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Effects of different doses of X-ray radiation on nerve regeneration after sciatic nerve injury in a rat model

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► ***Original article***

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ABSTRACT

Background: Physical agents, such as ultrasound, can promote functional restoration and regenerative processes of the peripheral nervous system. However, little is known about the effects of X-ray radiation on nerve regeneration after peripheral nerve injury. The aim of the present study was to investigate the effects of various doses of X-ray radiation on nerve regeneration after sciatic nerve injury in rats. ***Materials and Methods:*** The sciatic nerves of Sprague-Dawley rats were transected and repaired via epineurium end-to-end neurorrhaphy. Eighty rats each received single and local X-ray doses of 0 Gy, 0.2 Gy, 1 Gy, 7 Gy and 14 Gy. Functional and morphological assessments of the process of nerve regeneration were performed by using various measurement tools. ***Results:*** Compared with the 0 Gy, 0.2 Gy and 14 Gy groups, the 1 Gy and 7 Gy radiation groups experienced significantly increased sciatic functional index, motor nerve conductive velocity (MNCV), expression of S-100, mean diameter of axons, and thickness of myelin sheaths and decreased perineural scar tissue. There were no differences between the 1 Gy group and the 7 Gy group or between the 0 Gy group, the 0.2 Gy group and the 14 Gy group with the exception of MNCV and the expression level of S-100. ***Conclusion:*** X-ray radiation in doses of 1 Gy and 7 Gy promoted nerve regeneration after sciatic nerve injury in a rat model. The dose of 14 Gy exerted inhibitory effects, and 0.2 Gy exerted no significant effect on nerve regeneration.

Keywords: *Peripheral nerve injury, nerve regeneration, X-ray irradiation.*

INTRODUCTION

The treatment and rehabilitation of injured peripheral nerves currently represents a challenge. The reconstruction of nerve continuity is a prerequisite for nerve regeneration following transection. Despite continued technical improvements in microsurgical techniques, functional restoration is generally poor following repair of transected peripheral nerves [1].

Physical agents, such as electricity, magnetic fields, therapeutic shock waves and lasers, positively influence the functional recovery and regenerative process of peripheral nerves [2-5]. Low-dose irradiation (LDI) can inhibit the activity of fibroblasts and osteoblasts. LDI was safely used for many years for the prevention and treatment of various diseases in adults, such as heterotopic ossification and cheloid [6, 7]. LDI facilitates the restoration of ischaemic limbs and accelerates wound healing by stimulating blood vessel formation by increasing the expression of genes favouring angiogenesis [8, 9]. Low-dose external beam radiation can significantly reduce epidural fibrosis following laminectomy and prevent epineurial and intraneural scar formation after sciatic nerve injury in a rat

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model [6, 7, 10, 11]. However, the correlation between morphometry, electrophysiology and different doses of X-ray radiation has not been thoroughly to date. Jiang et al. revealed that low-dose radiation (1 Gy) could induce increased production of vascular endothelial growth factor (VEGF) and growth-associated protein-43 (GAP-43), thereby promoting nerve regeneration after peripheral nerve injury [12].

To the best of our knowledge, no studies have investigated how different low doses of radiation influence the regeneration of injured peripheral nerves. Therefore, the primary aim of the present study was to explore the effects of different doses of X-ray radiation on the nerve regeneration and electrophysiological recovery of injured peripheral nerves in an adult rat model.

MATERIALS AND METHODS

Animals

Eighty 6- to 8-week-old male Sprague-Dawley rats weighing between 200 to 250 g were provided by the Laboratory Animal Centre of the Medical College of Soochow University, China [SYXK (Su) 2013-0003]. Experiments were performed following the National Institutes of Health guide for animal experiments and were approved by the experimental animal care and use committee of Soochow University. The animals were anaesthetised by an intraperitoneal administration of chloral hydrate (350 mg per kg of the body weight) throughout the surgical procedures. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques.

