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RESEARCH ARTICLE
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NEURONAL DEGENERATION CAN BE DECELERATED BY PIRFENIDONE AFTER COMPRESSION SPINAL CORD INJURY

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The authors confirm their contribution to the paper as follows:
- Study conception and design: 1st Author.
- Data collection: 1st & 2nd Author;
- Analysis and interpretation of results: 1st & 3rd Author
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- Final approval: 6th Author

All authors reviewed the results and approved the final version of the manuscript.

ABSTRACT
Aim: To explore the neuroprotective effect of pirfenidone in rat's aneurysm clip compression spinal cord injury.

Methodology: A total of 30 healthy Sprague Dawley rats were randomly divided into spinal cord injury groups A, B and C. Group A received a placebo (n = 10), group B received 200 mg/kg/day of pirfenidone (n = 10) and group C received 500 mg/kg/day of pirfenidone. Based on the experimental duration of 14 and 28 days, each group was subdivided into groups 1 & 2 (n = 5 in each subgroup). An aneurysm clip with 70 g closing force was applied to the T7 level of the spinal cord for 1 minute to induce compression spinal cord injury. Immunohistochemistry by MAP2 anti-body for estimating viable neurons was performed.

Results: There were no normal viable neuronal cell bodies in the spinal cord injured areas in any groups. Nevertheless, neuronal cell body residues were detected in the injury sites and a statistically significant difference was witnessed within groups and between groups. The higher dose of 500 mg/kg/day of pirfenidone for 14 days slows down the process of neuronal degeneration in injury lesions compared to 200 mg/kg/day for a prolonged duration of 28 days.

Conclusion: Pirfenidone has no protective effect on neurons after spinal cord injury but due to its anti-oxidant and anti-inflammatory properties, it alters and delays the neurodegenerative process. This leads us to the future experimentation of pirfenidone in neurodegenerative diseases.

Keywords: Pirfenidone, Aneurysm Clip Model, Spinal Cord Compression Injury, neuronal degeneration, MAP2.

ABBREVIATIONS:
TGF: Tumour Growth Factor,
PDGF: Platelet-Derived Growth Factor,
MAP2: Microtubule Associated Protein,
DMSO: Dimethyl Sulfoxide

INTRODUCTION

Spinal cord injuries are one of the most serious health issues, affecting patients' overall quality of life. It has substantial consequences for the lives of individuals, their families, and society since it causes severe disability in the patient. Traumatic spinal cord injuries are a lifetime condition that must be addressed to reduce consequences (1). Immediately after direct primary injury which involves demyelination and neuronal/axonal necrosis, a cascade of secondary injuries starts which includes neuroinflammation, oxidative stress, edema, ischemic changes, cystic cavity formation, glial scar formation, and ultimately cell death (7; 16). Those who have suffered a spinal cord injury lose sensory information and muscle control because damaged neurons and nerve fibers cannot generate or carry signals. The neuronal cell death permanently causes sensorimotor and autonomic disabilities. These proceedings in both primary and secondary spinal cord injuries go through definite developmental stages that are immediate, acute, subacute, intermediate, and chronic phases (6).

During the sub-acute damage phase, phagocytes are heavily infiltrated, macrophages are invading, meningeal and perivascular fibroblasts are infiltrated, and reactive astrocytosis is caused by the activation of astrocytes by inflammatory mediators, which further leads to neuronal degeneration and oxidative stress-induced apoptosis in the intermediate phase (8; 13; 21; 23). Originally developed as an antihelminthic and antipyretic drug, Pirfenidone is a simple pyridine. Due to its tiny molecular size, it rapidly reaches most organs and crosses the blood-brain barrier when taken orally, reaching most organs (14). Pirfenidone holds anti-fibrotic, anti-inflammatory, and also antioxidative properties but still the exact mechanism of action is not well established (15). A cytokine growth factor such as TGF- or platelet-derived growth factor (PDGF) stimulates the production of fibroblasts, collagen, and extracellular matrix. Pirfenidone inhibits one or more of these processes (19). According to research, pirfenidone inhibits tumor necrosis factor, interleukin, and other inflammatory cytokines by blocking their release (3; 4; 10; 20).

