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# Potential risk of mitomycin C at high concentrations on peripheral nerve structure

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## Abstract

Although the local application of mitomycin C may prevent epidural adhesion after laminectomy, mitomycin C can induce neurotoxicity in optic and acoustic nerves at high concentrations. To determine the safe concentration range for mitomycin C, cotton pads soaked with mitomycin C at different concentrations (0.1, 0.3, 0.5, and 0.7 mg/mL) were immediately applied for 5 minutes to the operation area of rats that had undergone laminectomy at $L_1$. Rat sciatic nerves, instead of dorsal nerves, were used in this study. The results showed that mitomycin C at 0.1–0.5 mg/mL did not damage the structure and function of the sciatic nerve, while at 0.7 mg/mL, mitomycin C significantly reduced the thickness of the sciatic nerve myelin sheath compared with lower concentrations, though no functional change was found. These experimental findings indicate that the local application of mitomycin C at low concentrations is safe to prevent scar adhesion following laminectomy, but that mitomycin C at high concentrations (> 0.7 mg/mL) has potential safety risks to peripheral nerve structures.

***Key Words:*** *nerve regeneration; peripheral nerve injury; mitomycin C; myelin sheath; laminectomy; electrophysiology; nerve function; NSFC grant; neural regeneration*

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## Introduction

Epidural scar adhesion is understood to be a main contributing factor to undesirable postoperative symptoms following laminectomy. The scar that forms constrains nerve roots and impedes their normal motion in the vertebral column, causing the refractory leg and back pain that comprise the key features of 'failed back surgery syndrome' (Cauchoix et al., 1978; Aldrete, 1995; Guyer et al., 2006). Epidural scar adhesion also makes any subsequent operations in the same area dangerous and technically difficult (Chandler and Cappello, 2006; Jou et al., 2007). As a result, many surgical techniques and antiadhesion agents have been developed to minimize postoperative scarring (Ozer et al., 2006; Jou et al., 2007; Cemil et al., 2009; Rabb, 2010; Kasimcan et al., 2011).

Mitomycin C, a classical chemotherapeutic drug, is isolated from *Streptomyces caespitosus* or *Streptomyces lavendulae*. It is mainly used to treat upper (Keane et al., 1985; Wolf et al., 2010) and lower (Cummings et al., 1991; Ajani et al., 2008) gastrointestinal cancers and breast cancers (Konits et al., 1981; Vrdoljak et al., 2011) *via* intravenous infusion, as well as bladder tumors through bladder instillation (Mishina et al., 1975; Tolley et al., 1996). Accumulating evidence indicates that mitomycin C functions as an adjuvant therapy by preventing adhesion formation in ophthalmologic and otolaryngologic applications (Cruz, 1996; Schipper et al., 1997; Rahbar et al., 2000; Banthia and Selesnick, 2003). Recently, mitomycin C was shown to remarkably reduce epidural adhesion after laminectomy by decreasing scar formation (Lee et al., 2004, 2006a, 2006b; Yildiz et al., 2007; Liu et al., 2010). However, mitomycin C, as a chemotherapeutic agent, has inherent toxicity and other side effects. Several studies have demonstrated the side effects of mitomycin C, including decreased wound strength (Porter et al., 2006) and delayed wound healing (Ando et al., 1992; Demir et al., 2003; Su et al., 2012). The topical application of mitomycin C produced substantial sensorineural hearing loss and was ototoxic to the middle ear in gerbils (Moody et al., 2006). In addition, mitomycin C also had a toxic effect on the optic nerve and ciliary body (Mietz et al., 1997; Cetinkaya et al., 2008).

During topical application, mitomycin C soaks the dura mater, spinal nerve, and surrounding soft tissues directly. However, an intact blood-brain barrier in the brain and spinal cord prevented mitomycin C from producing adverse effects in the central nervous system (Schwartz and Philips,

