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Effect Of Rki-1447 On The Behavioral Aspect Post Compression Injury Model In Rats

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Abstract:
Objective: In this study a standardized spinal cord compression injury model with application of RKI-1447 to see whether RKI-1447 has the capacity to alleviate SCI related sensori-motor/ behavioral responses.
Methods: Sixty-four adult Sprague Dawley rats were used. The rats were divided into four groups A (Control group with only skin incision), B, C and D groups (all underwent laminectomy followed by compression injuries by applying the aneurysm clip of 70 gm for 01 minute. In addition, group C and D were given different doses of RKI-1447 (as 0.3 µg and 0.6 µg respectively). The animals were observed for sensorimotor behavioral responses on day 7, 14 and 28 post surgically using Basso, Beattie, Bresnahan (BBB) test, Cold sensation test, Von frey filaments, Hot plate test and tail flick test on each rats.
Results: The results acquired from all the groups were statistically analyzed by using repeated measure ANOVA followed by Tukey HSD test for groups comparison. Overall, the group A showed no neurological abnormalities, whereas the group B exhibited a clear neurological deficit, and group C and D showed significant improvements in their behavioral activities, comparable to group B at all evaluated time points ($p \leq 0.05$).
Conclusion: RKI-1447 appeared to improve the sensory as well as motor behavior of the rat undergoing spinal cord compression injury. Our results suggest a promising role for RKI-1447 in alleviating SCI-related impairments, offering potential avenues for further exploration and therapeutic development.

Introduction

Spinal cord injury (SCI) is a crippling neurological condition with significant socio-economic impact on affected individuals and the health system. At times sudden traumatic spinal lesions result from these injuries among which Vertebral fracture or dislocation of vertebrae are the most common causes of SCI [(2)].
The goal of SCI models is to mimic the characteristics of human SCI as precisely as feasible. These models differ in terms of animal use, the location of damage, and the mechanism of harm[(1, 3)]. Rats are often employed in the early investigations, because they are very affordable, generally available, and have shown functional, electrophysiological, and morphological results. Mice are especially valuable for genetic research [(4)]. There are many types of compression models developed for studying SCI including clip compression, balloon compression and spinal cord strapping techniques [(5, 6)].
Primary injury to spine is acquired when either the spine is fractured; disc is displaced, in cases with Ligamentous strain or a tear into the spinal cord substance. More than 90% of SCI cases are traumatic in nature, which are caused during traffic accidents, violence, sports etc [(7)]. The goal of the study was to see how effective RKI-1447 is in reducing inflammation and improving recovery in damaged spinal cords. After evaluating the behavioural alterations in rats following spinal cord injury. Rho-kinase inhibitors (also known as ROCK inhibitors) are a class of drugs that target the rho kinase protein (ROCK). RKI-1447 is an anti-invasive and anti-tumor medication that inhibits ROCK1 and ROCK2 with IC50 values of 14.5 nM and 6.2 nM, respectively.

As a result of a fast physical traumatic event, immediate SCI can arise in less than or up to two hours. Axon breakage, haemorrhagic grey matter, and a rise in pro-inflammatory cytokines are among the early pathogenic processes [(8)].

Because the RhoA/Rho kinase pathway is involved in so many pathophysiological processes, and therefore, the pharmacological inhibition of the RhoA/Rho kinase pathway could be a promising strategy for preventing cell death and promoting axonal regeneration. Rho-kinase inhibition led to axonal sprouting, and functional recovery of the spinal cord [(9)].
The dynamics of aqueous Humour outflow routes have been linked to Rho and ROCK signalling pathways. Furthermore, ROCK signalling causes oxidative stress in the Trabecular meshwork, which (Rho-kinase inhibitors) RKIs have been found to inhibit by boosting the catalase expression and lowering reactive oxygen species generation [(10)].

Nowadays the lab animal's i.e: rats are extensively used to estimate different types of behaviour tests that assess the neural functions especially in injury models. After a planned surgery of SCI at the desired site, changes in the spinal cord morphology and neural function can be confirmed with behavioural testing. Basically, behavioural testing involves the basic experiments after any medicinal intervention to determine the impact of SCI on different neural tracts and fasiculi of the Sensorimotor systems [11].