Surgical procedures

Following the anaesthesia, the left hindquarter of each rat was shaved and sterilised. The sciatic nerve was exposed and sharply transected with a microscissors at 10 mm above the sciatic nerve bifurcation. The two nerve stumps were sutured at the site without tension via standard epineurium end-to-end neurorrhaphy with 10-0 microsurgical sutures (Prolene, Ethicon, Somerville, NJ, USA). Then, the muscle was sutured with resorbable 4-0 sutures, and the skin was sutured with 4-0 nylon. Each rat was housed individually.

Groups and radiation procedure

At 24 hours after establishing the model of peripheral nerve injury, the eighty rats were randomly divided into five groups (n = 16). The animals in each group received local and single X -ray radiation doses of 0 Gy, 0.2 Gy, 1 Gy, 7 Gy or 14 Gy, at a dose rate of 200 cGy/min via the 6 MV photon beam of a medical electron linear accelerator (Primus, Siemens Medical Systems, Concord, CA, USA). The length × width of the field was 40 mm × 40 mm, which was centred at the incision site (along the posterior thigh and buttock) with a silicone gel bolus of 0.5 cm to obtain a 100% dose to the skin. The isodose distribution was such that the sciatic nerve tractus at a depth of 0.5 cm from the skin surface received at least 90% of the prescribed dose [6, 12].

The animals were sacrificed at the end of the 12th week postoperatively when electrophysiological examination, haematoxylin and eosin (HE) staining, immunohistochemical staining of S-100 and ultrastructural observation were performed. All measurement indicators were performed by an investigator blinded to the experimental allocation.

Walking track analysis of sciatic functional index (SFI)

An investigator who was blinded to experimental allocation performed walking track analysis 4, 8, 12 weeks post-neurorrhaphy. The plantar surface of both hindlimbs of each rat was dipped in black ink. After that step, the rats were allowed to run towards a dark tunnel on the white paper such that the footprints could be recorded. The lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second to the fourth toe (ITS) were measured on the experimental side (EPL, ETS, and EIT, respectively) and the contralateral (normal) side (NPL, NTS, and NIT, respectively) in each rat. The SFI value in each rat was calculated using following formula: SFI=−38.3 × (EPL − NPL)/

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NPL + 109.5 × (ETS − NTS)/NTS + 13.3 × (EITS − NITS)/ NITS−8.8. In general, the SFI value oscillates approximately 0 for normal nerve function, whereas around -100 represents total dysfunction [13].

Electrophysiological study

Twelve weeks post-neurorrhaphy, electrophysiological analysis was performed. Eight rats in each group were randomly selected and anaesthetised, and the repaired nerve was exposed as mentioned above. Stimulation electrodes were placed in the sciatic nerve trunk, the recording electrode was placed in the gastrocnemius muscle, and the ground electrode was placed in the subcutaneous tissue. An electrical stimulus was applied with a 1-20 mA intensity. The stimulus frequency was 1 Hz, and the duration was 0.1-0.2 ms. Data for the latency of onset and the peak amplitude of the compound muscle action potential (CMAP) were recorded, and the motor nerve conductive velocity (MNCV) value was calculated.

Evaluation of perineural scar tissue

Twelve weeks post-neurorrhaphy, after the electrophysiological evaluation, eight rats of each group were randomly selected for gross morphological observation of the anastomosis site. The fibrous connective tissue surrounding the repair site was examined using a surgical microscope. The numerical grading scheme of Petersen [14] for gross evaluation of scars was used to evaluate scar severity and nerve adherence as a macroscopic assessment.

Tissue harvest

Twelve weeks post-neurorrhaphy, after perineural scar tissue evaluations had been carried out, the rats were sacrificed by cervical dislocation. The regenerated nerves within 5 mm from the proximal anastomosis site, at the anastomosis site, and 5 mm from the distal anastomosis site were harvested for haematoxylin-eosin and immunohistochemical staining and ultrastructural observation.

