Modified Postoperative Strategy for Substantiation of Fibrotic Glial Scar Formation after Aneurysm Clip Compression Spinal Cord Injury in Rats

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ABSTRACT

Aim: To investigate the development of fibrotic glial scar with modified post-operative care in compression model of spinal cord injury in adult rats by using aneurysm clip compression.

Methodology: 10 healthy male adult Sprague Dawley rats, an average of 3 months old and weighing 250-300 gm, were randomly divided into AI & AII groups based on experiment duration. Group A1 rats were assessed after 14 days, and Group AII rats after 28 days of spinal cord injury. Rats were given isoflurane inhalation anaesthesia, and surgical exposure spinal cord at the T7 level was performed. An aneurysm clip having 70 g closing force was applied to the T7 level of the spinal cord for 1 minute and then released. Wound closure was done in separate layers, and rats were allowed to recover. Post-operative antibiotics administration was reduced to only one dose of ceftriaxone 2gm/kg and buprenorphine was replaced by tramadol intraperitoneal to avoid

injection site abscess and other complications. Ringer's lactate administration route was also changed from subcutaneous to intra-peritoneal to maintain hemostasis and prevent dehydration.

Results: Microscopic examination of the injury site stained with H & E and Masson's Trichrome stains, showed significant injury at the aneurysm clip application site in the spinal cord with disturbed cytoarchitecture and high inflammatory cell infiltration. Glial scar formation with central fibrotic core was prominent in injury lesions. All animals showed no sign of infection, surgical wound abscess or any other complication and survived well throughout the experimental duration after changing the route of drug administration from subcutaneous to intra-peritoneal.

Conclusion: Microscopic assessment shows that the aneurysm clip compression method for developing the spinal cord injury model is a reproducible, modest and low-cost model to study glial scar formation in rodents. Only a single dose of a strong antibiotic through intra-peritoneal route administration post-operatively is sufficient to avoid infections and injection site abscess on the condition that proper hygiene, diet, humidity and the surrounding temperature are maintained.

Keywords: Aneurysm Clip Compression Model, Masson"s Trichrome, Glial Fibrotic Scar

INTRODUCTION

Animal spinal cord injury (SCI) models can help understand the pathophysiological progress and test potential therapies. Contusion with weight drop, mechanical displacement with the electromagnetic device, compression with aneurysm clip or forceps, cutting injury with complete/partial transection and compression model with balloon inflation are declared and established. Because it resembles human traumatic spinal cord injury, the "compression injury model" is the best choice for translational research projects (1). In the "compression spinal cord

injury model" in rats, glial scar formation is the same as in SCI patients. The compression model of spinal cord injury is also a better choice for observing the therapeutic and neuroprotective effects of agents or drugs after spinal cord injury, neuronal sustainability, and for studying the secondary injury process steps and "cell transplantation" treatments (2).

Nevertheless, chronic progressive spinal stenosis caused by epidural spinal cord compression is very common clinically, accounting for 30–80% of non-traumatic spinal cord injuries. Compression can be caused by degeneration, tumour, tuberculosis, hematoma and traumatic injuries. Most of the models need detailed settings; such studies are laborious and require expensive equipment. Epidural compression with the aneurysm clip is a more straightforward, less expensive, and reproducible model (3). The purpose of this study was to evaluate the already designed aneurysm clip model and make necessary modifications in the whole methodology if needed.

MATERIALS AND METHODS

This experimental study was conducted in the "Department of Anatomy, Institute of Basic Medical Sciences, Khyber Medical University Peshawar, Pakistan" from 1st December 2020 to 30th May 2021, after approval from the Institution's ethical board. The minimum sample size (AI:- n=5 and AII:- n=5) was calculated using the resource equation approach (4). Only male rats were used in the experiment, and female rats were excluded due to breeding issues. Too young (less than 2 months) and old (more than 1 year) rats were omitted. Controlled environmental settings at 22–25 ^{0}C, proper humidity and a "light cycle" of 12 hours were maintained for each rat—free access excess to food and water *ad libitum*.