Because the RhoA/Rho kinase pathway is involved in so many pathophysiological processes, and therefore, the pharmacological inhibition of the RhoA/Rho kinase pathway could be a promising strategy for preventing cell death and promoting axonal regeneration. Rho-kinase inhibition led to axonal sprouting, and functional recovery of the spinal cord[1, 9]. ROCK molecules are found in all cell tissues and organs; however, the amount of expression varies depending on the cell tissue. Because of their function in cell proliferation, migration, and contraction, they might be used as therapeutic targets. The Rho-associated protein kinase (ROCK) is a serine-threonine kinase that belongs to the AGC family. By working on the cytoskeleton, primarily regulates the form and movement of cells. ROCKs (ROCK1 and ROCK2) are found in mammals (humans, rats, mice, and cows), Zebrafish, invertebrates (mosquitoes, flies), and chickens. ROCK1 is a key downstream effector of the small GTPase and has a molecular mass of 158 kDa[12].

Rat ROCKs were the first Rho effectors identified, and they phosphorylate MLC (Myosin light chain) for the development of stress fibres and focal adhesions[13]. Calcium ions trigger a myosin light chain kinase, which forces a contraction. Calcium ion intake is regulated by protein kinase C and Rho-associated protein kinase [14].
The aim of the current experiment was to determine the curative effect of RKI-1447 post surgically on the behaviour of the rats with SCI compression injury. These SCI models can be utilized to calibrate the behavioural aspects of rats and therefore may be used for the research related to diagnostic as well as therapeutic strategies.

Materials and Method
The current study was conducted of male albino rats after having a compression injury model for studying mechanism of spinal cord injury and its behavioural outcomes. The experimental study was carried out after the approval from Ethical Committee of Khyber Medical University, Peshawar. In our study, 24 healthy adult Sprague Dawley rats, average 08 to 10 weeks old and weighing 250-300 gm were included while any diseased or ill animal. All rats were divided in 02 groups, A and B. In group A, the skin incision was done only. The group B, C and D, animals were applied to an aneurysm clip with 70 g closing force was applied directly to the Dural sac at T7 level containing the spinal cord exposed by laminectomy of the T6 or T8 vertebra for 1 minute. At the end of the procedure, the clip was removed, and surgical wound closure was done in layers with silk.

Injection to the experimental Group
A sham group (A), of animals was treated with normal saline, while other groups group B, C and D, underwent laminectomy followed by compression. In addition, groups C, and D animals received RKI-1447, which were injected i.p. on daily basis (as 0.3 μg and 0.6 μg respectively) [15]. The RKI-1447 was purchased online, from EMD chemicals inc, San Diego CA 92121, an affiliated of MERK KGaA, Darmstadt, Germany; in a glass bottle of 10 mg powder with a temperature maintenance 0f -20°C. The drug was prepared in diluted form as was mentioned in protocol.

Behavioural Testing:
Behaviour tests were performed post surgically on day 7, day 14 and day 28. The behaviour assessment included analysis of locomotors and sensory changes.

The methodological framework used for behaviour assessment is shown in Fig (2).