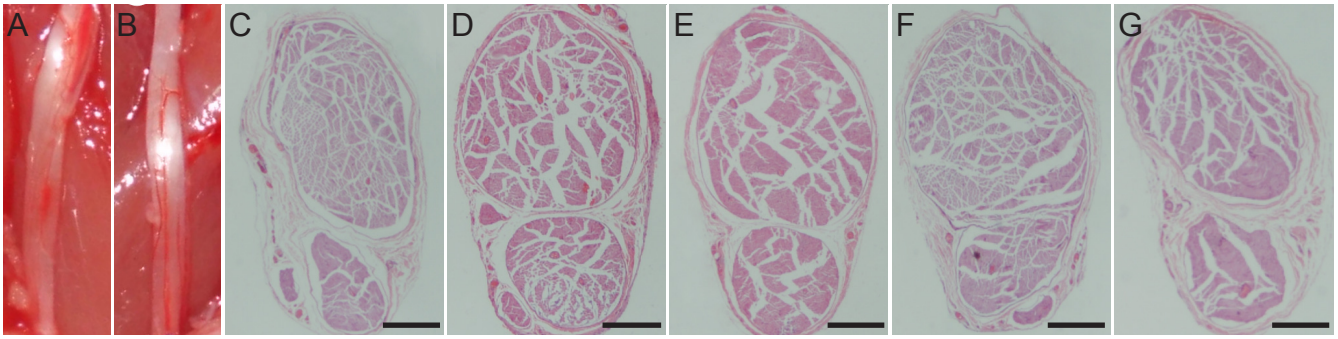


**Figure 1 Effect of mitomycin C on the structure of sciatic nerve.**
(A, B) Gross anatomy of the sciatic nerve after saline and mitomycin C treatments. (A) In the control group, 5 days after saline treatment, the normal diameter of sciatic nerve in rats was observed. (B) In the 0.7 mg/mL mitomycin C-treated group, 5 days after treatment, the diameter of the sciatic nerve remained similar to that in the control group. Photos were taken of anesthetized rats. (C–G) Hematoxylin-eosin staining of the sciatic nerve 5 days after treatment with saline and 0.1, 0.3, 0.5, and 0.7 mg/mL mitomycin C. There were no significant differences in the epineurium, diameter, or axonal density among the groups. Scale bars in C–G: 150 μm.

