The influence of low-level laser irradiation on spinal cord injuries following ischemia–reperfusion in rats[1]

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ABSTRACT

PURPOSE: To investigate if low level laser therapy (LLLT) can decrease spinal cord injuries after temporary induced spinal cord ischemia-reperfusion in rats because of its anti-inflammatory effects.

METHODS: Forty eight rats were randomized into two study groups of 24 rats each. In group I, ischemic-reperfusion (I-R) injury was induced without any treatment. Group II, was irradiated four times about 20 minutes for the following three days. The lesion site directly was irradiated transcutaneously to the spinal direction with 810 nm diode laser with output power of 150 mW. Functional recovery, immunohistochemical and histopathological changes were assessed.

RESULTS: The average functional recovery scores of group II were significantly higher than that the score of group I (2.86 ± 0.68, *vs* 1.38 ± 0.09; $p<0.05$). Histopathologic evaluations in group II were showed a mild changes in compare with group I, that suggested this group survived from I-R consequences. Moreover, as seen from TUNEL results, LLLT also protected neurons from I-R-induced apoptosis in rats.

CONCLUSION: Low level laser therapy was be able to minimize the damage to the rat spinal cord of reperfusion-induced injury.

Key words: Laser Therapy, Low-Level. Ischemic, Reperfusion. Spinal Cord. Rats.

Introduction

Neurologic injuries due to I-R of the spinal cord has an incidence of between 2.9% and 23%[1]. Pathogenic mechanisms of neuronal cell death after spinal cord I-R injury include energy failure, excitotoxicity, and oxidative stress[2,3].There are some applications which can reduce spinal cord I-R injuries such as hypothermia, vascular shunting, left heart bypass, drainage of cerebrospinal fluid, monitoring of somatosensory evoked potentials, single clamp technique and reimplantation of major intercostal arteries[4-6]. Also, there are experimental studies like ischemic preconditioning and adjunctive medications for reducing the incidence of this complication[7]. Despite several surgical modifications and pharmacologic approaches, postoperative spinal cord dysfunction has not been totally eliminated[8].

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[9,10]. LLLT has been clinically applied to treatment of rheumatoid arthritis, periodontal disease, pain management and healing of wounds and burns[11-13]. Many studies approved that LLLT has the potential to be an effective noninvasive therapy for spinal cord injury[14,15].

The aim of this study is to evaluate if LLLT can protect rats spinal cord from I-R injury, so we hypothesized that LLLT would attenuate immunohistochemical and histopathological changes and improve functional recovery after the ischemia/reperfusion-induced spinal cord injury in rats.

Methods

Animal care and experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80-23). Forty eight male Wistar rats weighing 400-450g were used in this study. Anesthesia was induced by intramuscular injection of ketamine hydrochloride 60 mg/kg and xylazine 10 mg/kg. A longitudinal incision was made through the skin on the abdominal region and the abdominal aorta was exposed through midline laparotomy. Heparin (250 UI/kg) was administered intravenously before aortic clamping. Spinal cord ischemia was induced by crossclamping for 60 min, using Bulldog forceps (Figure 1). Vascular clamps were placed under the left renal vein and above the bifurcation in the aorta. Then the forceps were removed and the chest closed routinely. Animals were placed in their cages after recovery. Rats were randomly assigned to two groups.

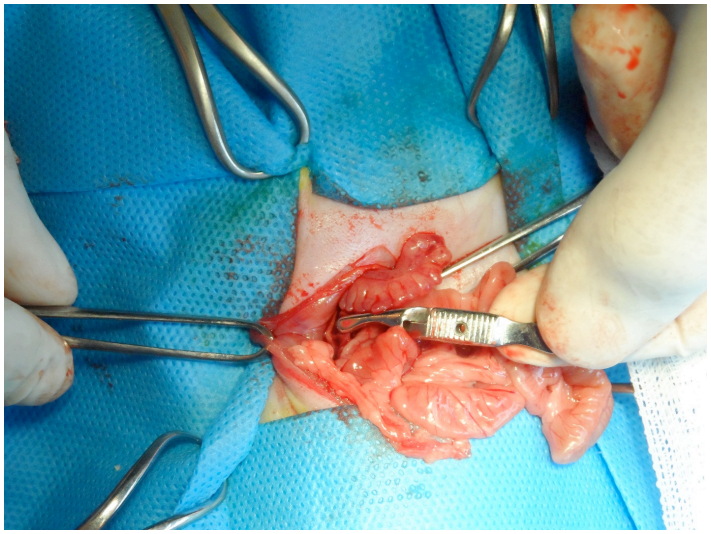

FIGURE 1 - Surgical site: the ventral aorta was exposed and clamped by Bulldog forceps for 60 minutes.