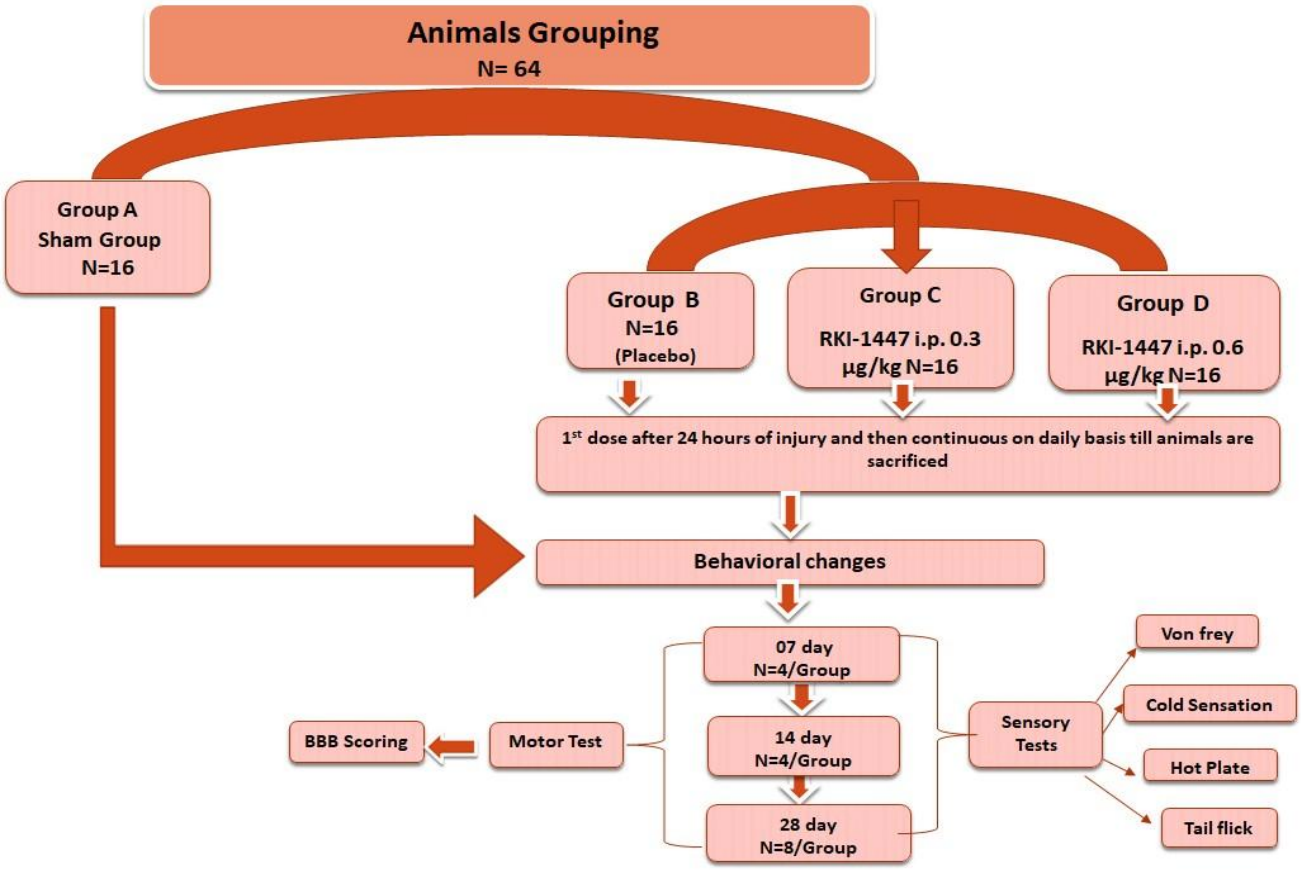

Figure 1. Methodological framework used for behaviour assessment.

Figure 2. Different types of behaviour tests like; BBB scoring apparatus, Von Frey Filaments, Tail Flick test and Hot plate test.

Statistical Analysis:

The data was entered and analyzed in SPSS version 25. For descriptive statistics, mean and standard deviations were computed. To assess the mean across and within groups, as well as multiple comparisons among groups, ANOVA and the post-Hoc Tukey HSD test were employed as inferential statistics. In our study, p value of <0.05 was considered as statistically significant in each statistical test.

RESULTS

All the animals used in the experiments survived their laminectomy, among which 02 of the rats died in group B post surgically after 07 days due to sepsis. The rats were sacrificed according to the grouping intervals (i.e: 7th, 14th and 28th days post surgically) after their behavioral studies. Urinary retention developed in only 4 rats from group B. Bladder massage was applied on daily basis twice (Credé's maneuver)[15] , to each rats for some days until they retained normalcy. Later on, the ability to void instinctively returned in most of the paraplegic animals. The incisional sites of the rats were completely healed on 7th post-operative day.

The results of the study were compared on two bases:

a. Group comparison on interval
b. Multiple comparison on intervals

Motor Test:

The BBB scoring or motor function of group A had no neurological deficits six hours' post-surgery as the effect of anesthesia vanished. Scoring of this group was maximum as there was no SCI in it. The scoring got better as the time frame was increased to 28 days' post op for measuring the behavior tests.

The compression group rats were either quadriplegic or had severed hamstring (BBB scores ranging from 0 to 16 points on different intervals i.e.: 2, 7 and 12 points respectively after the surgery**.** The comparison done for different intervals showed significant difference (0.154) among the groups except group A.

Sensory Tests:

The sensory test were multiple tests conducted to see the more accurate results of the behavioral outcomes. Hence the following tests are conducted.

