Pirfenidone Reduces Neurological Deficit by Promoting Axonal Growth after Compression Spinal Cord Injury

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ABSTRACT

Aim: This study aimed to determine whether pirfenidone can promote axonal growth and reduces the neurological deficit in rats following aneurysm clip compression spinal cord injury.

Methodology: Three main groups of thirty male Sprague Dawley rats were indiscriminately assigned (n = 10 in each group). Compression spinal cord injury was induced in all rats. Group "A" was given a placebo daily, group "B" was given a daily dose of pirfenidone 200 mg/kg/day, and group "C" was given a daily dose of pirfenidone 500 mg/kg/day. Using 14 & 28-day experimental durations, each group was subdivided into groups 1 & 2 (n = 5 in each). Von Frey test, hot plate test and acetone drop test were performed to evaluate sensitivity in the hind limbs of each rat. Immunohistochemistry was done using the GAP43 antibody to quantify axonal growth in the injury lesions.

Results: Scores of Von Frey test, hot plate test, acetone drop test and axonal growth were statistically different between the groups. Pifenidone-treated SCI groups showed improvements in all test scores and an increase in axonal growth compared to the non-pirfenidone-treated SCI group.

Conclusion: Pirfenidone improves sensory activity and promotes axonal growth after spinal cord injury, most probably by limiting collagen deposition in the inner central core of the glial scar and, providing passage and a favourable environment for axonal regeneration.

Keywords: Pirfenidone, Aneurysm Clip, Spinal Compression Injury, Von Frey test, GAP43

Abbreviations: SCI: Spinal cord injury, **PFD:** Pirfenidone, **DMSO**: Dimethyl sulfoxide

1. INTRODUCTION

Globally, there are 10.4–83 cases of spinal cord injury per million people per year and the condition remains a significant cause of morbidity and costs to society(16). Injuries to the spinal cord profoundly impact patients' overall quality of life. Spinal cord injuries caused by trauma are permanent conditions that need to be addressed to minimize their effects since it causes severe sensory and motor disability in the patient (1). The events of spinal cord injury can be classified into two types of physiological processes and timeframes (26). Physical concussions, sheer injuries, lacerations of the cord, and micro-haemorrhages are some of the consequences of a primary injury (9; 27; 31). Primary compression injuries, such as burst fractures and fracture-dislocation injuries, are among the most common examples of primary injuries (12; 23; 28). Subsequently, secondary injury is caused by complex interconnected signaling cascades and tissue

changes that lead to altered hemostasis, apoptosis, tissue devastation, neuronal death and glial scar formation (7; 27; 28).

Inflammatory reactions, macrophage invasion, meningeal and perivascular fibroblast infiltration, and reactive astrocytosis are responsible for the sub-acute damage phase lasting approximately two weeks. After that, oxidative stress increases leading to neuronal demyelination and apoptosis in the following intermediate stage. Glial scars form in the next stage, which suppresses axonal regeneration and repair. Essentially, this occurs because hypertrophic astrocytic processes around the lesion have been reconfigured unequally and collagen is being deposited by fibroblasts in the central core of glial scars. Axonal sprouting and regeneration can be promoted at the injured site by limiting the above-mentioned products (13; 17; 33; 35). These results will improve neurological rehabilitation and functional recovery. To date, neuroprotective and regenerative treatments for SCI have had limited clinical effectiveness despite a growing understanding of its pathophysiology (16).

In the beginning, pirfenidone (PFD) was used mostly for helminthic infections and pyrexia. Orally taken PFD crosses the blood-brain barrier quickly due to its small molecular size (20). A combination of anti-fibrotic, anti-oxidant, and anti-inflammatory properties are present in Pirfenidone. It inhibits fibroblast proliferation and cytokines and proteins associated with fibrosis. Additionally, it inhibits TGF- and platelet-derived growth factor (PDGF), which contribute to extracellular matrix formation and storage (30). A variety of inflammatory cytokines, including tumor necrosis factor, interleukin, and other inflammatory mediators, have been shown to be blocked by pirfenidone in several studies (3; 4; 15; 32).

2. MATERIALS AND METHODS

In accordance with institutional ethics review board and biosafety office approval, this study was conducted at the Institute of Basic Medical Sciences, Khyber Medical University Peshawar, Pakistan. In order to conduct this study, 30 healthy male Sprague Dawley rats were obtained from the National Institutes of Health (NIH). The animals were on average 3-4 months old and weighed 250-300 grams. During the experiment, rats were housed in a controlled environment with 22-25°C temperatures, 95% humidity, and 12 hours of daylight each day. As per the National Research Council's guidelines for laboratory animal care, the experiment was conducted.

2.1. Animals Grouping

Experimental animals were divided into A, B and C groups. Each of these groups were sub-divided into sub-group "1" with 14 days experimental duration and sub-group "2" with 28 days experimental duration (n = 5 in each sub-group). Groups A1 and A2 received DMSO (dimethyl sulfoxide) intra-peritoneally as a placebo daily. A compression spinal cord injury was induced in group B1 and group B2, and daily pirfenidone 200 mg/kg/day was administered daily, intra-peritoneally using DMSO as a solvent (29). Group C1 and C2 were subjected to compression spinal cord injury, and pirfenidone 500 mg/kg/day was administered intra-peritoneally every day, dissolved in DMSO (29).

2.2. Spinal Cord Injury Model

Following anaesthesia, a precise cut was made on the back of the rats at T7 vertebral body level to expose the spinous process and posterior lamina. Spinous processes were removed from the T7 vertebra as well as dorsal lamina were completely eliminated by laminectomy. A 70gm force aneurysm clip was applied approximately in the middle of the exposed T7 spinal cord segment with intact meninges. The clip was then removed gently after one minute and the wound was closed

in layers (14). Proper antibiotics and analgesics were given to overcome post-operative pain and infection.

2.3. Sensory Evaluation

Sensory evaluation of groups A1, B1 & C1 rats was done on the 15th day of experiment and group A2, B2 & C2 rats were evaluated on the 29th day of experiment.

