve progressively loses its ability to support regeneration during this

Abbreviations: ANOVA, analysis of variance; Artn, artemin; ATF3, activating transcription factor 3; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; Cdh1, cadherin 1; CNS, central nervous system; CREB, cAMP-response element binding protein; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; FBA, foot–base angle; Gap-43, growth-associated protein-43; GDNF, glial cell-derived neurotrophic factor; HRP, horseradish peroxidase; HTA, heel–tail angle; IF, immunofluorescence; LSD, least significant difference; MAG, myelin-associated glycoprotein; Mbp, myelin basic protein; MNCV, motor nerve conduction velocity; mNGF, mice-derived nerve growth factor; Mog, myelin oligodendrocyte glycoprotein; Mpz, myelin protein P0; NC, nitrocellulose filter; NF-200, neurofilament-200; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor-kappa B; NMJs, neuromuscular junctions; PBS, phosphate buffer saline; PF, paraformaldehyde; PNS, peripheral nervous system; RNA, ribonucleic acid; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SDS, sodium dodecyl sulfate; S.E.M., standard error of mean; SFI, sciatic functional index; Shh, sonic hedgehog; Sox11, sex-determining region Y-box 11; STAT3, signal transducer and activator of transcription; TEM, transmission electron microscopy; Trk, tyrosine kinase.

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process. To develop successful molecular therapies for nerve regeneration, we need to address the issues, which include improving the intrinsic regenerative ability of neurons to increase the speed of axonal outgrowth, preventing the loss of basal lamina and dealing with chronic denervation changes in denervated Schwann cells [8].

Quercetin, the most extensively studied flavonoid, has shown beneficial effects on various *in vivo* models of neural disorders, such as brain trauma [9,10], spinal cord injury [11,12], cerebral ischemia [13] and neurodegeneration [13–15]. Studies *in vitro* also revealed that quercetin increases neuron survival [16,17] and protects neurons by reducing toxicity and neuroinflammation [18]. In our previous study, we showed that quercetin promotes neurite growth through the elevation of intracellular cyclic adenosine monophosphate (cAMP) expression [19]. The well-known effects of quercetin on peripheral systems, including cardiovascular and inflammatory systems, may partly explain its beneficial effects on these *in vivo* neuronal disorder models [20]. However, research focusing specifically on intrinsic regeneration and distal hypotrophy is limited. In this paper, we report that quercetin could improve animal sensory and motor function recovery following peripheral nerve injury by increasing the intrinsic regeneration ability of neurons and preventing denervation at the distal stump.

2. Materials and methods

2.1. Animals and medicine

Adult male C57BL/6J mice (180 in total, from Aier Matt experimental animal Co. Ltd., Suzhou, JS, China) weighing 20–22 g were used in this research. All animals were maintained in a temperature- and humidity-controlled environment, with a 12:12-h light/dark cycle, and were allowed standard mouse chow and water *ad libitum*. The purity of quercetin was higher than 98% and was diluted in dimethyl sulfoxide to 0.4 g/ml and used as a stock preparation.

2.2. Surgery

All experimental procedures were approved by the Administration Committee of Experimental Animals, Jiangsu Province and China Pharmaceutical University. All surgical experiments were performed under isoflurane (1%) on adult male mice (8–12 weeks old). For left hind limb sciatic nerve-crush injury, the sciatic nerve was crushed with smooth forceps for 30 s, and the site of crush was marked with a 10-0 suture line (Ethilon) [21]. After injury, the wound was sutured in layers, and the mice were allowed to recover on heated pads. In the sham-operated controls, the sciatic nerve was exposed but not injured. The researcher who performed the crush injury surgery was blind to the group process.

2.3. Experimental design

The animals were separated into six groups in this study (n=10/group): (1) sham-operated control group, (2) vehicle group, (3) low-dose (0.2 $mg \cdot kg^{-1} \cdot day^{-1}$) quercetin group, (4) medium-dose (2 $mg \cdot kg^{-1} \cdot day^{-1}$) quercetin group, (5) high-dose (20 $mg \cdot kg^{-1} \cdot day^{-1}$) quercetin group, and (6) mice-derived nerve growth factor [mNGF; dosage and administration mode of mNGF selected in this study are based on the short half-life time of NGF in the local (quickly deactivated) and previous preclinical [22] and clinical trials [23], and we chose a dosage of 4.86 $\mu g \cdot kg^{-1} \cdot day^{-1}$ for this study; SINOBIOWAY Medicine, Xiamen, FJ, China] group. Based on previous pharmacodynamic experiments [24], animals were injected in the plantar muscle on the left hindlimb once daily with a volume of 0.1 ml. The sham group and vehicle groups were administered saline after surgery. The other groups were given quercetin or mNGF after surgery. Administration began on the first day after surgery and continued for 7, 14 or 35 days.

2.4. Behavioral test

2.4.1. Toe spreading test

To assess the motor recovery after nerve injury (sciatic nerve crush), movements of the toes were evaluated. Mice were gently covered with a piece of cloth and lifted by the tail. Under this condition, the digits spread, maximizing the space between them. The reappearance of this reflex was scored as previously described13: 0, no spreading; 1, intermediate spreading with all toes; and 2, full spreading. Mice were evaluated twice in each experimental session with at least a 45-min interval.