Grouping: All rats were divided into 2 groups, AI and AII groups, with 5 rats in each. This grouping was based on an experimental duration of 14 and 28 days respectively to assess spinal cord injury's acute and chronic outcomes (5-7).

1. **GROUP-A-I**: - Aneurysm clip compression of the spinal cord was performed, and rats were examined for behavioral and microscopic changes 14 days after injury.
2. **GROUP-A-II**: - compression injury of the spinal cord was performed using an aneurysm clip, and rats were examined for behavioral and microscopic changes 28 days after injury.

Surgical Preparation

Before surgery, all required surgical instruments and reagents were gathered, and all the tools were autoclaved. Sterilization of the surgical field by 70% isopropyl alcohol spray was performed. All subjects were given 0.05 mg/kg of buprenorphine subcutaneously before anesthesia. Before starting the surgery, induction of anesthesia with 5% isoflurane (through inhalation) in the induction chamber for 2 minutes and then Maintenance dose of 2% isoflurane (inhalation through face mask) during surgery with 1.5 percent oxygen. The rats were remain anesthetized till the completion of procedure successfully. Total surgery was about 15 to 20 minutes of duration. After the animal was anaesthetized, combining Polymyxin and bacitracin eye ointment was applied to the eyes to avoid corneal dehydration. Then the Subject was put on a "heating pad" at 37 °C to avoid hypothermia, and the head was placed correctly in the anesthesia cone. After the Subject was completely anaesthetized, isoflurane was reduced to a maintenance dose of 2%. The dorsal surface of the rat was shaved 1 cm around the planned incision location. The incision site was disinfected by applying 70% isopropyl alcohol spray and then 10% Povidone-iodine.

Dorsal Laminectomy

Before giving an incision, the animal was anaesthetized adequately by testing reflexes utilizing the toe and tail pinch technique. The incision was given along the dorsal spine with the scalpel. Retractors were applied to hold skin and fascia. The tissue on both sides of the spinal cord was cleared to uncover the muscles covering the spine. The top of the natural curve of the rat's spine was T12 and was used as a reference point.

Furthermore, a rib count was performed to identify the exact site for injury induction. T7 spinous process was identified, and a Cut was given just enough to expose the spinous process and posterior lamina of T7. Excessive tissue was removed from the lamina and spinous process to bare a small slice of the Spinal column.

Laminectomy was performed by introducing one side of a couple of small vana scissors sideways to the dorsolateral side of the vertebra and just underneath the lamina. Small, careful snips were performed to cut through the lateral side of the exposed vertebral lamina. It was confirmed that no pressure was applied to the spinal cord. The same procedure was repeated on the other side too.

Mild pressure was applied by a surgical sponge to stop bleeding, making sure that not apply pressure on the spinal cord. After bleeding was halted, the dorsal lamina of the vertebra was lifted, and any tissue attachments were cleared gently.

Spinal Cord Compression

Laminectomy forceps were used to free the lateral sides of the spinal cord from the vertebral bone so that the aneurysm clip for the compression injury was positioned on either side of the spinal cord. It was ensured that the arms of the aneurysm clip having 70 gm force were placed in the epidural space on adjacent sides of the spinal cord. The clip's tips reached the bottom of the

vertebral canal (8). An aneurysm clip was applied approximately in the middle of the exposed segment of the spinal cord. Aneurysm clip was applied for 1 minute and then removed.

Wound Closing

The muscle layer and fascia were sutured separately in layers carefully above the spinal cord with catgut 2.0. The skin over the wound was sutured with silk 2.0. To overcome the dehydration, Ringer's rate of 0.1 per 10 g body weight sub-cutaneously was administered. Rat was then placed in a bedding-free cage on the heating pad until recovery and then moved to another cage with bedding. Each rat was placed in a separate cage because of their eating habits and injuring each other. The whole surgical procedure is shown in figure 1.

