

# The mechanism of Naringin-enhanced remyelination after spinal cord injury

Wei Rong<sup>1</sup>, Yong-wei Pan<sup>1</sup>, Xu Cai<sup>1</sup>, Fei Song<sup>1</sup>, Zhe Zhao<sup>1</sup>, Song-hua Xiao<sup>1,\*</sup>, Cheng Zhang<sup>2,\*</sup>

1 Department of Orthopedics, Beijing Tsinghua Changgung Hospital, Medical Center, Tsinghua University, Beijing Key Laboratory of Bioelectromagnetism, Beijing, China

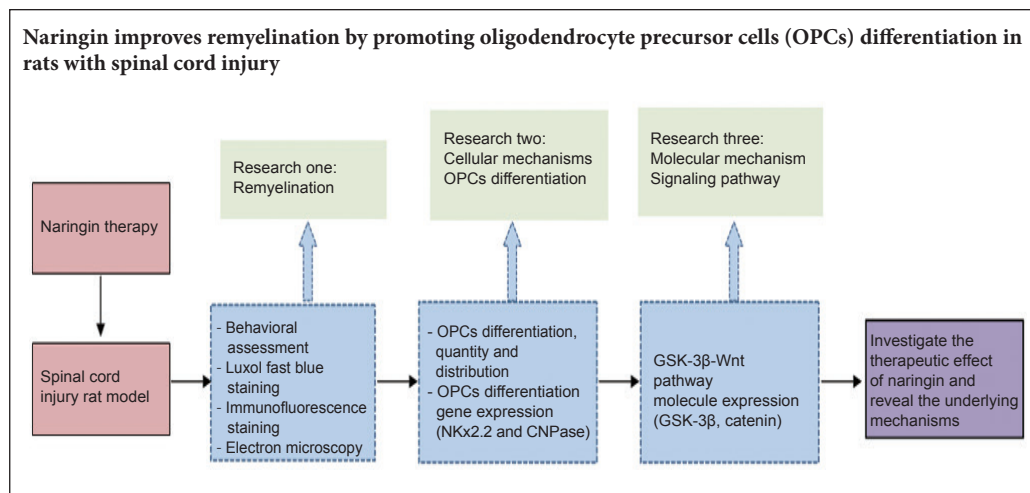
2 Beijing Key Laboratory of Bioelectromagnetism, Institute of Electrical Engineering, Chinese Academy of Sciences, Beijing, China

**How to cite this article:** Rong W, Pan YW, Cai X, Song F, Zhao Z, Xiao SH, Zhang C (2017) The mechanism of Naringin-enhanced remyelination after spinal cord injury. *Neural Regen Res* 12(3):470-477.

**Open access statement:** This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

**Funding:** This study was supported by the Natural Science Foundation of Beijing of China, No. 7164317; the Beijing Tsinghua Changgung Hospital Fund, No. 12015C1028; the National Natural Science Foundation of China, No. 31400717.

## Graphical Abstract



\*Correspondence to:

Song-hua Xiao, M.D. or  
Cheng Zhang, Ph.D.,  
xiaosh301@aliyun.com or  
zhangchengcc@mail.iee.ac.cn.

orcid:

0000-0002-7975-5259

(Song-hua Xiao)

0000-0002-9843-7977

(Cheng Zhang)

doi: 10.4103/1673-5374.202923

Accepted: 2017-01-20

## Abstract

Our previous study revealed that intragastric administration of naringin improved remyelination in rats with spinal cord injury and promoted the recovery of neurological function of the injured spinal cord. This study sought to reveal the mechanisms by which naringin improves oligodendrocyte precursor cell differentiation and maturation, and promotes remyelination. Spinal cord injury was induced in rats by the weight-drop method. Naringin was intragastrically administered daily (20, 40 mg/kg) for 4 weeks after spinal cord injury induction. Behavioral assessment, histopathological staining, immunofluorescence spectroscopy, ultrastructural analysis and biochemical assays were employed. Naringin treatment remarkably mitigated demyelination in the white matter, increased the quality of myelinated nerve fibers and myelin sheath thickness, promoted oligodendrocyte precursor cell differentiation by upregulating the expression of NKx2.2 and 2'3'-cyclic nucleotide 3'-phosphodiesterase, and inhibited  $\beta$ -catenin expression and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) phosphorylation. These findings indicate that naringin treatment regulates oligodendrocyte precursor cell differentiation and promotes remyelination after spinal cord injury through the  $\beta$ -catenin/GSK-3 $\beta$  signaling pathway.

**Key Words:** nerve regeneration; spinal cord injury; naringin; remyelination; oligodendrocyte precursor cells; oligodendrocytes;  $\beta$ -catenin; glycogen synthase kinase-3 $\beta$ ; NKx2.2; 2',3'-cyclic nucleotide 3'-phosphodiesterase; behavioral assessment; neural regeneration

## Introduction

Secondary injury processes are activated after spinal cord injury (SCI), including hemorrhage, ischemia, neural apoptosis and activation of various proteases. The myelin sheath is vital to neuronal function, providing trophic support to axons and enabling the rapid conduction of nerve impulses (Crawford et al., 2013). However, because of the high susceptibility of oligodendroglia to ischemic insult, the myelin sheath is more vulnerable to secondary injury than nerve fi-

bers. Prolonged and dispersed oligodendroglia death occurs after SCI, which results in widespread demyelination (Wu et al., 2012).

Adult oligodendrocytes (OLs) are generated from oligodendrocyte precursor cells (OPCs), which are widely distributed throughout the adult central nervous system (Levine et al., 2001). In response to various insults, local OPCs proliferate, migrate to the injury site, differentiate into mature OLs, and finally replace the injured myelin sheath; this process is

known as remyelination (Franklin and Ffrench-Constant, 2008a). It has been reported that remyelination is a beneficial process for promoting spinal cord reconstruction after SCI (Franklin and Ffrench-Constant, 2008b; Plemel et al., 2014; Hesp et al., 2015). At present, remyelination can be improved through two approaches: promotion of endogenous reconstruction and cell transplantation. Cell transplantation therapies are difficult to develop because of logistical and ethical concerns, and high costs are required for human experimental trials (Varma et al., 2013). Therefore, the development of novel therapeutic approaches that target endogenous OPC differentiation and improvement of remyelination is highly warranted to preserve and restore spinal cord function after injury.