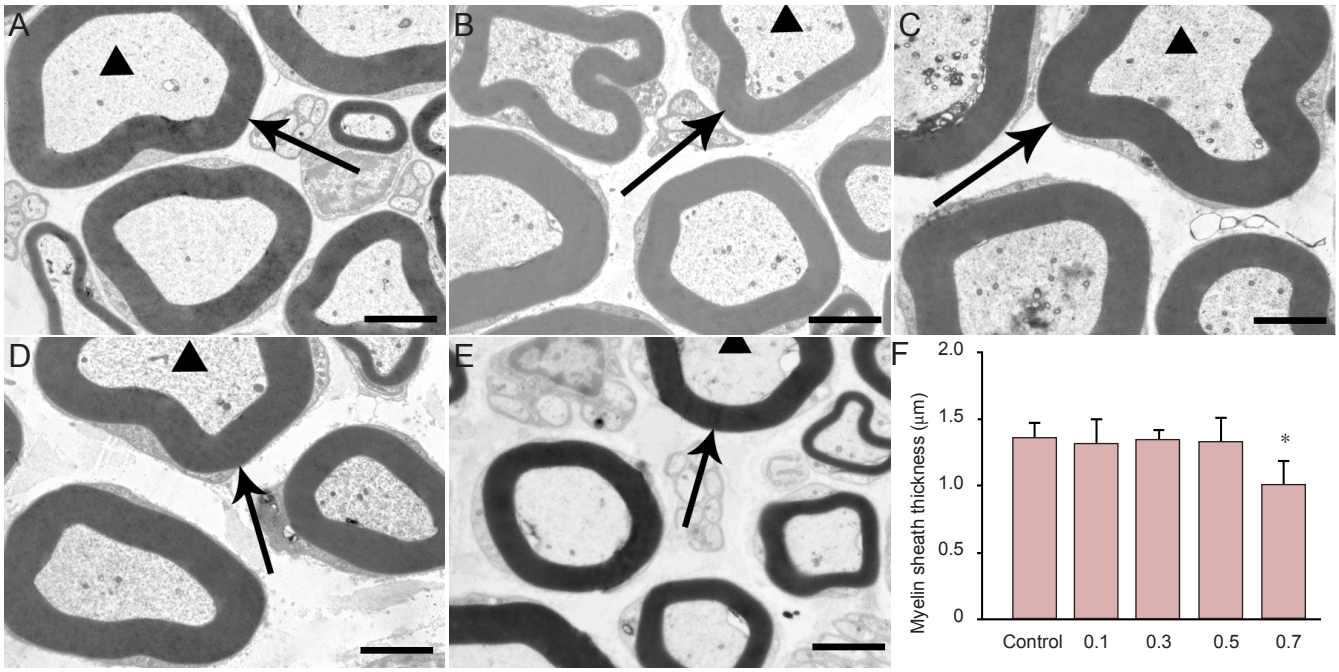


**Figure 2 Effect of mitomycin C on the myelin sheath and axons of the sciatic nerve.**
(A–E) Transmission electron microscope images of sciatic nerves at 5 days after saline and mitomycin C treatment. (A) The myelin sheath (arrow) from saline-treated rats was intact, and the axons (arrowhead) were arranged in a regular fashion. Similarly, the myelin sheaths (arrows) and axons (arrowheads) from the 0.1 (B), 0.3 (C), and 0.5 mg/mL mitomycin C groups (D) are shown. (E) The myelin sheath (arrow) and axons (arrowhead) from the 0.7 mg/mL mitomycin C group are intact. However, the myelin sheath thickness was significantly decreased. Scale bars: 2 μm.
(F) The myelin sheath thickness in the sciatic nerve for each treatment group. Data are expressed as mean ± SD of three rats in each group. $*P < 0.05$, *vs.* all other groups. Differences between groups were evaluated using two-tailed Student's *t*-tests. 0.1–0.7: 0.1, 0.3, 0.5, 0.7 mg/mL mitomycin C treatment groups.

1961; Lee et al., 2006b). Within the spinal canal, spinal nerves and the spinal cord are connected by the ventral and dorsal roots. The spinal nerve is part of the peripheral nervous system and is not protected from mitomycin C by the blood-brain barrier. Little is known about the safety risks of local application of mitomycin C on the function and morphology of peripheral nerves after laminectomy. In the present study, the spinal nerve and sciatic nerve of rats following laminectomy were used to investigate the safety risks of local application of mitomycin C on the function and morphology of peripheral nerves in rats. We aimed to assess the potential safety problems of topical application of mitomycin C after laminectomy.

## Materials and Methods

### Animals

One hundred and twenty adult, clean level Sprague-Dawley rats weighing 170–200 g were provided by the Experimental Animal Center of Nanjing Medical University, China (license No. SYXK (Su) 2008-0007). The animals were maintained

