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Research Article

# EFFICACY OF HELIUM - NEON LASER ON ACUTE SPINAL CORD INJURY IN RATS

**Zainab Hashim Radha[1], Abdulbari A. Alfaris*[2] and Batool S. Hamza[1],**

[1]*Department of Poultry Disease and Pathology, College of Veterinary Medicine, University of Basrah. Iraq.*

[2]*Department of Surgery and Obstetric, College of Veterinary Medicine, University of Basrah. Iraq.*

**Abstract**

The present study was carried out to evaluate the efficacy of Helium-neon laser therapy on the regeneration of spinal cord injury in laboratory rats. Twenty adult white rats were used in this study, divided into two groups, each group consists of ten animals and the two groups are Control group and Low level laser therapy group. Spinal cord had been transected in all animals of experiment in the group of Low level laser therapy, animals were exposed to Laser irradiation after one hour from surgical intervention at influx density 175 gauss, 2.5 cm spot area, the application was carried out during 14 days, once a day in a total of 14 sessions with each session lasting for 15 minutes. Animals of each group sacrificed on two periods of time after 6 and 12 weeks, clinical assessment for the motor and sensory functions were done throughout the time of experiment, macroscopic and microscopic examination of the spinal cord were done after sacrificing of the animals. The results indicate that the group of Low level laser therapy showed clear improvement in motor and sensory function, degree of spinal cord cooptation, also massive glial scar formation and axonal regeneration..

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## 1. Introduction

Spinal cord injury (SCI), a devastating condition affecting the central nervous system is associated with sensory, motor and visceral function impairment as well as chronic pain (Hagg and Oudega, 2006.

In spinal cord injury (SCI), complete or partial loss of autonomic, sensory, and motor functions is caused by interruption of neural signal conduction along the axonal tracts. There is generally poor recovery of these functions because of the difficulty of tissue regeneration in the central nervous system. Thus, SCI patients are left with serious residual disabilities, such as paralysis, respiratory difficulty, chronic pain, urinary problems and neurologic decline, leading to considerable decrease in quality of life. Various

* *Corresponding author*: Abdulbari A. Alfaris
*E.mail:* vetedu2013@gmail.com

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strategies have been examined for repair of SCI in animal models, including blockage of the endogenous growth inhibitory factors (Atalay *et al*., 2007). Infusion of neurotrophic factors, transplantation of growth promoting cells (Tsuji *et al*., 2010).

In recent times, low - level laser therapy (LLLT) constitutes a novel intervention shown to regulate neuronal function in cell cultures, animal models, and clinical conditions. Several studies have shown that near-infrared LLLT has the potential to be an effective non-invasive therapy for SCI, low level laser therapy (LLLT) has photochemical reactions with cell membranes, cellular organelles and enzymes. LLLT can induce a complex chain of physiological reactions by increasing mitochondrial respiration, activating transcription factors, reducing key inflammatory mediators, inhibiting apoptosis, stimulating angiogenesis and increasing neurogenesis to enhance wound healing, tissue regeneration and reduce acute inflammation (Hashmi *et al*., 2010). The detailed mechanisms of LLLT are still under investigation. However, the therapeutic efficacy relies fundamentally on the initial photochemical event, i.e., absorption of photons by photoacceptors or chromophores such as Cytochrome - c oxidase in the tissue (Huang *et al*., 2009). The aim of the present study was to evaluate the efficacy of LLLT on the regeneration of spinal cord injury.

## 2. Materials and Methods

Twenty laboratory rats (*Rattus norvegicus*) weighing 200±50 gm, were used. Animals accommodated in same laboratory condition by keeping them in special cages (4 animals per cage) for about 15 days before the operation for antimicrobial, antiparasitic drug administration and acclimatization. Animals were weighed immediately after buying them and weighed again before the beginning of the experiment, and then they were weighted weekly for one month after surgical intervention to induce spinal cord injury, then every two weeks till the end of experiment. **Control group:** The spinal cord of the animals was transected and the animal receives no treatment specific for the spinal cord injury. The data were recorded daily for four weeks then weekly till the end of the experiment at 12 weeks. **Low Level Laser Therapy Treated Group:** After incomplete transection of the spinal cord and after a surgical intervention the animals were exposed to low level laser therapy at influx density 632 nm. The application was carried out for 14 days. Once a day, in a total of 10 session, each session lasting 15 minutes while the animal was restrained with special restrainer.