Naringin (4',5,7-trihydroxy-flavonone-7-rhamnoglucoside) is a flavanone glycoside derived from grapefruit, tomato and other citrus fruits (Ho et al., 2000). Naringin can readily pass through the blood-brain barrier (Zbarsky et al., 2005). Naringin can affect many biological properties, such as anti-inflammatory, antioxidant and anti-apoptotic activities (Bailey, 2010), and potential therapeutic effects of naringin for central nervous system disorders have been described (Leem et al., 2014). We previously showed that naringin may be a promising therapy for SCI and that a neuroprotective effect may be associated with the inhibition of neural apoptosis (Rong et al., 2012). In the present study, we further explore the effects of naringin on differentiation and remyelination characteristics of OPCs, and investigate the underlying molecular mechanisms of these effects in an SCI rat model. This will provide new understanding of the mechanism by which naringin enhances spinal cord regeneration after injury.

## Materials and Methods

### Animals

Sixty adult female Sprague-Dawley rats weighing 200–220 g were purchased from Beijing HFK Bio-Technology, China [license number: SCXK (Jing) 2014-0004]. Experiments were performed in accordance with the National Guidelines for Experimental Animal Welfare (Ministry of Science and Technology of the People's Republic of China, 2006) and approved by the Animal Welfare Committee of Beijing Key Laboratory of Bioelectromagnetism.

Rats were randomly classified into four groups ( $n = 15$ ): sham group (received laminectomy only), vehicle group (SCI + vehicle), naringin 20 mg/kg group and naringin 40 mg/kg group (SCI + naringin 20, 40 mg/kg).

### Modeling and treatment with naringin

All rats were anesthetized with sodium pentobarbital (50 mg/kg; Abbott, North Chicago, IL, USA). Sham group rats only received a dorsal laminectomy at T<sub>10</sub>. Rats in the vehicle group, naringin 20 mg/kg group and naringin 40 mg/kg group were subjected to moderate SCI, which was induced by a modified weight-drop method as described by Perot et al. (1987). Following the procedure, rats were disabled

by hindlimb paralysis. Rats contused in a nonsymmetrical manner were excluded from the experiment. After surgery, the incisions were closed in layers. Bladders were manually expressed two or three times per day until the voiding reflex was re-established.

Naringin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.5% sodium carboxymethyl cellulose solution. Naringin-treated rats were given naringin intragastrically (5 mL/kg of body weight) daily for 4 weeks after the injury. Meanwhile, rats in the vehicle and sham groups ( $n = 15$ ) received the same volumes of 0.5% carboxymethyl cellulose solution at similar times.

### Behavioral assessment

Neurological assessments were performed before the operation, and on 1 day, and 3 days post-SCI, and then weekly until the time of sacrifice (4 weeks post-SCI) ( $n = 15$ ). The assessments were conducted by two independent experimenters who were not aware of the experimental conditions of the study. The open-field test assessed the rats' locomotion, weight support and coordination, and the results were evaluated using the Basso, Beattie, and Bresnahan (BBB) scale, as previously described (BASSO et al., 1995). A score of 0 represented no spontaneous movement, whereas a score of 21 represented complete mobility. For motor function, four groups of rats were evaluated by the modified Tarlov motor grading scale (Behrmann et al., 1992). Grade 5 meant the rats were able to walk normally and grade 1 meant the rats had no voluntary hind limb movement.

Four weeks post-injury, rats in all groups were sacrificed through administration of an overdose of sodium pentobarbital. The rats in each group were then randomly divided into three subgroups for the following experiments: Histological staining analysis ( $n = 5$ ); electron microscopy and morphometric analyses ( $n = 3$ ); and western blot assay ( $n = 7$ ).

### Histological staining

#### Tissue preparation

Four weeks post-SCI, rats ( $n = 5$  per group) were sacrificed, then transcardially perfused with PBS, followed by 4% paraformaldehyde. A 1 cm length of spinal cord around the lesion center was removed, placed in 4% paraformaldehyde for 12 hours, soaked in 15% and 30% sucrose until dehydrated, and then coated in OCT (Sakura Finetek USA, Torrance, CA, USA).

#### Luxol fast blue staining

To analyze myelin sheath loss, serial transverse sections (20  $\mu$ m) of spinal cord were stained with myelin sheath staining dye (Luxol fast blue) as previously described (Yune et al., 2007).

#### Immunofluorescence assessment

For myelinated nerve fiber staining, 5  $\mu$ m spinal cord sections

from the injury site were treated for immunofluorescence assessment using anti-neurofilament heavy (NF-H; high molecular weight neurofilament peptide), and anti-myelin basic protein (MBP) primary antibodies. Sections were incubated with anti-NF-H (1:800; Sigma-Aldrich, St Louis, MO, USA) at 4°C overnight and then with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (1:100; ZSGB-Bio, Beijing, China) at 37°C for 2 hours or with anti-MBP (1:100; Sigma-Aldrich) at 4°C overnight, followed by Texas Red-conjugated goat anti-mouse antibody (1:100; ZSGB-Bio) at 37°C for 2 hours.

#### Data quantification

For microscopic evaluations, a fluorescence microscope (Nikon E600, Tokyo, Japan) and a Leica microscope (Leica DM 4000B, Stuttgart, Germany) with a mounted camera were used. The spared areas and the numbers of myelinated nerve fibers were determined automatically using Image-Pro Plus software (version 5.0, Cybernetics, MD, USA). Manual quantifications were also performed by two independent examiners to verify the results provided by the automated analysis.

Spared myelin sheath areas were normalized against the total cross-sectional area, and the spared areas were calculated every 500  $\mu\text{m}$  within the central region of the injury.

The numbers of myelinated nerve fibers in the dorsal corticospinal tract were analyzed in three randomized fields (450  $\mu\text{m}$   $\times$  450  $\mu\text{m}$ ) of 10 sections per group (30 optical fields per group) and were exhibited as the mean quantity of nerve fibers per field in each group.