Post-operative Care

After the surgery, 0.05 mg/kg body weight buprenorphine was administered subcutaneously twice daily for the first 2 days. Ampicillin 33 mg/kg/day subcutaneous injection was given twice daily for 6 days (9). Low-concentration povidone-iodine ointment was ((Betadine, Mundi pharma (BD) Private Limited)) applied topically on the surgical wounds of all rats to enhance wound healing (10).

All rats were provided free access to traditional food and fresh water. Urinary bladder massage of all subjects by Crede manoeuvre was performed twice daily to empty the bladders. Animals were monitored regularly for autophagy, dehydration, and extreme weight loss.

Some rats showed injection site abscesses at the site where antibiotic and Ringer's lactate were administered. Due to the abscess formation, rats were noted doing autophagy, causing death. So

to avoid abscess formation and autophagy, an antibiotic was changed to 2gm/kg(11) ceftriaxone single intra-peritoneal dose after anaesthetizing the animal just before starting surgery. Similarly, Ringer's lactate was administered intra-peritoneal, 0.1 per 10 g body weight, just after surgery to maintain haemostasis. Buprenorphine was replaced by single dose injection of tramadol (brand name=Tramal) 40mg/kg through intra-peritoneal route and was used just after surgery. This switching over of drugs, its dose and route showed good results along with changing Ringer's route of administration from sub-cutaneous to intra-peritoneal. All the rats which were gone under the procedure were successfully recovered from anesthesia; so 0% mortality was observed.

At the end of the experiment, animals were sacrificed and Spinal cord tissue samples were collected from the injury site, fixed overnight in 10% buffered formalin and then processed for embedding and sectioning.

Staining

A 5µm thick longitudinal sections of the spinal cord were stained with Hematoxylin and Eosin for routine microscopy and Masson's trichrome staining for detecting collagen deposition at the injury site, a hallmark fibrotic glial scar formation after spinal cord injury.

Mounting of sections

After staining, tissue sections on glass slides were mounted with DPX solution and carefully coated with glass coverslips for easier handling, storage and preservation.

DATA ANALYSIS

For microscopy, 3 slides from each rat spinal cord tissue stained with Masson's Trichrome and H&E were examined. Percentage of deposited Collagen in the injury lesions was quantified using

image J Fiji software. The Mann-Whitney test was applied for statistical analysis of collagen measure using SPSS version 26.

RESULTS

Changing the antibiotic to a high single dose through the intra-peritoneal route and ringer lactate reduced the formation of injection site abscesses. In this way, rats' autophagy was stopped, preventing the loss of rats after surgery.

In groups A-I & A-II, a well-appreciated injury site with high cellularity due to inflammatory cell infiltration, disturbed cytoarchitecture and irregular deposition of collagen was noted, as shown in figures 3 & 4. Figure 1 shows longitudinal sections of normal spinal cord stained with masson's trichrome. Collagen deposition was more in group A-II than group A-I rats, demonstrating the time-dependent increase in collagen deposition and scar formation at the injury site.

Collagen deposition shows highly significant difference (P = .009) as shown in the table 1.

Groups	Mean	SD	Mean rank	Mann Whitney test value	df	p-value
AI	20.21	2.05	3	6.818	1	0.009
AII	36.31	6.91	8			

Tab. 1: Difference between collagen deposition in injury lesions of groups AI and AII showing highly significant P value.

