# Myelin-associated glycoprotein combined with chitin conduit inhibits painful neuroma formation after sciatic nerve transection

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## From the Contents



**Graphical Abstract** *Combined application of myelin-associated glycoprotein (MAG) and chitin conduit to inhibit painful neuroma formation after sciatic nerve transection*


## Abstract

Studies have shown that myelin-associated glycoprotein (MAG) can inhibit axon regeneration after nerve injury. However, the effects of MAG on neuroma formation after peripheral nerve injury remain poorly understood. In this study, local injection of MAG combined with nerve cap made of chitin conduit was used to intervene with the formation of painful neuroma after sciatic nerve transfection in rats. After 8 weeks of combined treatment, the autotomy behaviors were reduced in rats subjected to sciatic nerve transfection, the mRNA expression of nerve growth factor, a pain marker, in the proximal nerve stump was decreased, the density of regenerated axons was decreased, the thickness of the myelin sheath was increased, and the ratio of unmyelinated to myelinated axons was reduced. Moereover, the percentage of collagen fiber area and the percentage of fibrosis marker alpha-smooth muscle actin positive staining area in the proximal nerve stump were decreased. The combined treatment exhibited superior effects in these measures to chitin conduit treatment alone. These findings suggest that MAG combined with chitin conduit synergistically inhibits the formation of painful neuroma after sciatic nerve transection and alleviates neuropathic pain. This study was approved by the Animal Ethics Committee of Peking University People's Hospital (approval No. 2019PHE027) on December 5, 2019.

**Key Words:** autotomy; axon; chitin conduit; fibrosis; myelin-associated glycoprotein; painful neuroma; peripheral nerve; regeneration

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## Introduction

Traumatic neuroma is a common complication following peripheral nerve injury, which typically presents as disorganized bulbous tissue (Foltán et al., 2008). The common locations of painful neuroma are the lower extremities, radial nerve, and brachial plexus (Lu et al., 2018). The most disturbing clinical symptom of painful neuroma is persistent neuropathic pain, which not only seriously impairs patients' quality of life, but also has a heavy economic burden for society as a whole (Ducic et al., 2008; Liedgens et al., 2016). In recent decades, various attempts have been made to overcome painful neuroma formation, such as radiofrequency ablation, alcohol injection, and nerve stump shorting, but the long-term results are unsatisfactory (Burchiel et al., 1993; Matthews et al., 2019; Urits et al., 2020). With the development of nerve capping techniques, some progress has been made in inhibiting painful neuroma formation (Marcol et al., 2011; Chim et al., 2013). Nevertheless, the efficacy of this treatment modality needs to be further improved to meet the clinical demands.

Myelin-associated glycoprotein (MAG), which belongs to the immunoglobulin-like superfamily, is a transmembrane protein produced by myelinating glial cells. Gupta et al. (2006) reported that axonal sprouting in a rat model of peripheral nerve compression was related to down-regulated MAG expression. Tomita et al. (2007) found that local administration of MAG into the transected peripheral nerve can reduce redundant axonal branching. However, the effects of MAG on painful neuroma formation have not been investigated.

Chitin is a biocompatible material that is widely used in tissue engineering (Jayakumar et al., 2011; Yang, 2011; Wan and

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Tai, 2013; Li et al., 2014). In previous studies, we developed a chitin nerve conduit with good mechanical properties (Zhang et al., 2014, 2018a, b). Considering the complex mechanisms underlying painful neuroma formation, we hypothesized that application of MAG combined with a chitin conduit would effectively inhibit the formation of neuroma and relieve neuropathic pain. In this study, a rat model of sciatic nerve transection was adopted to test this hypothesis.

## Materials and Methods

### Conduit preparation

The chitin conduit was designed and provided by Peking University People's Hospital and the Chinese Textile Academy (Chinese patent No. 01136314.2). The length of chitin conduit was 10 mm, with an inner diameter of 2 mm and a wall thickness of 1 mm. After ultraviolet radiation, all chitin conduits were stored at 4°C for subsequent experiments.