Hot Plate Test

The outcome of the medullar and brain reflexes in hot plate test were used. The hot plate test was conducted on self-made hot plate digital instrument, shows Significant effects of procedures among the groups showed marked difference as depicted in tables (2 &3). Correspondingly, the hot plate test in group B showed functional defects when compared with other groups. The compression treated groups i.e: group C and D also showed improved reflexes in which RkI-1447 were used.

Von frey filaments

The Von frey analysis showed a substantial difference in the improvement of sensory functions. The behavioral outcomes expressed as the Von frey filament scores are presented in Figure (4). The noteworthy differences were found at all survival times longer than post 14 days of SCI by using Tukey HSD test (Table 2 &3). However, there was no result in the initial phase of testing as the response was nil in group B and D specifically (Table 1).

Cold Sensation Test

The outcome of this test seen the cold reflexes observed in the rats by using Tukey HSD test. Acetone evaporation measurements were taken at 30, 60, and 90 minutes at the tight region. The results showed a significant reduction in the time responding to acetone in the group B, but not in the group A. Statistically it indicates no significant effects of the difference in different time intervals as shown in the Figure (4) except the compression injury group (B) as shown in the Table (2 &3).

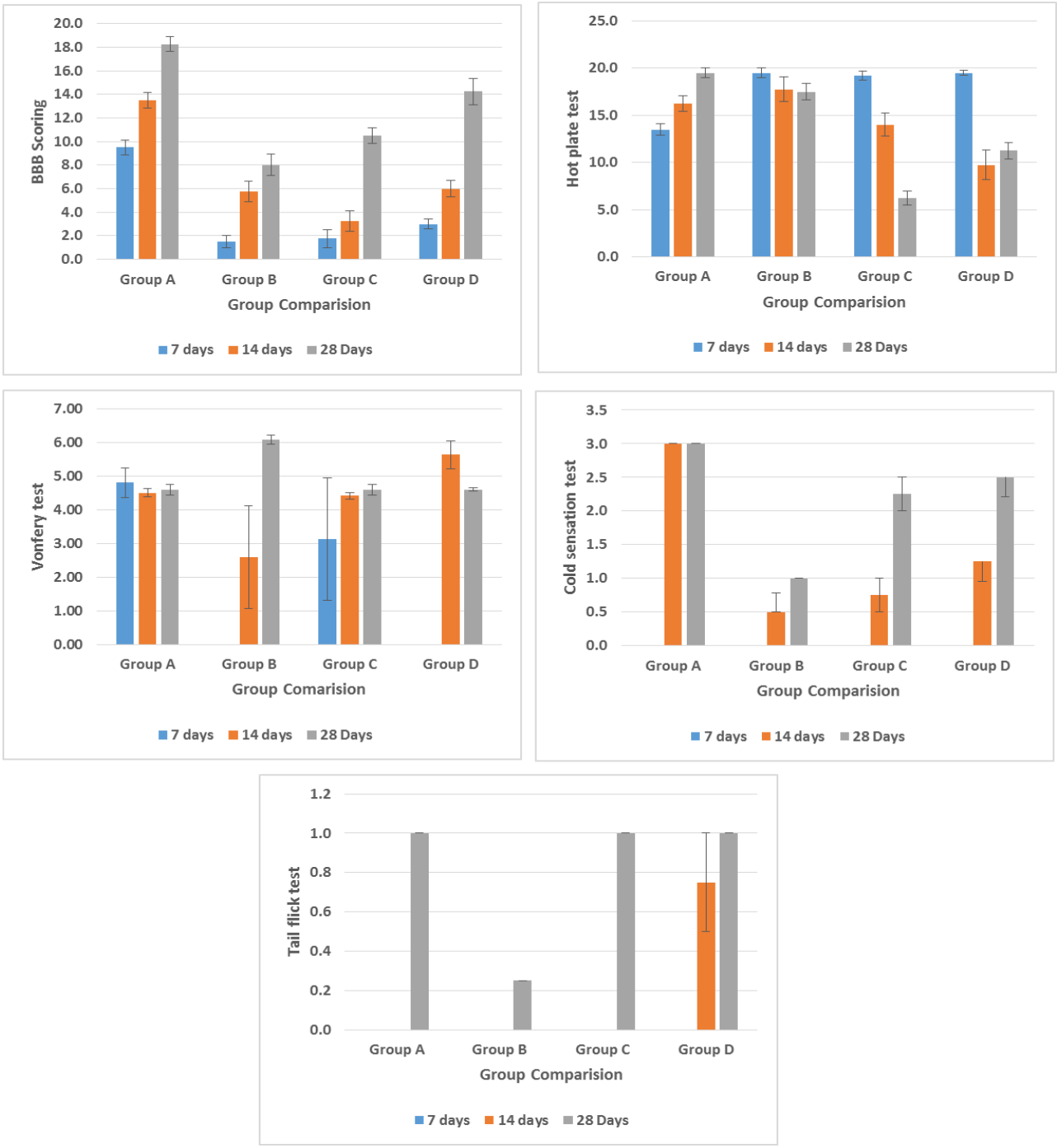

Figure 3. Mean and Standard Error of the sensorimotor test for all groups

Tail Flick test:

The end product of this assay was used to measure the thermal sensitivity by medullary reflexes. Afterwards the group A, rats showed increased tail flick reactions compared to other groups (Table 1), however, group B shoed significance non-significant Tukey HSD test is same in 7 and 14 post op days as compared to 28 post op day (Table 2&3). That the tail flick tests showed a decreased ability of the compression group B to respond to thermal stimuli.

Table 1. Group's comparison and multiple comparison for interval of 7 days

BBB scoring			
Groups	**Mean**	**SE**	**Sig.**
Group A	9.5	0.6	0.000