#### Electron microscopy and morphometric analyses

At 4 weeks after injury, spinal cords ( $n = 3$  per group) from T<sub>10</sub> were post-fixed in 2.5% glutaraldehyde. Ultrathin 70 nm thick sections were mounted on copper grids. Images of dorsal corticospinal tract were obtained by transmission electron microscopy (Hitachi-7650, Tokyo, Japan) at magnifications of 50,000 and 100,000. Approximately 100 myelinated fibers were observed. The diameters of axons were measured along the inner border of the myelin sheath, and the diameters of nerve fibers were measured along the outer border of the myelin. The myelin sheath thickness was equal to the diameter of the nerve fiber minus the diameter of the axon divided by two.

#### Western blot assay

Spinal cords encompassing the injury sites were removed, and dorsal white matter strips were isolated according to a previous method (Ouyang et al., 2010). The bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) was used to determine protein concentration and 20  $\mu\text{g}$  protein was loaded onto 12% polyacrylamide gels. The expression of NG2 and Olig2 (oligodendroglial lineage markers), NKx2.2 (a transcription factor associated with OPC differentiation), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (a myelin biosynthesis marker), Ser9-phosphorylated glycogen

synthase kinase-3 $\beta$  (p-GSK-3 $\beta$ ) and  $\beta$ -catenin (essential integrating molecules for differentiation signals) were detected. The antibodies used were: polyclonal rabbit anti-NG2 antiserum (1:1,000; Millipore, Bedford, MA, USA), monoclonal mouse anti-Olig2 (1:1,000; Abcam, Cambridge, MA, USA), monoclonal mouse anti-NKx2.2 (1:1,000; Abcam), monoclonal mouse anti-CNPase (1:500; Sigma-Aldrich), polyclonal goat anti-Ser9-pGSK-3 $\beta$  (1:200; Sigma-Aldrich), monoclonal mouse anti- $\beta$ -catenin (1:500; Abcam) and  $\beta$ -actin (1:10,000; Sigma-Aldrich). All the primary antibody incubations were at 4°C overnight. Secondary antibody incubations with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:200; ZSGB-Bio, Beijing, China) or horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:200; ZSGB-Bio) were performed at 37°C for 2 hours. The Odyssey<sup>®</sup> Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) was used for integrated optical density analysis.

#### Statistical analysis

All data are expressed as the mean  $\pm$  SD. Data analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Repeated-measures analysis of variance (Bonferroni's *post hoc* test) was used to compare the spared myelin sheath among groups at different time points. One-way analysis of variance (Tukey's *post hoc* test) was used to compare the spared myelin sheath among groups at different sites. Differences were considered statistically significant at  $P < 0.05$ .

## Results

#### Effect of naringin on locomotor activity

The rates of mortality, post-operative infection and hematuria in SCI rats were not significantly different among groups. All rats initially had a BBB score of 21, and the injury resulted in a BBB score of 0, which reflected total hindlimb paralysis. At 1 and 3 days post-injury, the injured rats had severe movement deficits. Compared to the vehicle group, rats in both naringin-treated groups showed significant locomotor activity that continued throughout the experimental period ( $P < 0.05$ ; **Figure 1A**).

All the rats were graded according to the modified Tarlov motor grading scale. The mean motor grading scores after injury are shown in **Figure 1B**. Before injury, the mean motor grading score was approximately 5.0 in all groups. After injury, the injury ranking decreased and then gradually increased. Four weeks post-injury, rats in both naringin-treated groups (20 and 40 mg/kg) displayed significantly better scores than the rats in the vehicle group ( $P < 0.05$ ).

#### Effect of naringin on spared myelin sheath

Within the stained sections, the blue stained white matter and butterfly shaped grey matter could be identified in rats from the sham group. Meanwhile, myelin sheath degeneration and cavities could be observed in rats of the vehicle, naringin 20 mg/kg, and naringin 40 mg/kg groups (**Figure 2A**). As shown in **Figure 2B**, the areas of myelin sheath in

both naringin groups were significantly larger than those in the vehicle group ( $P < 0.05$ ). These results indicate that naringin treatment could significantly reduce myelin sheath loss after injury.

#### Effect of naringin on the quantity of myelination

Fluorescent double-immunolabeling showed that axons in each group were surrounded by irregular myelin sheaths of different thickness (Figure 3A). More preserved myelinated axons in the dorsal corticospinal tract were present in naringin-treated rats compared with vehicle-treated rats ( $P < 0.05$ , vs. naringin 20 mg/kg;  $P < 0.01$ , vs. naringin 40 mg/kg) (Figure 3B).

Based on ultrastructure analysis, myelin sheaths in dorsal corticospinal tract of the sham group were healthy and contained intact membranes with dense layers (Figure 3C). The axons of rats in the vehicle group showed multiple pathological changes in the structure of myelin sheaths and axons, such as aberrantly swollen sheaths, myelin debris and axons wrapped by thinner myelin sheaths. Treatment with naringin (20 and 40 mg/kg) improved inter-laminar changes and myelin sheath thickness (Figure 3C). The resulting histogram demonstrated that, compared with the vehicle-treated rats, the mean myelin thickness was increased significantly in naringin-treated rats ( $P < 0.05$ , vs. naringin 20 mg/kg;  $P < 0.01$ , vs. naringin 40 mg/kg) (Figure 3D). These observations indicated that naringin therapy could preserve myelin, promote remyelination and exert protective effects on axons after SCI.

#### Effect of naringin on OLs and OPCs

The expression of oligodendroglial lineage markers, NG2 and Olig2, were determined through western blot assays (Figure 4A). Compared with the sham group, the expression of NG2 was significantly elevated in the vehicle group. Compared with the vehicle group, naringin therapy significantly increased the expression level of NG2 ( $P < 0.05$ , vs. naringin 20 mg/kg;  $P < 0.01$ , vs. naringin 40 mg/kg) (Figure 4B). Meanwhile, naringin treatment resulted in higher Olig2 protein levels compared with vehicle treatment ( $P < 0.05$ , vs. naringin 20 mg/kg;  $P < 0.01$ , vs. naringin 40 mg/kg) (Figure 4C). Hence, after SCI, naringin therapy could significantly increase OPC differentiation and OL quantity.