2.4.2. Pinprick assay

Mice were placed on wire mesh cages, habituated for three sessions the week before surgery, and tested on postoperative days accordingly. After a 30-min habituation period, an Austerlitz insect pin (size 000) (FST) was gently applied to the plantar surface of the paw. The most lateral part of the plantar surface of the hind paw was divided into five areas. The pinprick assay was applied from the most lateral toe (area E) to the heel (area A). A response was considered positive when the animal briskly removed its paw, and the mouse was graded 1 for this area, and then tested for the next one.13 Scoring was done by who blinded to the group design.

2.4.3. Reverse transcription quantitative polymerase chain reaction

Dissociated L4 and L5 spinal cords were prepared from experimental adult male mice at 7, 14 and 35 days after surgery. For tissue preparation, mice were anesthetized by chloral hydrate (7%) before an incision was made on the back to expose the spinal cord. L4 and L5 of the spinal cord were removed and then minced with eye scissors on ice. Total tissue mRNA was isolated using TRIzol Reagent (Invitrogen, Grand Island, NY, USA). RNA was then reverse-transcribed using the PrimeScriptRT reagent Kit (Takara, Tokyo, Japan). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed with the corresponding primers (Table 1) and SYBR Premix Ex TaqII (Takara) using a StepOnePlus System (Applied Biosystems, Grand Island, NY, USA). Gene expression was normalized to 18s RNA, and data were analyzed using StepOne Software.

2.4.4. LANCE cAMP assay

As previously discussed, the L4 and L5 spinal cords were removed and minced with eye scissors on ice. The samples were then lysed, and the pellet was incubated with lysis buffer on ice for 10 min before spinning at 14,000 rpm (16,000g) for 10 min at 4 °C. Finally, the supernatant was collected, and the cAMP concentration was assessed using a LANCE cAMP 384 Kit according to the manufacturer's instructions (PerkinElmer, American Fork, UT, USA).

2.4.5. Western blotting assay

Protein from the dislocated L4 and L5 spinal cords was extracted using RIPA buffer (Beyotime, Nanjing, JS, China) supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). After determining the protein concentration, we loaded equal amounts of protein into each lane; these were resolved on 10% sodium dodecyl sulfate polyacrylamide gradient gels and transferred onto a 0.45-μm nitrocellulose filter membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% bovine serum albumin and incubated with anti-Gap-43 (polyclonal antibody, 1:1000; Millipore), anti-p-STAT3 (monoclonal antibody, 1:1000; CST, Danvers, MA, USA), anti-STAT3 (monoclonal antibody, 1:1000; CST), anti-p-CREB (monoclonal antibody, 1:1000; CST), anti-CREB (monoclonal antibody, 1:1000; CST), anti-p-cjun (monoclonal antibody, 1:1000; CST), anti-cjun (monoclonal antibody, 1:1000; CST), anti-p-Trk (monoclonal antibody, 1:1000; CST), anti-Trk (monoclonal antibody, 1:1000; CST), anti-Mbp (monoclonal antibody, 1:1000; CST), anti-Mog (monoclonal antibody, 1:1000; CST), anti-MAG (monoclonal antibody, 1:1000; CST) and anti-β-actin (monoclonal antibody, 1:500; Santa Cruz, Dallas, TX, USA) overnight at 4 °C, washed, and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies. Immunoreactive bands were visualized by the Immobilon Western Chemiluminescent HRP Substrate (Millipore). Quantification of band intensity was performed by Quantity One software (Bio-Rad, Hercules, CA, USA). Experiments were repeated in triplicate.

2.4.6. Immunofluorescence

Sciatic nerves cut at day 14 post-surgery were analyzed. Nerves were removed as previously described and fixed in 4% fixed in paraformaldehyde/phosphate buffer saline for 24 h. Sections for immunostaining (20 μm) were cut with a cryostat (Leica, Wetzlar, Germany). After blocking with goat serum, primary antibodies against neurofilament-

Table 1
The primers designed for RT-qPCR

Gene	Forward PCR primer	Reverse PCR primer	Product length
p53	GCGTAAACGCTTCGAGATGTT	TTTTTATGGCGGGAAGTAGACTG	144
STAT3	CAATACCATTGACCTGCCGAT	GAGCGACTCAAACTGCCCT	109
NFAT	GACCCGGAGTTCGACTTCG	TGACACTAGGGGACACATAACTG	97
ATF3	GAGGATTTTGCTAACCTGACACC	TTGACGGTAACTGACTCCAGC	110
SOX11	TCATGGTGTGGTCCAAGATCG	GGTCCGTCTTGGGCTTTTTGC	216
NFκB	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTG	111
Jun	CCTTCTACGACGATGCCCTC	GGTTCAAGGTCATGCTCTGTTT	102
BDNF	TCATACTTCGGTTGCATGAAGG	AGACCTCTCGAACCTGCCC	137
GDNF	TATGGGATGTCGTGGCTGTC	CAGGCATATTGGCGGCGG	78
Artn	AGCTGTTTTGCAAGCTGCCG	GGCAGTGGGACAATGCAGTA	127
Shh	AAAGCTGACCCCTTTAGCCTA	TTCGGAGTTTCTTGTGATCTTCC	103
Gap43	TGGTGTCAAGCCGGAAGATAA	GCTGGTGCATCACCCTTCT	114
Mbp	AATCGGCTCACAAGGGATTCA	TCCTCCCAGCTTAAAGATTTTGG	74
Mpz	CGGACAGGGAAATCTATGGTGC	TGGTAGCGCCAGGTAAAAGAG	106
Cdh1	CAGGTCTCCTCATGGCTTTGC	CTTCCGAAAAGAAGGCTGTCC	175
CREB	AGCAGCTCATGCAACATCATC	AGTCCTTACAGGAAGACTGAACT	152

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