Group B	1.5	0.5	
Group C	1.8	0.8	
Group D	3	0.4	
Hot plate test			
Group A	13.5	0.6	0.000

Group B	19.5	0.5	
Group C	19.3	0.5	
Group D	19.5	0.3	
Von fery test			
Group A	4.8	0.4	0.007
Group B	0	0	
Group C	3.1	1.8	
Group D	0	0	

Multiple comparison					
Variable	**(J)**	**(I)**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**
BBB Score	Group A	Group B	8.000*	0.835	0
	Group B	Group C	-0.25	0.835	0.99
	Group C	Group D	-1.25	0.835	0.469
	Group D	Group A	-6.500*	0.835	0
Hot plate test	Group A	Group B	-6.000*	0.7	0
	Group B	Group C	0.25	0.7	0.984
	Group C	Group D	-0.25	0.7	0.984
	Group D	Group A	6.000*	0.7	0
Von fery test	Group A	Group B	4.803*	1.319	0.015
	Group B	Group C	-3.138	1.319	0.135
	Group C	Group D	3.138	1.319	0.135
	Group D	Group A	-4.803*	1.319	0.015

Table 2. Group's comparison and multiple comparison for intervals of 14 days.

BBB scoring			
Groups	**Mean**	**SE**	**Sig.**
Group A	13.5	0.6	0.000
Group B	5.8	0.9	
Group C	3.3	0.9	
Group D	6	0.7	
Hot plate test			
Group A	16.25	0.9	0.004
Group B	17.8	1.3	
Group C	14	1.2	
Group D	9.8	1.5	
Von fery test			
Group A	4.51	0.1	0.107
Group B	2.6	1.5	
Group C	4.4	0.1	
Group D	5.6	0.4	
Cold sensation test			
Group A	3	0	0.000
Group B	0.5	0.29	
Group C	0.75	0.25	
Group D	1.25	0.25	
Tail flick test			
Group A	0	0	0.002
Group B	0	0	
Group C	0	0	
Group D	0.75	0.25	

Multiple Comparison					
Variable	**(I)**	**(J)**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**
BBB Scoring	Group A	Group B	7.750*	1.09	0.000
	Group B	Group C	2.5	1.09	0.154
	Group C	Group D	-2.75	1.09	0.106
	Group D	Group A	-7.500*	1.09	0.000

Hot plate test	Group A	Group B	-1.5	1.782	0.834
	Group B	Group C	3.75	1.782	0.207
	Group C	Group D	4.25	1.782	0.133
	Group D	Group A	-6.500*	1.782	0.015
Von fery test	Group A	Group B	1.913	1.122	0.363
	Group B	Group C	-1.823	1.122	0.402
	Group C	Group D	-1.225	1.122	0.701
	Group D	Group A	1.135	1.122	0.746
Cold sensation test	Group A	Group B	2.500*	0.323	0.000
	Group B	Group C	-0.25	0.323	0.864
	Group C	Group D	-0.5	0.323	0.441
	Group D	Group A	-1.750*	0.323	0.001
Tail flick test	Group A	Group B	0.00	0.177	1
	Group B	Group C	-.750*	0.177	0.005
	Group C	Group D	.750*	0.177	0.005
	Group D	Group A	0.00	0.177	1

