

# Assessing the functional and histological effect of low-level laser therapy on spinal cord compression induced injuries on Wistar rats

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## ABSTRACT

Low-level laser therapy (LLLT) has become an increasingly mainstream modality, especially in the areas of physical medicine and rehabilitation. This study aimed to determine the functional and histological changes that occur at the lower thoracic - upper lumbar segments associated with the irradiation with LLLT in compression-induced injuries in Wistar rat's spinal cord. Eighteen rats were randomized into three groups; six rats in each. Group A (sham) with laminectomy only, group B laminectomy plus cord compression injury and finally group C had laminectomy plus cord compression injury plus irradiated with LLLT two times, one after 2 hours from the injury and another after 24 hours from the first dose, each of these doses lasted for 42 minutes. The lesion site for group C directly was irradiated to the spinal direction with a 905 nm diode laser with an output power of 25 mW. The functional recovery was measured by locomotor test (BBB scale) and the histopathological changes were assessed in all groups after two weeks. Rats treated with laser showed a significant improvement in the locomotor functions compared to spinally injured rats. While histopathological evaluations in group C showed mild changes in comparison with group B that suggested significant recovery of the spinal cord compression injury. These findings demonstrated that the 905 nm wavelength laser is a promising neuroprotective non-invasive treatment for improving functional recovery after spinal cord injury.

**Keywords:** Low-level laser therapy, spinal cord compression, Wistar rats, spinal cord injury.

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## INTRODUCTION

Spinal cord injuries (SCI) are one of the devastating causes of the paralysis worldwide (Ramotowski et al., 2019). Most countries report an annual incidence of SCI from 15 to 30 per million, and the prevalence ranges from 236 per million in India to 1800 per million in the United States (Jain et al., 2015; Kumar et al., 2018; Rahimi-Movaghar et al., 2013). Although a lot of trials had been conducted and up going researches are striving to give them hope of recovery, there is no definitive treatment for SCI to retain patients to their previous functional status. SCI could be a complete or partial loss of autonomic, sensory, and motor functions; that causes interruption of neural signal conduction along the axonal tracts (Atkinson, 2012). There is generally poor recovery of these functions because of the difficulty of tissue

regeneration in the central nervous system. Thus, SCI patients might develop serious residual disabilities, such as paralysis, respiratory difficulty, chronic pain, urinary problems, and neurologic decline, leading to a considerable decrease in quality of life. Furthermore, SCI historically has been associated with very high mortality rates (Atkinson, 2012).

The use of electrotherapeutics instruments with regenerative purposes is a common practice in physiotherapy, particularly using low-level laser therapy (LLLT) to accelerate regenerative processes to restore functionality (Paula et al., 2014). The beneficial mechanisms of LLLT is complex, it has photochemical reactions with cell membranes, cellular organelles, and enzymes, but essentially, it depends upon the absorption

of specific visible red and near-infrared wavelengths in photoreceptors within sub-cellular components. Particularly, the electron transport chain within the membranes of mitochondria, which is capable of enhancing the levels of Adenosine Triphosphate (ATP), as well as an increasing the electrical potential of the mitochondria membrane, alkalization of the cytoplasm, and activation of nucleic acid synthesis (Avci et al., 2013; Hashmi et al., 2010). Moreover, several studies proved the effectiveness of LLLT on neural regeneration, as it inhibiting the chemical mediators involved in the inflammatory process, accelerating the repairing mechanism, stimulating fibroblast proliferation, decreasing edema, regenerating peripheral nerve, as well as neovascularization processes (Alves et al., 2013; Paula et al., 2014; Piva et al., 2011).

Numerous studies proposed that the best treatment for spinal cord injury is to stimulate the repairing process. As, it is believed that, when receiving an external stimulus, the nervous system can be adjusted and reorganized using mechanisms to compensate the neuronal loss and promote, even though partial, restoration of remaining synaptic connections and consequently new neuronal sprouting (Oki et al., 2012; Silva et al., 2014). However, the studies involving the effects of this therapy for neural repair are scarce, with increasing evidence of the potential benefits of laser in recovery spinal cord injury (Alves et al., 2013; Piva et al., 2011). This study aimed to determine the functional and histological changes that occur at the lower thoracic - upper lumbar segments associated with the application of LLLT in compression-induced injuries of Wistar rat's spinal cord.