In control group (group I), I-R injury was induced but not irradiated with the laser beam. The irradiation protocol was applied as Byrnes described previously[16]. Briefly in treatment group (group II), 15 minutes after I-R induction on the spinal cord, the lesion site as a rectangular, about 3 cm^2 (3 cm length×1 cm width) was irradiated transcutaneously to the spinal direction with 810 nm diode laser (Thor International, UK;) with output power of 150 mW. The dosage applied to the surface of the skin was 1,589 J/cm2 per day (0.53 W/cm2, 450 J). Irradiation was repeated daily for the following 3 consecutive days. In each day, irradiation was applied 4 times about 20 minutes with contact mode.

Neurologic scoring system

The Neurologic deficits of animals were evaluated on postoperative 72 hour by a single trained blinded observer by using the following scoring:

Grade 0: paraplegia with no lower extremity motor function;

Grade 1: poor lower extremity motor function;

Grade 2: good movement of the hind limbs, but unable to stand;

Grade 3: able to stand but unable to walk normally;

Grade 4: complete recovery[17].

Spinal cord histopathologic examination

All animals were anesthetized with lethal dose of pentobarbital (25 mg/kg). Spinal cords were dissected totally and fixed in 10% formalin and embedded in paraffin with routine procedures. Sections from fourth to sixth lumbar segment were obtained. The spinal cord tissues were embedded in paraffin and serial transverse sections (5 µm) cut from paraffin blocks and stained with hemotoxylin and eosin for histopathologic examination. Histopathologic evaluations were performed with means of light microscopy by a neuropathologist who was blinded to experimental conditions.

TUNEL staining

TUNEL staining was performed by an in situ cell death detection kit (Roche, Germany). Hematoxylin was used to counterstain the sections. Quantitative analysis was performed blindly by counting the number of TUNEL positive neurons in the ventral horns in five microscopic fields as described previously[18].

Statistical analysis

All data are expressed as mean ± standard deviation. Statistical analysis of the neurologic scores were analyzed by using Kruskal-Wallis one-way analysis of variance (ANOVA). The investigators were blinded to the treatments. Values for statistical analyses were considered significant at $p<0.05$. All analyses were performed by using the SPSS software package (SPSS, Inc, Chicago, Ill).

Results

Neurological evaluations presented in Table 1. In the group I, the neurological scores was lower. Although in the group II, the Tarlov scale increased and showed a significant difference after 72h of reperfusion ($p<0.05$).

TABLE 1 - Neurologic status 72 hours after reperfusion as evaluated by the modified Tarlov neurologic recovery scale.

score	Group I (Control) N=24	Group II (Treatment) N=24
0	8	1
1	7	3
2	3	5
3	4	4
4	2	11
Mean ± SD	1.38 ± 0.09	2.86 ± 0.68*

*Mean neurologic scores showed a significant difference between control and treatment groups ($p<0.05$) both at 72h after reperfusion.

Histopathologic evaluations in group I, presented that had severe ischemic injury with inclusive necrosis of gray matter, which enclosed typically necrotic nuroglia cells with eosinophilic cytoplasm, and loss of cytoplasmic structures. In addition the numbers of normal nuroglia cells were apparently reduced in this group and neuronal structural alterations were observed, which included oligodendrocytes pyknosis, light staining tigroid body, nucleus's atrophy of nuroglia cell and nucleolus disappearance of oligodendrocytes. Furthermore, hemorrhagic macules were scattered into tissue structures and vacuolar changes were observed in the cytoplasm (Figure 2). The histopathologic changes in group II were milder than that observed in the group I, and the gray matter architecture was generally preserved, with most nuroglia cells appearing to have survived the ischemic consequences (Figure 3).