2.3.1. Von Frey Test

Manual Von Frey ascending stimulus method was applied, which approximates the mechanical pain withdrawal threshold. It involves applying Von Frey monofilaments in order with increasing force until a withdrawal response is triggered. As a result, the mechanical pain withdrawal threshold is determined by the strength (size) of the Von Frey filament generating the withdrawal response. The Von Frey monofilaments are calibrated with known size and force in newton (8).

Each rat was placed on a metal mesh individually. To prevent rat escape, an upside-down glass jar was placed over the rat, large enough to accommodate the rat easily. During testing, room temperature was maintained. In order to become accustomed and acclimatized to the new environment, rats were left free and unrestrained for 15 minutes. A persistent pre-determined force for 2–5 seconds was conveyed by sequentially applying Von Frey monofilaments perpendicularly to the hind paw plantar surface until it buckled. During the application of the filament or immediately after its removal, when the rat displayed spontaneous behaviors such as abrupt paw withdrawal, licking, or quaking of the paw, the response was deemed positive, and the force of the filament applied that generated the response was noted (8; 18; 24; 25).

2.3.2. Hot Plate Test

Hot plate test was used to measure heat thresholds. For performing this test, unrestrained rat was placed on preheated metal surface of the above mentioned equipment. A glass jar, big enough to accommodate the rat easily providing enough space for moment, was put upside down on rat to avoid rat escape. The temperature of the metal surface was maintained at a constant temperature, typically between 50°C and 55°C. 30 seconds time was set as cutoff point. The response latency was recorded in the form of time taken in seconds to notice any nocifensive behavior with in the cutoff limit. These Nocifensive behaviors were observed as fore-paw withdrawal, hind-paw withdrawal/licking, hind-paw stamping, slanting body posture and jumping (2; 8; 18). Time was recorded by digital stop watch and rats were immediately removed after noticing any nocifensive behavior initiation.

2.3.3. Acetone Drop Test

For evaluation of cold allodynia (Cold chemical thermal sensitivity), acetone drop test was carried (34). For performing this test, unrestrained rats were individually placed on a metal mesh surface. A glass jar, large enough to accommodate the rat easily providing enough space for moment, was put upside down on rat to avoid rat escape. Surrounding temperature during testing was maintained at room temperature. Rats were unrestrained and they were allowed for about 20 minutes to habituate in order to familiarize them with the new environment. After that when rat was in rest and not grooming itself, acetone drop approximately 50µl in volume was applied quietly on to the central region of the hind paw plantar surface. The rats reaction to acetone drop were recorded

within 20 seconds after delivering acetone drop and scored on a 4 point scale in which zero score for no response, 1 score for quick withdrawal, flick or stamp of the paw, 2 score for prolonged withdrawal or repeated flicking and 3 score for repeated flicking and licking of the paw were given accordingly. The responses were measured with a digital stopwatch. For each individual measurement, both paw planter surfaces of hind limbs were tested at least three times and then their means were calculated. Roughly 5 minutes break was given between each application of acetone drop (8; 18; 19; 21; 38).

2.4. End of Experiment

Group A1, B1 & C1 rats were euthanized on the 15th day of experiment and group A2, B2 & C2 rats were euthanized on the 29th day of experiment for collection of spinal cord injured segments for histological analysis. Euthanasia was done after performing all behavioral tests on each rat. After confirmation of death of rats, dissection of the dorsal spine was performed and spinal cord segments with injury sites in the center were taken out from vertebral column and were placed in freshly prepared 10% buffered formalin.

2.5. Tissue Processing & Immunohistochemistry

Tissues were processed through series of different reagents that performed tissue fixation, dehydration, clearing and then infiltration. Finally tissue were embedded in a medium (paraffin wax) that offers firm support to the tissue to be sectioned by microtome for staining and microscopy. 5 µm thin longitudinal tissue serial section ribbons were made by. Serial sectioning of spinal cord tissues was done from dorsal to ventral direction. Around 18 slides of each specimen, having three sections in a serial order on each slide, were prepared. Slides number 4, 8, 12 and 16 out of total 18 slides from each specimen were selected to estimate average measure of axonal

growths/sprouting in dorsal, central and ventral parts of the injury lesions. GAP43 (Growth Associated Protein 43) Recombinant Rabbit Monoclonal primary antibody was used for the detection of axonal sprouting/growth in the injury site of spinal cord.

2.6. Microscopy and Data Analysis

For microscopy and data analysis of all stained sections, Nikon Eclipse 80i microscope was used. Microscopy was done on 40X, 100X, 200X and 400X magnification and images were taken on all magnification. Multiple images in specific sequence were taken on 200X magnification in 1200µm diameter area around the center of the injury which covered entire injury area and surrounding tissue. These images were then stitched and grouped together using ICE software (Image Composite Editor 2.0). Axonal growth/sprouting in the injury lession was measured by image J Fiji software as percentage of area occupied by axonal sprouts in the total injury site per section.

2.7. Statistics

Analysis of the data was conducted using SPSS version 22 and for descriptive statistics, means and standard deviations were calculated. Data were compared between groups using the Kruskal Wallis test, and within groups using the Mann Whitney U test. Statistics were considered significant at a P value of <0.05.

3. RESULTS

Mean Von Frey test scores of subgroups A1 = 6.50 ± 0.24, A2 = 5.95 ± 0.36, B1 = 5.95 ± 0.38, B2 = 4.69 ± 0.26, C1 = 5.03 ± 0.68 and C2 = 3.63 ± 0.58. Von Frey test scores differed significantly between the groups A1 & A2, B1 & B2, and C1 & C2, as indicated in figure 1-A by P values of .025, .016, and .008 respectively. There was statistically significant difference in Von Frey test scores between A1, B1, C1 and A2, B2, C2 as P valve for these are .006 & .002 respectively, as

shown in figure 1-B. Improvement in the Von Frey test scores denoting the mechanical pain threshold of subgroups A1, A2, B1, B2, C1 and C2 was 97.7%, 89.4%, 89.4%, 70.5%, 75.6% and 54.5% respectively.