One day before the operation, the site of operation were clipped and shaved carefully. The animals were fasted for 12 hrs and the water withdrawn 2 hrs before operation. All surgical techniques were performed under aseptic conditions. Animals were anaesthetized before operation by intramuscular injection of a mixture of 50 mg/kg B.W ketamine hydrochloride and 10 mg/kg B.W xylazine. Adorsal incision was made in the mid-lumbar region of the anaesthetized rats muscle retracted in the Lumbar (L2- L3) to expose the vertebral bone plate which was still intact (Del-Bel *et al*., 2000). The vertebra was approached as described above and the vertebral bone of the L3 segment was removed bilaterally to expose the dura mater, which was left intact (Fig - 1) (Giglio *et al*., 2006).

**a) Reveal of vertebral colomn**

**b) Exposure of the spinal cord after laminectomy**

**Figure - 1: Lamioctomy**

After laminectomy, a sharp tiny blade was positioned perpendicularly to the tissue, touching the inferior side of the bone plate. An incomplete cut of the cord had been made with the blade running in the half of cord to the end of it (Fig - 2). The incision of the muscular layer had been closed by absorbable cat gut (Chromic catgut 2/0), while the incision of the skin was sutured by non-absorbable silk (3/0). Then, the animals were given pen-strept antibiotic and placed in a warm place in a single cage for each animal and the water and food remaining accessible.

**Figure - 2: Reveal incomplete spinal cord injury**

Laser was immediately irradiated after incomplete transaction of spinal cord according to the former study. A continuous 632 nm Helium neon laser beam was used in horizontal direction focus on the injury site of the spinal cord (Fig - 3). Laser treatment was continued for 15 minutes per rat daily for a total of consecutive 1 to 14 days in LLLT and combination group. The irradiation parameters have been identified safe and the energy could be effectively transmitted to the surface and the depth of the spinal cord (Byrnes *et al*., 2005).

**Figure – 3: Exposure of animal to laser irradiation**

After surgery, the rats were separated in single cages and routinely placed in heating places contain heating lamps for the first two weeks. The bladder was expressed twice daily for seven days, by massage at the lower abdominal region where the bladder was palpable, until it became no longer distended and impalpable, when an autonomous bladder voidance reflex was developing. Isotonic saline (0.9 %, 2 ml) administered subcutaneously twice daily in the first three post-operative days to supplement blood volume and prevent dehydration**.** Antibiotic was administered single dose daily for 10 days to prevent the complications (Talmadge *et al*., 2002). Inspection for skin irritation or decubitus ulcers or evidence of autophagia, was carried out daily.

**Assessment of Motor Function**

This depends on the ability of the animals to move their hind limb and on the development of the movement over weeks. This weekly assessment of the movement of the animals begins from the first time in which the animals became able to move the hind limb to the end of the study. Assessment of Sensory Function To establish the presence of sensory function the following examination were weekly recorded from the beginning of the hind limbs movement to the end of the study (12 weeks).

## 3. Results

Following spinal cord transected the symptoms which appeared below the level of injury include loss of movement in hind limb, loss of sensation and loss of bowel and bladder control.

### Control group

The clinical assessment of the animals of this group showed which was known as transected untreated. First week post – operation: Marked paralysis of the hind limb and the sensation was lost where there was no response to the pricking of the hind limbs and the animals showed weight loss and there is sever autonomic dysfunction (bladder and bowel functions). Second week post-operation: Loss of weight has been marked during this week. Animals of this group also developed stiffness of hind limb and joints. This clinical observations extend to the end of experiment where there was no improvement and the animals still paralyzed upto 12 weeks.