## MATERIALS AND METHODS

### Animals

This study was conducted in a special animal lab in the faculty of physiotherapy at Al Neelain University, including the rats handling, procedure, rats housing, and sample collection. The animals were treated following the ethical standards laid down in the US National Institutes of Health (NIH Publication No. 85-23) and its later revisions.

Female Wistar rats weighting (150 to 200 g) were housed at temperature-controlled room at 18-26°C with a 12- hour light/dark cycle. Bladders were emptied manually 2 times per day using the Credé maneuver, which is applying pressure over the bladder at the lower abdomen. Following the operation rats received tramadol with subcutaneous injection, 1.5 mg/kg every 12 hours for 2 days to relieve postoperative pain. During the first week, the rats received ceftriaxone 100 mg/kg with intraperitoneal injections for 2 days post-surgery to prevent infections. Rats that showed signs of a bladder infection (such as hematuria) were treated with amoxicillin-Clavulanate, at a 2 mg/100 g dose, with intraperitoneal injections 2 times a day until 24 hours after the signs disappeared (Scheff et al., 2003).

### Experimental protocol and study groups

Animal care and experiments were carried out under the Animal Research Review Panel (ARRP) Guideline. The study started with

thirty female Wistar rats divided as ten into each of the three groups but because of attrition causes, it ended up with 18 rats 6 in each group. The rats were divided into three groups: Group A: Laminectomy only (sham group), Group B: Laminectomy with compression induced SCI (control group), and finally Group C: Laminectomy with compression induced SCI + LLLT application within 2 h after the injury (operated group).

### Surgical procedure and tissue preparation

First, the rats were anaesthetized using a mixture of ketamine and xylazine 100 mg/kg and 10 mg/kg respectively delivered with intraperitoneal injections (Scheff et al., 2003). The fur overlying the thoracic vertebral column of the rats were removed using a shaver. Next, the skin and underlying muscles were also detached and retracted. Using iridectomy scissors, a laminectomy was made at the level of T12 and the spinal cord was exposed, after that, an injury was made using a modified aneurysm clip causing moderate to severe injury by pressure with a force of 35 g by the clip for one-minute time duration (Poon et al., 2007). The clip closes rapidly, cord contusion is produced by a compression force causing moderate to severe injury with this pressure, and the force of the clip was applied on the lateral to lateral aspects of the spinal cord (Figure 1).

### Laser treatment

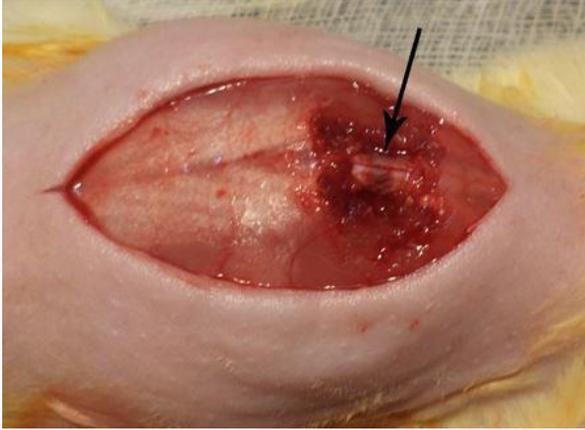
After two hours from the initial compression injury on the spinal cord, lesion sites in rats in group C were exposed and directly irradiated with a 905 nm diode laser, the beam transmitted through a polarizer (ASA.Volta 9-36057 Arcugnano (VI) – ITALIA). Then the incised skin was closed with sutures after laser irradiation. Exposure of the spinal cord, irradiation, and suturing for group C was repeated after 24 hours from the first exposure. The irradiation was done in one area of 1 cm<sup>2</sup>. The point was treated for 42 min. Power Density was calculated according to the equation = power / (3.14\*r<sup>2</sup>) = 25 / (3.14\*(0.5)<sup>2</sup>) = 25/12.56 = 1.99 mW/cm<sup>2</sup>, while energy Density = (power\*time)/beam area = (25\*2520)/12.56 = 63000/12.56 = 5015.9 mJ/cm<sup>2</sup> = 501.59 J/cm<sup>2</sup>