Mean hot plate test scores of subgroups A1 = 15.8 ± 2.0, A2 = 13.0 ± 2.0, B1 = 12 ± 1.9, B2 = 8.6 ± 1.36, C1 = 9.8 ± 1.3 and C2 = 6.8 ± 1.2. Difference in the hot plate test scores of subgroups A1 and A2 showed no significance as P = .091 while difference in subgroups BI and B2, and C1 and C2 showed significance as P = .027 and P = .015 respectively, demonstrated in figure 1-C. Difference between the hot plate test scores of subgroups A1, B1 and C1 showed significance as P = .01. Similarly, difference between the hot plate test scores of subgroups A2, B2 and C2 also showed high significance as P = .008, demonstrated in figure 1-D. In subgroups A1, A2, B1, B2, C1 and C2, the percentage of responsive heat threshold was 52%, 43%, 40%, 28%, 32% and 22% respectively.

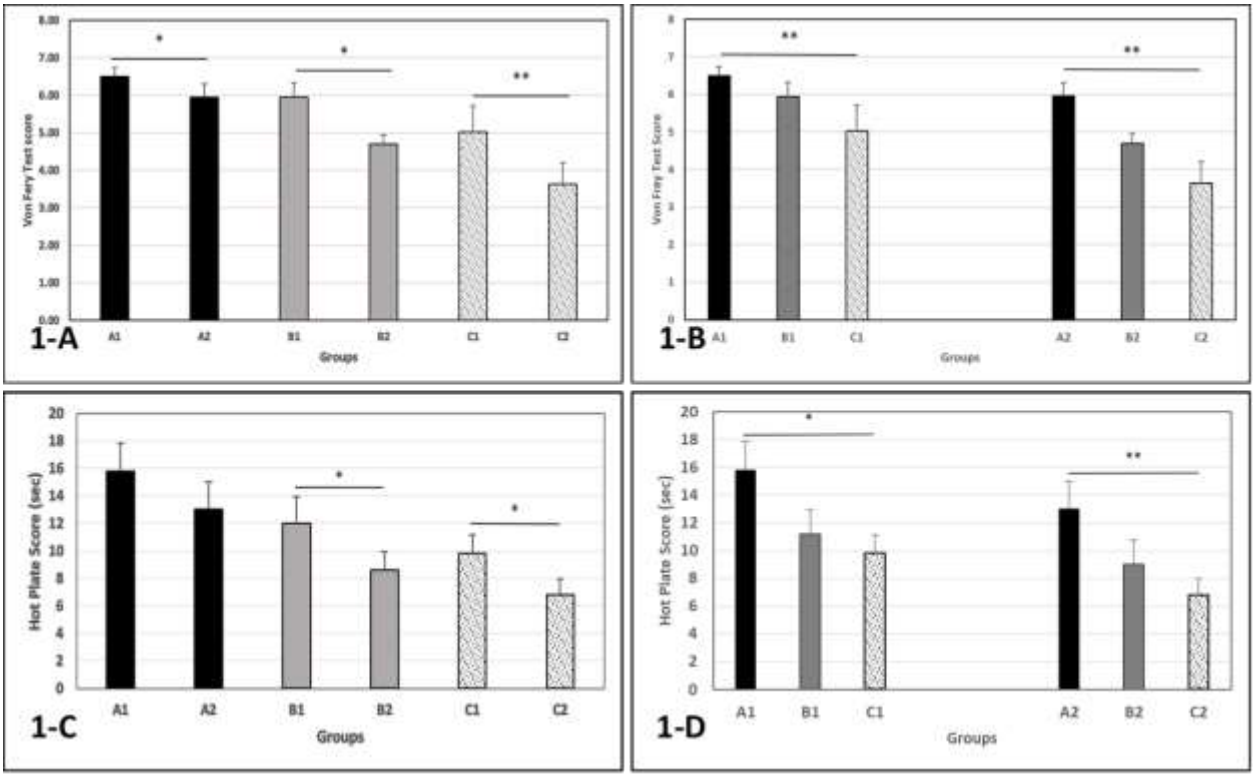

Figure 1: **(1-A)** Comparison of mean Von Frey test scores with in the groups. **(1-B)** Comparison of mean Von Frey test scores between the groups. **(1-C)** Comparison of mean hot plate test scores with in the groups. **(1-D)** Comparison of mean hot plate test scores between the groups. **"A1"** 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 presents ***P*** value <0.05 within & between the groups.

Mean acetone drop test scores of subgroups A1 = 0.0 ± 0.0, A2 = 0.2 ± 0.4, B1 = 0.2 ± 0.4, B2 = 1.2 ± 0.4, C1 = 0.8 ± 0.4 and C2 = 1.4 ± 0.5. Difference in the acetone drop test scores of subgroups A1 and A2 showed no significance as $P = .317$ while difference in subgroups BI & B2, and C1 & C2 showed significance as $P = .015$ and $P = .042$ respectively, shown in figure 2-A. Difference between the acetone drop test scores of subgroups A1, B1 and C1 showed significance as $P = .026$. Similarly, difference between the acetone drop test scores of subgroups A2, B2 and C2 also showed significance as $P = .01$, demonstrated in figure 2-B. Cold thermal sensitivity recovery of subgroups A1, A2, B1, B2, C1 and C2 was 0%, 10%, 10%, 60%, 40% and 80% respectively.

Mean percentage of area occupied by axonal sprouts in injury lesions of subgroups A1 = 4.09 ± 1.40, A2 = 8.19 ± 1.26, B1 = 12.08 ± 2.48, B2 = 16.95 ± 2.30, C1 = 19.63 ± 1.67 and C2 = 30.10 ± 2.00. Difference in the percentage of axonal growths in the injury lesions of subgroups A1 and A2, B1 and B2, and C1 and C2 showed significance as P values for these are $P = .009$, $P = .028$ and $P = .009$ respectively, shown in figures 2-C, 3, 4 & 5. Difference between the axonal growths in the injury site of subgroups A1, B1 and C1 and between subgroups A2, B2, and C2 showed high significance as P value for both was .002, shown in figures 2-D, 6 & 7.