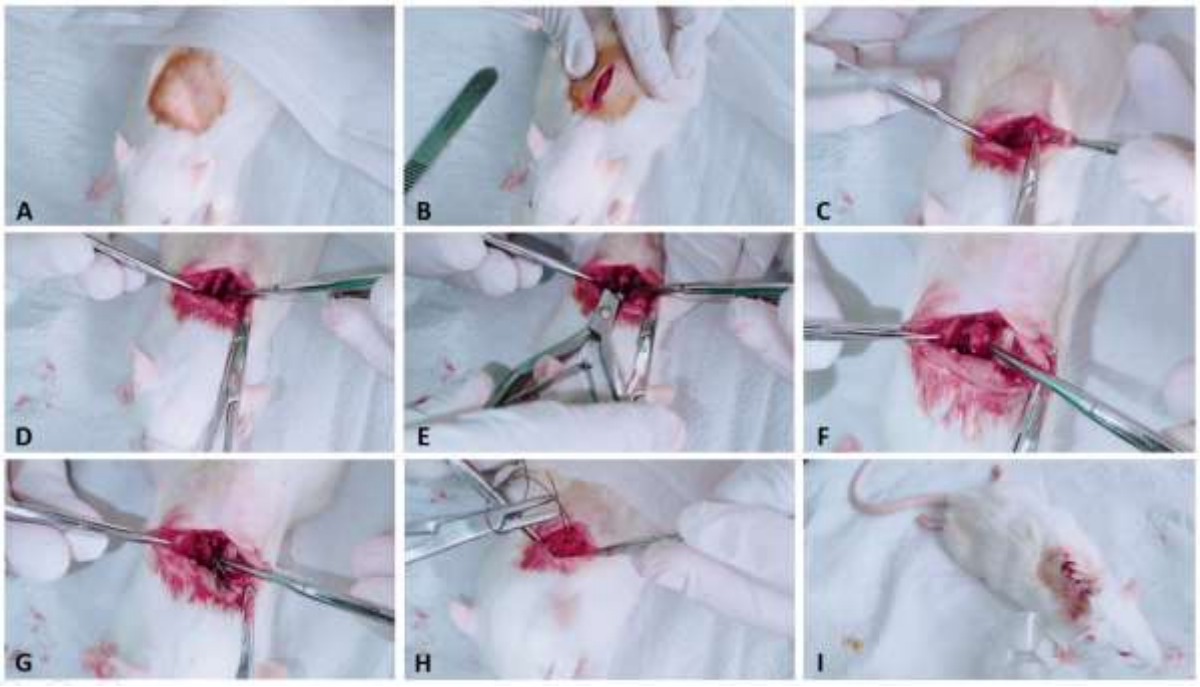

Figure 1: Surgical procedure and aneurysm clip compression. (A) Shaving and the disinfecting surgical area. (B) T7 vertebra was identified by counting ribs from the last 13th rib and surgical incision. (C) Back muscles and deep fascia were dissected. (D) Para-vertebral muscles were dissected on both sides of the T7 vertebra. (E) Dorsal laminectomy at T7 vertebra performed. (F) Spinal cord exposed with intact meninges. (G) Aneurysm clip with 70gm closing force applied for 1 minute. (H) Clip removed and all muscles sutured in layers. (I) Skin sutured, and rat allowed to recover.

