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Immunohistochemical Evaluation of RKI-1447 in Rat Thoracic Spinal Cord Compression Injury Model

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ABSTRACT

Background and Objectives: Compression spinal cord injury is a highly heterogeneous lesion, and tools to delineate pathophysiological recovery needed. Our objective was to profile the protective or reversal effects of RKI-1447 on neuron regeneration and its correlation with the degree of tissue damage in the rats with induced compression injury at the T7 level. **Methodology:** Albino rats of Sprague Dawley strain, 08 weeks of age, weighing 250 ±10gm (n= 64) were used in this study. The animals were procured from the Animal House of Khyber Medical University, IBMS campus. Every experiment was carried out following a protocol that the Institutional Animal Ethics Committee authorized in 09.09.2020 under the (DIR / (KMU AS & RB/BD/001187) under the 89th meeting. The study duration was for 03 years, in which the histological studies took 06 months. Two groups of animals were formed. The experimental group and the control group (A). The experimental groups were further divided into four subgroups i.e. B, C and D. Group A was a sham group, in which rats were incised at level T7 only. Spinal cord compression injury at T7 level was performed in groups B, C and D. Group B was treated only with placebo while the group C and D, were treated with RKI-1447, with a dose of 0.3 and 0.6 µg/kg/b.w. respectively. For histological analysis, the animals were sacrificed 7, 14 and 28 days after the completion of behavioral studies. The staining pattern of glial fibrillary acidic protein (GFAP) for Astrocytosis/Reactive astrocytes, GAP-43, for Axonal growth/sprouting and Neu N for neuronal number for viability of neurons were observed. **Results**: All the group animals were evaluated daily after surgery. Consequently, our research showed that rats in group A healed quickly with just the Laminectomy procedure. In experimental rats, both groups C and D showed improved behavioral and histological results. In contrast, group B demonstrated sluggish and inadequate recovery. Additionally, group D rats showed superior performance in behavioral assessments, and their histological scores significantly surpassed those of group C. Group "D" excelled over group "C," probably because of the high dosage of RKI-1447 (0.6 µg/kg) administered to group D. Regarding the time-related effects of RKI-1447, the 28-day survival treatment yielded promising outcomes compared to the 14-days treatment. **Conclusion:** The current research presents evidence suggesting that both functional and morphological outcomes following spinal cord compression injury can progress in a discontinuous, non-linear fashion, despite the injury levels being graded in a linear manner. Serum levels of GFAP, GAP-43 and NeuN, may serve as potential biomarkers for assessing the severity of traumatic conditions and traumatic limb injuries. However, further animal studies are necessary to translate these findings into clinical applications.

INTRODUCTION

Spinal cord injuries are life-altering conditions, with the first few days being the most crucial and the secondary complications being the leading cause of mortality and life-threatening.[1]

Spinal cord injury is a life disrupting conditions in which the initial days are the most critical conditions while secondary complications are the life threatening and the main cause of death. Many spinal cord injury studies conducted so far have focused on a small range of injury levels, often if the outcomes—whether functional, histological, or neurophysiological—progress in a consistent, graded manner as the severity of the injury increases.[2]

Spinal cord injuries are a major health concern that significantly impacts a person's quality of life. These injuries lead to profound effects not only on the individuals affected but also on their families and society, as they often result in severe disability. Traumatic spinal cord injuries are lifelong conditions that require ongoing attention to minimize their long-term effects.[3]

Individuals with spinal cord injuries experience a loss of sensory input and motor function due to the inability of damaged neurons and nerve fibers to transmit or generate signals. This damage leads to permanent sensorimotor and autonomic impairments. The progression of both primary and secondary spinal cord injuries occurs in distinct phases, including immediate, acute, subacute, intermediate, and chronic stages.[4]

The CNS contains a variety of glial cells, including oligodendrocytes, radial glia, Muller cells, resident and perivascular microglia, and astrocytes. In 1969, Dr. Eng identified and described glial fibrillary acidic protein (GFAP), a unique structural protein that distinguishes astrocytes (astroglia).[5]

GFAP (Glial Fibrillary Acidic Protein) monocl5nal antibody is used for the detection of reactive astrocytes surrounding the damage area of spinal cord. It's a type III gene protein which forms the cytoskeleton of the astrocytes.[6]

It is one of the fine markers for activated astrocytes. It also plays a vital signaling system that responds to different stressful conditions by regulation, migration and survival of cells via GAP-43 (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. This gene encodes a protein which has been labelled as a "growth or plasticity" protein because its expression is higher i neuronal growth cones throughout development and axonal regeneration process.[7]

This protein is considered as one of the most fundamental elements of an active regenerative response in the neuronal axons in nervous system. GAP-43 is a specific neuron protein and an important component of growth cone in axons. It is engaged in the axonal regeneration, neuronal growth and stabilization of synaptic functions. It also plays an important role in learning and memory functions of CNS.[8]

Neu N was initially introduced in 1992 by Mullen and colleagues, who developed a set of monoclonal antibodies targeting mouse antigens. Their primary goal was to identify immunological markers specific to mice, which could be used in transplantation studies.[9]