Histological examination

Twelve weeks post-neurorrhaphy, the regenerated nerve specimens of eight rats in each group were fixed in 4% paraformaldehyde solution, dehydrated, and embedded in paraffin. Six-micrometre-thick transverse sections were obtained, dewaxed, and stained with haematoxylin and eosin (Sinopharm, Shanghai, China). Images were captured using an Olympus BX51T-PHD-J11 light microscope.

Immunohistochemical analysis

Twelve weeks post-neurorrhaphy, the regenerated nerve specimens of eight rats in each group were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde solution at 4°C overnight. The specimens were dehydrated with an increasing alcohol series starting at 70% ethanol, followed by embedding in paraffin blocks. Five-micrometre-thick transverse sections were placed on poly-L-lysine -coated slides and stored overnight at 60°C. The slides were incubated with primary antibodies of rabbit monoclonal against S-100 (1:200, Sigma, St Louis, MO, USA) at 4°C overnight, washed with PBS and incubated with the appropriate secondary antibodies of biotin-conjugated goat anti-mouse IgG (1:100, Sigma, St Louis, MO, USA) at room temperature for 1 hour. After washing with PBS, the slides were incubated with horseradish peroxidase–conjugated streptavidin (1:100, Sigma, St Louis, MO, USA) for 1 hour. Then, the sections were incubated in haematoxylin (Sinopharm, Shanghai, China) for counterstaining of the nucleus. All sections were examined at five randomly selected fields at 400× magnification using the Olympus BX51 T-PHD-J11light microscope and photographed using a Canon EOS550D digital colour camera. The digital images were analysed using computer-based morphometry software (WinROOF, Mitani, Fukui, Japan). According to the automatically calculated parameters, the area of cells staining positive for nuclear or cytoplasmic S-100 was measured, expressed as a percentage of total area.

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Ultrastructural observation

Twelve weeks post-neurorrhaphy, the nerve specimens of eight rats in each group were washed with PBS and fixed in 4% glutaraldehyde solution for 4 hours, washed with PBS and immersed in 1% osmium tetroxide solution for 1 hour, and washed in PBS solution again. The nerves were then dehydrated using an increasing acetone series starting at 30% acetone. The tissues were embedded in Araldite CY212 (Agar, Stansted, UK) for 2 hours. The moulds were incubated at 40°C for 24 hours and at 60°C for 48 hours. Ultrathin sections with a thickness of 50 nm were obtained using a Leica EM UC6 Ultramicrotome. The tissue sections were then stained with 1% uranyl acetate and lead citrate (Baoman Biotechnology, Shanghai, China). All nerve sections were observed under a Hitachi H-600 transmission electron microscope (TEM), and images were captured using a Canon EOS550D camera.

Statistical analysis

All data are expressed as the mean values ± the standard deviation. Data were evaluated by one-way analysis of variance (ANOVA) with the post hoc Tukey t-test for comparisons between groups using Statistical Package for the Social Sciences ver. 17.0 statistics software (SPSS, Chicago, IL, USA). Probability values of less than 0.05 were considered to be significant.

RESULTS

Functional restoration of the repaired sciatic nerve

Four, 8 and 12 weeks post-neurorrhaphy, the sciatic nerve function index value of the five groups revealed improvement of various degrees. The SFI value significantly increased in the 1 Gy radiation group and the 7 Gy radiation group compared with the 0 Gy radiation group, the 0.2 Gy radiation group and the 14 Gy radiation group at both 4 and 8 weeks ($p<0.05$). No significant difference was observed between the 1 Gy radiation group and 7 Gy radiation group ($p>0.05$), nor among 0 Gy radiation group, 0.2 Gy radiation group and 14 Gy radiation group ($p>0.05$). At 12 weeks, no significant difference was observed among the 0 Gy radiation group, 0.2 Gy radiation group, 1 Gy radiation group and 7 Gy radiation group ($p>0.05$), but they all had a significantly better recovery compared with the 14 Gy radiation group ($p<0.05$; figure 1).