Table 3. Group's comparison and multiple comparison for intervals of 28 days

BBB scoring			
Groups	**Mean**	**SE**	**Sig.**
Group A	18.25	0.629	0.000
Group B	8	0.9	
Group C	10.5	0.6	
Group D	14.3	1.1	
Hot plate test			
Group A	19.5	0.5	0.000
Group B	17.5	0.9	
Group C	6.3	0.8	
Group D	11.3	0.9	
Hot plate test			
Group A	19.5	0.5	0.000
Group B	17.5	0.9	
Group C	6.3	0.8	
Group D	11.3	0.9	
Vonfery test			
Group A	4.6	0.162	0.000
Group B	6.1	0.1	
Group C	4.6	0.2	
Group D	4.6	0	
Cold sensation test			
Group A	3	0	0.000
Group B	1	0	
Group C	2.3	0.3	
Group D	2.5	0.3	
Tail flick test			
Group A	1	0	0.002
Group B	0.3	0	
Group C	1	0	
Group D	1	0	

Multiple Comparison					
Variable	**(I)**	**(J)**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**
BBB test	Group A	Group B	-2.5	1.199	0.213
	Group B	Group C	-3.750*	1.199	0.038
	Group C	Group D	-4.000*	1.199	0.026
	Group D	Group A	10.250*	1.199	0.000
Hot plate test	Group A	Group B	11.250*	1.07	0.000
	Group B	Group C	-5.000*	1.07	0.003

	Group C	Group D	-8.250*	1.07	0.000
	Group D	Group A	2.00	1.07	0.291
Von fery test	Group A	Group B	1.478*	0.191	0.000
	Group B	Group C	-0.005	0.191	1.00
	Group C	Group D	0.005	0.191	1.00
	Group D	Group A	-1.478*	0.191	0.000
Cold sensation test	Group A	Group B	-1.250*	0.27	0.003
	Group B	Group C	-0.25	0.27	0.792
	Group C	Group D	-0.5	0.27	0.298
	Group D	Group A	2.000*	0.27	0.000
Tail flick test	Group A	Group B	-.750*	0.177	0.005
	Group B	Group C	0.000	0.177	1.00
	Group C	Group D	0.000	0.177	1.00
	Group D	Group A	.750*	0.177	0.005

DISCUSSION

More than a decade of researchers, the traumatic lesions of spinal cord is denoting for the benefit of patients, their restraint to wheelchair and the various medical complications for years or even for the remaining of their lives. The defined treatment and the health care facilities for patients with SCI was, met with frustration in majority of cases [17, 18]. Continuous research studies and its Progression in this field has made the proposal, that eventually SCI will be corrigible and convincible[19].

In the research the use of animal models is a critical situation for devising the experimental approaches aimed to reinstate functions lost due to SCI [20]. Several novel treatments show early promise in SCI research fields [21].

These treatments should be carefully evaluated especially in clinical relevant research model of SCI foe selective strategies. A scheme of grading is recently developed to see the strength of preclinical studies [22].

The spontaneous grooming, bringing up and locomotion affair are the most sensitive for the detection of behavioral lacking occurred due to surgical procedure. Thus the rare limbs evaluating results and research event provides a sensitive indication of the imitate effect of SCI on different intervals. The foundation of any type of SCI study is the ability to acquire authentic, reproducible and useful behavioral data [23]. Among these test the most applicable tests to assess motor functions after a SCI is the 21 point BBB locomotor scale.

The BBB score points, represents a specific set of characteristics of locomotion of hind limb function after spinal cord trauma at different levels of the SCI in rats elicit during spontaneous open field locomotion [24]. The assessment of this test was to decide the useful effect of RKI-1447 with higher dose as 0.6 μg/kg/day along with lower dose of 0.3 μg/kg/day on different intervals as 7th, 14th and 28th day post-op duration. The result shows more effective result comparable to short duration treatment. The same pattern of study was conducted by researchers by using a modified Allen's method by using Puerarine as an anti-inflammatory is as a with higher doses shoed good results. These improvements were noted with similar results after induction of SCI [25].