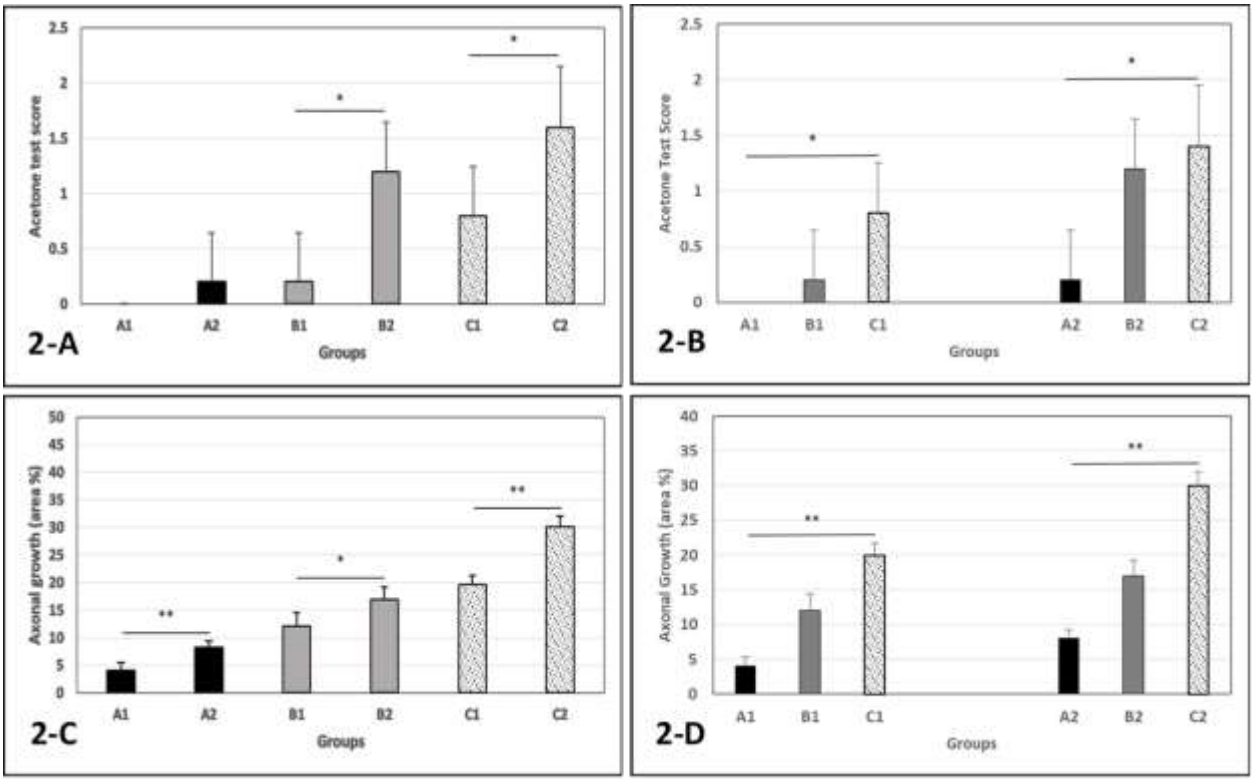

Figure 2: **(2-A)** Comparison of mean acetone drop test scores with in the groups. **(2-B)** Comparison of mean acetone drop test scores between the groups. **(2-C)** Within the groups comparison of mean percentage of injured area occupied by axonal growths/sprouts. **(2-D)** Between the groups comparison of mean percentage of injured area occupied by axonal growths/sprouts. **"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 presents ***P*** value <0.05 within & between the groups.