MATERIALS AND METHODS

After approval from the institutional ethical review board and the biosafety office, the study that was conducted at Khyber Medical University Peshawar, Pakistan in the department of anatomy and pharmacology. From the National Institutes of Health, 30 healthy male Sprague Dawley rats weighing 250 to 300 grams each were purchased. For each subject, measured environmental setups a 22 to 25 degrees Celsius, appropriate humidity and a 12-hour light cycle were maintained. Free access to water and food was given to all subjects.

GROUPING

There were three main groups A, B and C. Each group was sub-grouped into subgroup 1 (14 days experimental duration) and subgroup 2 (28 days experimental duration) on the basis of experimental duration (n = 5 in each subgroup). Compression spinal cord injury was performed in all groups by applying an aneurysm clip of 70m gram force for 1 minute at T7 spinal cord segment. After surgical induction of spinal cord injury, in subgroups A1 and group A2, DMSO was injected intra-peritoneal

as a placebo daily while rats in subgroups B1 & B2 were daily injected intra-peritoneal pirfenidone 200 mg/kg/day dissolved in DMSO and similarly subgroups C1 and group C2 animals were treated with pirfenidone 500 mg/kg/day in DMSO intra-peritoneal daily (18).
Group A1, B1 & C1 rats were euthanized on the 15th day of the experiment and group A2, B2 & C2 rats were euthanized on the 29th day of the experiment using an intraperitoneal injection of 200 mg/kg pentobarbitone sodium for collection of spinal cord injured segments for histological examination (9; 22). In order to fix and stiffen the spinal cord, the entire vertebral column and spinal cord were removed immediately and transferred to 10% neutral buffered formalin for 24 hours. After 24 hours, 1.5cm of undamaged spinal cord segment with injury site in the centre was removed from the vertebral column and processed and embedded in paraffin wax to make 5µm thin longitudinal tissue serial sections from the dorsal to the ventral direction.

Staining and Immunohistochemistry

Around 18 slides of each specimen in a serial order were prepared, out of which slides number 4, 8, 12 and 16 were selected from each spinal cord tissue section to quantify the average measure of viable neurons in injury lesions. MAP2 (Invitrogen, Thermofisher Scientific) monoclonal primary unconjugated antibody was used in 1:50 dilution for the detection of normal viable neurons in the injured area in the spinal cord by standardized immunohistochemistry technique (11; 12; 17).

MICROSCOPY & STATISTICS

Microscopy was done on 40X, 100X, 200X and 400X magnification by Nikon Eclipse 80i microscope and pictures were taken on all magnifications. Several images at 200x magnification in specific sequences were taken, covering a 1200m diameter area around the centre of the injury and its surrounding tissue. We then stitched and grouped these images together using Microsoft Research's Image Composite Editor 2.0 (ICE). Viable neurons (residues) were observed in the injured area and neuronal cell body residues were quantified by ImageJ Fiji software.
Using SPSS version 22, means & standard deviations were calculated, for descriptive statistics. For comparison between the groups, the Kruskal Wallis test was used and for comparison within the groups Mann Whitney U, test was applied (P value of < 0.05 was taken as statistically significant).

RESULTS

No normal viable neuronal cell body was identified in the injury site in any group but some neuronal cell body residues were identified and the percentage of the residues in the total injury area of each subject in all groups was estimated by image J. Difference in the percentage of neuronal cell bodies residues in the injury site of subgroups A1 and A2 showed no significance as $P = 0.32$. The difference between subgroups B1 & B2, and C1 & C2 showed high significance for both $P = .008$, demonstrated in figure 1-A. Differences between the neuronal cell bodies residues in the injury site of subgroups A1, B1 and C1 and between subgroups A2, B2, and C2 showed high significance as $P = .004$ & $.003$ respectively, shown in figure 1-B.