Neu N antibodies are commonly utilized to identify neurons, although they have some limitations. A PubMed search conducted in June 2024 using the term "Neu N" yielded more than 5000 results. Neu N immunoreactivity becomes more pronounced as neurons mature, usually following the downregulation of Doublecortin, a marker associated with the early phases of neuronal development.[10]

Spinal cord compression is a serious neurological condition that requires prompt and effective intervention to prevent permanent damage. The aim of this study is to evaluate the therapeutic potential of RKI-1447, a selective Rho-associated coiled-coil containing protein kinase (ROCK) inhibitor, which has shown promise in reducing inflammation and promoting neural recovery. By investigating the effects of RKI-1447, particularly at higher doses, this study seeks to explore its efficacy in improving outcomes following spinal cord compression.[11]

Objectives

The objectives of the study were:

- To evaluate the role of RKI-1447, in the reversal of inflammatory process in injured spinal cord.
- To assess the role of RKI-1447, in the recovery of the spinal cord injury

MATERIAL AND METHODS

The institutional ethical review board and the biosafety office of Khyber Medical University, IBMS Peshawar, gave their clearance for this lab-based experimental study. This experimental study was conducted in Khyber medical university IBMS campus pathology department and anatomy department. The rats were euthanized and kept for the tissue processing on which the antibodies were applied accordingly.

Animals

A total of 64 Sprague-Dawley rats, each with a weight range of 250±10 gm, were utilized in the investigation. The sample size was determined based on the resource equation method, which accounts for multiple behavioural observations and a single observation for molecular studies and histology. The rats were raised in cages with four rats each, and their light and dark cycles lasted for twelve hours. The rats had unlimited access to food and water, and the room temperature was kept at 22°C. Before the study started, they were given a week to get used to the living arrangements.

Grouping

Two main Groups were made to conduct the study. The sham group (Labelled as A), and the second group was experimental compression group which was further subdivided into groups B, C and D. Group B was treated with normal saline after compression while groups C and D received 0.3 µgm and 0.6 µgm doses of RKI-1447 daily, respectively.

The animals were kept under treatment and observation for three intervals i.e: 07th day, 14th day and 28th day. After their Behavioral studies[12] the animals were euthanized and were processed for Histological studies.

Optical Density

The optical density of GFAP, GAP-43 and Neu N expression were evaluated as an estimate of the quantity of immunopositively cells in the SCIs. Images were acquired at 40 X, 100X and 200X µm magnification on a Nikon E600 light microscope from three sections in each slide.[13]

Using Image-J and digital images, the injured area showed was identified. The mean gray values from three randomly chosen locations with or without immunoreactivity were used to calculate the background gray levels. By dividing the total immunoreactivity areas by the total area of the relevant sub-regions on the three sections, the total optical density value for each brain was determined.[14]

The Image J software, also known as Fiji, was employed to analyze the images that were acquired from the compound

microscope. The Optical density (OD) was employed to determine the average and standard deviation (SD) of the antibody expression intensity in each group. Meanwhile, GFAP, GAP-43 and Neu N, this method was used to determine each study group's mean and standard deviation of the total cell count. Using ImageJ software, the mean cell count was determined by following these steps: (Select image > set to 8-bit > Image > Adjust > Threshold (set minimum to 85 and maximum to 255) > Process > Make Binary > Convert to Mask > select areas > Analyze > Particles.)

The results were then exported to Excel and SPSS software for further analysis. (https://youtu.be/1PQprFZ2Byg, https://www.unige.ch/medecine/bioimaging/files/3714/1208/5964/CellCounting.pdf).

Optical density was calculated using a validated protocol in ImageJ: first, record a macro by selecting Image > Plugin > Macro > Record, and then draw a rectangular shape on the image. Save the macro item and proceed by opening the images individually from the file, selecting Image > Color > Deconvolution > H-DAB, and using Color 2 for intensity quantification. Then, select Plugin > Run > Macro.ijm and finally go to Analyze > Measure. The results were saved in an Excel sheet.

(https://www.researchgate.net/post/Does-anyone-have-a-protocol-for-quantifying-IHC-images-in-ImageJ).[15]

The optical density was computed using the following formula: The maximum intensity for each 8-bit image is 250, and OD is equal to log (maximum intensity / mean intensity).

Staining and Immunohistochemistry

The steps conducted for staining followed by IHC are sectioning the SCI site and then the slides preparation. Slides 3, 6, 9, 12 and 15 were chosen from each spinal cord tissue section to measure the average number of viable neurons in damage lesions out of about 15 slides of each specimen that were processed in a sequential order. GFAP, GAP-43, and NeuN monoclonal antibodies were used in 1:50 dilution to detect change in astrocytic count, normal viable neurons, and neuronal count in spinal cord injury sites.[16]

Microscopy & statistics

For immunohistochemistry, slides number 3, 6, 9, 12 and 15 out of total slides were selected from each spinal cord tissue specimen to evaluate average measure of reactive Astrocytosis by using GFAP antibody, Axonal growth in injury site by GAP-43 antibody and noticeable viable neurons in the injury site by NeuN antibody. Each antibody was applied to one section per slide due to the three tissue sections on each slide. Hydrophobic barriers were made by PAP pen (ab2601, Abcam UK) between the sections to prevent mixing of antibodies and other reagents used during staining process. The commercially available IHC kit (Dako, En Vision TM FLEX Mini Kit, High PH, Code# K8024) was utilized for IHC staining.

