X-irradiation for inhibiting glial scar formation in injured spinal cord

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Abstract

X-irradiation has a beneficial effect in treating spinal cord injury. We supposed that X-irradiation could improve the microenvironment at the site of a spinal cord injury and inhibit glial scar formation. Thus, this study was designed to observe the effects of 8 Gy X-irradiation on the injury site at 6 hours and 2, 4, 7, and 14 days post injury, in terms of improvement in the microenvironment and hind limb motor function. Immunohistochemistry showed that the expression of macrophage marker ED-1 and the area with glial scar formation were reduced. In addition, the Basso, Beattie and Bresnahan score was higher at 7 days post injury relative to the other time points post injury. Results indicated that X-irradiation at a dose of 8 Gy can inhibit glial scar formation and alleviate the inflammatory reaction, thereby repairing spinal cord injury. X-irradiation at 7 days post spinal cord injury may be the best time window.

Key Words

neural regeneration; spinal cord; glial scar; X-irradiation; functional recovery; astrocytes; grants-supported paper; neuroregeneration

Research Highlights

(1) The glial scar is the main inhibitor of axon regeneration and functional recovery in the central nervous system. X-irradiation has been shown to inhibit the formation of glial scars post injury and can promote injury repair.
(2) This study investigated the effects of X-irradiation at different time points on the formation of the glial scar and its influence on the neurological function in rats post injury. Results confirmed that X-irradiation at a dose of 8 Gy can inhibit glial scar formation at the injury site and alleviate the inflammatory reaction. Day 7 post injury may be the optimal time window for topical X-irradiation.

INTRODUCTION

Spinal cord injury has been regarded clinically as irreversible damage caused by tissue contusion due to trauma and can result in axonal degeneration, nerve cell death, and microvasculature destruction[1]. These events subsequently trigger a cascade of pathological changes called secondary damage, including vascular and biochemical changes, hemorrhagic necrosis, inflammatory processes and demyelination. Morphological changes are manifested principally in the death of neurons, glial cells and inhibition of axon regeneration in the acute stage[2].

In the subacute or chronic stages, secondary damage continues to occurThis includeproliferation and fibrosis of neuroglial cells, formation of glial scars and the irreproducible compensatory growth of neuroaxons[3-4]. The findings from most
biomedical studies have indicated that spinal cord injury is usually accompanied by the formation of cystic cavities surrounded by glial scars, which severely impede the regeneration of severed axons. Glial scars are composed of extracellular matrices and various types of cells, and astrocytes in particular play key roles in glial scar formation[5].

However, some studies have reported that astrocytes play a favorable role during the period of early injury through the release of neurotrophic factors[6]. Although reactive astrocytes may exert some beneficial effects by regulating local immune responses and promoting tissue repair, mounting evidence indicates that glial scars constitute a major component of the post-injury environment that discourages spontaneous axonal regeneration[5]. Reactive astrocytes hypertrophy over time forming a physical barrier; they also secrete a variety of inhibitory chondroitin/keratin sulfate proteoglycans, forming a more immediate molecular barrier that contributes to a long-term dystrophic state in the vast majority of non-regenerating fibers. Most studies had reported that reducing astrogliosis and the proliferation of glial cells possibly promotes axon outgrowth and functional recovery after injury[6-7].

X-irradiation was first studied in neonatal rats for investigating the influence of X-irradiation on the spinal cord in 1963. Gilmore[8] found that X-irradiation had a significant impact on the spinal cords of neonatal rats. Changes in the nervous system were observed in 70% of neonatal rats that had undergone X-irradiation; these changes were found to be less pronounced when older rats were irradiated[9].

Some studies have indicated that X-irradiation can influence both neurons and glial cells, while neurons are more insensitive to X-rays as compared with glial cells. X-irradiation at an optimal dose does not damage nerve cells, but can inhibit the growth of glial cells especially oligodendroglia, thereby promoting the regeneration of axons[10-11]. Kalderon and Fuks[12] found that X-rays mitigated against the death and degeneration of neurons, and at least partially improved the recovery of locomotor function following spinal cord transection in rats. The beneficial effects of X-rays was found to be associated with the prevention of gliosis, cavitation, and loss of normal spinal cord morphology, which appear after a delay of several weeks following transaction. However, the mechanism remains unclear and there is no uniform view on the optimal radiation dose and time window reported in published studies.

The aim of the present study was to evaluate the effects of X-irradiation after spinal cord injury, especially with regard to the inhibition of glial scar formation and improvement of functional recovery; a key objective was to find the optimal radiation dose and time window. Hence a rat model of spinal cord injury was established. Rats were divided into different time window groups. The inhibitory effect of X-irradiation on glial cells was observed using immunohistochemistry. The Basso, Beattie and Bresnahan (BBB) score was used to evaluate functional recovery.