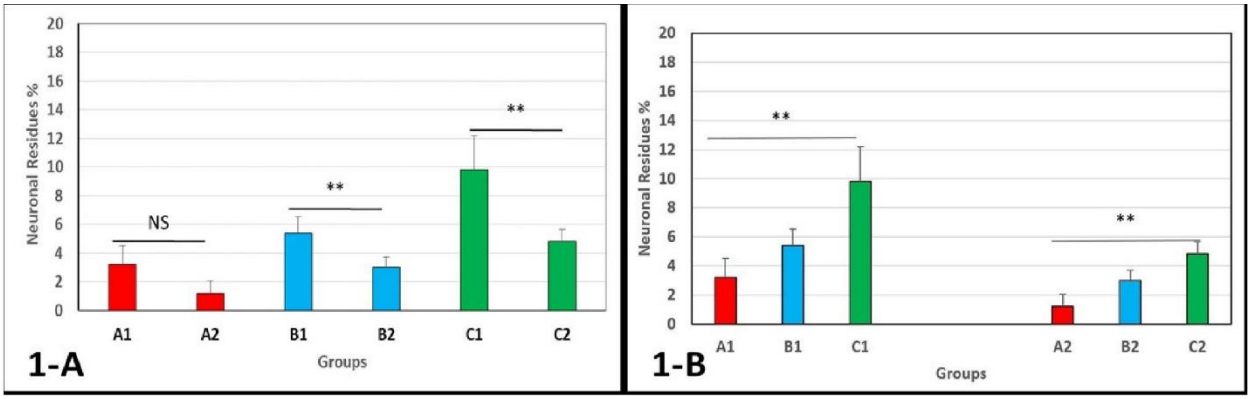

Figure 1:- (1-A) within the group comparison of mean percentages of neuronal cell bodies residues. **(1-B)** Between the groups comparison of mean percentage of neuronal cell bodies residues. **A1:-**

Non-Pirfenidone treated group with 14 days experimental duration. **A2**:- Non-Pirfenidone treated group with 28 days experimental duration. **B1**:- 200 mg/kg/day Pirfenidone treated group with 14 days experimental duration. **(B2)** 200 mg/kg/day Pirfenidone treated group with 28 days experimental duration. **C1:-** 500 mg/kg/day Pirfenidone treated group with 14 days experimental duration. **C2:-** 500 mg/kg/day Pirfenidone treated group with 28 days experimental duration. ⊤ denotes SDEV. Mann Whitney U test and Kruskal Wallis test P value <0.05 in all groups except between A1 & A2, P value >0.05.

Neuronal cell body residues in the injury site were more pronounced in pirfenidone-treated groups as compared to non-pirfenidone-treated groups. Furthermore, between high and low-dose pirfenidone-treated groups, high dose (500 mg/kg/day) group showed more residual neuronal bodies as compared to low dose (200 mg/kg/day) group. These residual neuronal bodies were more prominent in 14 days subgroups as compared to 28 days subgroups within the groups, shown in figures 3 & 4. These findings show that pirfenidone has slowed down the process of neuronal degeneration after spinal cord injury, especially in the high dose group.