Electrophysiological study

The latencies of CMAP onset in the 1 Gy radiation group and 7 Gy radiation group were significantly shorter than those in the 0 Gy radiation group, 0.2 Gy radiation group and 14 Gy radiation group ($p<0.05$), and CMAP onset in the 0 Gy radiation group and 0.2 Gy radiation group was significantly shorter than that in the 14 Gy radiation group ($p<0.05$). No statistically significant differences were found between th e 1 Gy radiation group and 7 Gy radiation group or the 0 Gy radiation group and 0.2 Gy radiation group (figure 2A). The peak amplitudes of CMAP and MNCV in the 1 Gy radiation group and 7 Gy radiation group were significantly higher than those in the 0 Gy radiation group, 0.2 Gy radiation group and 14 Gy radiation group ($p<0.05$), and those in the 0 Gy radiation group and 0.2 Gy radiation group were significantly higher than those in the 14 Gy radiation group ($p<0.05$). No statistically significant differences were found between the 1 Gy radiation group and 7 Gy radiation group or between the 0 Gy radiation group and 0.2 Gy radiation group ($p>0.05$; figure 2B and C).

Evaluation of perineural scar tissue

In the 0 Gy radiation group, 0.2 Gy radiation group and 14 Gy radiation group, thick, tenacious epineurial scar tissue enveloped and tethered the nerves. Nerve isolation and separation often required strong, blunt, and sometimes violent dissection. The regenerated nerves in the 1 Gy radiation group and 7 Gy radiation group were encircled by a very slight and lucent membrane. Less scarring was present in these two groups, and the nerve was also less tenacious and could be easily separated from the surrounding tissue compared with the 0 Gy radiation group, 0.2 Gy radiation group and 14 Gy radiation group. Significantly decreased

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scores for nerve adherence and nerve separability in the 1 Gy radiation group and 7 Gy radiation group were observed compared with the 0 Gy radiation group, 0.2 Gy radiation group and 14 Gy radiation group (p<0.05). No statistically significant differences were found between the 1 Gy radiation group and 7 Gy radiation group (p>0.05) or between the 0 Gy radiation group, 0.2 Gy radiation group and 14 Gy radiation group (p>0.05; figure 3A).

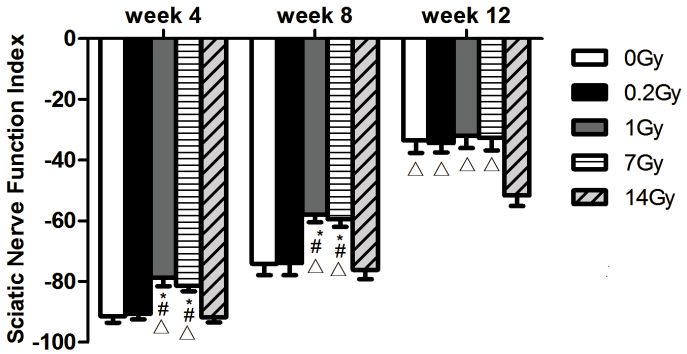

Figure 1. Motor function recovery assessed with walking track analysis. *P < 0.05 vs 0 Gy group, #P < 0.05 vs 0.2 Gy group, ΔP < 0.05 vs 14 Gy group.

