Effects of 660nm Low-Level Laser Therapy on P2X3 Expression of Lumbar DRG of Adult Male Rats with Neuropathic Pain

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ABSTRACT

Background: Neuropathic Pain (NP) is a serious suffering medical condition that frequently leads to disability and life style changes. Although the exact mechanisms of NP are still unknown, recently the role of reactive oxygen species (ROS) reported as an important factor for NP. Apoptosis, increase of ATP production and reduction of antioxidants are also the other factors influencing in NP. There are certain therapeutic procedures for NP among them using laser therapy newly received more attention. In the present research we studied the molecular effects of Low Level Laser Therapy (LLLT) on a rat model of NP.

Methods: Thirty adult male Wistar rats (200-250 g) that randomly divided into three groups including chronic constriction injury (CCI), CCI+LLLT and control were used in this study. CCI technique was used to induce NP. Laser therapy was done by using laser beam of 660 for 14 days following CCI. After that, expression of P2X3 of the DRG, Bax and Bcl2 in lumbar spinal segments measured by Western Blotting. Level of glutathione (GSH) was also measured in lumbar spinal cord segments by Continuous Spectrophotometric Rate Determination method. For behavioral study the mechanical and thermal hyperalgesia were evaluated in days 7 and 14 after CCI.

Results: LLLT for two weeks increased expression of Bcl 2 and GSH, whereas decreased Bax and P2X3 expression significantly. Comparing the results of behavioral study showed significant differences in the mechanical and thermal threshold showed between CCI and CCI+ LLLT groups.

Conclusion: Based on our findings, the therapeutic effects of LLLT for NP act throughout cellular and molecular mechanisms which improve mitochondrial function that in turn improve cell function and prevent apoptosis.

Keywords: Low level laser therapy; P2X3, Glutathione; Apoptosis

INTRODUCTION

Neuropathic pain (NP), known as a serious suffering medical condition usually results from certain damage to the peripheral or central nervous system including peripheral nerve compression, chemotherapy side effects, diabetes, spinal cord injury and infection 1.
Epidemiological studies showed that at least 3% of the word population suffering from NP. Neuropathic pain as a lifelong chronic condition with no definite treatment could affect the patient’s life quality. Allodynia and hyperalgesia are two primary and dominant symptoms of NP. The main causative reasons and underlying mechanisms of NP are still unknown; therefore, discovery of effective therapy is challenging. There are certain theories about etiology and pathophysiology of NP, among them the production of reactive oxygen species (ROS) via mitochondria following injury received more attention recently. In pathological condition ROS that naturally produced by cells used for cell signaling and hemostasis over expressed lead to oxidative stress and cell damage. ROS damages mitochondrial function via destruction to electron transport chain that in turn increases of proapoptotic gene expression such as bax, bcl-xL, bcl-xS, p53, E2F1. By over production of ROS following injury the needs for ROS scavenger increases, anti-oxidants such as Superoxide dismutase (SOD) catalase and glutathione will decreased, so the pathological conditions get worse and deterioration will continue. Neuro-inflammation that happens following injury also play important role in NP progress. It is shown that increasing synthesis and releasing of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, by glia cells leads to pain feeling. In addition, inflammatory mediators influence neuronal expression of nociceptors and ion channels, in turn contributing to spontaneous activity of sensory fibers. Neuronal and non-neuronal cell crosstalk is controlled by neuronal ATP and action of ATP receptors like P2X3. Following nerve injury neural and non-neuronal cells produced vast amount of ATP that leads consequently to P2X3 receptors up regulation of DRG and spinal cord that finally result to pain induction. Although the mechanisms of NP are not clearly understood, certain therapeutic procedures suggested for pain relief. During past two decades low level laser therapy (LLLT) has its own valuable and advantages. LLLT of different variations opened new horizon for therapy of wide spectrum of human diseases. Regarding pain relief the exact mechanism of LLLT is still unknown. It is reported that the LLLT acts via photochemical action to decrease inflammatory agent such as PGE2, interleukin IL-1β, IL-6, and IL-10. There are also growing evidences on the effect of LLLT on prevention of apoptosis. Due to important role of P2X3 receptors in NP induction and lack of enough evidences about therapeutic effects of the expression of these receptors and the advantages of LLLT we decided to design the present research work in order to see the effects of LLLT on P2X3 expression in NP model. We also studied effects of LLLT on Bax, BCl2, and glutathione (GSH). For behavioral study hyperalgesia threshold also evaluated.