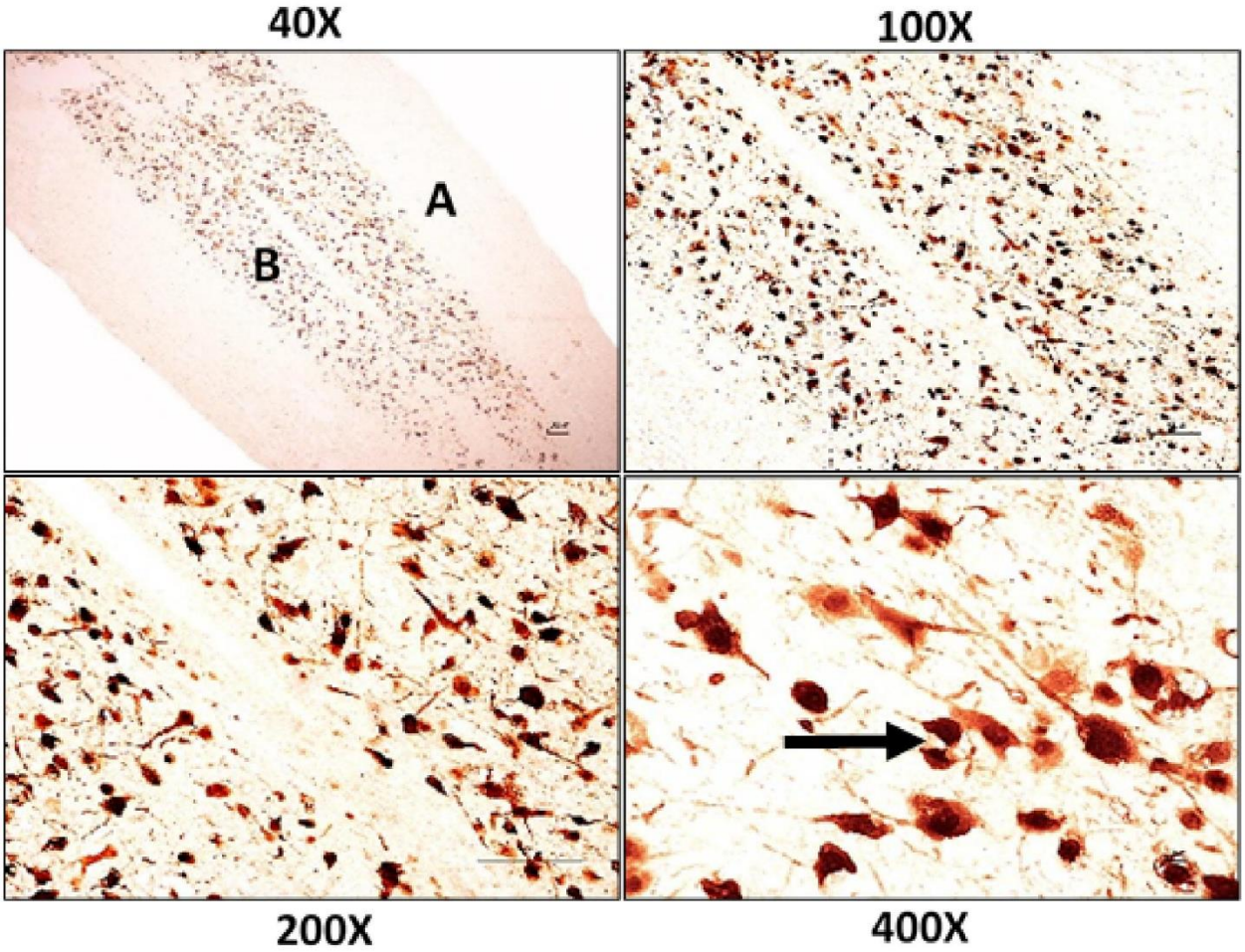

Figure 2: - MAP2 antibody immunohistochemistry photomicrographs of 5 µm thick normal rat spinal cord longitudinal section at 40X, 100X, 200X & 400X. **"A"** at 40X demonstrates normal peripheral white matter and **"B"** shows central grey matter tracks having neuronal cell bodies. Arrow at 400X shows normal neuronal cell bodies.