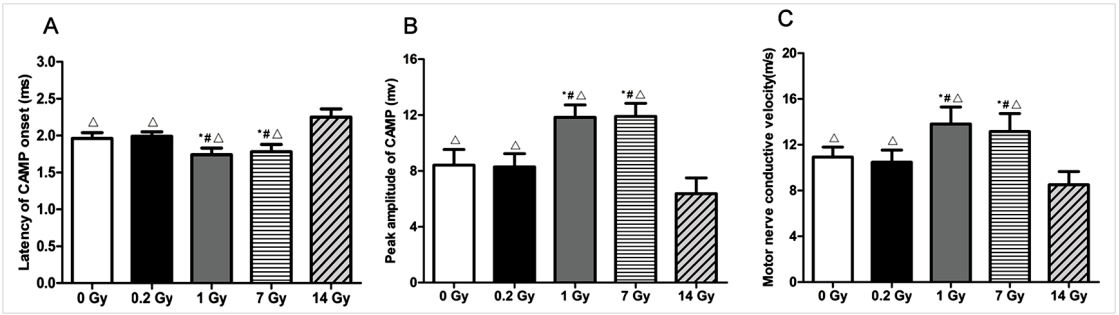

Figure 2. Results of the electrophysiological study among groups. **(A)** The latency of onset of CAMP. **(B)** The peak amplitude of CAMP. **(C)** The motor nerve conductive velocity. *P < 0.05 vs 0 Gy group, #P < 0.05 vs 0.2 Gy group, ΔP < 0.05 vs 14 Gy group.

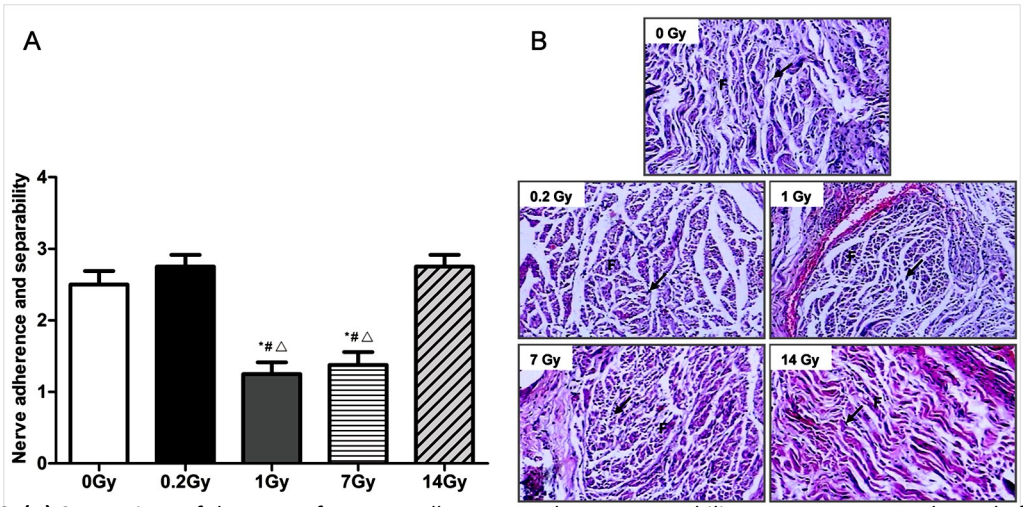

Figure 3. (A) Comparison of the scores for nerve adherence and nerve separability among groups at the end of 12 weeks post-neurorrhaphy. **(B)** Light micrographs showing cross-sections of the regenerated nerves stained with HE (magnification × 40). *P<0.05 vs 0 Gy group, #P<0.05 vs 0.2 Gy group, ΔP<0.05 vs 14 Gy group. Black arrows show inflammatory cell, F: The regenerated nerve fibre.

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Histological examination

HE staining demonstrated that the tissue structure was slightly loosened in the 1 Gy radiation group and 7 Gy radiation group. The regenerated nerve fibres were densely dispersed and a few inflammatory cells were scattered around the axons. In the 0 Gy radiation group, 0.2 Gy radiation group and 14 Gy radiation group, the tissue structure was loosened. The nerve fibres were sparsely dispersed and numerous inflammatory cells infiltrated around the axons (figure 3B).

Immunohistochemical analysis

The immunohistochemical staining showed different degrees of S-100 expression, shown by brown-stained nuclei, in the five groups (figure 4A). The level of positive expression of S-100 was the highest in the 1 Gy radiation group, medium in the 7 Gy radiation group, lower in the 0 Gy radiation group and 0.2 Gy radiation group, and lowest in the 14 Gy radiation group (p<0.05; figure 4B).