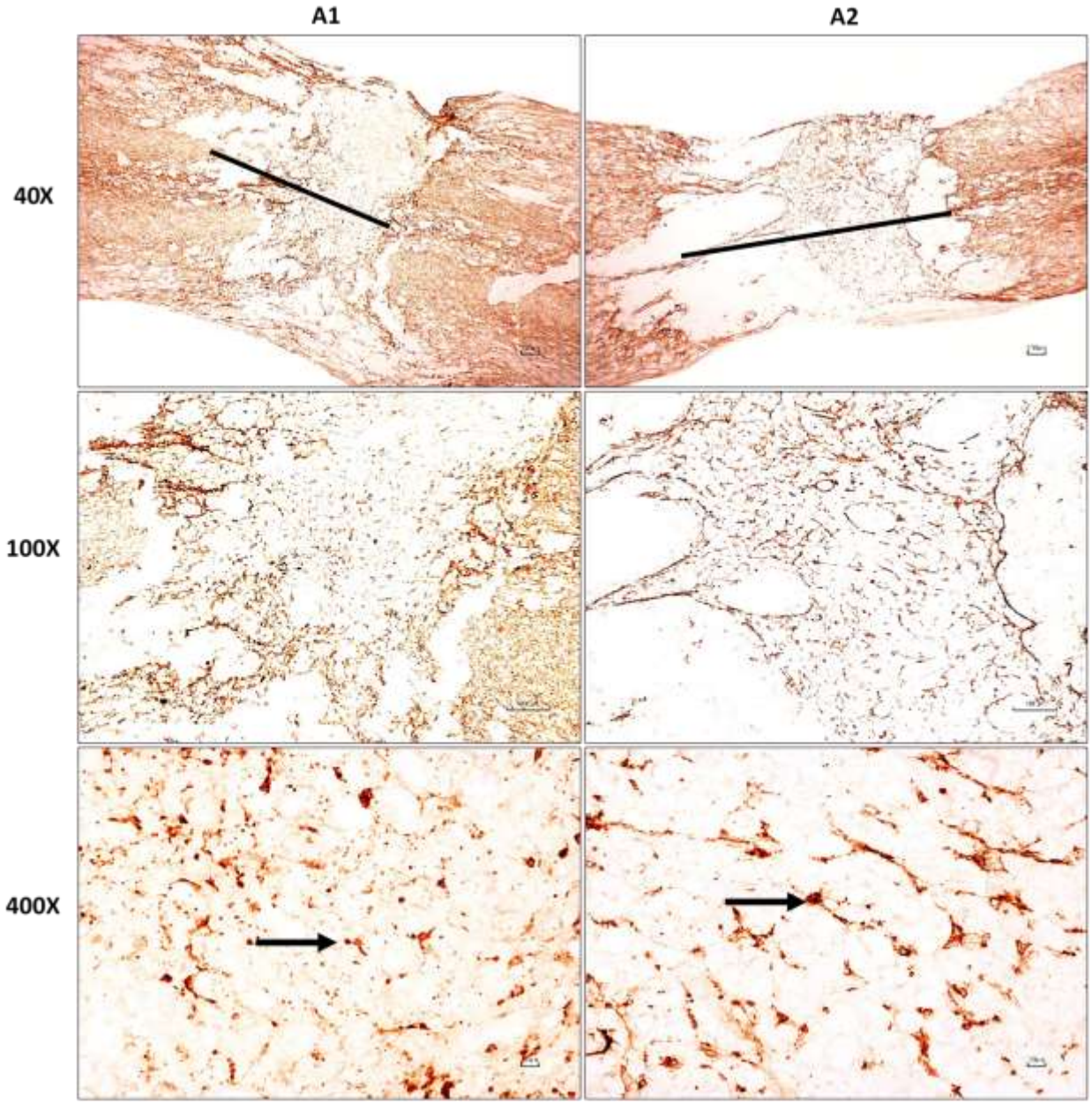

Figure 3: Photomicrographs of 5 µm thick rat spinal cord longitudinal sections showing Immunohistochemical staining for the expression of GAP43, from non pirfenidone treated groups at 40X, 100X, 200X & 400X . **"A1"** represents 14 days experimental duration group while **"A2"** shows 28 days experimental duration group. At 40X, prominent injury site marked by black lines can be appreciated. At 400X, axonal growths/sprouts in the injury lesions are marked by arrows.

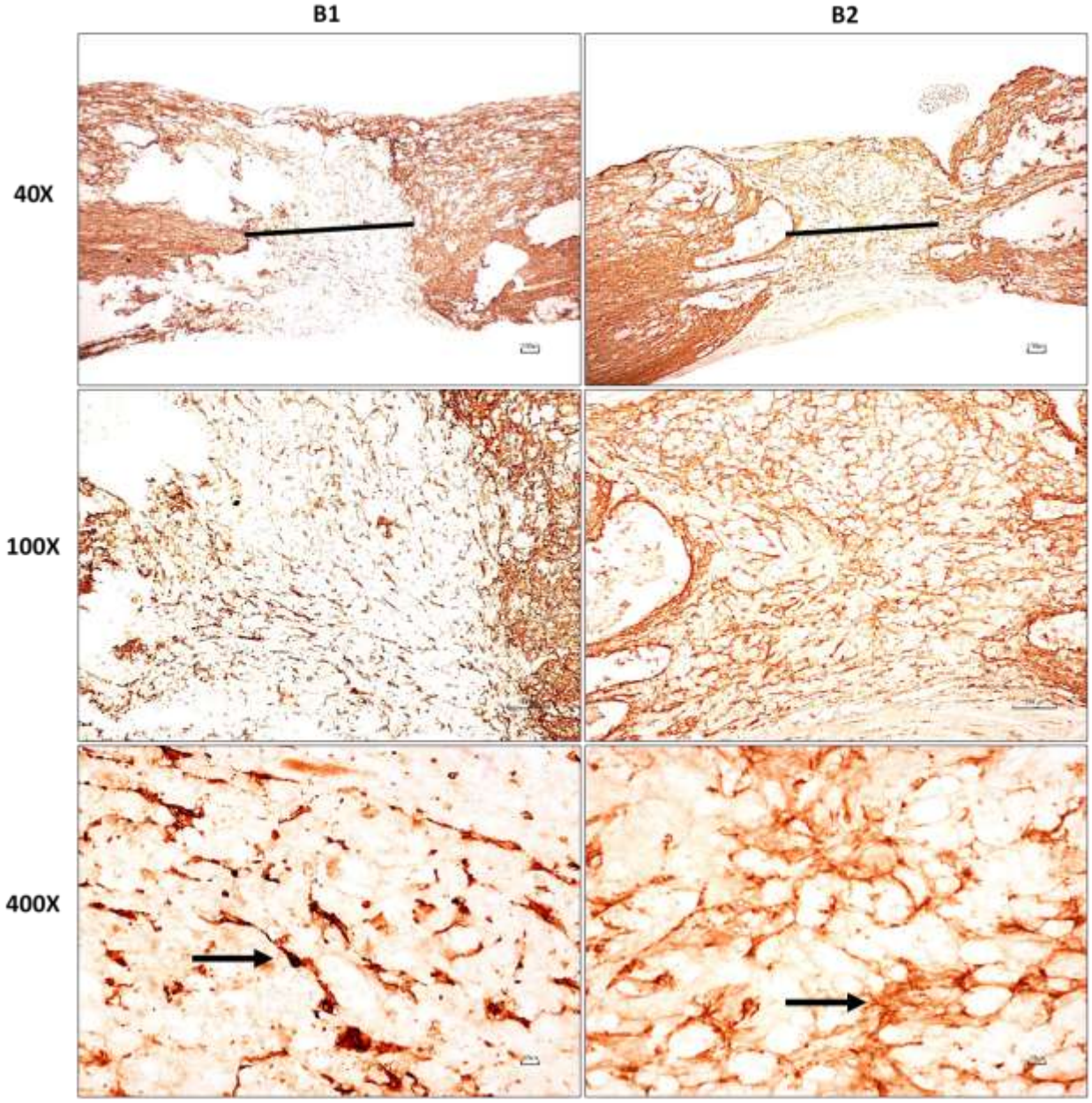

Figure 4: Photomicrographs of 5 µm thick rat spinal cord longitudinal sections showing Immunohistochemical staining for the expression of GAP43, from 200 mg/kg/day pirfenidone treated groups at 40X, 100X, 200X & 400X . **"B1"** represents 14 days experimental duration group while **"B2"** shows 28 days experimental duration group. At 40X, prominent injury site marked by

black lines can be appreciated. At 400X, axonal growths/sprouts in the injury lesions are marked by arrows. More axonal growths can be seen in **"B2"** group compared to **"B1"**.