### Ethical approval

All animals were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China; license no. SCXK (Jing) 2016-0006). The experimental protocols were approved by the Animal Ethics Committee of Peking University People's Hospital on December 5, 2019 (approval No. 2019PHE027) and performed in the Laboratory Animal Unit of Peking University People's Hospital. All experiments were designed and reported according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

### Animals and surgical procedures

Mutual attack in male rats may affect the accuracy of experimental results, so in this study, 30 specific-pathogen-free female Sprague-Dawley rats (6–7 weeks old, weighing 180–200 g) were used. All rats were randomly divided into the following three groups ($n$ = 10/group): the chitin conduit capping group (conduit group), MAG local injection and capping group (combination group), and no treatment group (model group). All animals were anesthetized with 3% isoflurane (500 mL/min) (RWD Life Science, Shenzhen, China) inhalation. In the sterile operating room, the right sciatic nerve was exposed and transected at the mid-femur level, and a 10 mm gap between the proximal and distal segments was left to avoid nerve reconnection. Subsequently, the proximal nerve stump in the combination group was injected with 5 μL of recombinant rat MAG protein (final concentration: 0.25 μg/μL, R&D Systems, Minneapolis, MN, USA), while 5 μL of physiological saline was injected at the same site in the conduit group. After injection, the proximal nerve stump was sutured to a depth of 2 mm into the chitin conduit (10 mm length) with 8/0 nylon sutures in the combination and conduit groups. In the model group, the proximal nerve stump was left in situ without any management. Finally, the skin and muscle incisions in all groups were closed with 4/0 nylon sutures.

### Autotomy behaviors observation

Autotomy behaviors were measured by two blinded observers 8 weeks after the operation. The quantitative analysis of autotomy was carried out using the modified Wall Scale (Wall et al., 1979; Zeltser et al., 2000). In brief, a score of 1 was given if two or more nails were removed. An additional score of 1 was added if each half toe was attacked. The maximum score for this scale was 11. The higher the score, the more severe the injury.

### Specimen preparation

Eight weeks post-operation, all animals were euthanized by carbon dioxide inhalation. The carbon dioxide replacement rate was set to 30% per minute. After harvesting the proximal nerve stumps, half of the specimens in each group were selected at random for histological studies and fixed in 4% paraformaldehyde at 4°C. One cubic millimeter of tissue was taken from the center of every remaining specimen for transmission electron microscope observation. Then, the remaining specimens were immediately stored at –80°C for quantitative assessment of nerve growth factor (NGF) and alpha-smooth muscle actin (α-SMA) gene expression as markers of pain and fibrosis (Herzberg et al., 1997; Atherton et al., 2006; Yan et al., 2012; Weng et al., 2016).

### Quantitative real-time polymerase chain reaction

Total RNA was extracted from every stored nerve specimen using a tissue RNA extraction Kit (ES Science Biotech, Shanghai, China) and reverse transcribed into complementary DNA using complementary DNA Reverse Transcription Kit (Toyobo, Osaka, Japan). Quantitative real-time polymerase chain reaction was performed in triplicate using SYBR Green Realtime polymerase chain reaction Master Mix (Toyobo) on a real-time polymerase chain reaction system (Applied Biosystems, Foster City, CA, USA). The relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalized by the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The primers used in this study are as follows: glyceraldehyde-3-phosphate dehydrogenase: forward: 5′-ATG GTG AAG GTC GGT GTG AAC G-3′, reverse: 5′-TTA CTC CTT GGA GGC CAT GTA G-3′; *NGF*: forward: 5′-GAT CGG CGT ACA GGC AGA AC-3′, reverse: 5′-GGC TCG GCA CTT GGT CTC AA-3′; *α-SMA*: forward: 5′-GCT CCT CCA GAA CGC AAA TAT-3′, reverse: 5′-GGG CCA GCT TCG TCA TAC TC-3′.