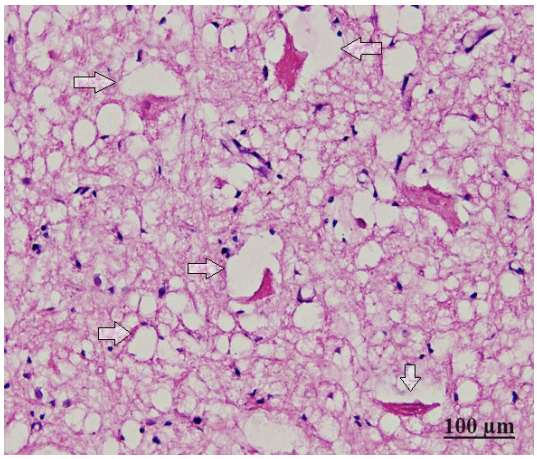

FIGURE 2 - The neurons of spinal cord anterior horn of group I were assessed by H&E staining and viewed at the magnification of 200 times which presented group necrotic changes with prominent vacuolization, intensely eosinophilic cytoplasm, Nissl granule loss, and pyknosis (arrows) as well as by the presence of infiltrating neutrophils and mononuclear phagocytes severe percellular edema and glial cell proliferation.

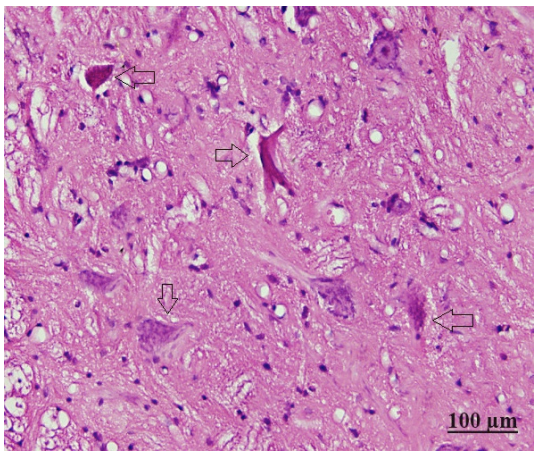

FIGURE 3 - The neurons of spinal cord anterior horn of group II were assessed by H&E staining and viewed at the magnification of 200 times which showed relative preservation of tissue architecture along with almost complete protection of the neurons, vascular structures, and glial cells along with only mild per cellular edema. The arrows indicate ischemia neuron cells showing mildly eosinophilic cytoplasm, Nissl body loss, and pyknosis.

Average TUNEL-positive cell counts are shown in Table 2. These data show that the group II exhibited significantly fewer TUNEL-positive cells compared with the group I.

TABLE 2 - Quantitative analysis of the number of TUNEL-positive cells in the ventral horn of spinal cord of all groups, 72h after reperfusion.

Group	I (Control)		II (Treatment)	
Number of TUNEL-posetive motor neurons	Mean (n=24)	SD	Mean (n=24)	SD
	73.04	0.3	36.50**	0.6

*Mean Quantitative analysis showed a significant difference between control and treatment groups ($p<0.05$) both at 72h after reperfusion.

It is understandable that the number of TUNEL-positive neurons decreased significantly after laser therapy, suggesting that LLLT may protect spinal cords from I-R apoptosis. Spinal cord sections were stained with TUNEL and observed at the light microscopic level (400 times magnification). In the spinal cord ventral horn of the group I, amount of vacuoles appeared and numerous TUNEL-positive neurons were observed (Figure 4). By contrary, very few positively stained neurons were observed in group II (Figure 5).

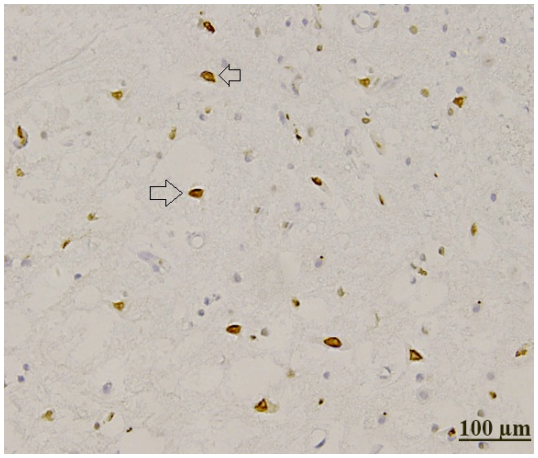

FIGURE 4 - TUNEL staining and quantification of apoptotic motor neurons after reperfusion (×400). Many TUNEL-positive neurons with intense nucleus staining were visible in group I. The arrows indicate TUNEL-positive motor neurons.