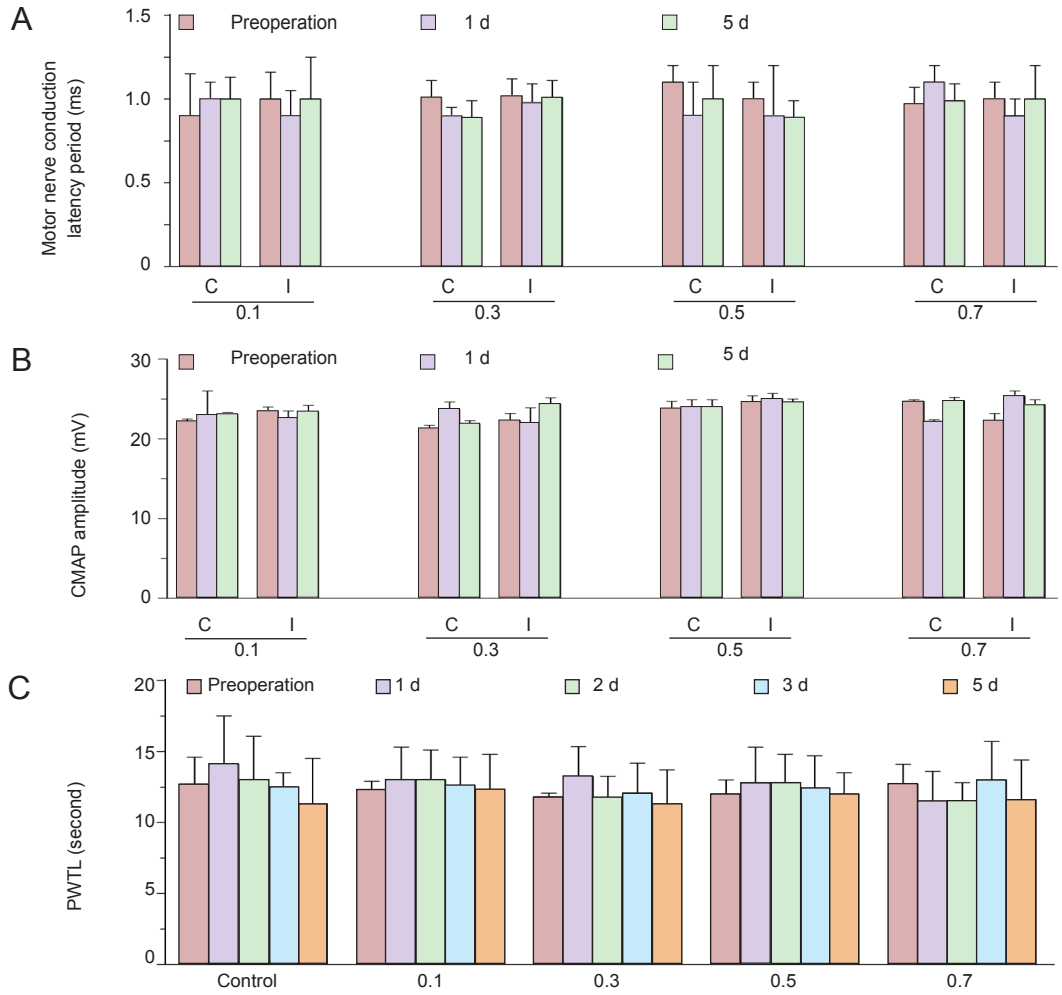


**Figure 3 Effects of topical application of mitomycin C (0.1, 0.3, 0.5, and 0.7 mg/mL) on motor nerve conduction latency (A), amplitude of the compound muscle action potential (CMAP) (B) and paw withdrawal thermal latency (PWTL) (C) before and after surgery.**
Data are expressed as mean ± SD of three rats in each group. Differences between groups were evaluated using two-tailed Student's *t*-tests. C: Sciatic nerve at the non-injured side; I: sciatic nerve at the injured side; 0.1–0.7: 0.1, 0.3, 0.5, 0.7 mg/mL mitomycin C treatment groups; 1, 2, 3, and 5 d: 1, 2, 3, and 5 days after surgery.

under standard laboratory conditions (12-hour light/dark cycle, 18–26°C, 40–70% humidity). All rats were housed in a conventional animal facility and were randomly divided into five groups: a control group and 0.1, 0.3, 0.5, and 0.7 mg/mL mitomycin C treatment groups. All experimental procedures conformed to the Guideline for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and were approved by the Ethics Committee of Nanjing Medical University, China.

**Rat models of laminectomy and sciatic nerve exposure**

The rat laminectomy model was performed essentially as described previously (Lee et al., 2004). In brief, rats were anesthetized with pentobarbital (50 mg/kg) *via* intraperitoneal injection. After the hair around $L_{4–5}$ was shaved, the skin was sterilized with an iodine solution. A dorsal skin incision was made and continued deep to the spinous process. The paraspinal muscles were stripped away from the lamina and spinous process. Left laminectomies of $L_{4–5}$ were carried out using rongeurs. Next, a laminectomy defect was created, leaving the dura mater clean and fully exposed. This model was used for the paw withdrawal thermal latency assay.

The left sciatic nerve exposure rat model was also performed essentially as described previously (Ilbay et al., 2005). In brief, after anesthesia, the rat sciatic nerve was exposed and isolated from the surrounding tissues, using aseptic technique to bluntly separate the peroneal and tibial components back toward the sciatic foramen. This model was used for the neural electrophysiological recordings and histological evaluations.

**Topical application of mitomycin C**

After hemostasis was achieved, pieces of cotton wool (1 cm × 1 cm) were soaked with 0.1, 0.3, 0.5, and 0.7 mg/mL mitomycin C (powder purity: 100%, dissolved in saline; Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) at previously reported concentrations (Su et al., 2010; Lee et al., 2006a, b) or saline. After soaking for 1 minute, the impregnated cotton wool pieces were taken out of solution and allowed to drip until

no further water droplets formed. The surgeons then placed the impregnated cotton wool around the surgical area for 5 minutes. Next, the soaked cotton pads were removed and the laminectomy area or the exposed sciatic nerve was washed with saline to rinse away any leftover reagent. The wound was closed layer by layer in all rats using the same suture material. No prophylactic antibiotics were used.