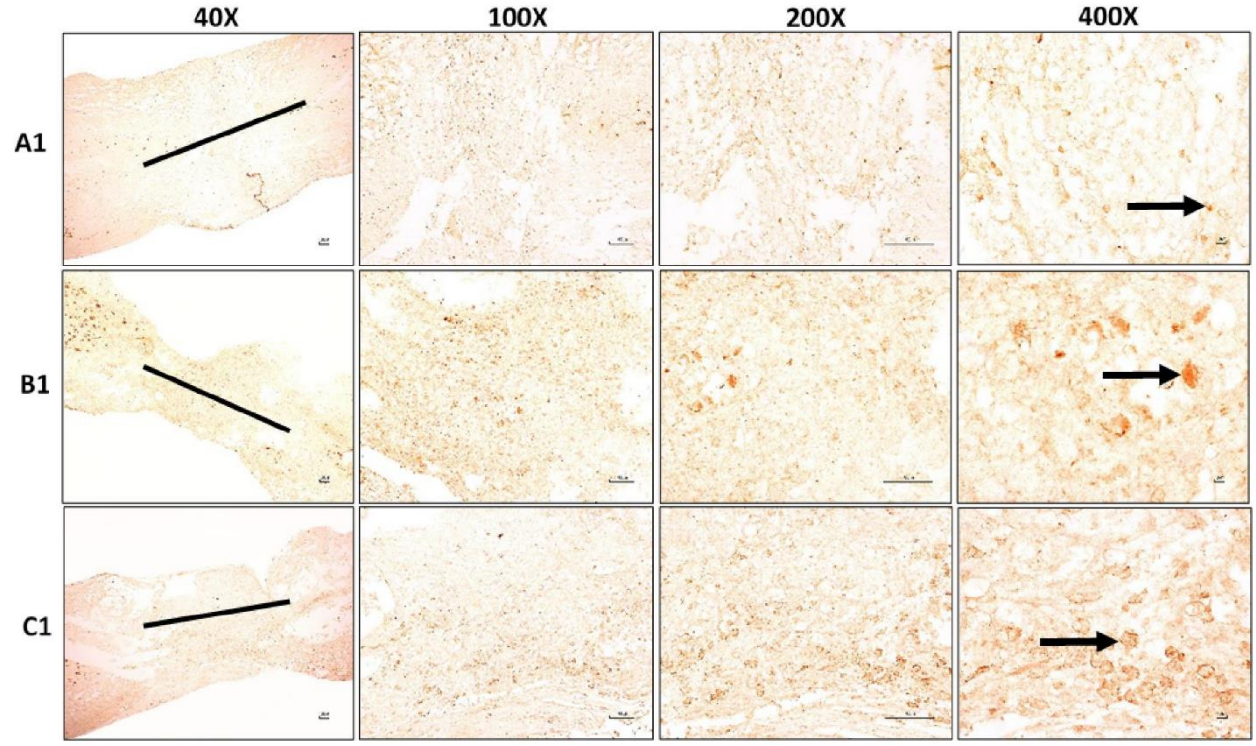

Figure 3: - MAP2 antibody immunohistochemistry photomicrographs of 5 µm thick rat spinal cord longitudinal sections from 14 days experimental duration groups at 40X, 100X, 200X & 400X showing prominent injury site marked by black lines at 40X and comparison of the percentage of neuronal cell bodies residues in the injury lesions marked by arrows between groups. More neuronal residues can be seen in prifenidone treated groups (B1 & C1) compared to non pirfenidone treated group (A1). Between pirfenidone treated groups, 500 mg/kg dosage group (C1) shows more residues compared to 200 mg/kg group (B1).