#### Effect of naringin on NKx2.2 and CNPase protein expression

Western blot assays revealed the protein levels of NKx2.2 and CNPase in the different groups 4 weeks after injury (Figure 5A). NKx2.2 is a transcription factor associated with OPC differentiation and a marker of OPC activation. As shown in Figure 5A, B, naringin treatment increased NKx2.2 protein levels more effectively than vehicle treatment ( $P < 0.01$ , vs. naringin 20 mg/kg;  $P < 0.01$ , vs. naringin 40 mg/kg). CNPase is an oligodendroglial marker and is implicated in myelin biosynthesis. CNPase levels were decreased in the vehicle group (Figure 5A, B). In contrast, naringin therapy remarkably increased the levels of CNPase ( $P < 0.05$ , vs. naringin 20

mg/kg;  $P < 0.01$ , vs. naringin 40 mg/kg). Therefore, naringin exhibited the potential to promote remyelination.

#### Effect of naringin on protein expression of GSK-3 $\beta$ and $\beta$ -catenin

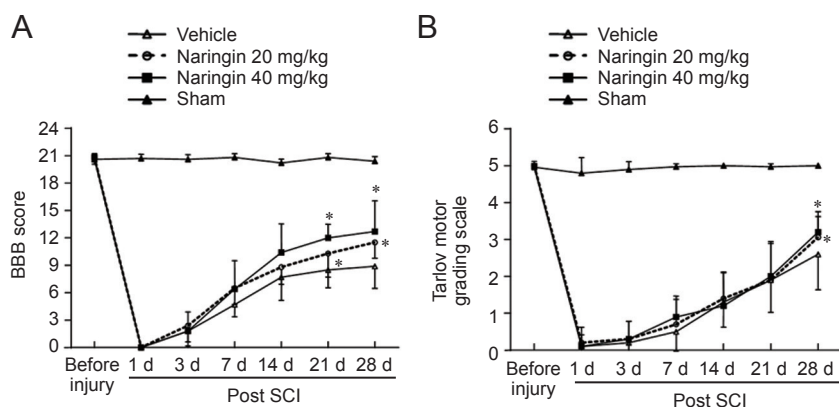
Western blot assays revealed the expression of GSK-3 $\beta$  and  $\beta$ -catenin in the different groups (Figure 6A). Compared with the vehicle group, naringin treatment significantly increased GSK-3 $\beta$  phosphorylation at Ser9 ( $P < 0.05$ , vs. naringin 20 mg/kg;  $P < 0.05$ , vs. naringin 40 mg/kg) (Figure 6B). Naringin treatment significantly increased  $\beta$ -catenin levels compared with vehicle treatment ( $P < 0.05$ , vs. naringin 20 mg/kg;  $P < 0.05$ , vs. naringin 40 mg/kg) (Figure 6C).

## Discussion

Chronic, high dose naringin therapy may cause side effects, such as increased risk of gene mutation, in patients with SCI (Havsteen, 2002). Our previous study demonstrated that low-dose administration may prevent the potential harmful side effects of naringin (Rong et al., 2012). In the present study, no increase in mortality rate, post-operative infection or hematuria were observed. Nevertheless, further investigations must be performed to confirm the safety of naringin treatment.

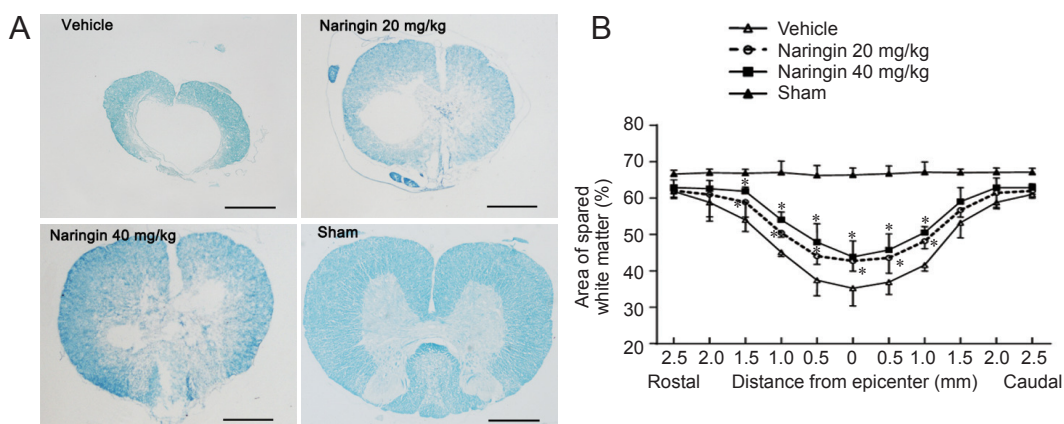
Previous studies in the field of spinal cord regeneration mainly focused on devising therapeutic measures to enhance neural regeneration (Hulsebosch, 2002; Yang et al., 2015). Recently, research emphasis has shifted to promoting remyelination after injury (Zhang et al., 2015). In the present study, Luxol fast blue staining, double-immunofluorescence staining, electron microscopy and morphometric analyses were conducted on the dorsal corticospinal tract of spinal cord 4 weeks post-injury. Results demonstrated that the quality of myelinated axons, the extent and the thickness of the myelin sheath were significantly improved in the groups that received naringin treatment, indicating improved remyelination after naringin treatment.