### Laser treated group

The clinical assessment of the animals of this group shows: First week post - operation: Mild weight loss, motor and sensory dysfunction of the hind limb. The animals regain the autonomic function in second week, there were also marked weight loss in all animals in the second to third Fourth week post – operation. At the end of this week some animals showed the onset of hind limb movement, while the others showed the onset of movement during the fifth week. However, the assessment of the sensory function revealed no obvious sensation because the animals didn’t respond to the pricking of the hind limbs, but the animals start to regain weight. In the fifth to sixth week, there was marked movement in the hind limb in which the animals start to move their hind limb during movement. Eighth week post-operation: movement has still been developed and the animals had the ability to stand on hock joint. Moreover, there was mild to moderate response to pricking of the hind limbs. Tenth week post-operation: three animals start to use their paw in the movement (the other two animals use their paws in the eleventh week and have good muscle contraction and twelfth week post-operation all animals depend in their movement on the paw and have a good muscular contraction and moderate sensory reflex.

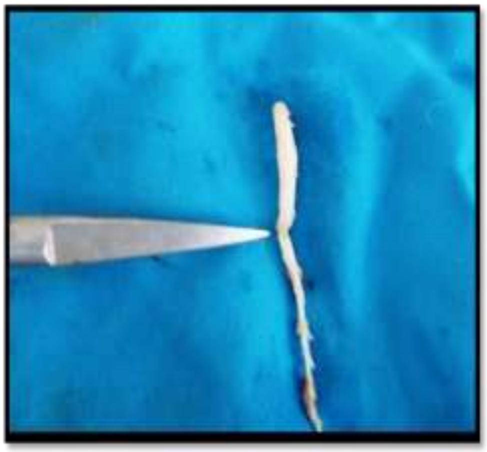
### Macroscopic findings

### Control Group

After 6 weeks, re-exploration of spinal cord revealed that there was no coaptation in the site of transection and there was marked Wallarian degeneration (in which the spinal cord being in thread like structure) in the distal part of the spinal cord with complete disappearance of dorsal root ganglia in the area under transaction (Fig- 4a). In the proximal part, there was also evidence of Wallarian degeneration in the area near to the transection while there was normal appearance of spinal cord in the area far from transection. Exploration of the thigh muscle, revealed that there was marked atrophy in this muscle these changes were more severe in the animals of 12 weeks post operation (Fig - 4b).

### Laser Treated Group

After 6 weeks, re-exploration of the spinal cord of this group revealed that there was an evidence of coaptation between the two sides of the transected spinal cord (Fig - 5a). The site of transection appears congested and the dorsal root ganglia were present. There was moderate atrophy of the thigh muscle; there was also marked reduction in Wallarian degeneration in comparison with the positive group (Fig - 5b). After 12 weeks re-exploration of the spinal cord revealed good coaptation between the sides of transection and there was no evidence of Wallarian degeneration. The general view of the spinal cord appears congested especially in the site of transection. In addition to the presence of dorsal root ganglia in the distal part which appears slightly atrophied and the thigh muscle was slightly atrophied.



**a) After 6 weeks reveals no coaptation in the area of transection and presence of Wallarian degeneration**

**b) After 12 weeks which reveals slight coaptation in the area of transection and Wallarian degeneration in the post transection area**

**Figure – 4: The spinal cord of positive control**

**a) After 6 weeks reveals congestion in the area of transection and presence of the ganglia in the post transection area and no evidence of Wallarian degeneration**

**b) After 12 weeks reveals good status and complete absence of Wallarian degeneration**

**Figure – 5: The spinal cord of laser group**

In the sixth week histopathology, Control Group of the spinal cord revealed marked vacculation in the white matter which indicate the degeneration of the nerve fibers and some of them appear oedematous. In the gray matter, there were some atrophied neurons and others appear necrotized, minimal proliferation of astrocytes and oligodendrocytes in the site of transaction were apparent (Fig - 6, 7 and 8). In the twelfth week, there were also marked vacculation in the white matter due to degeneration of the nerve fibers, some of them appear oedematous. In the gray matter, some neurons appeared atrophied and others showed complete absence at the site of transection. There was minimal glial scar formation due to proliferation of reactive astrocytes and still there is a gap between pre and post transection area (Fig - 9).