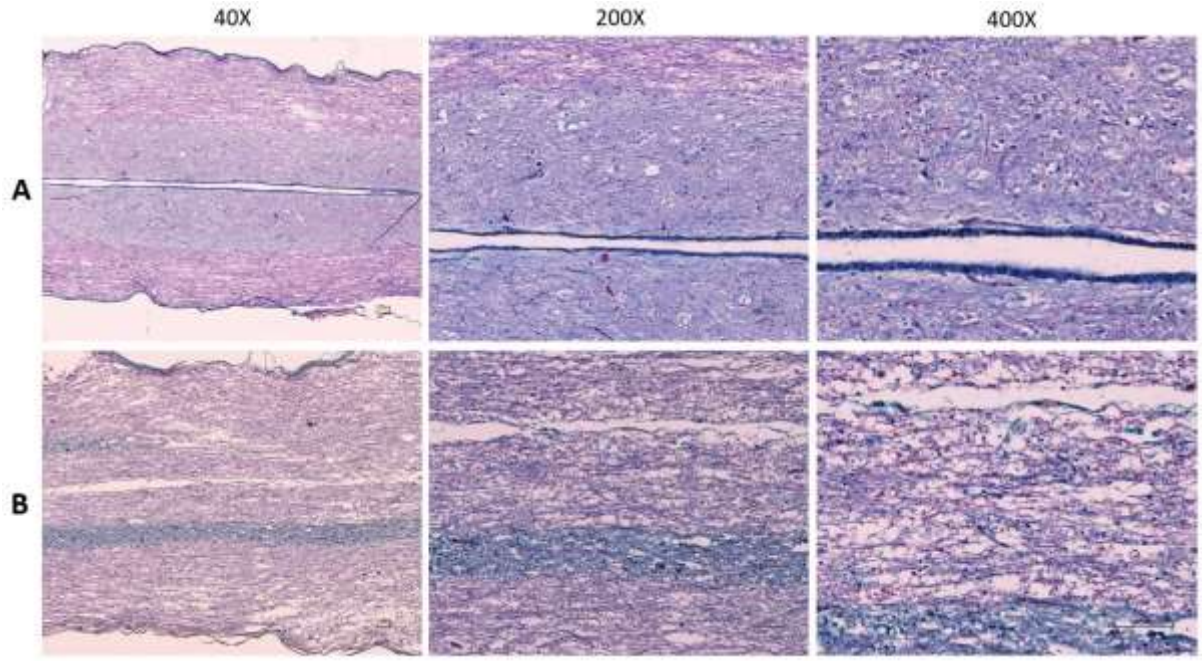

Figure 2: Histological micrograph of normal rats' 5µm thick longitudinal spinal cord sections. (A) & (B) both Sections stained with Masson's Trichrome at 10x, 20x & 40x magnifications show normal spinal cord structure. Undamaged pathways with neuronal cell bodies and axons, an intact central canal lined with ependymal cells and unvoiced collagen.