In the central nervous system, OLs are myelin-forming cells. OPCs are precursor OLs that are widely distributed throughout the central nervous system (Sim et al., 2002; Wei and Xiao, 2016). After injury, OPCs are recruited through migration and proliferation to the injury site (Horky et al., 2006). These cells are then capable of differentiating into OLs under favorable conditions (Hesp et al., 2015). OPC differentiation and oligodendrocyte survival after traumatic SCI are important in achieving successful remyelination (Mekhail et al., 2012). Mekhail et al. (2012) claimed that therapeutic measures are needed to reduce OL death, improve endogenous OPC differentiation, enhance remyelination, and ultimately preserve neurological function after SCI. Our results demonstrated that naringin increased the protein level of the oligodendroglial lineage markers, NG2 and Olig2. These results indicated that naringin treatment could effectively reduce OPC death and enhance endogenous OPC survival after SCI. CNPase is the third most abundant protein in central nervous system myelin and its expression increas-



**Figure 1** Effect of naringin on locomotor activity.

(A) Open field assessment (BBB scores) before and after injury. (B) Tarlov's motor grading scale before and after injury. \* $P < 0.05$ , vs. vehicle group (SCI + vehicle). Data are expressed as the mean  $\pm$  SD ( $n = 15$ ; repeated-measures analysis of variance and Bonferroni's *post hoc* test). SCI: Spinal cord injury; d: day(s); BBB: Basso, Beattie, and Bresnahan.



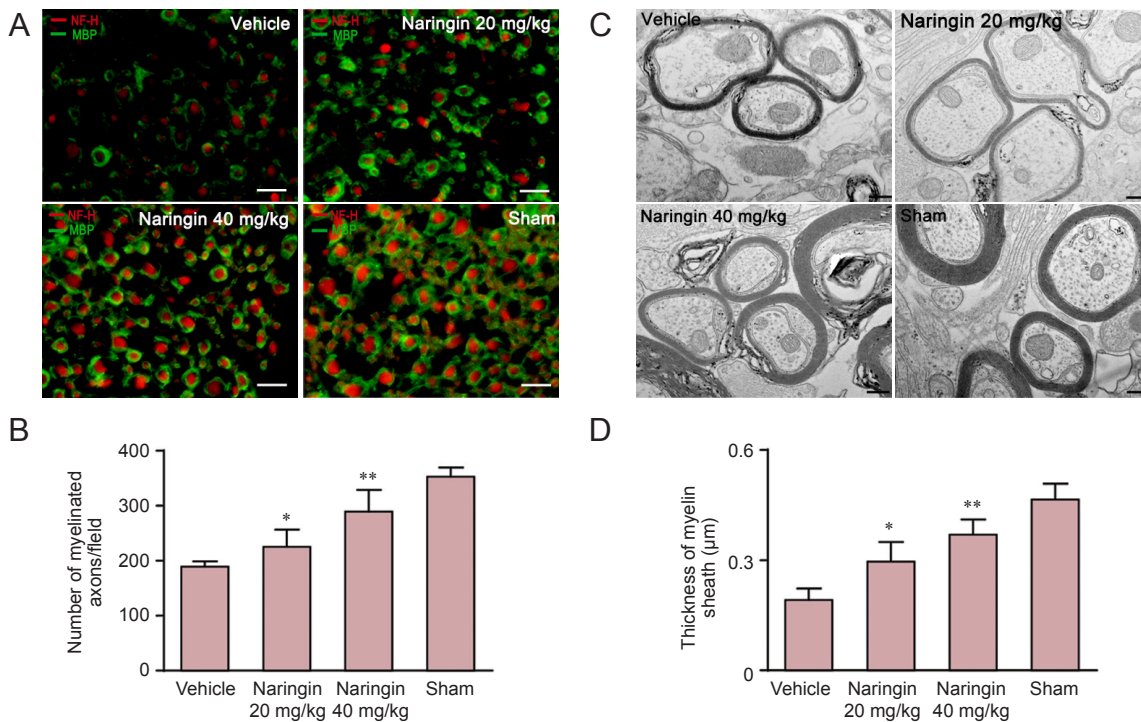
**Figure 2** Effect of naringin on spared myelin sheath in rats 4 weeks post-injury.

(A) Representative Luxol fast blue stained spinal cord sections taken at the lesion epicenter. Scale bars: 500  $\mu$ m. (B) Quantitation of spared myelin sheath in each group. \* $P < 0.05$ , vs. vehicle group (SCI + vehicle). Data are expressed as the mean  $\pm$  SD ( $n = 15$ ; repeated-measures analysis of variance with Bonferroni's *post hoc* test was used to compare the spared myelin sheath among groups; one-way analysis of variance with Tukey's *post hoc* test was used to compare the spared myelin sheath among groups at different sites).

es with myelination in cultured nerve tissues and *in vivo* (Birgbauer et al., 2004). Moreover, CNPase is a pivotal component of the molecular complex that mediates early stage myelination (Yin et al., 2012). Nkx2.2 induces the differentiation of OPCs into mature oligodendrocytes in mature spinal cord tissue (Fancy et al., 2009) and is a key factor in remyelination after traumatic SCI (Zhu et al., 2014). Accordingly, in the present study, we detected the expression of CNPase and Nkx2.2 in SCI rats and naringin administration significantly enhanced CNPase and Nkx2.2 expression, indicating that naringin has the potential to promote OPC differentiation and remyelination.

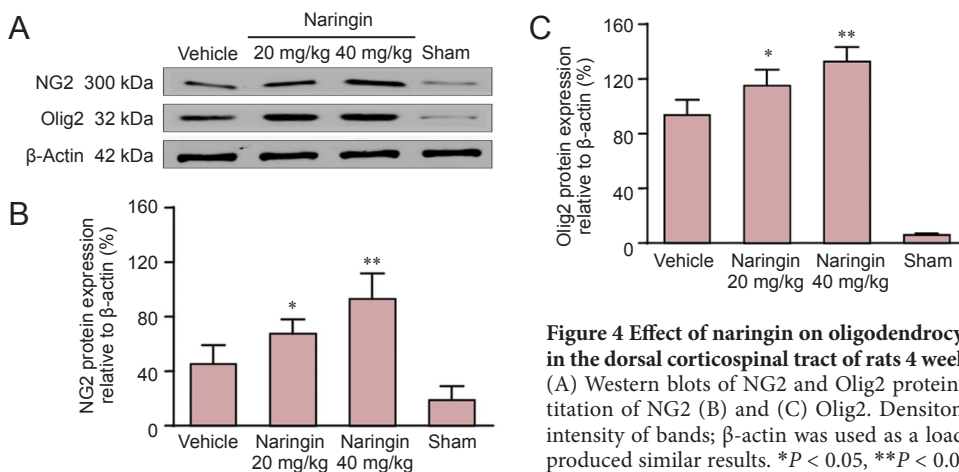
Wnt signaling plays an important role in different cellular processes, such as neural stem cell proliferation, migration and motility (Sethi and Vidal-Puig, 2010). In the canonical Wnt signaling pathway, Wnts bind to their receptors to inhibit the phosphorylation of  $\beta$ -catenin by GSK-3 $\beta$ . Conversely, GSK-3 $\beta$  inactivation (by phosphorylation of Ser9) stabilizes and causes the accumulation of free cytosolic  $\beta$ -catenin (Boonchai et al., 2000). GSK-3 $\beta$  plays a negative role in the regulation of OL differentiation *in vivo* (Bartzokis, 2012). GSK-3 $\beta$  inhibition not only stimulates