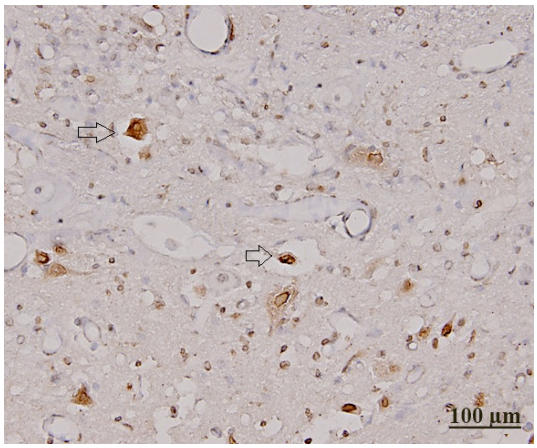

FIGURE 5 - TUNEL staining and quantification of apoptotic motor neurons after reperfusion (×400). Only a small number of positively stained neurons were observed in the group II. The arrows TUNEL-positive motor neurons.

Discussion

Our results showed that LLLT will be able to reduce the damages of spinal cord after I-R in rats. This result was verified by both neurological and histological and observations. Additionally, Functional recovery of LLLT group was significantly improved when compared with control group.

Spinal cord I-R injury is a persistent clinical problem in surgical repair of thoracic and thoracoabdominal aneurism surgeries[19,20]. The major cause of spinal cord injury, during and after aortic surgery to the occurrence of one or more of the three following events: (I) the duration and degree of ischaemia; (II) failure to re-establish blood flow to the spinal cord after repair; (III) a biochemically mediated reperfusion injury[21]. Reperfusion is the restoration of blood flow to the organ after a period of ischaemia. Reperfusion of ischaemic neuronal tissues leads to release production of oxygen derived free radicals, produced as a result of incomplete oxygenation during the period of ischaemia[22]. Inflammatory response with production of cytokines by microglia and activated neutrophils also contributes to generation of these radicals[23,24]. Several different surgical strategies and laboratory studies have been developed in attempt to decrease the risk of this devastating complication[25-27]. However, neurological injury in thoracoabdomial surgery remains one of the greatest unsolved mysteries[28-30].

The therapeutic effects of LLLT have been reported, being associated with production of anti-apoptotic, pro-proliferative, antioxidant, and angiogenic factors[31-33]. LLLT also known as photobiomodulation, is an emerging therapeutic approach in which cells or tissues are exposed to low-levels of red and near-IR light. Its experimental applications have broadened to include serious

diseases such as heart attack, stroke, and spinal cord injury. Oron et al, suggested that a transcranial application of LLLT after traumatic brain injury provides a significant long-term functional neurological benefit and decreases brain tissue loss[34]. In another research applied LLLT in acute Spinal cord injury caused by of trauma which promotes axonal regeneration and functional recovery[35].

LLLT may have beneficial effects in the acute treatment of I-R by reducing inflammatory mediators, inhibiting apoptosis, stimulating angiogenesis, and increasing neurogenesis[9]. Transcranial LLLT applied after ischemic stroke in rats caused a significant improvement of neurological score compared to sham animals[36].

We hypothesized that LLLT would effectively protect spinal cord by its antioxidant and anti-inflammatory. To our knowledge, the present study probably is the first study to evaluating the neuroprotective effects of LLLT in attenuating I-R induced neurologic injury to the rat spinal cord. It is known that functional recovery after I-R is highly correlated with the volume of remaining normal nerve fibers in spinal tissue[37]. Adno *et al*.[11], demonstrated transcutaneous application of 810-nm nonpolarized laser significantly promoted axonal regrowth, our results are in agreement with that and show association of improved neurologic status.

Byrnes *et al*.[16], found that 810 nm light, at a dosage of 1.589 J/cm^2, significantly improves axonal regrowth, functional improvement and statistically significant suppression of immune cell invasion and pro-inflammatory cytokine and chemokine gene expression. Similarly we documented that LLLT had efficient protection on neural cells from apoptosis or necrosis. Also decreased inflammatory cell accumulation in the spinal cords of animals that received LLLT as compared with the control group also supports LLLT proposed anti-inflammatory property and may contribute to neuroprotection.

Conclusion

Low level laser therapy protects the spinal cord from ischemia-reperfusion injury spinal cord ischemia and provide better locomotor function in rats which may be related to anti-inflammatory properties of that.

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