**Histological evaluation**

*Hematoxylin-eosin staining*

The sciatic nerve exposure model rats were used for histological assessment of the 0.1, 0.3, 0.5, and 0.7 mg/mL mitomycin C groups and the control group by blinded surgical dissection. At 5 days after the topical application of mitomycin C or saline to the sciatic nerve, all rats were killed by intraperitoneal injection of an overdose of pentobarbital (60 mg/kg). The separated sciatic nerve was fixed with 10% formalin and embedded in paraffin for sectioning. Nine successive transversal sections of 5 μm thickness were obtained from the sciatic nerve and stained with hematoxylin-eosin. The sciatic nerve structure and morphology were evaluated under a light microscope (FDX-35, Nikon, Tokyo, Japan).

*Transmission electron microscope*

For electron microscopy (JEM-1010, JEOL Ltd., Tokyo, Japan), samples were excised, rinsed three times in buffer, and then fixed in 5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.3) overnight at 4°C. The samples were then postfixed in 1% osmium tetroxide for 2 hours at 4°C. After dehydration and embedding in spurr resin, thick sections (50 μm thickness) were stained with 1% toluidine blue in 1% borax at 60°C. Finally, thin sections (50 to 80 nm thickness) were cut and stained in uranyl acetate and lead citrate.

**Neuroelectrophysiological recording**

Electrophysiological evaluation, including motor nerve conduction latency (Snooks et al., 1985; Tankisi et al., 2007) and measurement of compound muscle action potential (Krarup et al., 2002; Laughlin et al., 2011) was used to assess neurological function before mitomycin C treatment, and at 1 and 5 days after mitomycin C or saline treatment in the sciatic nerve exposure model rats. A bipolar stimulating probe (Medtronic, Jacksonville, FL, USA) connected to a constant current stimulator that delivered monophasic square wave pulses was selected to perform the electrical stimulation. The stimulating electrode was placed in the sciatic nerve with a 2-mm separation between the electrodes proximally at the spine. The motor nerve conduction latency and compound muscle action potential were recorded using similar bipolar probes placed in the belly of the gastrocnemius. To assure supramaximal stimulation at the beginning of the experiment, the electrical stimulation was in the range of 2–15 mA. The duration of each stimulus was 0.1 ms.

**Paw withdrawal thermal latency assay**

The laminectomy model rats from each group were used for the paw withdrawal thermal latency assay to assess neurological function before the operation and during the acute phase at 1, 2, 3, and 5 days after mitomycin C or saline treatment. The paw withdrawal thermal latency assay was performed as previously described (Li and Chen, 2004). In brief, the rats were placed in a transparent plexiglass chamber to measure their heat sensitivity using a plantar test apparatus (IITC Life Science Inc., Los Angeles, CA, USA). Stimuli were repeated four or five times to calculate the mean paw withdrawal thermal latency for each rat. The inter-stimulus interval was more than 10 minutes. If the latency exceeded 25 seconds, the stimulus was stopped to avoid excessive tissue injury, and the region was considered to have had no response.

**Statistical analysis**

Data are expressed as the median and statistical range of the *H*-score for each group. All other results are expressed as mean ± SD. Differences between the groups were evaluated using two-tailed Student's *t*-tests. The statistical analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). *P*-values less than 0.05 were considered to be statistically significant.

## Results

**Effect of mitomycin C on general conditions of laminectomy model rats**

No remarkable differences were found in the signs of hind limb neurological function after saline or mitomycin C treatment, and no treated rats exhibited a full-thickness wound dehiscence.

**Effect of mitomycin C on sciatic nerve morphology of laminectomy model rats**

Hematoxylin-eosin staining revealed no damage to the sciatic nerve structure or morphology in the control and 0.7 mg/mL mitomycin C-treated group rats. The epineurium remained intact, and the diameter and axonal density of the sciatic nerve were similar in the control and all mitomycin C-treated groups during the first 5 days after the operation (Figure 1).