### Behavioral analysis

After surgery the rats exhibited hindlimb paralysis and loss of bladder function. For two consecutive weeks the right and left hindlimb locomotor function were evaluated and graded using Basso, Beattie, and Bresnahan (BBB) scale (Aziz et al., 2014; Martinez et al., 2009). The locomotor evaluation was made in an open field in a wooden box (50x80x40) diameter, with a smooth surface for four mints. One week before the surgery each animal was acclimated to the open field test. The analysis of this test was analyzed according to limb movement, paw placement, stepping, coordination, toe clearance, and tail position. A score ranged from 0 (no hind limb movement) to 21 (normal locomotion) this was recorded and scored by one investigator who was blinded to the experiment.

### Histological procedure

Rats were euthanized using deep anaesthesia by inhaling an overdose of isoflurane, then a 1.5 cm specimen was taken from the spinal cord. The fixed specimens were held in 10% neutral buffered formalin then cut into three equal halves to be processed, embedded in paraffin wax, sectioned by a microtome (5 µ), and stained with hematoxylin and eosin by the conventional and microwave methods (Malatesta, 2016).



**Figure 1.** Surgical site: the lateral to the lateral cord was exposed and clamped by Bulldog forceps for 1 minute (Arrow points to the compressed cord).

### Ethics approval

Ethical clearance was obtained from the Institutional Review Board at Al Neelain University.

### Data analysis

Data from the BBB scale were expressed as mean ranks and sum of ranks. Statistical analysis of the neurologic scores was analyzed by using the Wilcoxon Signed Ranks Test, and Mann-Whitney Test. The investigators were blinded to the treatments. Values for statistical analyses were considered significant at  $p < 0.05$ . All analyses were performed using the SPSS software package version 22.

## RESULTS

In this study; we used a modified aneurysm clip to produce the compression model in the rat's spinal cord; which was sustained for a 1-minute duration. Wilcoxon Signed Ranks test; shown in Table 1; revealed that

negative ranks on the right and left BBB scale were found to be significant in both control and operated groups; which meant that scoring of BBB scale after the surgery becomes less for both groups. On the other hand, ties rank on the right and left BBB scale were found in the sham group was equal before and after the laminectomy. These results indicated that this compression model was significantly effective in causing the SCI model in rats.

Twelve days post-surgery an open field BBB scale scored for all groups for both right and left hindlimb. As shown in Table 2, a comparison between mean ranks of BBB results in both right and left post-surgery scores in both control and operated groups, using Mann-Whitney Test, showed that there was no significant difference between these two groups.

However, the sum of ranks for the operated group is to some extent higher than the control group being 31 for right and 27 for the left hindlimb in the control group, while the operated group being 47 for right and 50 for the left hindlimb.

Further evaluations were performed by a neuropathologist, who was blinded to the experimental procedures. The spinal cord injured area was captured, through a Sony camera connected to the microscope, (Figures 2, 3 and 4). As shown in Figure 2, the photo of the spinal cord at H&E X 100 power, the sham-operated rats demonstrated normal structure with no neuronal loss, edema, or gliosis, while the SCI group showed no neuron with wide gliosis, as well as liquefactive necrosis. Moreover, rats treated with LLLT demonstrated minimal neuronal death and minimal neuropil edema. Furthermore, Figures 3 and 4 were captured at higher power H&E X 400, they illustrated that group A (sham group) had a normal structure with no neuronal loss, edema or gliosis, with 16 neurons seen, whereas group B rats cord showed neuronal death with wide gliosis and polymorph nuclear leukocyte infiltrated necrosis with no neurons seen. Nevertheless, group C rats treated with LLLT showed focal neuronal vacuolation with minimal neuropil edema, and perineuronal edema with 12 neurons seen.