Ultrastructural observation

The transmission electron microscopic examination showed the shape of the regenerated myelin sheaths in the 1 Gy radiation group was regularly round or oval. The layers could be observed compactly, clearly and thickly without swelling in the myelin sheath. In the 7 Gy radiation group, the shape of the regenerated myelin sheaths were partially contorted. In the 0 Gy radiation group and 0.2 Gy radiation group, the shape of the regenerated myelin sheaths was more contorted, and the layers of myelin sheath were moderate vacuole degeneration. In the 14 Gy radiation group, the regenerated axons underwent obvious axonal swelling, myelin sheath vacuolisation and distortion. The layers were distinctly separate, and the structure was obscure, with vacuole degeneration (figure 5). The average thickness of the myelin sheath and axon diameter in the 1 Gy radiation group and 7 Gy radiation group were significantly higher compared with the 0 Gy radiation group, 0.2 Gy radiation group and 14 Gy radiation group (p<0.05). No statistically significant differences were found between the 1 Gy radiation group and 7 Gy radiation group (p>0.05) or between the 0 Gy radiation group, 0.2 Gy radiation group and 14 Gy radiation group (p>0.05; figure 6).

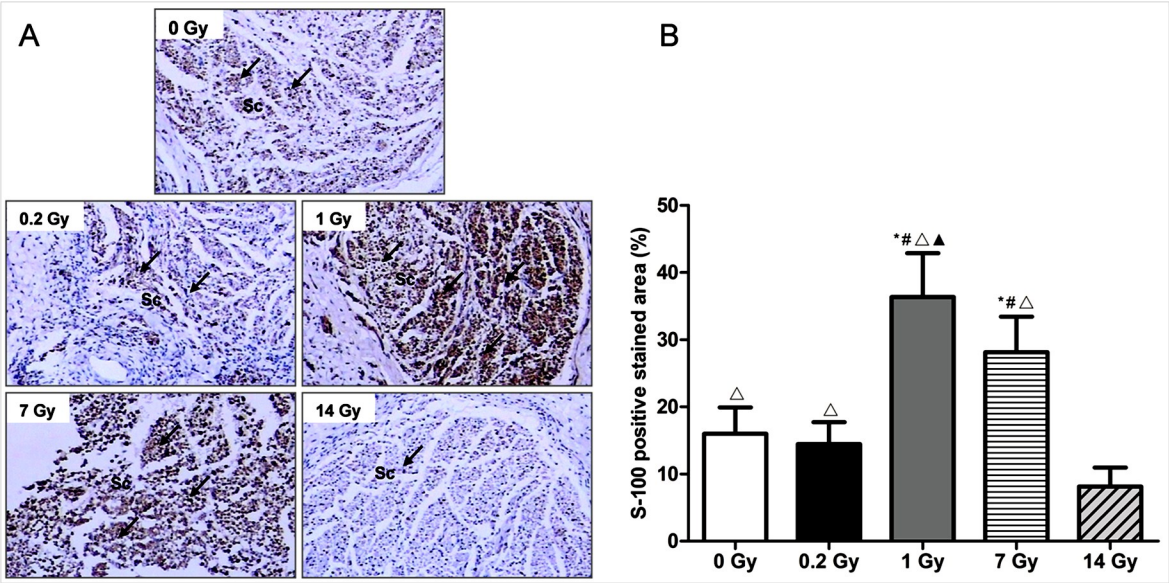

Figure 4. (A) Light micrographs showing immunohistochemistry of S-100 of the regenerated nerves at the end of 12 weeks post-neurorrhaphy. **(B)** Quantitative analysis of immunostaining for S-100 among groups (magnification × 40). *P<0.05 vs 0 Gy group, $^{\#}$P < 0.05 vs 0.2 Gy group, $^{\blacktriangle}$P<0.05 vs 7 Gy group, $^{\triangle}$P<0.05 vs 14 Gy group. Black arrows show positive expression of S-100, Sc: Schwann cell.