The following materials (solutions and reagents) were provided in the kit.[17]

Peroxidase Blocking Agent

Phosphate buffer including hydrogen peroxide, 15 mmol/L NaN3 and detergent.

- **HRP**: - Dextran combined with goat secondary antibody molecules and peroxidase molecules to combat mouse and rabbit immunoglobulin in a buffered solution including a preservative and stabilizing protein.
- **DAB+ Chromogen**: Tetrahydrochloride of 3-diaminobenzidine (in organic solvent).
- **Substrate Buffer**: - hydrogen peroxide and preservative in a buffered solution.
- **Target Retrieval Solution, High pH (50x concentration)**: - Tris/EDTA buffer, pH 9.
- **Wash Buffer (20x concentration)**: - Tris buffered saline with, pH 7.6.
- **Antibody Diluent**: - Protein and 15 mmol/L NaN3 are present in Tris buffer, which has a pH of 7.2.

After applying the antibodies to the slides, microscopic examination was conducted using a Nikon Eclipse 80i microscope at magnifications of 10X, 40X, and 100X. Multiple attempts were made at each magnification to capture clear and precise images. Viable neurons within the injured region were examined and quantified through optical density analysis using ImageJ Fiji software.

Table 1

Details of Primary Antibodies Used in Immunohistochemistry.

Antibody	Supplier	Species Reactivity	Host/ Isotype	Class	Clone
GFAP	Thermofisher Scientific	Human, rats, pig and chicken	Mouse/ IgGIM	Monoclonal	ASTR-06
GAP43	Invitrogen Thermofisher Scientific	Human, Mouse and rat	Rabbit /IgG	Recombinant/ Monoclonal	SC60-06
Neu N	Invitrogen Thermofisher Scientific	Mouse	Rat / IgG1, kappa	Monoclonal	A-60

Statistical Analysis

Microsoft Excel 18 and SPSS version 25 were used for the statistical analysis. Descriptive statistics were computed using means and standard deviations. The Kruskal Wallis test was employed to make comparisons across the groups. Additionally, the ONE WAY ANNOVA test was utilized to conduct comparisons between the individual groups (with a P value of less than 0.05 being deemed statistically significant). All data appears as the mean ± SD, with a significance level of $p < 0.05$.

RESULTS

At regular postoperative intervals, the rats were meticulously monitored. Rats were acting a little drowsy, sleepy, and inactive during the first few days after surgery. They also consume less food and liquids. Some rats displayed dark crimson secretions from their eyes and nostrils on the first to third post-operative days.

With each passing day, the rats' activity levels progressively increased, and their food consumption returned to normal. The activity levels of the rats in group C, and especially in group D, showed much greater improvement compared to those in group B.

Reactive Astrocytes Count (Glial Scar Outer Core)

It was found that as the severity of the trauma increased, there was a rise in the number of apoptotic cells, especially

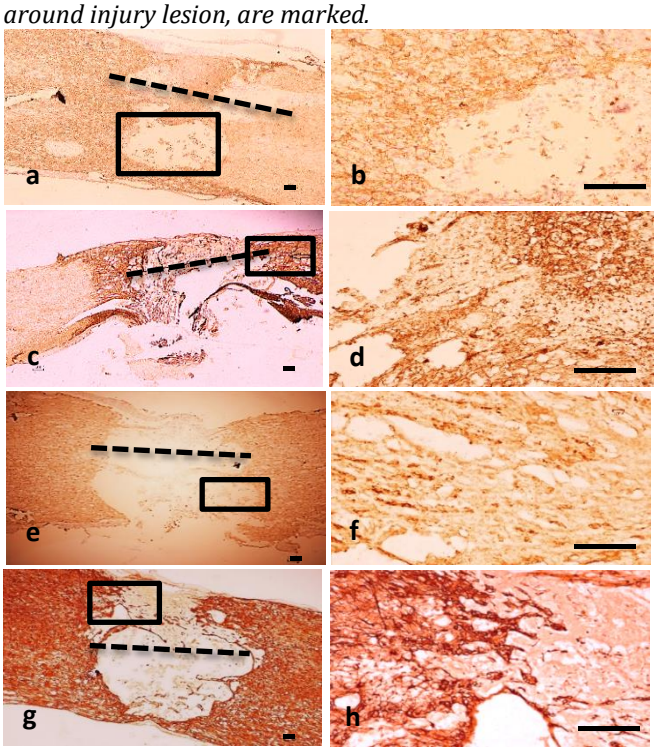
GFAP (+) protoplasmic astrocytes in the perilesional area of the cortex. After processing the tissue and applying the GFAP antibody to tissue sections, reactive astrocytes were counted around the injury site using optical density.