**Laser Treated Group**

After six weeks, histopathology of the spinal cord revealed that there were number of vacculated degenerate nerve fibers, some of them appear oedematous at the site of transection. There were some regenerate axons with formation of glial scar (astrocytes, oligodendrocytes and microglial cells) (Fig - 10 and 11), also some atrophied degenerating neurons in the gray matter (Fig - 12). After twelve weeks, histopathology of spinal cord revealed that there was a marked reduction in the degenerated nerve fibers. In the site of transaction, there were glial scar due to proliferation of astrocytes and oligodendrocytes and there were heavy number of regenerated axons arranged in transverse and longitudinal directions associated with proliferation of oligodendrocytes and microglial cells (Fig - 13).

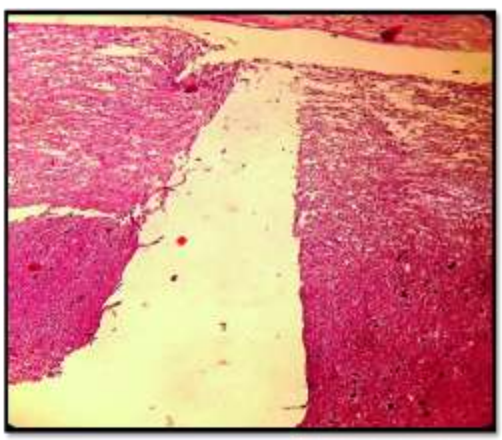


**Figure – 6: Spinal cord of positive control group after 6 weeks reveals a little proliferation of astrocytes in the site of transection (arrows) but still there is a gap between two sides of transection. H & E 100 X**

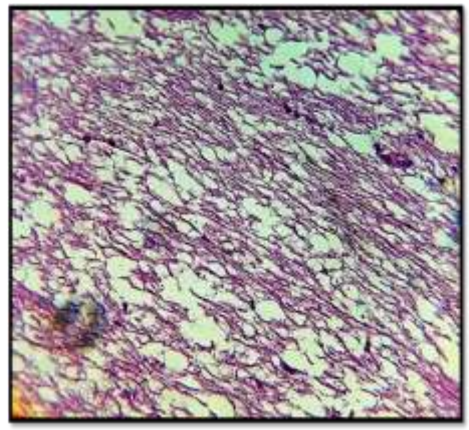


**Figure – 7: White matter of positive control group after 6 weeks shows sever degenerative vacculated nerve fibers (Arrows). H & E 400 X**

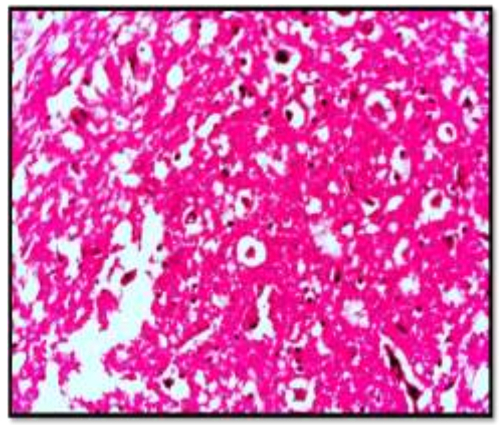


**Figure – 8: Positive control group reveals absence of neurons in the gray matter with severe vacculation (arrows) H & E 400 X**

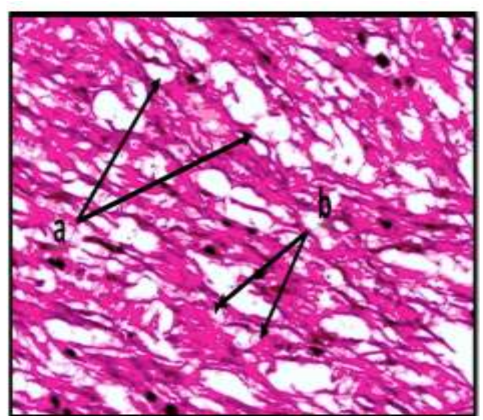



**Figure - 9: Positive control group after 12 weeks shows a) Vaculated degenerated nerve fibers; b) Presence of few glia in white matter. H & E 400 X**