**RESULTS**

**Quantitative analysis of experimental animals**

Thirty-six Wistar rats were used to establish the model of spinal cord injury at vertebra T10 using the vertical weight drop method. Rats were randomly and equally assigned to six experimental groups: unirradiated control group (no X-irradiation) and groups subjected to 8 Gy X-irradiation at 6 hours and 2, 4, 7 and 14 days after spinal cord injury. All 36 rats were included in the final analysis.

**X-irradiation reduced glial scar formation post spinal cord injury**

To verify whether X-irradiation could reduce the number of glial cells and decrease glial scar formation, we evaluated the expression of ED-1, mononuclear macrophage antigen, after irradiation. Immunohistochemical analysis showed that X-irradiation at 7 days post spinal cord injury using a dose of 8 Gy significantly reduced the number of glial cells ($P < 0.001$). This finding confirmed that X-irradiation at an appropriate dose and time window can effectively inhibit glial scar formation (Figures 1, 2).

Hematoxylin-eosin staining was used to observe the morphology and pathological changes in the glial scar at the center of the lesion by means of optical microscopy. Results showed that the syringomyelia and glial scar were significantly reduced in the group irradiated at 7 days post injury, while a large amount of glial scar and structural disorder was observed in the other groups (Figure 3).

**Changes in locomotor function in rats with spinal cord injury following X-irradiation**

Finally, we tested whether inhibiting the growth of the glial scar was correlated with the functional recovery in rats.
We evaluated the effect of X-irradiation on recovery of locomotor function after spinal cord injury according to a 21-point locomotor BBB scale. All rats showed posterior limb paralysis after spinal cord injury, and the mean BBB score was 0–1 points at 3 days after surgery. A gradual recovery of hind limb locomotion was seen over the following 4 weeks in all rats, and there was no statistical difference between the groups at 2 weeks; starting from the 3rd week, the recovery of hind limb locomotion presented a significant difference in the group irradiated at 7 days post injury (P < 0.05), and no statistical difference was observed between the other groups (Figure 4).

**DISCUSSION**

Most experimental investigations documenting the effects of irradiation on the central nervous system have been carried out in normal animals[13]. The present study, in which X-rays were delivered to the injured spinal cord, demonstrated that X-irradiation can both reduce the formation of a glial scar and enhance the recovery of locomotor function following contusion injury.
The glial cell was most sensitive to X-irradiation at the 7th day after spinal cord injury, as compared with higher sensitivities of glial cells and neurons to X-rays. We concluded that the mechanism was related to the mechanism of action of X-irradiation in spinal cord injury, as there has not been elucidated.

Significantly superior treatment effects were observed after X-irradiation at the injured site in the time window of 7 days post contusion injury, compared with irradiation at 6 hours and 2, 4, or 14 days post injury. The reason for this difference is not known because the reason for this difference is not known because the mechanism of action of X-irradiation in spinal cord injury has not been elucidated.

We concluded that the mechanism was related to the differing sensitivities of glial cells and neurons to X-rays. The glial cell was most sensitive to X-irradiation at the 7th day after spinal cord injury, resulting in significantly decreased glial cell numbers; glial scar formation was also reduced.

X-irradiation has also been used in a study of compression injury in another rat model of spinal cord injury at doses ranging from 2 to 20 Gy [13]. Modest improvement and a reduction in syringomyelia and gliosis were observed after 4 weeks with a low dose of 2 Gy, but superior results were not observed after higher doses of 5–20 Gy. Although the improved locomotor and histological outcomes due to low-dose X-irradiation were consistent with the present study, toxic effects of higher doses were observed by these authors [13]. Hence, in our study we utilized a dose of 8 Gy, because this dose is not harmful to the central nervous system and is effective for functional recovery [14-15].

Microglia, white blood cells and astrocytes participate in inflammation after spinal cord injury, and white blood cells and macrophages are the main cells related to injury in the vascular system; the aggregation of microglia and macrophages may be the primary mechanism of secondary injury after spinal cord injury [16]. The intrinsic microglia in the spinal cord can transform rapidly into macrophages after spinal cord injury, and produce toxic molecules that mediate tissue damage due to lipid peroxidation and some other mechanisms [17]. However, microglia and macrophages also have the function of promoting axon regeneration, such as clearing fragments of the medullary sheath and neurons, producing cell factors to promote regeneration, and increasing axonal sprouting.

In the early stage of spinal cord injury, the promoting function of active glial cells plays a more essential role than their inhibitory effects. We assumed that X-irradiation can improve the local microenvironment, promote axon regeneration, and inhibit glial scar formation after spinal cord injury. To verify the hypothesis, we observed cells that were positive for the ED-1 mononuclear macrophage antigen, to detect whether or not X-irradiation could reduce the formation of a glial scar. ED-1 can specifically bind to the lysosome membrane of activated macrophages, and can be used to detect the functional activity of macrophages after spinal cord injury.