To compare the reactive astrocyte counts within the groups, the Kruskal Wallis test was used. Significant differences in astrocyte counts were observed between groups B and A, and C and D, as shown in (Figure 2). This indicates that there was greater astrocyte reactivity around the injury site in the 28-day groups compared to the 14-day groups. Reactive Astrocytosis increased in group C and D which were treated by RKI-1447.

These results suggest that the non-RKI-1447 treated group showed more reactive Astrocytosis around the injury, forming a denser outer core of the glial scar compared to the RKI-1447-treated groups. Additionally, the data also highlights the greater effectiveness of a higher dose of RKI-1447 (0.6 µg/kg/day) compared to a lower dose (0.3 µg/kg/day) in reducing reactive Astrocytosis.

Figure 1

- 5 µm thick rat spinal cord longitudinal sections showing IHC staining for the expression of GFAP in reactive astrocytes, at 10X, 40X prominent injury site marked by black lines can be appreciated. Highly proliferated astrocytes with increase in number, size and GFAP content around injury lesion, are marked.



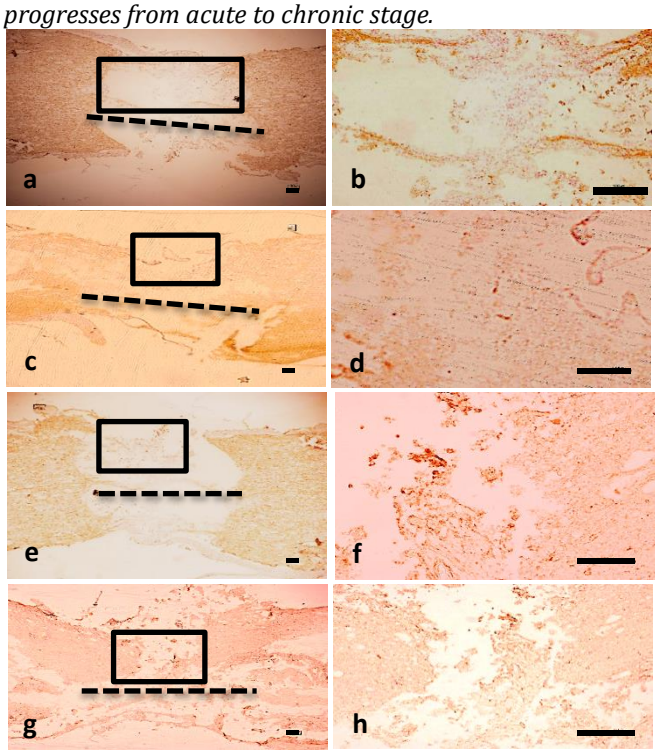
GAP-43 (Axonal Growth or Sprouting)

After tissue processing and the application of the GAP-43 antibody to tissue sections, the injury site was analyzed, and axonal growth sprouting was quantified using optical density. This was done by measuring the intensity of the area occupied by axonal sprouting at the injury site across multiple attempts on each slide.

To assess differences in the intensity of the total injury area occupied by axonal growth/sprouts within the groups, a ONE WAY ANNOVA test was used. Significant differences were observed in the axonal growth at the injury sites between groups A and B, C and D, with P values of P = .007, P = .028, and P = .009, respectively (Figure -2). These results confirm that there was greater axonal growth in the injury sites of the 28-day groups compared to the 14-day groups.

Figure 2

- 5 µm thick rat spinal cord longitudinal sections showing IHC staining for the expression of GAP-43 in Axonal growth, at 10X, 40X prominent injury marked by black Boxes can be appreciated. More axonal growths/sprouts can be identified in group "D" compared to group "B" which indicates increase in axonal growths/sprouts in injury lesions, as the injury progresses from acute to chronic stage.



These images indicate that there was less axonal growth/sprouting in the injury site of not treated group as compared to RKI-1447 treated groups. These results also reveal the effectiveness of RKI-1447 in a higher dose of 0.6 µg/kg/day as compared to lower dose of RKI-1447 0.3 µg/kg/day to enhance axonal sprouting more in the injury site, after spinal cord injury, shown in figure -2.

Neu N (Viable Neuron Count)

After applying the NeuN antibody to tissue sections, viable neurons at the injury sites were examined. But only some residual neuronal cell bodies were detected, but no normal, viable neuronal cell bodies were found at the injury site in any group except for the non-compression group i.e: Group A. As a result, no statistical data regarding neuronal viability or functional neurons within the injury site post-spinal cord injury was obtained.

Optical density was used to estimate the optical density of neuronal cell body residues inside the entire damage area for each participant in all groups. In the injury lesion, we observed the presence of residues and traces of neuronal cell bodies across all the groups. There was a clear distinction in the number of neuronal residues between the RKI-1447 treated group and the non-RKI-1447 treated group. Our study also unveils the efficacy of RKI-1447 in a higher dose of 0.6 µg/kg/day as well as in lower dose of 0.3

µg/kg/day in both 14 days and 28 days duration.
These remaining neuronal bodies were more noticeable in 28-day subgroups than in 14-day subgroups within the groups. shown in figures -3.