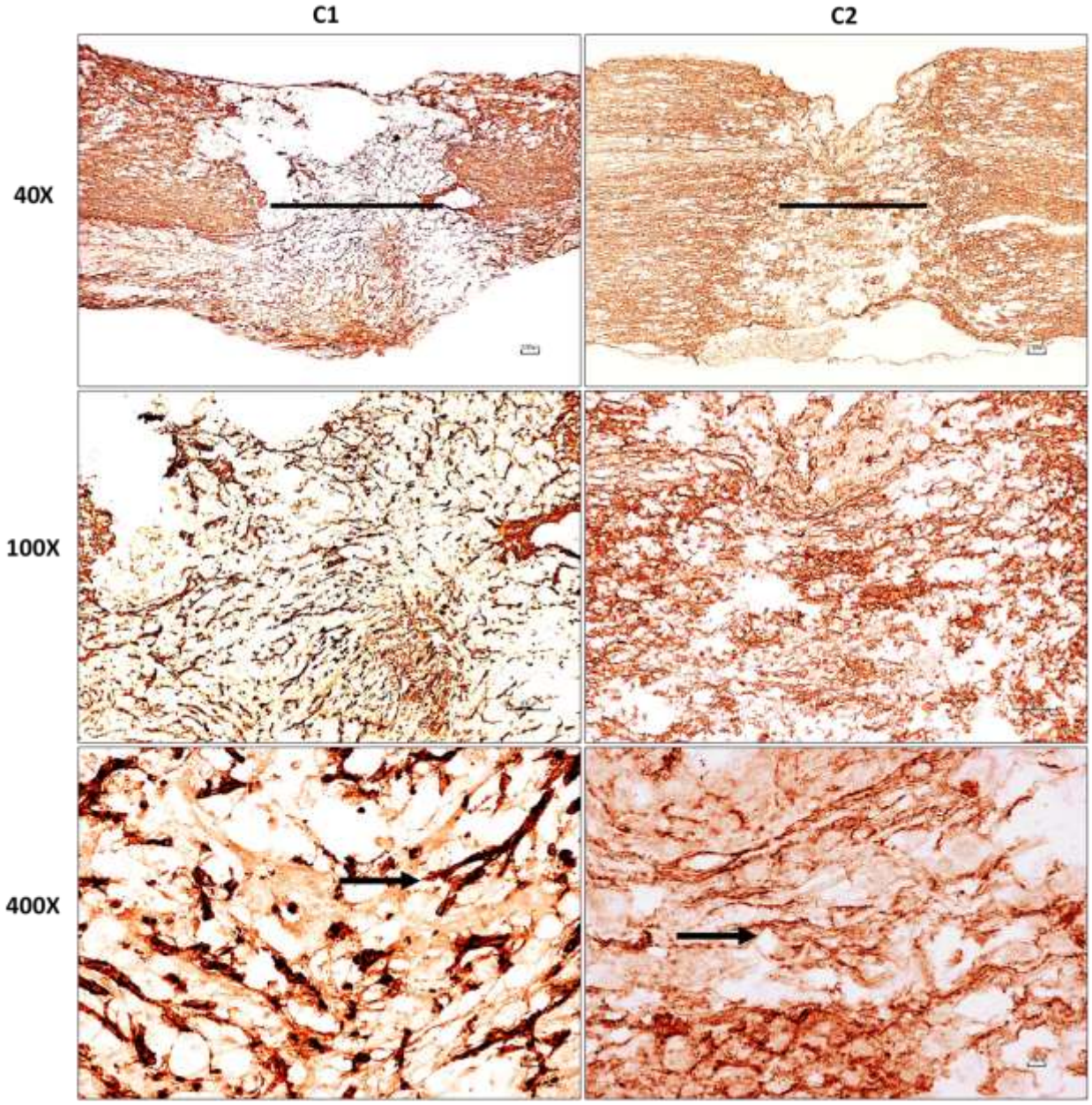

Figure 5: Photomicrographs of 5 µm thick rat spinal cord longitudinal sections showing Immunohistochemical staining for the expression of GAP43, from 500 mg/kg/day pirfenidone treated groups at 40X, 100X, 200X & 400X . **"C1"** represents 14 days experimental duration group

while **"C2"** shows 28 days experimental duration group. At 40X, prominent injury site marked by black lines can be appreciated. At 400X, axonal growths/sprouts in the injury lesions are marked by arrows. More axonal growths can be seen in **"C2"** group compared to **"C1".**