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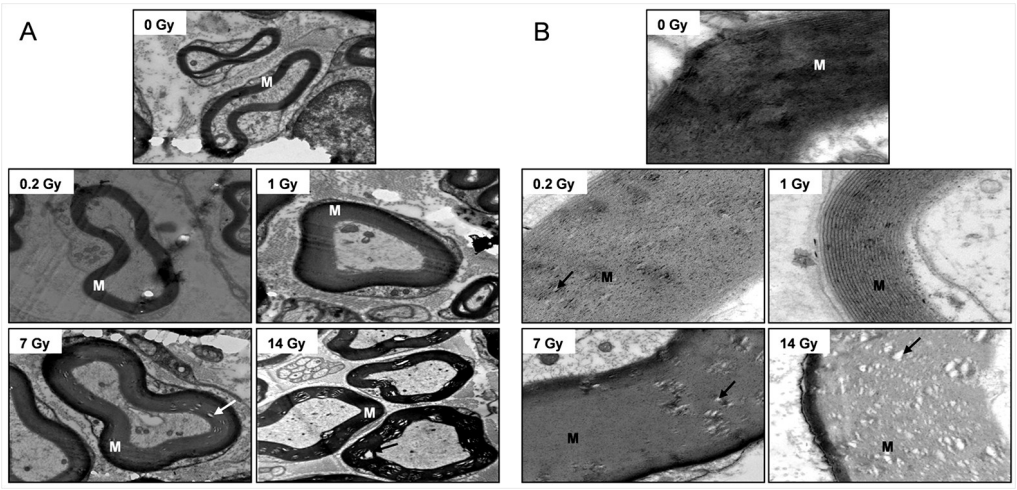

Figure 5. TEM images showing the morphology of axons and myelin sheaths at the end of 12 weeks post-neurorrhaphy (**A:** magnification × 4000, **B:** magnification × 30000). Black and white arrows show vacuole degeneration, M: Myelinated nerve fibre.

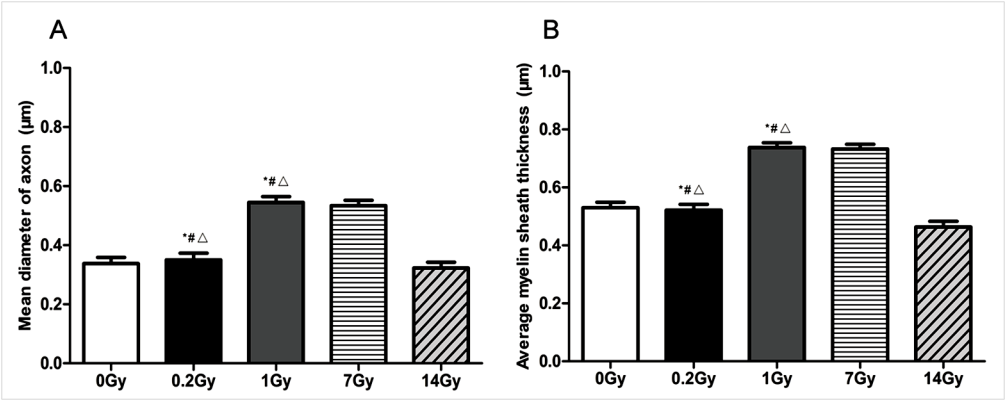

Figure 6. (A) Comparison of the mean axon diameter among groups. **(B)** Comparison of the average of myelin sheath thickness among groups. *P<0.05 vs 0 Gy group, #P<0.05 vs 0.2 Gy group, △P<0.05 vs 14 Gy group.