Figure 3

- 5 µm thick rat spinal cord longitudinal sections showing IHC staining for the expression of Neu N in Neuronal Count, at 10X, 40X prominent injury site marked by black lines can be appreciated. Neuronal content around injury lesion, are marked.

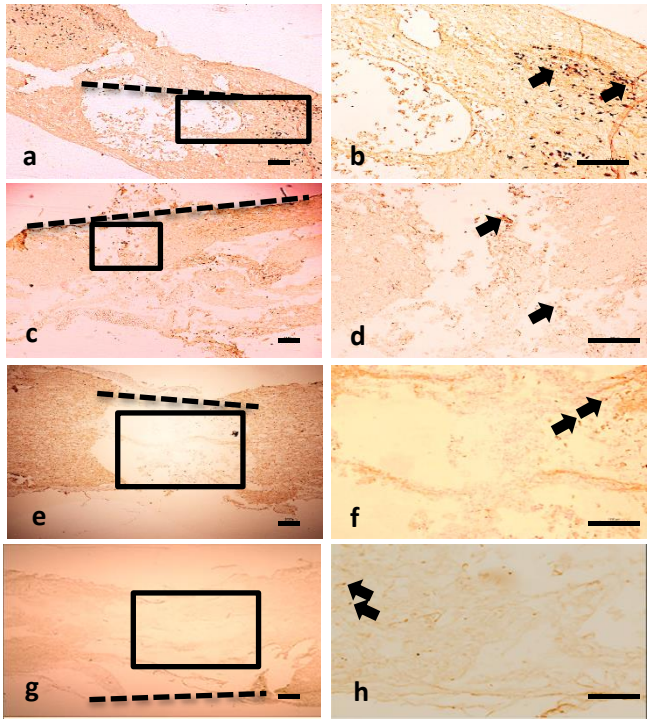
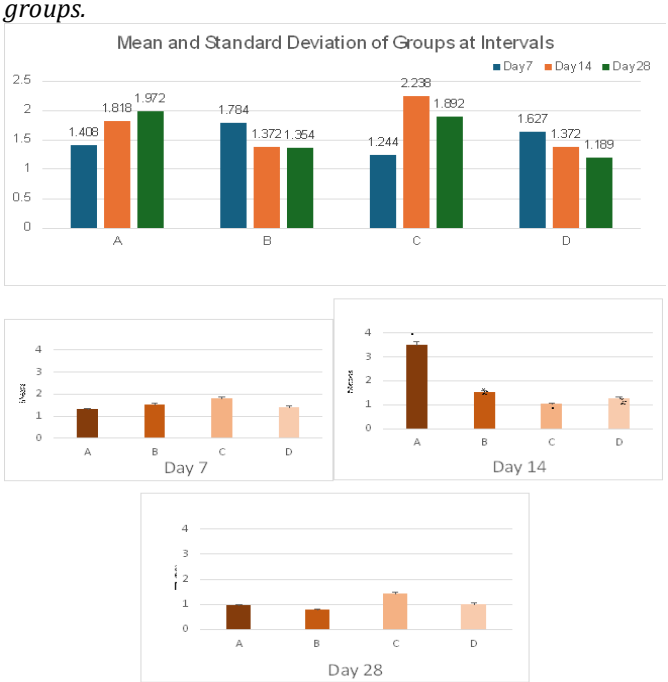


Figure 4

- Comparison of the mean and standard deviation between groups at various points in time, such as 7th, 14th and 28 days after the rats were placed to sleep and their histological parameters were examined. (I) represents SDEV. The P value from the Kruskal Wallis test is less than 0.05 across all groups.



The Graph shows that RKI-1447 treatment had a time-dependent and dose-sensitive influence on the evaluated outcome after compression injury, according to the graphical analysis conducted over 7th, 14th, and 28th days. On day 7th, the compression-untreated group displayed the lowest mean value, demonstrating the detrimental effects of compression damage, whereas the sham group displayed the highest mean value, showing normal baseline function.

All groups showed a time-dependent reaction to RKI-1447 therapy after compression injury (Figure -4).

In comparison to the sham group (A), all compression groups (B, C, and D) displayed lower outcome values on Day 7, suggesting an early injury-related deterioration. Nevertheless, there was no statistically significant difference between any of the treatment groups, indicating that neither RKI-1447 dose had a meaningful short-term impact at this point.

By Day 14, the therapy response became more apparent. While all compression groups, including untreated (B = 1.001), low-dose RKI-1447 (C = 0.964), and high-dose RKI-1447 (D = 1.014), were considerably lower than sham ($p < 0.05$), the sham group (A) maintained the greatest mean value (1.918). This demonstrates that compression has a long-lasting detrimental effect and that by day 14, neither RKI-1447 dose had a discernible protective effect.