the proliferation and survival of OPCs, but also enhances oligodendrocyte differentiation and myelination *via* multiple mechanisms (Rodriguez et al., 2014). GSK-3 $\beta$  inhibition also produces equivalent effects on the adult nervous system and stimulates regeneration of OPCs and remyelination following demyelination (Azim and Butt, 2011). Moreover, GSK-3 $\beta$  inhibition increases the activity of cAMP response element-binding protein (Barresi et al., 2015), which is a positive regulator of OL differentiation and myelination, and suppressed the inhibition of OL differentiation *in vitro* (Azim and Butt, 2011). Naringin treatment enhanced intracellular  $\beta$ -catenin accumulation and GSK-3 $\beta$  phosphorylation in mouse melanoma cells (Huang et al., 2011). Meanwhile, naringin (100 mg/kg per day) administration significantly induced GSK-3 $\beta$  phosphorylation in a mouse model of Alzheimer's disease (Wang et al., 2012). Furthermore, the present results are consistent with significantly increased phosphorylation of GSK-3 $\beta$  and  $\beta$ -catenin expression in naringin-treated rats. Thus, treatment with naringin may promote the survival of oligodendrocytes and stimulate the differentiation and remyelination of OPCs by inhibiting the  $\beta$ -catenin/GSK-3 $\beta$  pathway.



**Figure 3** Effect of naringin on the myelinated axons and myelin sheaths of rats 4 weeks post-injury.

(A) Representative double-immunostaining (MBP and NF-H) photographs of the dorsal corticospinal tract from each group. Scale bars: 50 μm. (B) The number of myelinated axons in each group ( $n = 5$ ). (C) Representative transmission electron microscopy photographs of the dorsal corticospinal tract from each group. The myelin sheaths in the sham group contained intact membranes; however, the myelin sheaths in the vehicle group showed aberrantly swollen sheaths. Treatment with naringin (20 and 40 mg/kg) improved the inter-laminar organization and myelin sheath thicknesses. Scale bars: 500 nm. (D) The thickness of myelin sheaths in the dorsal corticospinal tract of spinal cord ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , vs. vehicle group (SCI + vehicle). Data are expressed as the mean ± SD (one-way analysis of variance and Tukey's *post hoc* test). MBP: Myelin basic protein; NF-H: neurofilament heavy.



**Figure 4** Effect of naringin on oligodendrocytes and oligodendrocyte precursor cells in the dorsal corticospinal tract of rats 4 weeks post-injury.

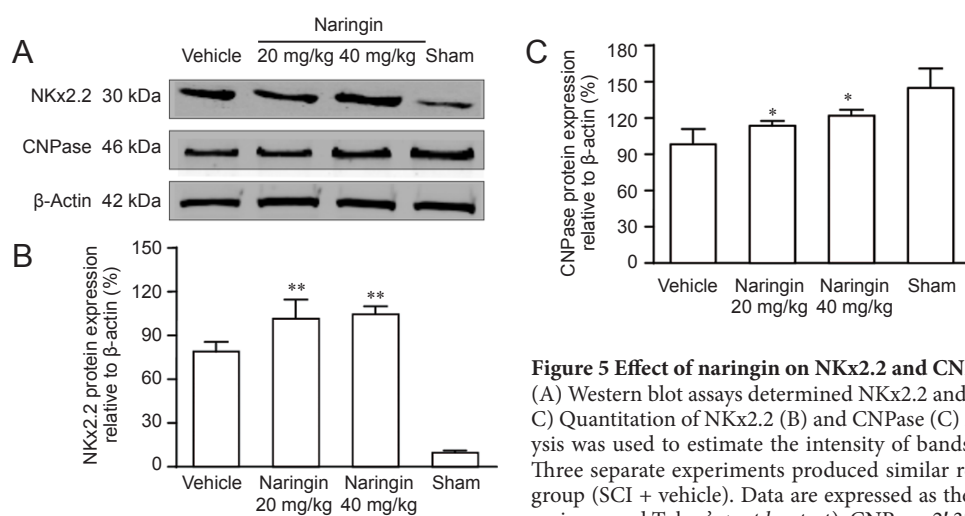
(A) Western blots of NG2 and Olig2 protein levels in different groups. (B, C) Quantitation of NG2 (B) and (C) Olig2. Densitometric analysis was used to estimate the intensity of bands; β-actin was used as a loading control. Three separate experiments produced similar results. \* $P < 0.05$ , \*\* $P < 0.01$ , vs. vehicle group (SCI + vehicle). Data are expressed as the mean ± SD ( $n = 7$ ; one-way analysis of variance and Tukey's *post hoc* test).