### Histological analysis

After ethanol dehydration, xylene transparency, and paraffin embedding, the middle parts of the nerve specimens were cross-cut into 5-μm-thick sections. At least ten sections were obtained from each specimen after poorly cut sections had been removed. Then, 15 sections were randomly selected from each group and every fifth section was used for Masson's trichrome staining to evaluate the distribution of collagen fibers, immunohistochemical staining to assess the immunopositivity of α-SMA, and immunofluorescence staining to examine the regenerated axons. For Masson's trichrome staining, the sections were deparaffinized and stained with Masson's Trichrome Stain Kit (Solarbio, Beijing, China) in accordance with the manufacturer's instructions. For immunohistochemical staining, the sections were deparaffinized, rehydrated, and incubated with 3% hydrogen peroxide for 10 minutes. After antigen retrieval, the sections were blocked with 5% goat serum (Solarbio) for 30 minutes, incubated with rabbit anti-α-SMA antibody (1:2000, Cat# 14395-1-AP, RRID: AB_2223009, Proteintech, Beijing, China) overnight at 4°C, and incubated with biotinylated anti-rabbit IgG secondary antibody (1:200, Cat# ZB-2010, ZSGB-BIO, Beijing, China) for 1 hour at room temperature. Immunoreactivity was visualized by 3,3′-diaminobenzidine (ZSGB-BIO) after incubating with horseradish peroxidase-conjugated streptavidin (ZSGB-BIO). For immunofluorescence staining, the sections were deparaffinized and rehydrated, and underwent antigen retrieval. After blocking with 5% goat serum (Solarbio) for 30 minutes, the sections were incubated with mouse anti-neurofilament 200 antibody (1:200, Cat# N4389, RRID: AB_260781, Sigma, St. Louis, MO, USA) overnight at 4 °C and stained with Alexa Fluor 594 anti-mouse IgG (1:200, Cat# ZF-0513, ZSGB-BIO) for 2 hours at room temperature. The nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma).

All images were captured using a Leica DM4 B microscope (Leica, Wetzlar, Germany). Five fields of each section were randomly selected and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

The percentage of collagen fiber area was calculated using the following equation: percentage of collagen fiber area = collagen fiber area / total image area × 100. The percentage

of α-SMA positive staining area was calculated as the α-SMA positive staining area / the total image area × 100. The density of axons was defined as the number of axons within an area of 5000 μm$^2$.

### Transmission electron microscope observation

The nerve samples were fixed in cold buffered 2.5% glutaraldehyde solution for 4 hours. After being washed by 0.01 M phosphate buffered solution, the samples were fixed with 1% osmium tetroxide, dehydrated in graded concentrations of ethanol, embedded using resin, and cut into 70 nm ultrathin sections. Five sections were randomly selected from each group. After being stained with 3% uranyl acetate-lead citrate, images of the ultrathin sections were taken with a transmission electron microscope (JEM-1400Plus, JEOL, Tokyo, Japan). Five fields of each section were randomly selected and analyzed using Image-Pro Plus 6.0 software to calculate myelin sheath thickness and the ratio of unmyelinated to myelinated axons.

### Statistical analysis

All numerical data are presented as the mean ± standard deviation (SD). The experimental results were analyzed with SPSS 22.0 software (IBM, Armonk, NY, USA) using one-way analysis of variance followed by Tukey's *post hoc* tests. Between-group differences were considered statistically significant at $P < 0.05$.

## Results

### Combination of MAG and chitin conduit reduces autotomy behaviors in a rat model of sciatic nerve transection

As shown in **Figure 1A**, the autotomy score in the combination group was significantly lower than that in the conduit and model groups (both $P < 0.01$). The autotomy score in the conduit group was significantly lower than that in the model group ($P < 0.01$).