MATERIALS AND METHODS
Biological model
Thirty adult male Wistar rats (200-250 g) were used in this study purchased from Iran Pasture Institute. The animals kept in separated cages with food and water ad libitum. All procedures in this study approved by Animal Experimentation Ethics Committee of Iran University of Medical Sciences. The animals randomly divided into three groups (n=10) as follows: Control group (intact animals), CCI group: subjected to surgical procedure, & laser therapy group (660 nm): subjected to surgical procedure and laser irradiation with energy density of 4 J/cm2 and intensity of 0.354 W/cm2. All the steps of this study were done in a single-blind pattern.

Surgery
To induce neuropathic pain we used Benet and Xie method. Briefly animals underwent of anesthesia by mixture of ketamine-xylasin (10/1), right sciatic nerve exposed, four loosely tie put around the nerve by 4/0 cut gut. All the animals were subjected to the behavioral test before surgery.

Laser therapy
A couple of CW diode laser emitter with the following specification was used in this study. One is a laser with a wavelength of 660 nm, power of 100mW (Heltschl, modelME-TL10000-SK), energy density of 4 J/cm2, and power density of 0.354 W/cm2. The beam area on the samples was ~0.238 cm2. The irradiation time was 11.3s for visible wavelength and 16.13s for NIR one. Laser calibration was done routinely before use. Three points on the surgical incision were irradiated transectaneously with no direct skin contact as follows: two points on two ends of surgical incision and another at their midpoint. The laser therapy was started on the first day after the surgery and was continued for 2 weeks daily at the same time between 10 and 12 a.m.

Behavioral Tests
Behavioral study was done in three steps including before CCI, day 7 and 14 after CCI surgery as follows:
- Thermal withdrawal threshold
  By using a plantar test apparatus (Ugo Basile, Italy),
thermal hyperalgesia, the latency to withdrawal of the hind paws from a focused beam of radiant heat applied to the plantar surface, was studied \[(17, 18)\]. The animals were placed in an acrylic box with glass floor, and the plantar surface of their hind paw was exposed to a beam of infrared radiant heat. The paw withdrawal latencies were recorded at infrared intensity 50; three trials for the right hind paws were performed, and for each reading, the apparatus was set at a cutoff time of 25 s. Each trial was separated by an interval time of 5 min.

- Mechanical withdrawal threshold
Mechanical paw withdrawal threshold assessed with the Randall–Selitto method by using an Analgesy-meter apparatus (Ugo Basile, Italy; \[(17, 18)\]). This instrument exerts a force that increases at a constant rate. The force was applied to the hind paw of treat, which was placed on a small plinth under a cone-shaped pusher with a rounded tip (1.5 mm in diameter). The rat was held upright with the head and limb to be tested free, but most of the rest of the body cradled in the hands of the experimenter. The paw was then put under the pusher until the rat withdrew its hind paw. Each hind paw was tested twice, with a 10-min interval between the measurements, and mechanical paw withdrawal thresholds were calculated as the average of two consecutive measurements.

Molecular study

**Western Blotting**

By using western blot technique expression of Bax, Bcl2 and P2X3 proteins studied. Frozen spinal cord tissue and DRG homogenized in 0.5 ml of RIPA buffer 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA,1% Triton X-100, 0.1%ripa buffer sodium dodecyl sulfate, and 0.5% sodium deoxycholate) containing protease and phosphatase cocktails (Sigma). After centrifugation at 13,000 g for 20 min at 4°C, the supernatant was collected. Protein concentrations determined by Bradford protein assay, and equivalent amounts of total cellular protein were separated by 10% SDS–PAGE. The gels then electro blotted onto nitrocellulose membranes. Subsequently, membranes were blocked 1 h with 5% nonfat skim milk in TBS containing 0.1% (v/v) Tween-20, and probed with specific primary antibodies overnight at 4°C. After three washes in TBS-T, membranes were incubated with secondary antibodies -conjugated by alkaline phosphatase Proteins then visualized with ECL. By using Total Lab Soft wear western blot bundle measured according to amount of protein staining and quantifies.