All compression groups, including treated ones (C = 0.906, D = 1.039), continued to exhibit statistically significant decreases in comparison to sham ($p < 0.05$), while the trend remained constant on Day 28: sham values remained at their greatest (mean = 1.089). The low-dose RKI group (C) notably improved slightly compared to the untreated group (B = 0.889), but this difference was not statistically significant.

The compression-untreated group continuously performed the worst during the study. With the 0.3 µg therapy offering the most long-term benefit, these results point to a non-linear, delayed therapeutic effect of the lower RKI-1447 dose, underscoring the significance of dose selection and time in attaining the best possible recovery after compression damage.

DISCUSSION

Spinal cord injuries are a significant concern that has long been the subject of research. These injuries involve both initial and subsequent damage, with the focus of treatment being to prevent or reduce further harm. Within hours of the injury, macrophages are activated and release various inflammatory substances, including TNF-α, IL-6, IL-1β, CD-16, CD-32, and matrix metalloproteinases. In addition, activated astrocytes and migrating meningeal fibroblasts at the injury site contribute to the development of fibrosis in the affected area. As a result, the formation of a glial scar occurs, which ultimately impedes axon growth and recovery, leading to poor healing after spinal cord injuries. The current study on RKI-1447, a powerful inhibitor of ROCK 1 and ROCK 2, highlights its potential as part of a new class of drugs for treating various eye conditions like ocular hypertension. It also shows promises in reducing the migration, invasion, and anchorage-independent growth of breast cancer cells.[18]

Since Rho pathways play a significant role in spinal cord

injuries (SCI), inhibiting these pathways may aid in SCI recovery. Although RKI-1447 is an effective Rho pathway inhibitor, it has not yet been tested for SCI treatment

Reactive Astrocytosis (Glial Scar Outer Core)

Astrocytes showing positive GFAP staining were counted within a 1200 µm area extending from the injury centre on both sides, using immunohistochemical methods. We found a significant difference in the number of reactive astrocytes between the RKI-1447-treated and non-treated groups. Our research also demonstrates the effectiveness of RKI-1447 at both doses (0.6 µg/kg/day) and lower (0.3 µg/kg/day) doses, over periods of 7th, 14th and 28th days. Notably, the higher dose administered for longer time resulted in a more significant reduction in reactive astrocyte counts after spinal cord injury compared to the lower dose at 28 days post-injury. Upon reviewing existing literature, we did not find any studies that specifically calculated and compared reactive astrocytes in RKI-1447 treated and non-treated rats with compression spinal cord injury.

Our findings are consistent with those of Yamazaki K and colleagues, who reported the anti-inflammatory effects of FTY720. Their study demonstrated that FTY720 mitigated local inflammatory responses and reduced glial scar formation by influencing reactive astrocytes in rats with compression spinal cord injuries. Specifically, the area with GFAP-positive reactive astrocytes, located 4 mm caudal to the injury site, was significantly smaller in the FTY720-treated group compared to the vehicle-treated group.[19]

The results of the current study are like those of a recent investigation, particularly in terms of the anti-inflammatory effects of RKI-1447 and alendronate. The study observed that alendronate exhibited anti-inflammatory properties by reducing inflammatory responses in a spinal cord injury (compression model). Specifically, it found that GFAP-positive reactive astrocytes were present in both the control group and rats with spinal cord injuries treated with either a vehicle or alendronate. However, the number of reactive astrocytes, as indicated by GFAP positivity, was significantly lower in the spinal cord injury group treated with alendronate compared to the group treated with the vehicle on day 28 post-injury.[20]

The study concluded that CCIII functions as an anti-inflammatory agent by reducing the expression of TNF-α, COX2, and CD68, as well as an anti-fibrotic agent by inhibiting MMP-9. Statistically significant differences were found in the GFAP-positive reactive astrocytes between the SCI+CCIII, SCI+ Riluzole, and sham groups. Both the SCI+CCIII and SCI+ Riluzole groups showed a notable decrease in reactive astrocytes on the seventh day after the injury compared to the sham groups.[21]

Additionally, our study supports the findings of Kang S et al. (2018), who established the anti-inflammatory and neuroprotective effects of Baicalin in a rat compression spinal cord injury model. Their research demonstrated that Baicalin reduced the levels of TNF-α, IL-1β, and IL-6. Statistically significant differences in the number of GFAP-positive reactive astrocytes were found between the SCI group and the Baicalin-treated group. The Baicalin-treated rats, particularly those in the higher-dose group, exhibited a significant reduction in GFAP-positive reactive astrocytes compared to the SCI group 30 days after the injury.[22]

The study showed a notable reduction in GFAP-positive reactive astrocytes in the curcumin-treated group compared to both the control spinal cord injury group and the methylprednisolone-treated group. These findings are consistent with the work of Lin B et al. (2014), who explored the anti-inflammatory effects of U0126 in a rat spinal cord contusion model. Their research demonstrated a reduction in glial scar formation and enhanced sensorimotor recovery following spinal cord injury. Additionally, their study found a significant decrease in GFAP-positive reactive astrocytes in the U0126-treated group compared to the control group. However, 28 days after spinal cord injury, the number of GFAP-positive reactive astrocytes was lower than at 14 days in both the U0126-treated and non-treated groups.[23]