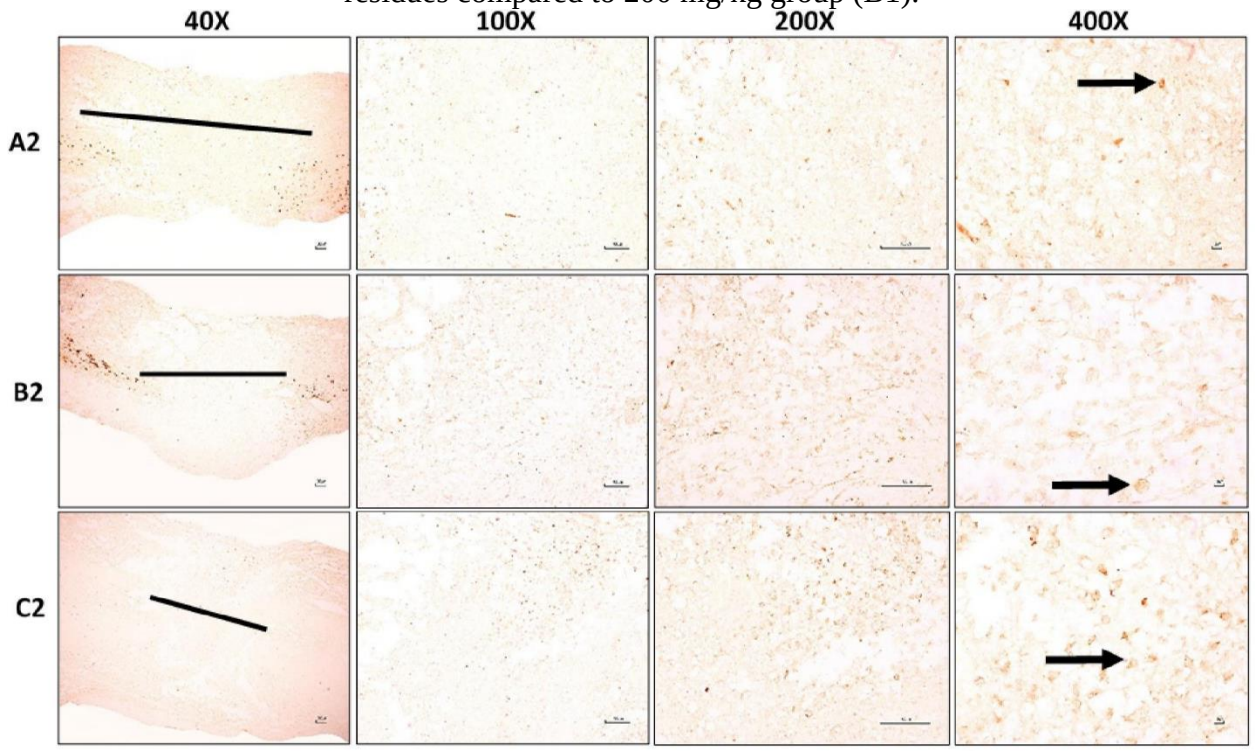

Figure 4: - MAP2 antibody immunohistochemistry photomicrographs of 5 µm thick rat spinal cord longitudinal sections from 28 days experimental duration groups at 40X, 100X, 200X & 400X showing prominent injury site marked by black lines at 40X and comparison of the percentage of neuronal cell bodies residues in the injury lesions marked by arrows between groups. More neuronal

residues can be seen in prifenidone treated groups (B2 & C2) compared to non pirfenidone treated group (A2). Between pirfenidone treated groups, 500 mg/kg dosage group (C2) shows more residues compared to 200 mg/kg group (B2).

DISCUSSION

As a universal problem, the treatment of spinal cord injury has constantly been the point of attention for researchers. Neuronal degeneration and the ultimate death of neurons as a result of primary injury to the spinal cord is one of the main causes of enduring post-injury motor and sensory disabilities. In our present study, we used pirfenidone for the first time to evaluate its effect on stabilizing and protecting neurons after spinal cord injury by decreasing oxidative stress, inflammation and fibrosis. According to our observations, pirfenidone has no direct neuro-protective effect as there were no viable neurons detected in pirfenidone-treated rats after spinal cord injury. However, the presence of neuronal cell bodies residues especially more in high dose pirfenidone-treated group signifies that pirfenidone somehow decelerates the process of neuronal degradation and degeneration. On review of the literature, we haven't found any study in which viable neurons were calculated and compared in pirfenidone and non-pirfenidone-treated rats having compression spinal cord injury. Because of the anti-inflammatory, anti-fibrotic and anti-oxidant effects of pirfenidone, we compared our study with recent studies in which pirfenidone was used in CNS other than the spinal cord for neuro-protection purposes.

Our present study is in contrast with the study conducted by Buzkurt I et al (2022) in which they demonstrated the neuroprotective effects of 500 mg/kg dose of pirfenidone in a weight drop traumatic brain injury model in rats. They reported significant differences in the loss of neurons between the pirfenidone-treated group and the non-pirfenidone-treated group after 1 week of the injury. Pirfenidone treated group presented less neuronal loss as compared to the non-pirfenidone-treated group (2). Our present study results show a resemblance to the results of a study conducted by Castro-Torres RD et al (2014), in which they demonstrated the effectiveness of a single dose of pirfenidone in attenuating neuronal degeneration by reducing lipid-peroxidation after kainic-acid-induced toxicity in rat hippocampus. They reported significant differences in the degenerating neurons in between the 350 mg/kg pirfenidone dose treated group and other SCI/lower doses groups, detected by Fluoro-Jade B staining 72 hours after treatment. 350 mg/kg dose treated rats showed a high number of surviving neurons than other groups (5).

CONCLUSION

An injury to the spinal cord usually results in a wide range of sensory, motor, and autonomic neuropathy due to the damage caused by a variety of processes, including neuronal cell death, occurring during the healing process. These injuries remain a serious public health problem around the world. Clinicians face more challenges because of SCI and its devastating effects. In the aftermath of a primary SCI, neuronal cell death could have a critical impact on the mechanisms that produce the ultimate neurological deficit. Various systems in the spinal cord respond to trauma, including inflammation, angiogenesis, neurogenesis, plasticity, and spontaneous regenerative mechanisms, but they are unable to prevent damage from progressing. By modulating or improving these mechanisms, new therapeutic options may be made possible, which could open up a wider range of possibilities. Using an experimental compression spinal cord injury model, Pirfenidone was evaluated for its antifibrotic, anti-inflammatory, and antioxidative properties. Our study with limitations has concluded that pirfenidone has anti-oxidant and anti-inflammatory effects and slows down the process of neuronal degeneration and degradation. More research is required to investigate the efficacy of pirfenidone in neuro-degenerative disorders and traumatic spinal cord/CNS injuries.

Author contribution

All the authors contributed equally.

Conflict of interest
The authors declare no competing interest.

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