**Table 1.** Comparison between the mean ranks of BBB results in both Right & Left hindlimb in all of three group (pre & post to the operation).

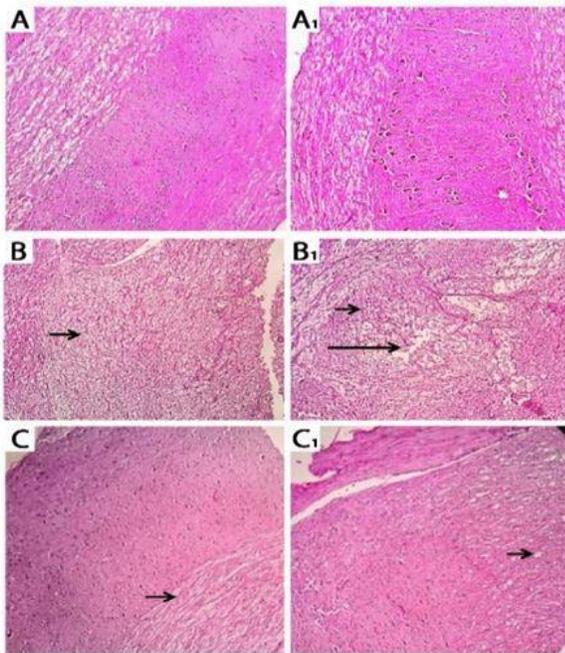
Wilcoxon signed ranks test		Number	Mean rank	Sum of ranks	P value
BBB scale results Rt Post - BBB scale results Rt Pre	Negative ranks	12 A	6.5	78	0.002**
	Positive ranks	0 B	0	0	
	Ties	6 C			
	Total	18			
BBB scale results Lt Post - BBB scale results Lt Pre	Negative ranks	12 A1	6.5	78	0.002**
	Positive ranks	0 B1	0	0	
	Ties	6 C1			
	Total	18			

\*A: BBB scale results Rt Post < BBB scale results Rt Pre, B: BBB scale results Rt Post > BBB scale results Rt Pre, C: BBB scale results Rt Post = BBB scale results Rt Pre, A1: BBB scale results Lt Post < BBB scale results Lt Pre, B1: BBB scale results Lt Post > BBB scale results Lt Pre, C1: BBB scale results Lt Post = BBB scale results Lt Pre.

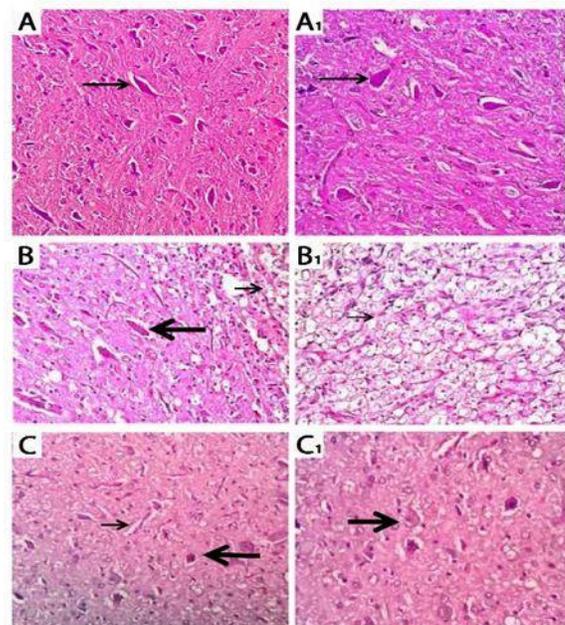
**Table 2.** Comparison between the mean ranks of BBB results in both Right & Left hindlimb post-surgery scores in group B & C.

Mann-Whitney Test	Group	Number	Mean rank	Sum of ranks	P value
BBB scale results Rt Post	B	6	5	31	0.240*
	C	6	8	47	
	Total	12			
BBB scale results Lt Post	B	6	5	28.5	0.093*
	C	6	8	49.5	
	Total	12			

\*\* P value > 0.05 that's considered as statistically insignificant.