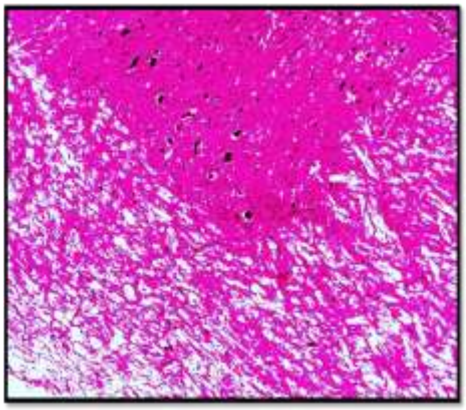


**Figure – 10: Spinal cord of laser group reveals the area of white and gry matter. H & E 100X**

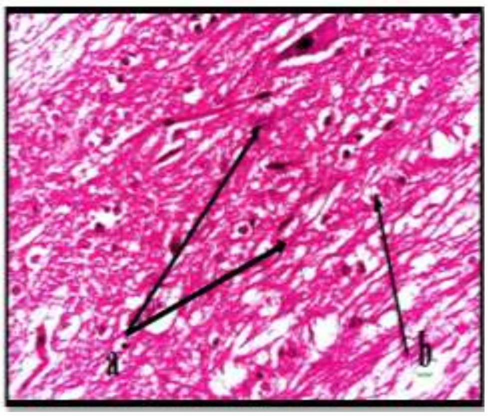



**Figure – 11: Spinal cord of laser gray matter of 6 weeks shows a) Normal neurons b) Degenerated neurons (arrows) H & E 400 X**

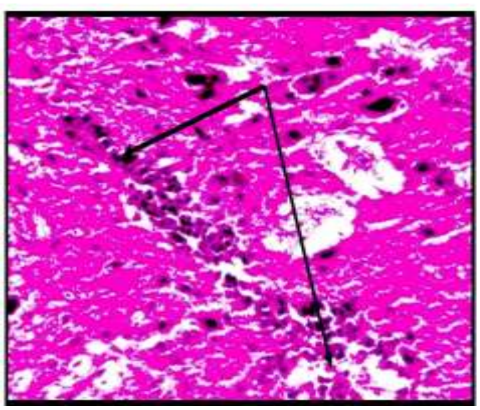


**Figure – 12: Spinal cord of Laser group after 6 weeks shows vaculated nerve fibers with afocus of glia cells (astrocyte and oligodendrocyte) (arrows) H & E 400 X**

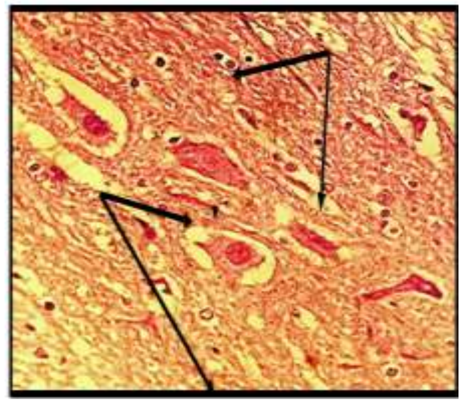


**Figure – 13: Spinal cord of laser gray matter at 12 weeks shows slightly atrophied neurons with gliosis (arrows) H & E 400X**

## 4. Discussion

Currently, there is no treatment available to restore motor and sensory function after debilitating SCI. However progress is being made in research, numerous treatment strategies are being investigated to repair damaged axons after SCI in rats (Nash *et al*., 2002). In the present study, following incomplete transection of the of the spinal cord, both groups showed signs of partial paralysis in hind limb, loss of sensation ,involuntary urination, difficult defecation and decrease weight (sensory, motor and autonomic dysfunction), but this signs gradually disappeared at an earlier time in combination of LLLT compared with control. Moreover, several studies proved that LLLT had beneficial effects on SCI rehabilitation, in particular inflammation alleviation (Byrnes *et al*., 2005). The study applied a laser beam (810 nm wave length, 150 mW output power, 0.3 $cm^2$ spot) focused on the surface of the skin to the spinal cord, this supported by Karu (2008) which mention that the wavelengths which are commonly used in LLLT are in the red and near infrared band (600 – 1000 nm) which immediately irradiated after spinal cord transection this is supported by Takahiro *et al*. (2013), who was suggested that LLLT should be initiated as soon as possible. Laser treatment was continued for 15 minutes per rat daily for a total of 14 days in Laser and combination group. This was agreed with Byrnes *et al.* (2005) which mentioned that the LLLT should be irradiated from 1to14 days with 50 minutes per day.