In the present study, we found that X-irradiation at 7 days can reduce the number of ED-1 positive cells present and promote functional recovery. This suggested that X-irradiation may mitigate the inflammation process. X-irradiation at 6 hours and 2 and 4 days elicited no beneficial effects on functional recovery. The reason for this may have been the inhibition of phagocytosis of cell debris by microglia. However, irradiation did not suppress the recovery. Thus, the early phase glial cells may not be sensitive to X-rays; however, the precise mechanisms involved require further investigation.

After spinal cord injury, a series of cells and molecules are activated and participate in the formation of the glial scar, which mainly consists of astrocytes, and the coloboma due to injury is finally filled with astrocytes [18-19]. Studies have indicated that the formation of a glial scar can lead to complicated consequences such as

![Figure 4: Effects of X-irradiation on hind limb locomotion function in rats with spinal cord injury.](image)
necrocytosis and an inflammatory reaction, and the space occupied by the glial scar can also act as an inhibitory boundary to axon regeneration\cite{20}. Our study suggested that X-irradiation at 7 days could significantly improve motor function, and we assumed that the X-rays promoted the apoptosis of glial cells and reduced the formation of the glial scar.

After central nervous system injury, the formation of astrocytic glial scars functions to minimize secondary tissue damage and to restore post-injury tissue homeostasis\cite{21-22}. However, these beneficial effects arise at the expense of reducing the regenerative potential of damaged axons. Astrocytic glial scars impede the growth of regenerating axons both physically and chemically. They promote axon regeneration by releasing neurotrophic factors and by phagocytizing cell debris at an early stage, while the over proliferation of glial cells has the opposite effect on axon regeneration\cite{23}.

Our study found that day 7 was the dividing point between two opposite effects; rats undergoing X-irradiation after spinal cord injury at this time can achieve a better recovery of neurological function. The minimal efficacy of X-irradiation at 14 days may have been because glial cells were less sensitive to X-rays at this time.

There were several advantages to our study. We discussed in depth the optimal treatment time window and dose of X-irradiation for spinal cord injury. The therapeutic mechanism involved in the use of X-irradiation to restore spinal cord function after injury was preliminarily explored. However, there were some limitations to the study. No dosage effect was discussed; more groups involving different X-ray doses should be evaluated in a future study. In addition, more multiple experimental methods should be applied to obtain objective conclusions.

X-irradiation at an appropriate dose was found to be essential for functional and structural recovery after spinal cord injury, while the mechanism involved remains unclear. Some authors have considered that neovascularization can help to improve the survival and regeneration of neurons, and that X-rays can reduce the formation of the neural scar that influences neovascularization\cite{24-25}. X-rays have also been demonstrated to improve cellular reactions in mammals after spinal cord injury, and promote continuity of spinal structure.

Some studies have indicated that a potential pathway of tissue degeneration is activated in spinal cord injury, and that the pathway induces the formation of syringomyelia\cite{26}. Unfortunately, the mechanism involved in this pathway remains poorly understood, although one or more neuroglial cells have been found to play a role\cite{27}. An appropriate dose of X-rays can ablate the cells mediating the tissue degeneration pathway, without affecting the internal recovery process.

Our study confirmed that irradiation with a dose of 8 Gy X-rays at 7 days post injury can inhibit the formation of the glial scar, and significantly improve the functional recovery of rats. We conclude that activated glial cells were most sensitive to X-rays on the 7th day after spinal cord injury; however, the specific mechanisms and methods involved require further study.

**MATERIALS AND METHODS**

**Design**
A randomized controlled preclinical animal study.

**Time and setting**
Experimental investigations were carried out in a laboratory at Tianjin Medical University General Hospital, China from 2009 to 2010.

**Materials**
Thirty-six female adult Wistar rats aged 8 weeks and weighing 200 ±  30 g, were provided by Beijing HFK Bioscience Co., Ltd., China (license No. SCXK (Jing) 2009-0004).

The procedures involving animals were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China\cite{28}.

**Methods**

*Establishment of the rat model of spinal cord injury*

Moderate spinal cord injury was induced using the Impact Model II device\cite{29-31}. Rats were subjected to laminectomy at the T_{9-11} level under anesthesia with intraperitoneal injection of 10% chloral hydrate (300 mg/kg). The dorsal surface of the cord at T_{10} was exposed and subjected to a weight drop impact using a 10 g rod (2.5 mm in diameter) dropped from a height of 25 mm. Subsequently, rats spasmed several times, and this was followed by flaccid paralysis, indicating a successful model\cite{30-31}.
After injury, penicillin was injected and the muscles and skin were closed layer by layer. Rats were raised in a chamber at 22–25°C, with regular ventilation. The bedding in the rearing cages was regularly changed to keep it dry. Physiological saline was administered by subcutaneous intraperitoneal injection at a volume of 5 mL, once or twice a day to prevent fluid and electrolyte disorders. Manual evacuation of the urinary bladder and hind limbs were performed twice per day to prevent intestinal obstruction and pressure ulcers. Extrusion massaging of the urinary bladder was performed three times per day until the rats were able to regain normal bladder function.