Axonal Growth/Sprouting

GAP-43 positive axonal growths/sprouts were assessed within the injury lesion using immunohistochemistry. A significant difference in axonal growth was observed between the RKI-1447 treated and non-treated groups. Our research highlights the effectiveness of RKI-1447 at both a higher dose of 0.6 µg/kg/day and a lower dose of 0.3 µg/kg/day, across both 14- and 28-day treatment periods. However, the higher dose and longer treatment duration of 28 days resulted in a greater degree of axonal sprouting compared to the lower dose and shorter 14-day period. These findings suggest that RKI-1447 promotes axonal regeneration following spinal cord injury.

After reviewing the available literature, we found no studies that specifically measured and compared axonal growth or sprouting in rats with spinal cord compression injuries, both with and without RKI-1447 treatment.

Our findings are consistent with a recent study by Chandran P and his team, who assessed the potential of an ethanolic extract from *Mucuna Pruriens* (MP) as an anti-inflammatory and antioxidant agent. This study aimed to evaluate its effectiveness in alleviating neurological impairments following weight-drop contusion spinal cord injuries in rats. The results showed a significant difference in GAP43 mRNA levels, as measured by real-time PCR, between the MP-treated group and the SCI model group. Specifically, the GAP43 mRNA levels were higher in the MP-treated group compared to the untreated SCI group 10 weeks after the injury, highlighting the efficacy of the ethanolic extract of *Mucuna Pruriens* in promoting axonal regeneration following spinal cord injury.[24]

The current study is supported by a recent investigation where researchers demonstrated the neuroprotective and anti-inflammatory effects of Aucubin in a rat model of spinal cord injury induced by clip compression. The results revealed a significant difference in GAP-43 expression at the injury site between the Aucubin-treated group and the SCI model group, as observed through immunofluorescence. GAP-43 positive regions were notably more prominent in the Aucubin-treated group compared to the untreated SCI group on days 7, 14, and 28 post-injuries, suggesting Aucubin's role in promoting

axonal regeneration following spinal cord injury.[25]

Our research is similar to the work of Liu X and colleagues, who investigated the potential of Resveratrol in a rat contusion model of spinal cord injury. Their findings highlighted Resveratrol's anti-inflammatory, antioxidant, anti-apoptotic, and neuroprotective properties, which align with the objectives of our study. The results indicated a significant difference in GAP-43 intensity at the injury site between the Resveratrol-treated group and the SCI model group, detected through immunohistochemistry. GAP-43 positive regions were much more pronounced in the Resveratrol-treated group compared to the untreated SCI group on days 14 and 35 post-injury, further confirming Resveratrol's effectiveness in supporting axonal regeneration after spinal cord injury.[26]

The results of this study further corroborate previous research demonstrating that Baicalin has anti-inflammatory and neuroprotective effects, as evidenced by its ability tom reduce levels of TNF-α, IL-1β, and IL-6 in a rat model of compression spinal cord injury. The study found a statistically significant difference in GAP-43 expression between the SCI group and the Baicalin-treated group. Rats treated with Baicalin showed a notable increase in GAP-43 expression, particularly in the high-dose group, compared to the SCI group, on day 30 after the injury.[27]

Neuronal Sustainability

Nau N positive viable neurons were examined and counted inside the injury lesion. We observed no viable neuron in the injury lesion of any group. Therefore, no statistical evaluation was performed. However, we have noticed traces and residues of neuronal cell bodies in the injury lesion of all groups. There was obvious difference in the number of neuronal residues between RKI-1447 treated and non-RKI-1447 treated groups.

Our study also unveils the efficacy of RKI-1447 in a higher dose of 0.6 µg/kg/day as well as in lower dose of 0.3 µg/kg/day in 7th ,14th and 28th days duration. However, higher dose and in 14 days duration, RKI-1447 showed more neuro-protective effect by slowing down the neuronal apoptosis/degeneration in injury lesion after spinal cord injury as compared to lower dose and in 28 days post-injury duration. This shows the efficacy of RKI-1447 in decelerating the process of neuronal degeneration and apoptosis after spinal cord injury.

After conducting a thorough review of the existing literature, no studies were discovered that had calculated and compared the presence of viable neurons in rats with spinal cord compression injuries, distinguishing between those treated with RKI-1447 and those not treated with RKI-1447.