### Combination of MAG and chitin conduit reduces the relative mRNA expression levels of *NGF* and *α-SMA* in proximal nerve stumps in a rat model of sciatic nerve transection

As shown in **Figure 1B** and **C**, the relative mRNA expression levels of *NGF* and *α-SMA* in the combination and conduit groups were significantly lower than those in the model group (all $P < 0.01$). In addition, the relative mRNA expression level of *NGF* in the combination group was significantly lower than that in the conduit group ($P < 0.01$). However, the difference in the relative mRNA expression level of *α-SMA* between the combination and conduit groups was not significant ($P > 0.05$).

### Combination of MAG and chitin conduit improves the pathology of regenerated nerve fibers in proximal nerve stumps in a rat model of sciatic nerve transection

The Masson's trichrome staining analysis revealed that plenty of collagen fibers and regenerated nerve fibers were disorderly distributed in the model group. In the conduit group, nerve fibers were also distributed in a disorganized manner. However, nerve fibers showed regular distribution in the combination group (**Figure 2A**). As shown in **Figure 2B**, the percentage of collagen fiber area in the combination group was significantly lower than that in the model group ($P < 0.01$), while the difference between the combination and conduit groups was not significant ($P > 0.05$).

### Combination of MAG and chitin conduit reduces the expression of α-SMA in proximal nerve stumps in a rat model of sciatic nerve transection

As shown by the immunohistochemical analysis, α-SMA was abundantly detected in the model group. In the conduit and combination groups, α-SMA was mainly expressed in the walls of blood vessels (**Figure 2C**). As shown in **Figure 2D**, the percentage of α-SMA positive staining area in the combination group was significantly lower than that in the model group ($P < 0.01$), while the difference between the combination and conduit groups was not significant ($P > 0.05$).

### Combination of MAG and chitin conduit reduces the density of regenerated axons in proximal nerve stumps in a rat model of sciatic nerve transection

As shown by the immunofluorescent analysis, regenerated axons were densely distributed in the model and conduit groups, while regenerated axons were sparsely distributed in the combination group (**Figure 2E**). Results of the axon density analysis are shown in **Figure 2F**. Axon density in the combination group was significantly lower than that in the conduit and model groups (both $P < 0.01$), while there was no significant difference between the conduit and model groups ($P > 0.05$).

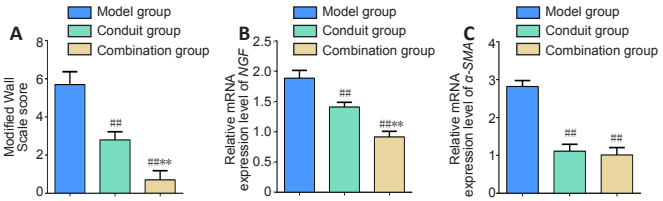


**Figure 1 | Effect of combination of myelin-associated glycoprotein and chitin conduit on autotomy behavior and *NGF* and *α-SMA* gene expression in proximal nerve stumps 8 weeks postoperatively.**

(A) Modified Wall Scale ($n = 10$ animals per group). The higher the score, the more severe the injury. (B, C) Relative mRNA expression levels of NGF (B) and α-SMA (C). The relative mRNA expression level ($n = 5$ animals per group) was normalized by *GADPH*. Data are expressed as the mean ± SD. ##$P < 0.01$, *vs*. model group; **$P < 0.01$, *vs*. conduit group (one-way analysis of variance followed by Tukey's *post hoc* test). GADPH: Glyceraldehyde-3-phosphate dehydrogenase; NGF: nerve growth factor; α-SMA: alpha-smooth muscle actin.