**Antioxidant assay**

**Glutathione peroxidase**

In order to assay GSH peroxidase (GSH-Px; EC 1.11.19) level in spinal cord we used Continuous Spectrophotometric Rate Determination method \[19\]. Briefly, we mixed Glutathione Reductase Enzyme Solution,1.0 mM Sodium Azide Solution (Buffer w/ Azide) R),and 200 mM Glutathione, reduced (GSH) with 8-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form (β-NADPH).This cocktail were mixed whit 10.0 mM Sodium Phosphate Buffer and Glutathione Peroxidase Enzyme Solution. Preperd supernatant aded to second cocktail. First OD read by Spector photometer in 340 nm wave length then fresh 0.042% (w/w) Hydrogen Peroxide (H2O2 added, final OD read as the same wave length with interval 30s during 5 min.

**Statistical analysis**

Data exposed to statistical analysis by using SPSS 19.0, the results presented as means ± SD, \(P\) less than 0.05 considered significant.

**RESULTS**

**Plantar Test**

Statistically comparing the results among the groups considered significant. There was significant difference between the 7th , 14th post-surgery days of the LLLT 660nm group and the CCI group \((P<0.001)\); also there was significant difference between the 7th , 14th post-surgery days of the control group and the CCI group \((P<0.001)\); and there were no significant difference between the 7th , 14th post-surgery days of the LLLT 660nm group and the control group (Figure 1).

**Figure 1.** Mean values of the Thermal Withdrawal Threshold obtained from the groups during the study period (before surgery (control), the 7th & 14th day after surgery).
Randall–Selitto method
For the mechanical withdrawal threshold statistical analysis showed that the difference between control group and the CCI group on the 7th, 14th post-surgery days, was significant (P<0.001); the difference between LLLT 660nm group and the CCI group on the 7th, 14th post-surgery days, was also significant (P<0.001); and there was significant difference between the 7th, 14th post-surgery days of the LLLT 660nm group and the control group (P<0.01), (P<0.001) respectively (Figure 2).

P2X3
P2X3 expression showed that the difference between control group and the CCI group, was significant (P<0.001); also difference between LLLT 660nm group and the CCI group, was significant (P<0.001); and there was significant difference between the LLLT 660nm group and the control group (P<0.001) (Figure 3).

Bax
For Bax protein expression in different groups the statistical analysis indicated significant difference between control group and the CCI group, (P<0.001); also the difference between LLLT 660nm group and the control group, was significant (P<0.001); but there was no significant difference between the LLLT 660nm group and the CCI group (Figure 4).

Bcl2
For expression of Bcl2 the statistical analysis showed that there was no significant difference between the CCI group and the control group; and the difference between CCI group and the LLLT 660nm group, was significant (P<0.001); and there was significant difference between the LLLT 660nm group and the control group (P<0.001) (Figure 5).

Bax/Bcl2 ratio
The ratio of Bax/Bcl2 also studied and statistical analysis revealed the significant difference among all
the groups ($P<0.001$) (Figure 6).

**Glutathione**

Statistical analysis indicated that the difference between control group and the CCI group, was significant ($P<0.001$); also difference between LLLT 660nm group and the CCI group, was significant ($P<0.001$); and there was no significant difference between the LLLT 660nm group and the control group (Figure 7).

**DISCUSSION**

Our study showed that 660nm LLL therapy is able to relieve certain symptoms of pain such as thermal hyperalgesia and also decrease the rate of apoptosis, alters P2X3 receptor expression as well as preventing glutathione reduction. It is shown that NP is accompanied by inflammation process. Following nerve injury pro inflammatory cytokines that released from local sources, generate hypersensitivity and pain 20. Schwann cells are the first cells react to injury by activating extra cellular signal related kinases (ERK) and mitogen activating protein (MAP) kinas signaling pathway that leads to demyelination and Wallerian degeneration 21,22. Consequently, inflammation destroys blood brain barrier and blood-nerve barrier 23,24. There are unknown cellular and molecular mechanisms for NP that may influence outcome of therapy. Based on our results LLLT was useful for NP; however the mechanisms of effectiveness of that are not well-known. It is reported that LLLT stimulate Schwann cells proliferation and help to nerve regeneration 25. LLL of 808-nm is shown to be able to increase myelin sheath thickness and improve the regeneration and functional recovery after sciatic nerve crash, likewise it accelerate GAP43 expression on distal part of crashed region in sciatic nerve that could be a marker for regeneration 26.