**Figure 2.** A: Photomicrograph of the spinal cord, H&E X 100, of normal control rat showing normal structure with no neuronal loss, edema or gliosis. A1: H&E X 200, of normal control rat showing a normal structure with no neuronal loss, edema or gliosis. B: Photomicrograph of the spinal cord of group B rat cord, H&E X 100, showing neuronal death with wide gliosis (short arrow). No neurons are seen. B1: H&E X 200, showing neuronal death with wide gliosis (short arrow) and liquefactive necrosis (long arrow). C: Photomicrograph of the spinal cord of group C rat cord, H&E X 100, showing minimal neuronal death with minimal neuropil edema (short arrow). C1: H&E X 200, showing minimal neuronal death with minimal neuropil edema (short arrow).

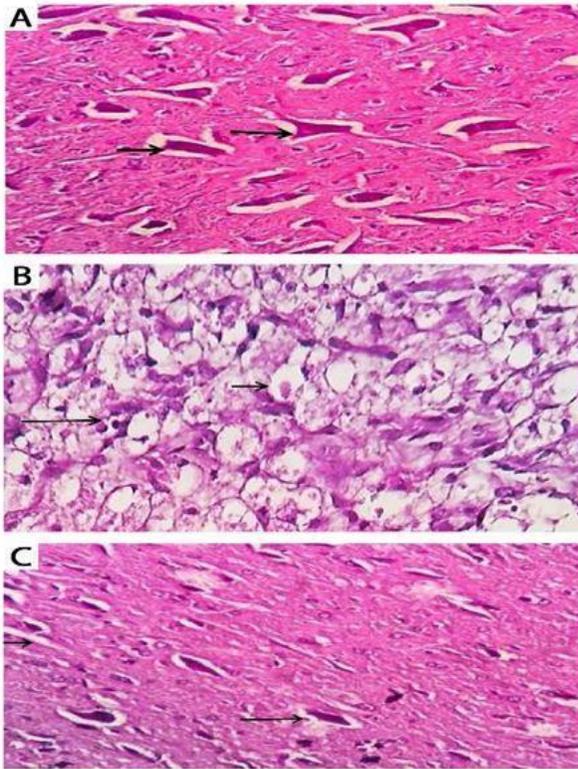


**Figure 3.** A: Photomicrograph of the spinal cord, H&E X 400, of normal control rat showing a normal structure with no neuronal loss, edema or gliosis, 16 neurons are seen (thin arrow). A1: H&E X 400, of normal control rat showing normal structure with no neuronal loss, oedema or gliosis, 16 neurons are seen (thin arrow). B: Photomicrograph of spinal cord of group B rat cord, H&E X 400, showing neuronal death (thick arrow) with wide gliosis (short arrow). Only 6 neurons are seen. B1: H&E X 400, showing neuronal death with wide gliosis (short arrow). No neurons are seen. C: Photomicrograph of the spinal cord of group C rat cord, H&E X 400, showing focal neuronal pyknosis (thick arrow) with minimal perivascular edema (short arrow). 12 neurons are seen. C1: H&E X 400, showing focal neuronal vacuolation (short arrow). 12 neurons are seen.

## DISCUSSION

The current study evaluated the effect of LLLT on recovery after spinal cord compression in Wistar rats. We treated rats with 905nm laser after SCI, and they demonstrated a significant improvement in both

behavioral and histological analysis. These findings provide further support to the neuroprotective capability of LLLT in reducing the damage to rats after spinal cord compression-induced injuries. Several studies proved the efficacy of laser therapy in different neurological diseases



**Figure 4.** A: Photomicrograph of the spinal cord, H&E X 400, of normal control rat showing a normal structure with no neuronal loss, edema or gliosis, 16 neurons are seen (thin arrows). B: Photomicrograph of the spinal cord of group B rat cord, H&E X 400, showing neuronal death with wide gliosis (short arrow) and polymorph nuclear leukocyte infiltrate necrosis (long arrow). No neurons are seen. C: Photomicrograph of the spinal cord of group C rat cord, H&E X 400, showing focal neuronal vacuolation with minimal neuropil edema (short arrow) and perineuronal edema (long arrow). 12 neurons are seen.