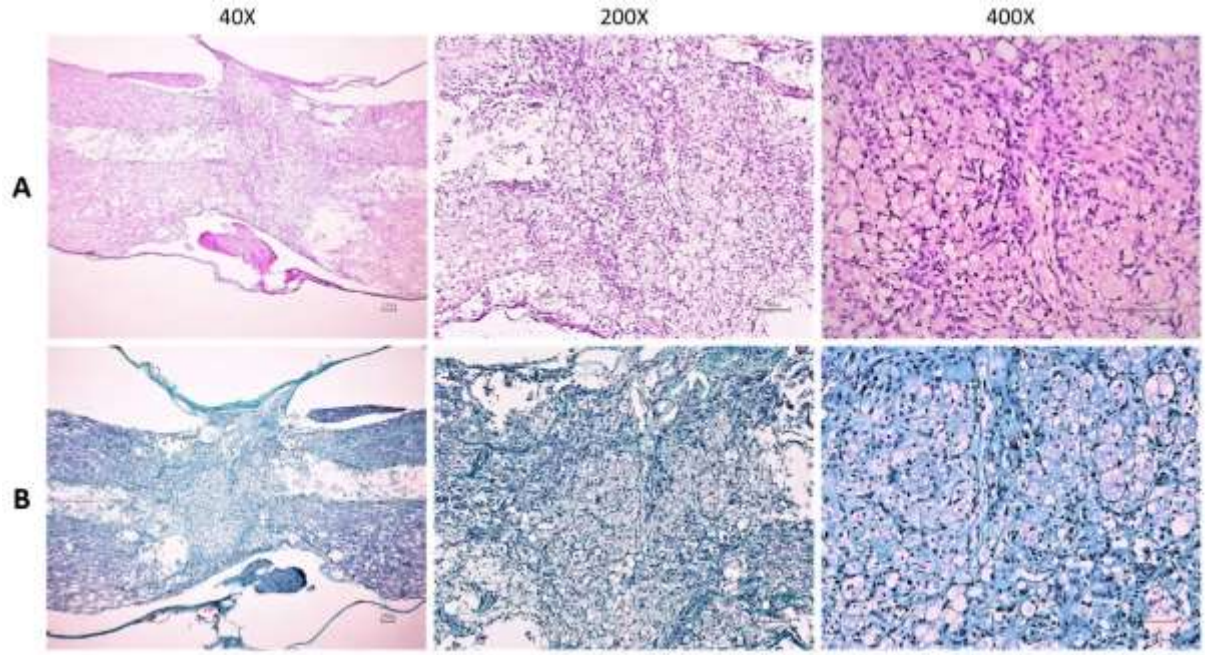

Figure 3: Photomicrograph of 5µm thick longitudinal section of the spinal cord of group AI rat. (A) Section stained with H&E at 10x, 20x & 40x magnifications showing injury site with disrupted architecture, cavitation and inflammatory cells infiltration. (B) Masson's Trichrome stained spinal cord longitudinal section showing significant injury with pronounced fibrosis (collagen deposition) at the injury site at 10x, 20x & 40x magnifications.

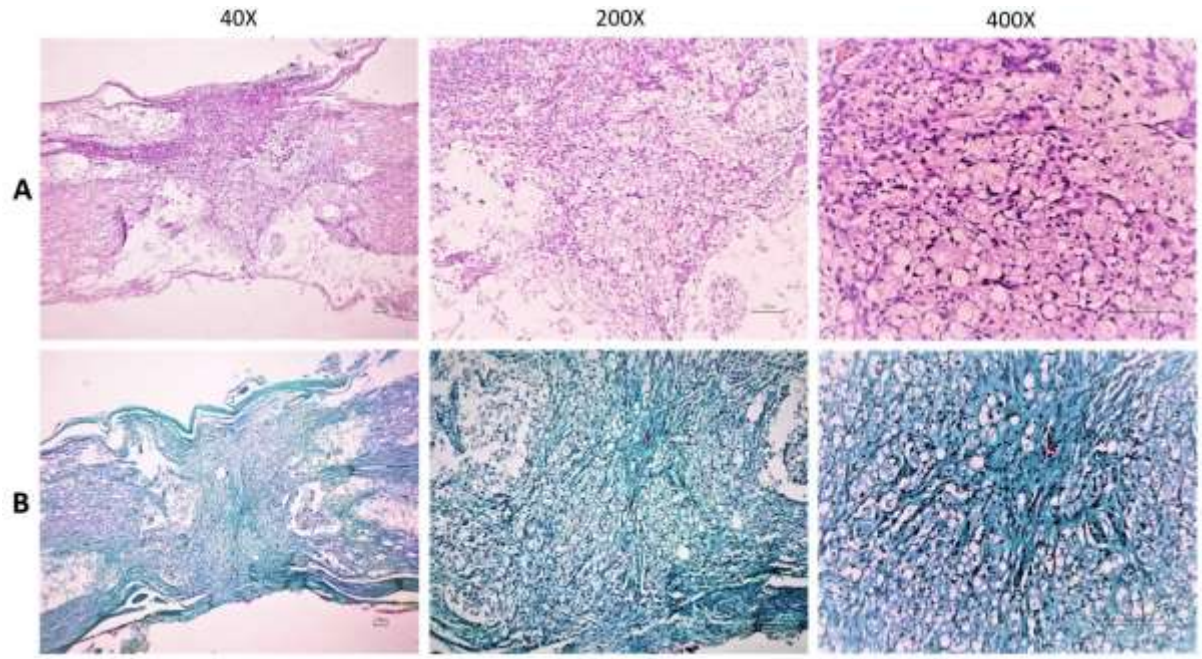

Figure 4: Microscopic image of a 5µm thick longitudinal section of group AII rat spinal cord. (A) Section stained with H&E at 10x, 20x & 40x magnifications showing injury site with the interrupted anatomical plane of the spinal cord, vaculation and high inflammatory cells penetration. (B) Masson's Trichrome stained spinal cord longitudinal section showing protuberant injury with highly marked fibrosis (collagen deposition) at the injury site at 10x, 20x & 40x magnifications.