DISCUSSION

In 1982, Luckey [15] first proposed that LDI had beneficial effects on animal health and survival. From then on, various studies have been performed to investigate the effects of LDI on biological systems [16, 17]. Görgülü *et al.* [6] demonstrated that a single 700 cGy cobalt-60 dose reduced epineurial scar formation in a rat sciatic nerve injury model and did not exert any fibrotic effects on the nerves. Zhou *et al.* [18] demonstrated that LDI differed from high- and medium-dose radiation and produced a beneficial effect on fracture healing in a rat femoral closed fracture model. Chen *et al.* [19] reported that low dose X-ray radiation induced beneficial effects, such as promoting proliferation and differentiation of osteoblasts, and accelerating fracture healing. Karimipour *et al.* [20] found that LDI has the potential to promote neovascularization to improve flap survival.

In this study, various doses of X-ray radiation were used to radiate injured peripheral nerves in adult rats. Various measurements were used to assess the effects on nerve regeneration. The results of the walking track analysis showed that the SFI recovery of the 1 Gy radiation group and 7 Gy radiation group were quicker and better than those of the 0 Gy radiation group, 0.2 Gy radiation group and 14 Gy radiation group at 4 and 8 weeks post-neurorrhaphy. By 12 weeks after neurorrhaphy, no significant difference in SFI value was observed among the 0 Gy radiation group, 0.2 Gy radiation group, 1 Gy radiation group and 7 Gy radiation group, but

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they all had a significantly better recovery compared with the 14 Gy radiation group. These results reveal that 4-8 weeks was the crucial time for nerve regeneration. During this time, 1 Gy and 7 Gy radiation had a significant role in accelerating axonal movement across the repaired site and promoting the recovery of motor function. This phenomenon was probably correlated with the regenerated axons which had crossed the impaired loci in this period. The nerve regeneration substances produced by the distal nerve of impaired loci could retrogradely transport to the neuron to accelerate the neuron synthesis and protein secretion to accelerate the nerve regeneration. The 14 Gy radiation caused irreversible damage to the nerve, which manifested as a delayed neuronal injury. Thus the SFI recovery of the other groups was superior to the 14 Gy radiation group. We found that 1 Gy and 7 Gy radiation significantly reduced the latency of onset, elevated the peak amplitude of CMAP and improved sciatic nerve motor conduction velocity. Therefore, some functional recovery was observed under 1 Gy and 7 Gy radiation. The 1 Gy and 7 Gy radiation prevented and decrease perineural scar tissue; however, 0 Gy, 0.2 Gy and 14 Gy radiation exerted no effects. According to S-100 immunocytochemical analysis, 1 Gy and 7 Gy effectively promoted Schwann cell migration and proliferation. In ultrastructural observations, we found that 1 Gy and 7 Gy radiation benefitted axon growth and the formation and maturation of regenerated myelin sheaths; however, 0.2 Gy radiation had no significant effect, and 14 Gy radiation resulted in vacuolisation and lamellar separation.

The precise mechanism responsible for axon regeneration and electrophysiological recovery after the dose of 1 Gy and 7 Gy X-ray radiation is not fully understood. One possible mechanism is that the 1 Gy- and 7 Gy-irradiated tissue enhances the production of neurotrophic factors and promotes the expression of VEGF, which could promote angiogenesis and increase blood circulation to the injury site. The 1 Gy and 7 Gy doses of radiation could have reduced the number of fibroblasts and inhibited the fibroblasts from secreting growth factors, thereby reducing the formation of epineurial scars after peripheral nerve surgery.

In summary, our data demonstrate that a single and local dose of 1 Gy or 7 Gy X-ray radiation promoted nerve regeneration after sciatic nerve injury in a rat model. The dose of 14 Gy exerted inhibitory effects, and 0.2 Gy exerted no significant effect on nerve regeneration. Further large-scale studies with long-term follow-up are needed to clarify the exact mechanisms of the effects that the 1 Gy and 7 Gy doses of radiation exert on peripheral nerve regeneration.

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***Conflicts of interest*:** *Declared none.*

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