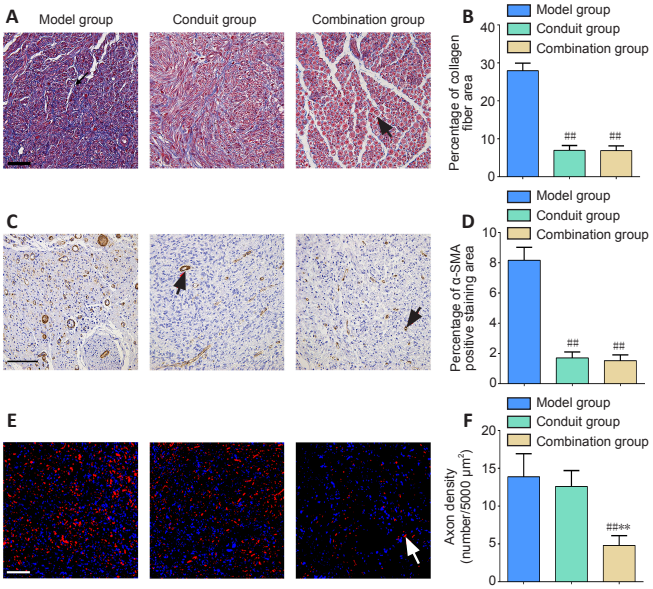


**Figure 2 | Effect of combination of myelin-associated glycoprotein and chitin conduit on the pathology of regenerated nerve fibers in proximal nerve stumps 8 weeks postoperatively.**

(A) Masson's trichrome staining of cross sections of the proximal nerve stump. Collagen fibers are shown in blue and nerve fibers in pink. More collagen fibers (black arrow) were observed in the model group, and regular distributed nerve fibers (red arrow) were observed in the combination group. (B) Quantitative results of the percentage of collagen fiber area. (C) α-SMA immunohistochemical staining (brown) of cross sections of the proximal nerve stump. In the conduit and combination groups, α-SMA was mainly expressed in the walls of blood vessels (red arrow). (D) Quantitative results of the percentage of α-SMA positive staining area. (E) NF200 immunofluorescence staining of cross sections of the proximal nerve stump. The fluorescent indicator used was Alexa Fluor 594 for NF200 (red). Nuclei are shown in blue. Little axons (red arrow) were observed in the combination group. Scale bars: 50 μm in A, 100 μm in C and E. (F) Quantitative results of the density of regenerated axons. The axon density was defined as the number of axons within an area of 5000 μm$^2$. Data are expressed as the mean ± SD ($n = 5$ animals per group). ##$P < 0.01$, *vs*. model group; **$P < 0.01$, *vs*. conduit group (one-way analysis of variance followed by Tukey's *post hoc* test). NF200: Neurofilament-200; α-SMA: alpha-smooth muscle actin.

**Combination of MAG and chitin conduit improves the ultrastructure of regenerated axons in proximal nerve stumps in a rat model of sciatic nerve transection**

The ultrastructure characteristics of regenerated axons under the transmission electron microscope are illustrated in **Figure 3A**. As shown in **Figure 3B**, myelin sheath thickness in the combination group was significantly higher than that in the other two groups (both $P < 0.01$). Furthermore, myelin sheath thickness in the conduit group was significantly higher than that in the model group ($P < 0.01$). As shown in **Figure 3C**, the ratio of unmyelinated to myelinated axons in the combination group was significantly lower than those in the other two groups (both $P < 0.01$), and the ratio in the conduit group was lower than that in the model group ($P < 0.01$).

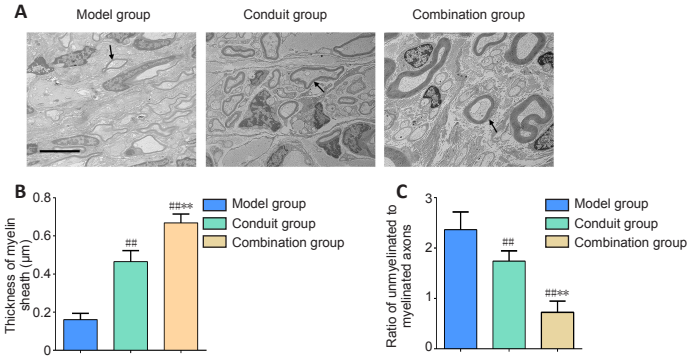


**Figure 3 | Effect of combination of myelin-associated glycoprotein and chitin conduit on the ultrastructure of regenerated axons in proximal nerve stumps 8 weeks postoperatively.**

(A) Transmission electron microscopy of cross sections of proximal nerve stumps in the model, conduit, and combination groups. Scattered axons with thick myelin sheaths were observed in the combination group. Black arrows indicate the myelin sheath. Scale bar: 5 μm. (B, C) Quantitative results of myelin sheath thickness (B), and the ratio of unmyelinated to myelinated axons (C). Data are expressed as the mean ± SD ($n = 5$ animals per group). ##$P < 0.01$, *vs*. model group; **$P < 0.01$, *vs*. conduit group (one-way analysis of variance followed by Tukey's *post hoc* test).