By electron microscopy, the axons were arranged in a regular fashion in both the saline and mitomycin C-treated groups. The astrocytes displayed normal morphology. The small and large vessels presented a regular appearance with a smooth, thin basement membrane. The collagen surrounding the vessels was distributed and the pial septa did not show consistent differences between the treated and control nerves. The thickness of the myelin sheaths in the 0.7 mg/mL mitomycin C-treated group was significantly lower than those of the other mitomycin C-treated groups and the control group 5 days after treatment ($P < 0.05$; Figure 2).

**Effects of mitomycin C on the behavior of laminectomy model rats**

*Motor nerve conduction latency*

Square wave pulse stimulation was applied to the injured (left) sciatic nerve in the 0.1 mg/mL treatment group before and at 1 and 5 days after mitomycin C treatment. The motor nerve conduction latencies on the right side were similar to those on the left side ($P > 0.05$). For pulse stimulation to the left and right sciatic nerve in the 0.3, 0.5, and 0.7 mg/mL groups before and at 1 and 5 days after mitomycin C

treatment, the motor nerve conduction latency recorded at the gastrocnemius was similar in all groups ($P > 0.05$). The motor nerve conduction latency on both the injured and control sides was also similar in all groups before and at 1 and 5 days after the operation ($P > 0.05$; Figure 3A).

*Compound muscle action potential amplitude*

After stimulation to the injured (left) sciatic nerve in the 0.1, 0.3, 0.5, and 0.7 mg/mL mitomycin C-treated groups before and at 1 and 5 days after the operation, the amplitudes of the compound muscle action potential at the gastrocnemius on the injured side were not significantly different among the groups ($P > 0.05$). During stimulation to the right side, the mean amplitude of the compound muscle action potential recorded was similar to those of the injured side ($P > 0.05$; Figure 3B).

*Paw withdrawal thermal latency*

As shown in Figure 3C, the paw withdrawal thermal latencies were all within the normal range (Dirig et al., 1997) before and at 1, 2, 3, and 5 days after saline or mitomycin C treatment. The paw withdrawal thermal latency showed no significant differences in any groups before and at 1, 2, 3, and 5 days after saline or mitomycin C treatment ($P > 0.05$).

## Discussion

One of the major problems in failed back surgery syndrome is epidural scar formation (Burton et al., 1981). The epidural scars that form can lead to intractable pain and sensory and motor deficits because of compression and/or tethering of the nerve roots. Many studies have attempted to prevent epidural scar formation by applying various drugs and materials, such as recombinant tissue-plasminogen activator gel, bioelastic polymers, hyaluronan, and Adcon-L (Henderson et al., 1993; Alkalay et al., 2003; Ganzer et al., 2003; Massie et al., 2005).

Since it was first reported that mitomycin C prevented epidural fibrosis after laminectomy (Henderson et al., 1993), a number of studies have shown its ability to decrease fibroblast proliferation and induce fibroblast apoptosis, which leads to the reduction of scar formation (Henderson et al., 1993; Dogulu et al., 2003; Massie et al., 2005; Lee et al., 2006a, b). With accumulating experience, however, several adverse effects of mitomycin C treatment have appeared, such as delays in wound healing and neurotoxicity (Rubinfeld et al., 1992; Moody et al., 2006; Su et al., 2012). Still, the safety of local application of mitomycin C on peripheral nerves after laminectomy is not completely understood. In the present study, a rat model was used to investigate the impact of topical mitomycin C application on peripheral nerves.

The sciatic nerve is a large nerve in both humans and animals that is derived from the $L_{4-5}$ spinal nerves and runs cross the buttocks and down the lower limb. It contains both the anterior and posterior fibers of the lumbosacral plexus (Schmalbruch, 1986; Rigaud et al., 2008). The sciatic nerve is widely used to evaluate the effects of various agents on nerve function. In the present study, sciatic nerve was used to perform neural electrophysiological tests and histological observations to evaluate the effects of the local application of mitomycin C on peripheral nerves.