Thigh muscle atrophy was noticed in all animals of control and treated groups with variable degrees of severity in control group, it was very severe because of disuse atrophy which is usually related to the spinal cord transection and this agreed with Talmadge *et al*. (2002), it seems that the muscle mass and muscle force contraction were related to the development of motor function in which the muscle mass developed to nearly normal in the group of combination treatment after development of motor function and more than the other treated groups.

The neuro-histopathological inspection of the spinal cord in longitudinal section in the positive control group revealed sever vacculation of the nerve fibers in the white matter this attributed by key pro-inflammatory cytokines which lead to the secondary cascades of events that accurse after several hours to days of spinal cord injury which include the mitochondrial dysfunction which lead to failure of aerobic energy metabolism and finally lead to production of free oxygen radicals which cause lipid peroxidation and lead to increase vascular permeability, local ischemia, and intraneuronal edema. Fiber deformation and local demyelination which refer to degenerate axons this agreed with Tsai *et al*. (2008) and this what called Wallarian degeneration which results from separation of axons and their myelin sheath from the neuronal cell body this agreed with Becera (1995).

The vacculation in the gray matter result from degenerated and necrotized neurons in both subgroups of $6^{th}$ and $12^{th}$ week, the neuronal cell necrosis result from ischemia which occur after spinal cord secondary injury. Ischemia result from inadequate blood supply to the tissue lead to hypoxia and reduction in perivscular PH from accumulation of acid metabolites such as lactate this tissue perfusion may increase cellular damage by promoting the influx of free radicals and other toxic byproducts, this agreed with (Amar, 2007).

The present study revealed that the laser group were vacuolated degenerate nerve fibers in the white matter after six weeks post operation , in the gray matter the neurons appear slightly atrophied , in the area of transection there were moderate axonal regeneration, this changes being prominent in the subgroup of $12^{th}$ week in which the regenerate axons being more massive as well as presence of glial scar (proliferation of astrocytes and oligodendrocytes) also presence of cells with foamy cytoplasm which may be the oligodendrocytes which engulf the degenerate myelin also proliferation of new regenerate blood vessels , the proliferation of astrocytes in the site of transection and the presence of cells with foamy cytoplasm were supported by (Becerra *et al*., 1995 ).

The prominent changes which were noticed in the group of combination may give indication about the synergistic effect of both treatments to be an anti-inflammatory drug and responsible about the reduction of edema, as a result relief. Although, the mechanism by which LLLT cause axonal regeneration still currently unknown, one of the suggestions to the LLLT-induced biologic effects is the contribution of intracellular calcium as a signaling molecule. Absorption of photons by intracellular photoacceptors, such as cytochrome oxidase, leads to electronically excited states and consequently can lead to acceleration of electron transfer reactions (Pastore *et al*., 2000). More electron transport necessarily leads to increased production of ATP (Karu, 2010), the cellular level has been ascribed to the acceleration of electron transfer reactions, resulting in increase of reactive oxygen species and $Ca_2^-$ as versatile second messengers (Lavi *et al*., 2003).

The stimuli which activate microglia within the spinal cord have not been definitively elucidated but believed to change oxygen tension, alter the level of extracellular metabolites (e.g., glutamate), and cytokines or chemokines secreting by neighboring cells (endothelia, neurons & glia) specially (Interleukin 1, Interleukin 6 & Tumor necrosis factor - α), upon activation of microglia alter their phenotype and secretary properties and have rounded phagocytic morphology and secrete proinflammatory cytokines in rounded milieu (Gordon and Martinez, 2010). The conclusion of this study was enhanced the regeneration spinal cord injury.

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