There are limitations in the present study. To confirm that naringin improves remyelination, a detailed time course should be followed to first show demyelination occurred equally in all injured groups and then that remyelination occurred to a greater extent in treated rats. Also, changes in body weight following SCI are very important to clarify the therapeutic effect of naringin. Further work is required to

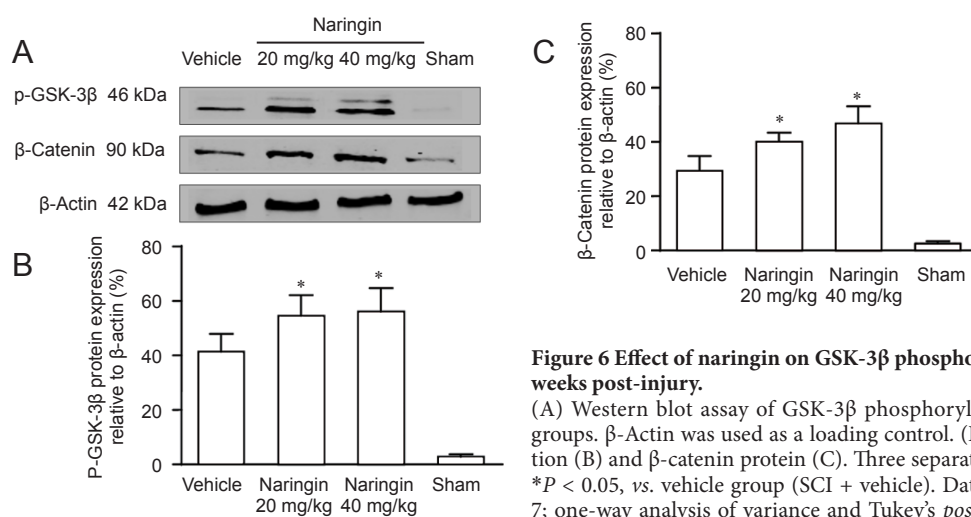
evaluate changes in body weight and general well-being after treatment with naringin.

Collectively, these results suggest that naringin treatment is a potential therapy for SCI aimed at remyelination.

**Acknowledgments:** The authors are grateful to technical staff in our lab for assistance. The authors thank Dr. Chen Huang and Li Chen from the Third Hospital of Peking University in China for valuable discussions and



**Figure 5 Effect of naringin on NKx2.2 and CNPase protein levels 4 weeks post-injury.** (A) Western blot assays determined NKx2.2 and CNPase protein levels in each group. (B, C) Quantitation of NKx2.2 (B) and CNPase (C) levels in each group. Densitometric analysis was used to estimate the intensity of bands;  $\beta$ -actin was used as a loading control. Three separate experiments produced similar results. \* $P < 0.05$ , \*\* $P < 0.01$ , vs. vehicle group (SCI + vehicle). Data are expressed as the mean  $\pm$  SD ( $n = 7$ ; one-way analysis of variance and Tukey's *post hoc* test). CNPase: 2',3'-Cyclic nucleotide 3'-phosphodiesterase.



**Figure 6 Effect of naringin on GSK-3 $\beta$  phosphorylation and  $\beta$ -catenin levels in rats 4 weeks post-injury.** (A) Western blot assay of GSK-3 $\beta$  phosphorylation and  $\beta$ -catenin levels in different groups.  $\beta$ -Actin was used as a loading control. (B, C) The level of GSK-3 $\beta$  phosphorylation (B) and  $\beta$ -catenin protein (C). Three separate experiments produced similar results. \* $P < 0.05$ , vs. vehicle group (SCI + vehicle). Data are expressed as the mean  $\pm$  SD ( $n = 7$ ; one-way analysis of variance and Tukey's *post hoc* test). GSK-3 $\beta$ : Glycogen synthase kinase-3 $\beta$ ; p-GSK-3 $\beta$ : phosphorylated glycogen synthase kinase-3 $\beta$ .

experimental help.

**Author contributions:** YWP, XC and SHX designed the study. FS and ZZ made animal models. WR and CZ did histological staining and biological experiments, analyzed the data and wrote the paper. WR and CZ obtained funding. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Plagiarism check:** This paper was screened twice using CrossCheck to verify originality before publication.

**Peer review:** This paper was double-blinded and stringently reviewed by international expert reviewers.

## References

- Azim K, Butt AM (2011) GSK3 $\beta$  negatively regulates oligodendrocyte differentiation and myelination in vivo. *Glia* 59:540-553.
- Bailey DG (2010) Fruit juice inhibition of uptake transport: a new type of food-drug interaction. *Br J Clin Pharmacol* 70:645-655.
- Barresi V, Mondello S, Branca G, Rajan TS, Vitarelli E, Tuccari G (2015) p-CREB expression in human gliomas: potential use in the differential diagnosis between astrocytoma and oligodendroglioma. *Hum Pathol* 46:231-238.
- Bartzokis G (2012) Neuroglial pharmacology: myelination as a shared mechanism of action of psychotropic treatments. *Neuropharmacology* 62:2137-2153.
- Basso DM, Beattie MS, Bresnahan JC (1995) A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 12:1-21.
- Behrmann DL, Bresnahan JC, Beattie MS, Shah BR (1992) Spinal-cord injury produced by consistent mechanical displacement of the cord in rats - behavioral and histologic analysis. *J Neurotrauma* 9:197-217.
- Birgbauer E, Rao TS, Webb M (2004) Lysolecithin induces demyelination in vitro in a cerebellar slice culture system. *J Neurosci Res* 78:157-166.
- Boonchai W, Walsh M, Cummings M, Chenevix-Trench G (2000) Expression of beta-catenin, a key mediator of the WNT signaling pathway, in basal cell carcinoma. *Arch Dermatol* 136:937-938.
- Crawford AH, Chambers C, Franklin RJ (2013) Remyelination: the true regeneration of the central nervous system. *J Comp Pathol* 149:242-254.
- Fancy SP, Baranzini SE, Zhao C, Yuk DI, Irvine KA, Kaing S, Sanai N, Franklin RJ, Rowitch DH (2009) Dysregulation of the Wnt pathway inhibits timely myelination and remyelination in the mammalian CNS. *Gene Dev* 23:1571-1585.
- Franklin RJ, Ffrench-Constant C (2008a) Remyelination in the CNS: from biology to therapy. *Nat Rev Neurosci* 9:839-855.
- Havsteen BH (2002) The biochemistry and medical significance of the flavonoids. *Pharmacol Therapeut* 96:67-202.
- Hesp ZC, Goldstein EA, Miranda CJ, Kaspar BK, McTigue DM (2015) Chronic oligodendrogenesis and remyelination after spinal cord injury in mice and rats. *J Neurosci* 35:1274-1290.
- Ho PC, Saville DJ, Coville PF, Wanwimolruk S (2000) Content of CY-P3A4 inhibitors, naringin, naringenin and bergapten in grapefruit and grapefruit juice products. *Pharm Acta Helv* 74:379-385.