(Chen et al., 2015; Hakimiha et al., 2020; Pinto Faria et al., 2020).

In the present study, we used a modified aneurysm clip model to injure rats' spinal cord at the lower thoracic - upper lumbar segments. This model has given reliable and consistent results for studying acute SCI in rats and has been used by numerous researchers in the field. It worth mentioning, the clip edges exert force on the spinal cord bi-directionally and simultaneously from the ventral and dorsal surfaces, which simulates the common mechanisms in human SCI such as fracture-dislocation and burst-compression fractures (Poon et al., 2007; Sharif-Alhoseini et al., 2017).

In laser-treated rats, we used LLLT 905 nm at a dosage of 501.59 J/cm<sup>2</sup>, we believed that our wavelengths penetrate all the tissue layers surrounding the spinal cord, and this is consistent with Byrnes et al. finding, as they reported that 6% of the incident power of 810 nm laser light can penetrate the spinal cord (Byrnes et al.,

2005).

The current data demonstrated that LLLT improved functional recovery after cord injury, as illustrated in table 1, the results from the BBB test showed that group C (laser-treated group) achieved a significant improvement compared to SCI rats. Similarly, considerable researches reported comparable findings. Svobodova et al. reported that laser-treated rats with a simultaneous 808 nm continuous emission and 905 nm demonstrated a significant improvement in the first week following SCI (Svobodova et al., 2019). Moreover, other studies found that 810 nm light, at a dosage of 1.589 J/cm<sup>2</sup>, significantly improves axonal regrowth, functional improvement, and statistically significant suppression of immune cell invasion and pro-inflammatory cytokine and chemokine gene expression (Anders, 2009; Veronez et al., 2017).

Concerning the current data, the histological findings correlate with the observed behavior analysis, as rats treated with LLLT demonstrated a minimal neuronal death, with minimal neuropil edema, and twelve neurons seen, compared to SCI rats that showed neuronal death, wide gliosis and polymorph nuclear leukocyte infiltrated necrosis with no neurons seen (Figures 2, 3 and 4). Therefore, it is evident from the current finding that the major effect of LLLT is due to its neuroprotective potential. Similarly, several other studies support the efficacy of LLLT in SCI regardless of the polarization direction, with a reduction in the formation of glial scar and cavity (Hamblin et al., 2018; Sotoudeh et al., 2015). Moreover, Xuan et al. documented that LLLT had efficient protection against neural cells from apoptosis or necrosis (Xuan et al., 2013). Also, Wong-Riley et al. confirmed these findings when an in vitro study established that laser treatment can reduce the death of functionally inactivated primary neurons by restoring the function of mitochondrial enzyme cytochrome C oxidase (Wong-Riley et al., 2005). However, Ramotowski et al. stated no significant difference in the cavity area between the different laser groups (Ramotowski et al., 2019). Furthermore, it had been reported that LLLT decreases the inflammatory cell accumulation in the spinal cords of animals that received LLLT as compared with the control group, further supporting the anti-inflammatory property of LLLT (Chen et al., 2015).

Moreover, several studies demonstrated that transcutaneous application of non-polarized laser significantly promoted axonal regrowth (Anders et al., 2015; Paula et al., 2014), our results are in agreement with that and showed an association of improved neurologic status from the increased number of survivals of the neurons in the operated group over the control one (Figures 2, 3 and 4).

Nevertheless, our study had several possible limitations, such as the small sample size, the estimation of the most accurate minimal dose of the LLLT application, which eventually needs more studies to overcome.

## Conclusion

We concluded from this study that the use of LLLT 905 nm at a dosage of 501.59 J/cm<sup>2</sup> in rats' spinal cord compression model, had a potential neuroprotective effect with the possibility of using as a treatment method for spinal cord injury, due to its positive impact on the spinal cord, in decreasing the neuronal loss and minimizing the inflammatory process that accompanies SCI.

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