The current research authenticate a recent study conducted by application of subcutaneous injection of 0.8 mg/kg of Ac-SDKP peptide as a stimulant for anti-inflammatory effect by using weight drop contusion SCI, showing progress in the BBB score post 07 day [26].

The same result was also noted in the study conducted by Sun C and fellows, in which they demonstrated the effectiveness of BBB scoring by applying neurotropin as neuroprotective and anti-inflammatory therapy in contusion SCI, showing significant improvement of BBB scoring in sham and treated group post 14 and 28 days [27].

The sensory functions were assessed below the level of injury (Hind paw) by measuring the thermal and mechanical in contrast to supraspinally mediated escape behavior. We did not observe hyperalgesia in SCI induced in hind pa, which is in line with observation reported from other studies [27,28].

To see the pain sensitivity threshold, the Von frey test was performed in our current study. The study was conducted among with sham group comparable to compression and treated groups which showed statistically significant results in the higher dose rather than the other groups.

The current study showed similarity, in which anti-inflammatory and antioxidant effect of Tacrolimus (TAC) on a polyethylene glycol modified maghnemite nanospheres in weight drop SCI model. TAC-PEG-MNs treated animal showed steady improvement in the pain sensitivity threshold then the only TAC or PEG MNs treated control groups, tested post injury days [29].

The current results of our research also goes parallel with another recent study conducted in which the anti-inflammatory effect of FTY 720, which inhibit local inflammation and reduced scar formation by by modulating reactive astrocytes in SCI. It also showed significant and gradual enhancement in neuropathic pain threshold and the vehicle treated groups post op 7th,14th,28th,35th and 42nd post injury days [30].

The similar conduction of tests and results were also shown in the studies of Chio Y et al (222) and melatonin both showing similarity as an anti-inflammatory effect which demonstrate statistically significant decline in the pain threshold tested by von frey filaments in treated group on compression SCI groups compares to control and compressed groups post of 28 days [31,32].

According to the literature, there was no study conducted regarding treatment of RKI-1447 about the cold sensation of rats in Spinal cord compression injury groups. However, we concluded our study with recent researches in which other anti-inflammatory and anti-oxidative therapies were used to improve the neurological outcomes after SCI.
Our study can be validated with the study conducted Fakhri S et al, in which intrathecal administration of Naringenin, it was applied in compression SCI model, which showed statistically significance difference in acetone drop test scores between treated and untreated groups post op 28 day. [31]
For estimation of hot sensitivity threshold, the hot plate test most sensitive tests to reflex function for noticing of sensorimotor shortage post SCI, for which our current study shows application of RKI-1447 in treated groups compressed with un treated and control groups, showing better results in accordant to higher doses with more post op duration.
Our study was in accordance to the study unveil the effect of Alendronate reducing inflammatory response also reduced the heat sensitivity threshold in SCI = Alendronate group comparing to others, showing significant difference between these groups post 28 days [32].

CONCLUSIONS

The laboratory rats are well accepted in experimental SCI models. They are frequently used for SCI studies. This study confirms the re-creatibility and reliability and sensitivity tests in spinal cord compression model in rats. The behavioral characteristic indicates that BBB and other sensory tests are similar to the conditions of medullary pathology in different experimental animals as well as in humans.
The compression model can prove its suitability for preclinical testing for new therapeutic technique to modulate and rehabilitate the neuropathological process participated in secondary injury mechanisms and to improve its aftermath in para- or quadriplegic patients.
For successful clinical researches shows new interventions for SCI for which appropriate plans of experiments are must. Nevertheless the 3Rs should be in consideration for the ethical and animal welfare and for the acceptance of its outcomes[33].

Limitations:
There are certain limitations in our study along the use of rodents in our experiments.

1.Smaller sample size:
Small sample size is used as compared to human clinical trials, which limits the statistical value of the study.

2. Limited experimental duration:
Rodents experiments are usually of limited duration as compared to clinical trials. It may not capture the long term effect, safety profile or chronic conditions.

3. Translation to clinical Practice:
Many preclinical results may not coincide with the clinical trials, hence it is challenging to practice the trail directly used in rodents.

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