Increase in pro inflammatory cytokines like TNF- $\alpha$, IL-6, and IL-1$\beta$ in CNS are main inducers of hyperalgesia and allodynia 27. There are certain studies demonstrated that LLL 660 nm significantly reduces the overexpression of TNF-$\alpha$, and IL-1$\beta$ occurring after NP 27. Using of LLL of different frequencies also recovers local blood circulation through photolysis of NO - hemoglobin complex 28. Moreover, dorsal root ganglia (DRGs) which carry sensory information to dorsal horn are involved in pain process. Regarding the role of DRG and P2X3 receptors in NP, we know that any type of painful stimulation leads to more ATP production and over expression of P2X3 receptors 20. The important role of ATP in pain induction has been confirmed previously 30. In addition, high level of P2X3 receptor is accompanied by the vanilloid VR1 receptor expression, which is supposed to have a role in thermal hyperalgesia 31. Also, P2X3 receptors are expressed on the central terminals of C-fibers innervating dorsal horn lamina II. The activation of these receptors leads to enhance release of excitatory neurotransmitter such as glutamate which causes sensitization 32. Briefly, ATP is released to increase glutamate secretion, and is responsible for the development of inflammation and hyperalgesia through activation of P2X3 receptors. As illustrated in this study P2X3 receptors are highly expressed in DRG at the day of maximum pain and LLL 660nm reduced its expression. The effect of LLL on expression of P2X3 has not been studied previously. However; it seems ATP production is controlled by LLL and this effect might influence release of glutamate so reduction of excitatory neurotransmitter attenuated hypersensitivity. On the other hands, some studies have been proved infra-red laser inhibits vanilloid VR1 and VR4 receptors which is attributed to decrease capsaicin release, Ca2 influx and
Apoptosis is another causative reason of NP. The role of ROS in induction of apoptosis has been proven. ROS is generated by several elements including over production of glutamate, impairment in buffering of calcium, increased lipid peroxidation and decreased antioxidants like SOD and GSH. This study illustrated that Bax, as a marker of apoptosis, dramatically increased and GSH decreased 14 days after CCI. Bax increases mitochondrial membrane permeability via opening of the mitochondrial permeability transition pore (MPTP), allowing to release of cytochrome c and creation of the apoptosome. This leads to activation of caspase-9 and caspase-3 that result in cell death. In present study, most actual anti-apoptotic effect of LLL 660nm was on increase of Bcl2 and GSH. After irradiation of LLL 660nm Bax expression was not changed significantly. Zhang et al showed Low-power laser irradiation could prevent Bax translocation and caspase-3 activation; therefore, inhibit apoptosis. Bcl2 that known for its anti-apoptotic effect acts as an upstream regulator of the mitochondrial pathway and plays an essential role in determining whether a cell will live or die. Bax is in the cytosol of living cell, moves to the mitochondrion during apoptosis. Bcl2 could bind to activated Bax and inhibit increase of membrane permeability. Another speculated LLL mechanism to rescue apoptosis is its capability to restore cellular respiration in damaged mitochondria from iron-sulfur nitrosyl complexes. LLL is absorbed by components of the cellular respiratory chain and leads to acceleration of electron transport, ATP synthesis, oxygen consumption, membrane potential, and enzymatic activity to generate ROS. In this study GSH increased after LLL therapy. The effect of LLL on the CNS antioxidants and its mechanisms were not studied much before. This rise possibly indicates improvement in mitochondrial function. GHS as an antioxidant removes H2O2, a type of ROS, by coupling its reduction to water. ROS that naturally act as a cell signaling molecule, regulator of nucleic acid and protein synthesis, could be further generated by LLL and lead to transcriptional changes such as an increased production of proliferative factors like nuclear factor B (NF-B). But there is any report about generation of oxidative stress.

**CONCLUSION**

Based on our results it is speculated that Low Level Laser Therapy for NP acts via different cellular and molecular mechanisms that prevent apoptosis, increasing antioxidants and improving mitochondrial function. Whether these effects are long-lasting need more study.

**REFERENCES**


Low-Level Laser Therapy on P2X3 Expression of DRG—Kerdari et al.