DISCUSSION

Several animal models have been established for research purposes, allowing us to understand the multidimensional biomedical mechanisms of spinal cord injuries and to advance therapeutic approaches for this illness. A perfect animal model should have numerous characteristics comprising its significance to the pathophysiology of humanoid spinal cord injury, reproducibility,

availability, and prospective to produce several severities of injury (12). Results of our study showed that the aneurysm clip model is more effective and less expensive than the contusion model by Kathryn A. Harman et al. They used the Infinite Horizons device, which is an expensive device and needs trained expertise (13). Aneurysm clip also shows more actual outcomes compared to the transection model done by Chen Li et al. (14). Although transection is simple to perform but is less applicable to human spinal cord injuries as a complete transection of the spinal cord seldom happens. Aneurysm clip yields calculated force for injury induction, as we used in our experiment. At the same time, Longbing Ma et al. (15) applied a cotton strip under T13 lemina to achieve extradural compression, which exerted unknown pressure over the spinal cord.

Aneurysm clip gives a precise injury with calculated force at an exact desired location within 1 minute while Elena E. Foditsch et al. (16) induced injury by inserting balloon catheter with the help of guide wires and dilators which id time required technique and exact location for injury site can be missed. Chen Guang et al. used Infinite Horizon SCI Impactor on female Sprague–Dawley (SD) rats for producing contusion spinal cord injury at the T10 level, but again this infinite horizon spinal cord injury impactor is a costly method. It must require professional and skilful hands for precise and meaningful outcomes (17). The same Infinite Horizons device has been used by Mathew et al. to obtain spinal cord injury (18). Zhang et al. modified Allen's method to establish in male Sprague Dawley rats by dropping a 10gm rod from a distance of 5 cm above the spinal cord and then leaving the rod to rest on the lesion spot for 3 minutes (19). Allen's II impactor was operated by Xiao-Jing et al. studied spinal cord injury induction in their experimental work. They dropped a 10gm rod 25-cm above the exposed cord to achieve the injury (20). Limin Liu et al. used the same impactor for spinal cord injury initiation. However, the rod dropped from a height of 12.5mm (21). Yaguang et al. dropped a 20gm rod on a rat fixed in a stereotaxic system with an

exposed spinal cord to produce injury (22). All these contusive methods can produce doubts about the transmission of required true force to the spinal cord and the exact desired injury construction. Therefore, the reliability of this contusive model is questionable.

Like our study model, Wangying et al used a vascular clip in female rats to achieve spinal cord injury at T9 vertebral level. They achieved a successful injury with less effort and expense (23). Seung-Dam Heo et al. also applied a vascular clip similar to the aneurysm clip. They achieved a well-appreciated and accurate desired spinal cord injury for the research purpose (24). After giving antibiotics and Ringer's Lactate subcutaneously, rats developed injection site abscess, which caused death due to autophagy. Changing the route of administration from subcutaneous to intra-peritoneal and a single-dose antibiotic solved the problem. Nabil et al. also demonstrated the effective use of ceftriaxone in diabetic rats to avoid infection and for active wound healing (25). In a study, Mustafa et al. used ceftriaxone in traumatic brain injury models in rats. They noted that ceftriaxone treatment avoids Cortical Inhibitory Interneuron dysfunction via Transient Salvage of GLT-1 expression (26).

CONCLUSION

In animal studies for the assessment of neural repair and regeneration after induction of spinal cord injury, aneurysm clip compression gives much better results as it is comparatively low-priced, readily available and has proven almost similar functional, electrophysiological and morphological results to humans. Because it resembles human traumatic spinal cord injury, the "aneurysm clip compression injury model" is the best choice for translational research projects. In this model, glial scar formation is the same as in spinal cord injury patients. The aneurysm clip compression model of spinal cord injury is also appropriate for observing the therapeutic and neuroprotective effects of different drugs and other agents. It also gives a good base for studying the secondary injury

process steps and "cell transplantation" treatments after spinal cord injury. Intra-peritoneal route for antibiotics and other drug administration is the best choice as it diminishes the risk of injection site abscess and loss of experimental animals.

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Conflict of interest: The authors declare no competing interest

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