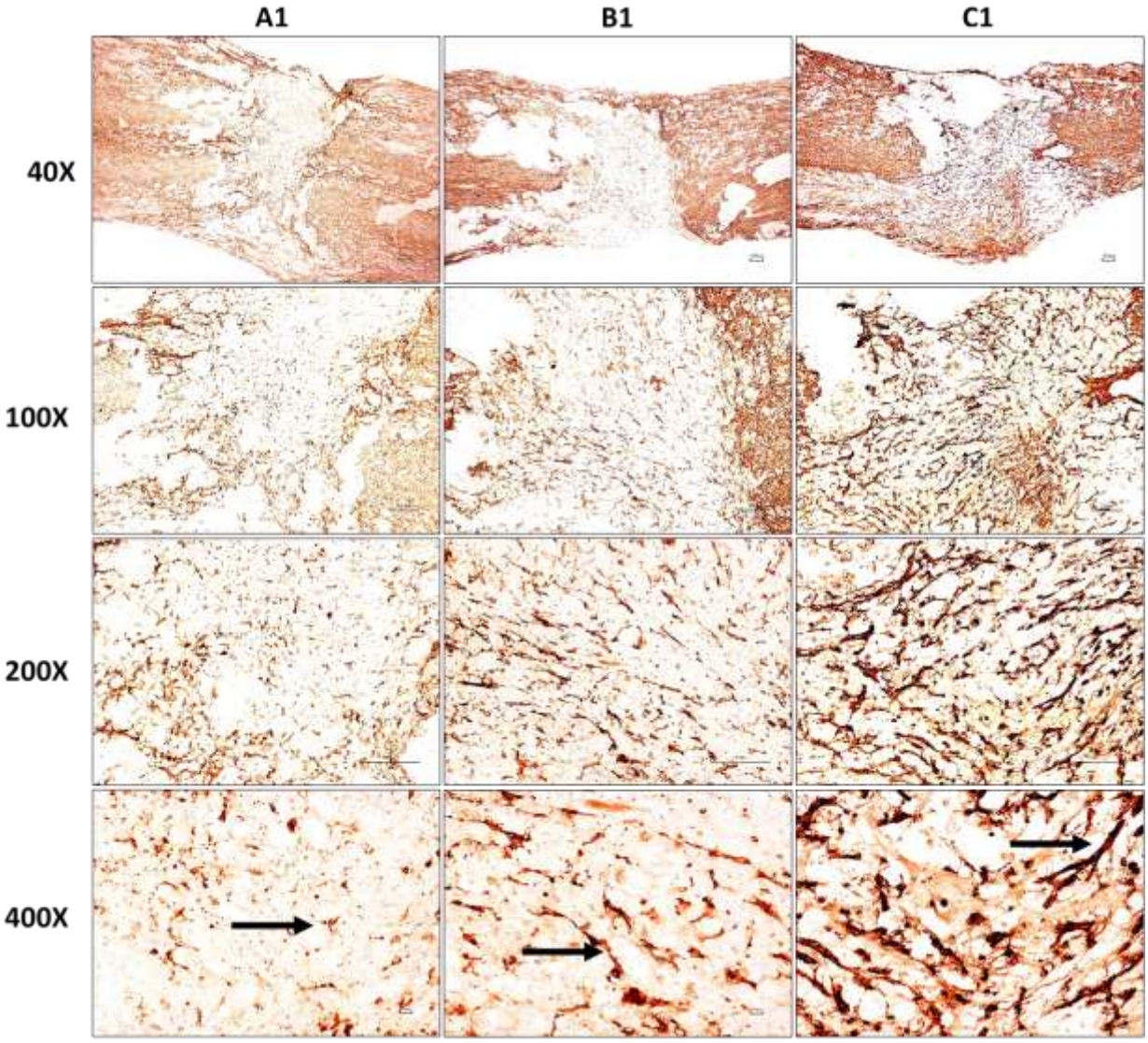

Figure 6: Photomicrographs of 5 µm thick rat spinal cord longitudinal sections showing Immunohistochemical staining for the expression of GAP43, from 14 days experimental duration groups at 40X, 100X, 200X & 400X. On comparison, more axonal growths can be seen in prifenidone treated groups **"B1" & "C1"** compared to non pirfenidone treated group **"A1"** marked

by arrows at 400X. Between pirfenidone treated groups, 500 mg/kg dosage group **"C1"** shows upsurge in axonal growths compared to 200 mg/kg group **"B1".**

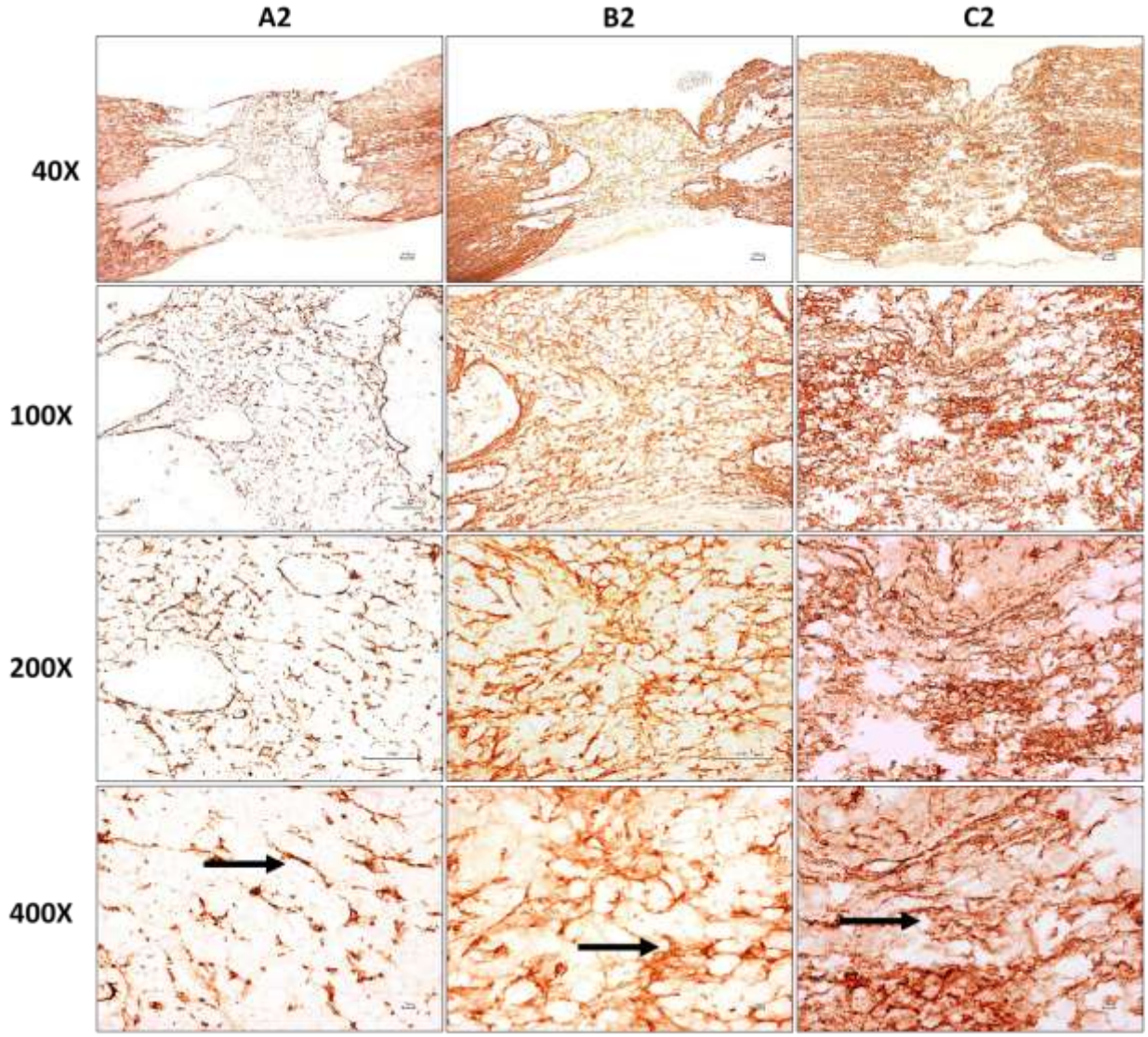

Figure 7: Photomicrographs of 5 µm thick rat spinal cord longitudinal sections showing Immunohistochemical staining for the expression of GAP43, from 28 days experimental duration groups at 40X, 100X, 200X & 400X. On comparison, more axonal growths can be seen in prifenidone treated groups **"B2" & "C2"** compared to non pirfenidone treated group **"A2"** marked

by arrows at 400X. Between pirfenidone treated groups, 500 mg/kg dosage group **"C2"** shows upsurge in axonal growths compared to 200 mg/kg group **"B2".**