- Horky LL, Galimi F, Gage FH, Horner PJ (2006) Fate of endogenous stem/progenitor cells following spinal cord injury. *J Comp Neurol* 498:525-538.
- Huang YC, Yang CH, Chiou YL (2011) Citrus flavanone naringenin enhances melanogenesis through the activation of Wnt/beta-catenin signalling in mouse melanoma cells. *Phytomedicine* 18:1244-1249.
- Hulsebosch CE (2002) Recent advances in pathophysiology and treatment of spinal cord injury. *Adv Physiol Educ* 26:238-255.
- Leem E, Nam JH, Jeon MT, Shin WH, Won SY, Park SJ, Choi MS, Jin BK, Jung UJ, Kim SR (2014) Naringin protects the nigrostriatal dopaminergic projection through induction of GDNF in a neurotoxin model of Parkinson's disease. *J Nutr Biochem* 25:801-806.
- Levine JM, Reynolds R, Fawcett JW (2001) The oligodendrocyte precursor cell in health and disease. *Trends Neurosci* 24:39-47.
- Mekhail M, Almazan G, Tabrizian M (2012) Oligodendrocyte-protection and remyelination post-spinal cord injuries: a review. *Prog Neurobiol* 96:322-339.
- Ministry of Science and Technology of the People's Republic of China (2006) Guidance Suggestions for the Care and Use of Laboratory Animals.
- Ouyang H, Sun W, Fu Y, Li J, Cheng JX, Nauman E, Shi R (2010) Compression induces acute demyelination and potassium channel exposure in spinal cord. *J Neurotrauma* 27:1109-1120.
- Perot PL, Jr., Lee WA, Hsu CY, Hogan EL, Cox RD, Gross AJ (1987) Therapeutic model for experimental spinal cord injury in the rat: I. Mortality and motor deficit. *Cent Nerv Syst Trauma* 4:149-159.
- Plemel JR, Keough MB, Duncan GJ, Sparling JS, Yong VW, Stys PK, Tetzlaff W (2014) Remyelination after spinal cord injury: is it a target for repair? *Prog Neurobiol* 117:54-72.
- Rodriguez JP, Coulter M, Miotke J, Meyer RL, Takemaru K, Levine JM (2014) Abrogation of beta-catenin signaling in oligodendrocyte precursor cells reduces glial scarring and promotes axon regeneration after CNS injury. *J Neurosci* 34:10285-10297.
- Rong W, Wang J, Liu X, Jiang L, Wei F, Hu X, Han X, Liu Z (2012) Naringin treatment improves functional recovery by increasing BDNF and VEGF expression, inhibiting neuronal apoptosis after spinal cord injury. *Neurochem Res* 37:1615-1623.
- Sethi JK, Vidal-Puig A (2010) Wnt signalling and the control of cellular metabolism. *Biochem J* 427:1-17.
- Sim FJ, Zhao C, Penderis J, Franklin RJ (2002) The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. *J Neurosci* 22:2451-2459.
- Varma AK, Das A, Wallace Gt, Barry J, Vertegel AA, Ray SK, Banik NL (2013) Spinal cord injury: a review of current therapy, future treatments, and basic science frontiers. *Neurochem Res* 38:895-905.
- Wang D, Gao K, Li X, Shen X, Zhang X, Ma C, Qin C, Zhang L (2012) Long-term naringin consumption reverses a glucose uptake defect and improves cognitive deficits in a mouse model of Alzheimer's disease. *Pharmacol Biochem Behav* 102:13-20.
- Wei F, Xiao XL (2016) Oligodendrocyte transplantation for acute spinal cord injury: insulin-like growth factor 1 induces bone marrow mesenchymal stem cells differentiating into oligodendrocytes. *Zhongguo Zuzhi Gongcheng Yanjiu* 20:3419-3424.
- Wu B, Sun L, Li P, Tian M, Luo Y, Ren X (2012) Transplantation of oligodendrocyte precursor cells improves myelination and promotes functional recovery after spinal cord injury. *Injury* 43:794-801.
- Yang JH, Lv JG, Wang H, Nie HY (2015) Electroacupuncture promotes the recovery of motor neuron function in the anterior horn of the injured spinal cord. *Neural Regen Res* 10:2033-2039.
- Yin LL, Lin LL, Zhang L, Li L (2012) Epimedium flavonoids ameliorate experimental autoimmune encephalomyelitis in rats by modulating neuroinflammatory and neurotrophic responses. *Neuropharmacology* 63:851-862.
- Yune TY, Lee JY, Jung GY, Kim SJ, Jiang MH, Kim YC, Oh YJ, Markelonis GJ, Oh TH (2007) Minocycline alleviates death of oligodendrocytes by inhibiting pro-nerve growth factor production in microglia after spinal cord injury. *J Neurosci* 27:7751-7761.
- Zbarsky V, Datla KP, Parkar S, Rai DK, Aruoma OI, Dexter DT (2005) Neuroprotective properties of the natural phenolic antioxidants curcumin and naringenin but not quercetin and fisetin in a 6-OHDA model of Parkinson's disease. *Free Radic Res* 39:1119-1125.
- Zhang SQ, Wu MF, Liu JB, Li Y, Zhu QS, Gu R (2015) Transplantation of human telomerase reverse transcriptase gene-transfected Schwann cells for repairing spinal cord injury. *Neural Regen Res* 10:2040-2047.
- Zhu Q, Zhao X, Zheng K, Li H, Huang H, Zhang Z, Mastracci T, Wegner M, Chen Y, Sussel L, Qiu M (2014) Genetic evidence that Nkx2.2 and Pdgfra are major determinants of the timing of oligodendrocyte differentiation in the developing CNS. *Development* 141:548-555.

Copyedited by Allen J, Raye W, Wang J, Li CH, Qiu Y, Song LP, Zhao M