## Discussion

Although the peripheral nervous system has a certain ability to regenerate, complete structural and functional recovery following peripheral nerve injury is rarely achieved (Scheib and Höke, 2013; Gordon et al., 2015). Furthermore, regenerated nerve fibers can interweave with collagen connective tissue, forming painful neuromas (Neumeister and Winters, 2020). Although the exact mechanisms underlying painful neuroma formation are not well understood, many preventative methods have been proposed. These methods can be classified into two main categories – medication and surgical intervention (Yao et al., 2017; Lu et al., 2018).

In this study, we attempted to develop a combined method to inhibit traumatic neuroma formation. The autotomy score is frequently employed to evaluate the degree of neuropathic pain following peripheral nerve injury (Marcol et al., 2011; Yan et al., 2015; Yi et al., 2018). Eight weeks after surgery, we found that the autotomy score in the combination group was significantly lower than that in the conduit and model groups. However, many studies have argued that autotomy behaviors may be also associated with the external environment and psychological stress (Carr et al., 1992; Al-Qattan, 1999). NGF is a neurotrophic factor that is known to contribute to persistent neuropathic pain (Herzberg et al., 1997; Atherton et al., 2006). In this study, the NGF mRNA expression level was investigated to further compare the pain status between different groups; it was lowest in the combination group. This indicates that combined application of MAG and chitin conduit can effectively relieve neuropathic pain after sciatic nerve transection.

Masson's trichrome staining was carried out to assess the extent of nerve stump fibrosis in each group. A typical neuroma structure was found in the model group, with disorganized nerve fibers mixed with massive collagen tissue. With the aggravation of fibrosis, the regenerated axons can be squeezed by myofibroblasts, resulting in persistent mechanical stimulation (Foltán et al., 2008; Yan et al., 2012). To reflect myofibroblast activity precisely (Cui et al., 2021), the expression level of α-SMA mRNA and the distribution of α-SMA were investigated in this study; α-SMA was widely distributed and its mRNA expression level significantly higher in the model group than in the other two groups. Comprehensive analysis of these fibrosis indicators revealed that combined application of MAG and chitin conduit can effectively reduce fibrosis after sciatic nerve transection.

Axonal regeneration was investigated using neurofilament 200 immunofluorescence staining (Li et al., 2021), which revealed that the density of regenerated axons in the combination group was significantly lower than in the other two groups. One of the most important pathologic characteristics of painful neuroma is a high proportion of unmyelinated axons (Battista and Cravioto, 1981; Vora et al., 2005). The absence of a myelin sheath can seriously impair the electrical insulation of axons and allow cross-talk between adjacent axons. The degree of myelination was highest in the combination group, as revealed by the ratio of unmyelinated to myelinated axons and myelin sheath thickness of the regenerated axons.

This study has some limitations. The main limitation is the lack of comprehensive neuroma-related pain evaluation. In addition to NGF, there are other pain markers, such as substance P and c-fos (Yan et al., 2014; He et al., 2020). Further studies could adopt these markers to comprehensively evaluate neuroma-related pain after sciatic nerve transection. Another limitation is that we only investigated the effects of MAG local injection combined with chitin conduit on painful neuroma formation 8 weeks after surgery. The long-term effects of MAG local injection combined with chitin conduit on painful neuroma formation will be investigated in further studies.

In conclusion, combined application of MAG and chitin conduit can exert synergistic effects on inhibiting painful neuroma formation. This finding may help develop an effective therapy for painful neuroma in the clinic.

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