X-irradiation at the injury site at different time points
Rats in the unirradiated control group were subjected to the surgical procedure without X-irradiation (CLINAC600C: Varian, Tianjin Medical University General Hospital, Tianjin, China). The remaining rats were subjected to spinal cord injury and treated with a single X-ray dose of 8 Gy at 6 hours and 2, 4, 7 or 14 days after spinal cord injury.

Rats were fixed to an irradiation table on a linear accelerator after anesthesia using an intraperitoneal injection of 10% chloral hydrate (300 mg/kg, Tianjin Medical University; Figure 5).

Figure 5  Rats were irradiated on a CLINAC 600C linear accelerator after spinal cord injury.

Irradiation was focused on the center of the lesion encompassing an area 1.5 cm in length and 0.8 cm in width. The dose exposure rate was 3.2 Gy/min, and the integral radiation dose was 8 Gy.

Functional assessment of rats with spinal cord injury
The BBB scoring test was used to evaluate the functional recovery of hind limbs in rats. The scale ranged from a score of 0 to a maximum score of 21. Score 0 indicates no observable hind limb movement, and a gradually increasing score indicates the improved function of the hind limb; a score of 21 represents normal function in the hind limb.

The BBB score was evaluated at 3, 7, 14, 21, and 28 days after injury to assess the severity of spinal cord injury after X-irradiation. The rats were subsequently tested weekly for 4 weeks. All animals were included in the analysis without death.

Immunohistochemistry and hematoxylin-eosin staining for glial scar formation in the spinal cord injury site
Rats were sacrificed at 4 weeks post injury and the injured spinal cords (T10, 3 cm length) were fixed in 4% (v/v) paraformaldehyde solution. Sections cut at a thickness of 5 um were prepared from paraffin-embedded tissues. Dried slices were immersed in dimethylbenzene I and II for 30 minutes for deparaffinization, and washed with 0.01 mol/L PBS solution (pH 7.3) twice, for 5 minutes per session. Endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide for 5–10 minutes.

Sections were washed three times with PBS and incubated with goat anti-rabbit biotinylated secondary antibody (ectoderm, polyclonal; Boster, Wuhan, China; 1:50) at 4°C. Sections were washed with PBS and incubated with strept avidin-biotin complex (immunoassay kit, Boster) for 20 minutes at 37°C. Sections were incubated overnight with rabbit anti-rat monoclonal antibody ED-1 primary antibody (ectoderm, polyclonal; Boster, Wuhan, China; 1:50) at 4°C. Sections were washed and incubated with goat anti-rabbit biotinylated secondary antibody (ectoderm, polyclonal; Boster) for 20 minutes at 37°C. Sections were incubated with strept avidin-biotin complex (immunoassay kit, Boster) for 20 minutes, followed by four PBS washes.

3,3′-Diaminobenzidine reagent was applied for 5 minutes and the sections were then washed with distilled water.

Sections were counterstained with hematoxylin for 2 minutes and disunited in 70% alcoholic solution mixed with 1% hydrochloric acid. The reaction was terminated using ammonia wash water. Sections were immersed in dimethylbenzene for transparency, and neutral gum was applied to seal the slices.

The dewaxing process involved in the hematoxylin-eosin staining was similar to the above-mentioned process. The sections were counterstained with hematoxylin for 2 minutes in Harris hematoxylin solution (Germany) for 5–10 minutes and washed in distilled water until they were blue. Hydrochloric acid and alcohol were used for color separation. The sections were washed and mixed with 1% eosin solution for 5–10 minutes, then washed by distilled water followed by evaporation, clearing and mounting.

Acquisition and analyses of data and images
Immunohistochemical analysis was carried out with the aid of a CH30 microscope (Olympus, Tokyo, Japan), and
Olympus digital camera was used to take photographs, which were stored in CMP format. Digital images of immunohistochemically stained sections were evaluated using a Pathologic Image Analysis System (Beihang University, Beijing, China) at Beihang University to calculate tissue areas that were positive for ED-1.

**Statistical analysis**

Data were expressed as the mean ± SD. All data were statistically processed using SPSS 15.0 software (SPSS, Chicago, IL, USA). Statistical significance was determined using one-way analysis of variance followed by the least significant difference test. A value < 0.05 indicated statistical significance.

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**Conflicts of interest:** None declared.

**Ethical approval:** This study was approved by the Animal Ethics Committee of Tianjin Medical University, China.

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