According to these above-mentioned values, the pirfenidone-treated group progressed more than the non-pirfenidone-treated group on sensory evaluation. Similarly, pirfenidone treated groups showed increase quantity of axonal sprouts/regeneration in injury lesions compared to non-pirfenidone treated group. Additionally, these results show that 500 mg/kg/day dose of pirfenidone for a 28-days period was more effective in improving sensory recovery after spinal cord injury than 200 mg/kg/day dose for a 14-days period.

4. DISCUSSION

Researchers have always focused on the treatment of spinal cord injuries as a universal issue. The main objective of treatment in spinal cord injuries is to prevent and reduce secondary injuries. One of the main hindrances to the growth of damaged axons is the glial scar, a product of secondary injury. Consequently, spinal cord injuries do not recover well and have poor outcomes. The purpose of this study was to reduce oxidative stress, inflammation, and fibrosis after spinal cord injury to improve functional impairment by pirfenidone treatment. So pirfenidone can be used to reduce neurological deficits and improve functional recovery following spinal cord injury as an anti-inflammatory and anti-fibrotic agent. We found that pirfenidone is effective in improving sensory recovery following spinal cord injury.

Based on a review of the literature, we have been unable to find any study in which the pain sensitivity threshold (Von Frey test), heat threshold (Hot plate test) and cold thermal sensitivity threshould (acetone drop test) of rats with compression spinal cord injury was measured and

compared between rats treated with and without pirfenidone. Therefore we compared our study results with recent studies in which other anti-inflammatory, anti-fibrotic and anti-oxidant pharmacological interventions were used to improve the neurological outcomes after spinal cord injury. The findings of this study support those of Choi Y et al (2022), who demonstrated that alendronate suppresses spinal cord injury (compression model) induced inflammatory responses. In comparison to the SCI+alendronate group, the SCI+vehicle group showed a decreased response to Von Frey filaments, hot plate test, acetone drop test and an increase in BBB scores (6).

Tacrolimus (TAC) packed on polyethene glycol-modified maghemite nanospheres (PEG-MNs) was shown to have anti-inflammatory and anti-oxidant effects in the rat weight drop spinal cord injury model by Wang J et al (2022). When tested by von frey monofilaments 05, 10, 15, 20, 30, and 40 days after spinal cord injury, the TAC-PEG-MNs treated animals showed statistically significant and steady improvements in pain sensitivity thresholds (36). According to Masoudi A et al (2021), astaxanthin has anti-inflammatory and anti-oxidant effects in rat spinal cord injury models. After 28 days of spinal cord injury, AST treatment rats showed significant improment in neuropathic pain sensitivity compared to control groups (22).

As far as anti-inflammatory effects of both pirfenidone and melatonin are concerned, our study results are similar to those of Fakhri S et al (2021). On 28 days following the SCI, the melatonin-treated group showed a statistically significant decline in their pain threshold as compared to the control group (10). FTY720 has been found to be anti-inflammatory in a study by Yamazaki K et al (2020). Using von Frey monofilaments at 7, 14, 21, 28, 35, and 42 days post-injury, FTY720-treated animals showed statistically significant improvement in their neuropathic pain thresholds compared with their vehicle-treated control group (39). Our present study show similarity in heat and cold sensitivity recovery results with the study conducted by Fakhri S (2022), in which they

revealed anti-inflammatory and anti-oxidant effects of intrathecally administered naringenin in aneurysm clip compression rat spinal cord injury model (11).

Our present study results show sameness with the results of a recent study conducted by Chandran P et al (2022) in which ethanolic extract of Mucuna Pruriens (MP) was used as an anti-inflammatory and anti-oxidant agent to check its ability to overcome the neurological deficit after weight drop contusion spinal cord injury in rats. The study result showed significant difference in the GAP43 mRNA levels detected by real time PCR in the injury lesion in between MP treated group and SCI model group. GAP43 mRNA levels were higher in the MP treated group as compare to non-treated SCI group after 10 weeks post injury which describe the efficacy of ethanolic extract of Mucuna Pruriens in axonal regeneration after spinal cord injury (5).

Our present study is supported by the study conducted by Xiao S et al (2022) in which they have demonstrated neuro-protective and anti-inflammatory effects Aucubin in clip compression model of rat spinal cord injury. The study result showed significant difference in the GAP43 expression in the injury lesion in between Aucubin treated group and SCI model group detected by immunofluorescence. GAP43 positive areas were much obvious in the aucubin treated group as compare to non aucubin treated SCI group on 7, 14 and 28 post injury days which shows the effectiveness of Aucubin in axonal regeneration after spinal cord injury (37).

5. CONCLUSION

Axonal growth and neurological recovery are hindered by glial scarring that develops after spinal cord injury. Sensorimotor impairment after spinal cord injury can be reduced by reducing the central fibrotic core and outer reactive astrocytic core of glial scars. It was concluded that pirfenidone, an anti-fibrotic and anti-inflammatory drug, significantly improved functional

neurological recovery. This was likely due to its ability to inhibit activation and migration of meningeal fibroblasts, as well as proliferation and reactivation of astrocytes, which prevent glial scar formation following spinal cord injury. In this way, space and a favourable environment are created for the regeneration of axons.

Authors Contribution Statement

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 Author
- Draft manuscript preparation: 1st Author.
- Supervision: 3rd Author
- Final approval: 2nd Author

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

Conflict of interest: The authors declare no competing interest.

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Submission declaration: The work described above has not been published formerly and it is not under consideration for publication somewhere else. All authors approved the publication and if accepted in this journal, will not be published elsewhere.

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