These results confirm that the local application of mitomycin C at less than 0.5 mg/mL was safe for rat peripheral nerve. The wound healing was complete in all groups, and no muscle cavity was found in any of the mitomycin C treatment groups. The gross anatomy and hematoxylin-eosin staining showed that mitomycin C did not damage the structure or epineurium of the sciatic nerve. However, the electron microscopy of the sciatic nerve after mitomycin C treatment demonstrated that the thickness of the myelin sheath was significantly decreased in the 0.7 mg/mL group compared with the control and other concentrations groups. It is well known that the essential parameters for nerve conduction efficiency include the myelin sheath length and diameter (Waxman, 1997). In addition, numerous peripheral neuropathies can affect myelin sheath thickness and cause compact myelin deposition (Suter and Scherer, 2003). To determine whether this decrease affected the function of the sciatic nerve, we conducted the neural electrophysiological test and paw withdrawal thermal latency assay.

Peripheral nerve and nerve root injuries that increase the excitability of neurons and lead to central sensitization in the spinal dorsal horn are thought to constitute the conditions of allodynia and hyperalgesia (Wagner et al., 1998). Hyperalgesia results in notably shorter paw withdrawal latencies compared with normal conditions (Hargreaves et al., 1988; Zhang et al., 2008). In the present study, the laminectomy model rat was used for the paw withdrawal thermal latency assay. The assay results showed no significant differences between any groups either before or at 1 or 5 days after the operation. The paw withdrawal thermal latency assay results suggest that hyperalgia was not an effect of the topical application of mitomycin C on spinal nerve function.

Alterations to motor nerve conduction latency and compound muscle action potential amplitude reflect the function of the myelin sheath and the axons of motor neurons during acute nerve injury (Sun et al., 2007). The values of these neural electrophysiological parameters measured in the present study on sciatic nerve did not show any changes with increasing concentration of mitomycin C. The local application of mitomycin C at concentrations below 0.7 mg/mL does not appear to harm myelin sheath function or the axons of the sciatic nerve in the short term.

The local or systemic applications of mitomycin C may cause dose-dependent complications such as inflammation, hypervascularization, hematoma, and necrosis due to its antiproliferative effects (Rubinfeld et al., 1992; Kureshi et al., 2006; Mearza and Aslanides, 2007). In the present study, no harmful effects of mitomycin C on the function of sciatic nerve were observed after treatment. This lack of any differences may be caused by the low mitomycin C doses, short application times, and washing of the operation site with plenty of normal saline. However, treatment with the 0.7 mg/mL mitomycin C reduced the myelin sheath thickness, though this reduction did not influence the function of the sciatic nerve. The magnitude of this reduction may

not have passed the threshold necessary to effect a change in function, or the repair of the myelin sheath could have been too rapid for this change to affect nerve function. The topical use of mitomycin C below 0.5 mg/mL appears to have no effect on the spinal nerve after laminectomy in the short term because this dosage is nontoxic to the sciatic nerve in rats. However, a longer follow-up time must be assessed in future studies before drawing a firm conclusion. A large animal model, such as the dog laminectomy model, can help surgeons to minimize the iatrogenic spinal nerve injury during the operation. In a future study, the dog laminectomy model will be used to investigate the effects of local application of mitomycin C on the spinal nerve in the spinal canal. A better way to control the dose administered would help to improve the reproducibility of the experiment.

In conclusion, the local application of mitomycin C below 0.5 mg/mL for preventing post-laminectomy epidural scar formation was demonstrated to have no effect on the peripheral nerve. The local application of mitomycin C at concentrations over 0.7 mg/mL, however, may have potential adverse effects on the peripheral nerve by decreasing the thickness of the myelin sheath. A follow-up study using a large animal model is needed to confirm the effects of the local application of mitomycin C on the sciatic nerve.

**Author contributions:** *Sui T and Zhang JH conducted the experiments, collected and analyzed data, and wrote the manuscript. Cao XJ was in charge of funds, guided the study, and provided technical support. Du SH, Su CH and Que J participated in data analysis and provided technical support. All authors approved the final version of the manuscript.*

**Conflicts of interest:** *None declared.*

**Peer review:** *Mitomycin can prevent adhesion, the existing hypothesis that it can affect the central nervous system and peripheral nervous system has insufficient theoretical evidence. This study aimed to discuss the influence of mitomycin at different concentrations on the morphology and function of peripheral nervous system, providing clinical guidance value.*

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