The outcomes of our current research align with those of a previous study, wherein researchers illustrated the efficacy of a solitary RKI-1447 dose in mitigating neuronal degeneration. This was accomplished by reducing lipid peroxidation in the rat hippocampal region after kainic acid-induced toxicity. Fluoro-Jade B staining 72 hours after treatment revealed a significant difference in the degenerating neurons between the groups treated with 0.6 µ mg/kg RKI-1447 and other SCI/lower dosage groups. Rats given a dosage of 0.3 µg/kg had more surviving neurons than rats in other groups.[28]

The present investigation is supported by a recent study carried out by Wang C and colleagues. Their study showcases the efficacy of a bioactive, versatile citrate-based hydrogel therapeutic system that enables an extended release of extracellular vesicles derived from mesenchymal stromal cells (referred to as FE@EVs). This system serves as a promising treatment strategy for mitigating fibrosis and inflammation, ultimately facilitating the restoration of both functional and structural aspects following a complete transection spinal cord injury. The study results showed statistically significant difference in neuronal apoptosis in FE@EVs treated group as compared to other groups, indicated by MAP-2 immunofluorescence staining on 21, 35, 49 and 56 days post injury. FE@EVs treated group proved significant reduction in neuronal degeneration compared with other groups.[29]

In a rat model of spinal cord injury, they accomplished this by lowering the expression levels of COX-2, TNF-α, IL-1β, and IL-6. Their study results revealed that myelination in spinal cord white matter and motor neuron number in grey matter was significantly conserved in astaxanthin treated rats as compared to the control group rats. Our present research investigation is corroborated by a recent study that delineated the protective effects of intrathecally administered naringenin on neurons in the dorsal and ventral regions of the spinal cord following spinal cord injury induced by aneurysm clip compression in rats, emphasizing its anti-inflammatory and antioxidant properties. Statistically significant difference in viable neurons in both dorsal and ventral horns was noted in between the naringenin treated group and control spinal cord injury group on day 28 post SCI.[30]

The current findings from our research exhibit resemblances with a previous study that demonstrate that the anti-inflammatory and antioxidant properties of Selenium-doped carbon quantum dots (Se-CQDs) in a rat model of contusion spinal cord injury. According to a study, one of the main causes of subsequent damage in the injured spinal cord is the increased production of ROS (reactive oxygen species) following spinal cord injury. Se-CQDs could scavenge ROS. The study results show a statistically significant difference in the viable neurons at the lesion site in between Se-CQDs treated group and saline treated group. Se- CQDs treated group showed significantly higher number of neurons at lesion site as compared to saline treated group, detected by anti-Nue N immunofluorescence technique after 08 weeks of injury.[31]

CONCLUSION

A suitable model for rodents is a laboratory rat with spinal cord injury. Globally, they are widely used for translational research pertaining to SCI. Laboratory rats have been used to develop and test a variety of surgical techniques, including compression, contusion, and transection models. Since the purpose of pre-clinical research is to get ready for clinical scenarios, it is critical to replicate all controllable factors as closely as possible in order to guarantee successful therapeutic translation. It will be challenging to successfully replicate any noteworthy advancements made in pre-clinical trials in SCI patients if

this is not accomplished. Currently, the majority of pre-clinical SCI models do not account for many of the variables that may influence how the body reacts to spinal cord trauma, such as age, sex, drug use before and after injury, and head trauma. Clinical trials for SCI will fail more frequently if pre-clinical animal models don't accurately reflect the clinical environment.

The accuracy of investigations depends on selecting the right SCI model. All behavioral tests must be universal, authentic, and reliable in order to be considered valid. In addition to illustrating various models and assessing spinal injury tests, this article discusses selection criteria, benefits, and drawbacks.

Recommendation

Traumatic spinal cord injury is a ruinous condition that leaves most of the victims with everlasting neurological deficits. Glial scar formed following spinal cord injury is considered to be one of the main factors that makes hindrance and unfavorable conditions for axonal growth and neurological recovery. In response to inflammatory mediators after spinal cord injury, meningeal fibroblasts activation and migration towards the injury site play role in establishing central fibrous core of glial scar. Furthermore, astrocytes become hypertrophic, proliferate, remodel and express an overexpression of GFAP, forming the outer core of the glial scar. These migrated meningeal fibroblasts and proliferated hypertrophic reactive astrocytes are mainly responsible for secreting collagen fibers and other fibrotic components like extra cellular matrix in the glial scar.

According to the results of our study, RKI-1447 demonstrates the RhoA/Rho kinase pathway, which is triggered by a variety of signals and sets off a chain of subsequent events that lead to functional deficits, such as inflammation, neuropathic pain, demyelination, cell death, and axon degeneration. Can effectively reduce the formation of glial scars following spinal cord injuries. This is achieved by inhibiting the activation and migration of meningeal fibroblasts, as well as the proliferation and reactivation of astrocytes. As a result, RKI-1447 creates a conducive environment that facilitates axonal regeneration, leading to enhanced neurological recovery characterized by improved sensory and motor functions.

In order to improve the aftermath of secondary injury mechanisms and to modulate and rehabilitate the neuropathological process involved, the compression model can demonstrate its suitability for preclinical testing of novel therapeutic approaches in patients who are paralyzed or quadriplegic.

Clinical research must have suitable experimental plans to successfully demonstrate novel interventions for SCI. However, for the sake of ethics, animal welfare, and the acceptance of its results, the three Rs should be taken into account.

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