Low-Level Laser Therapy Applied Transcranially to Rats After Induction of Stroke Significantly Reduces Long-Term Neurological Deficits

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Abstract

Background and Purpose—Low-level laser therapy (LLLT) modulates various biological processes. In the present study, we assessed the hypothesis that LLLT after induction of stroke may have a beneficial effect on ischemic brain tissue.

Methods—Two sets of experiments were performed. Stroke was induced in rats by (1) permanent occlusion of the middle cerebral artery through a craniotomy or (2) insertion of a filament. After induction of stroke, a battery of neurological and functional tests (neurological score, adhesive removal) was performed. Four and 24 hours poststroke, a Ga-As diode laser was used transcranially to illuminate the hemisphere contralateral to the stroke at a power density of 7.5 mW/cm².

Results—In both models of stroke, LLLT significantly reduced neurological deficits when applied 24 hours poststroke. Application of the laser at 4 hours poststroke did not affect the neurological outcome of the stroke-induced rats as compared with controls. There was no statistically significant difference in the stroke lesion area between control and laser-irradiated rats. The number of newly formed neuronal cells, assessed by double immunoreactivity to bromodeoxyuridine and tubulin isotype III as well as migrating cells (doublecortin immunoactivity), was significantly elevated in the subventricular zone of the hemisphere ipsilateral to the induction of stroke when treated by LLLT.

Conclusions—Our data suggest that a noninvasive intervention of LLLT issued 24 hours after acute stroke may provide a significant functional benefit with an underlying mechanism possibly being induction of neurogenesis.

Introduction

Low-level laser therapy (LLLT) has been found to modulate various biological processes such as increasing mitochondrial respiration and ATP synthesis, facilitating wound healing and promoting the process of skeletal muscle regeneration and angiogenesis. It was previously shown in an experimental model of the infarcted heart that LLLT had a profound cardioprotective effect, resulting in a 50% to 70% reduction in infarct size 4 to 6 weeks postleft descending coronary artery chronic occlusion. This effect was partially attributed to a significant increase in the number of intact mitochondria and ATP content as well as
inducible heat shock proteins and catalase in laser-irradiated infarct induced hearts of rats and dogs as compared with nonirradiated ones.\textsuperscript{4-6}

LLLT has also been shown to biomodulate processes in the nervous system. Anders et al\textsuperscript{8} recently reviewed the beneficial effects of LLLT on functional recovery of injured peripheral nerves. The effect of LLLT on stroke has been investigated to a limited extent. Leung et al\textsuperscript{9} have shown that LLLT in stroked rats causes suppression of nitric oxide synthase activity and upregulation of TGF-β1, which are considered neurotoxic and neuroprotective, respectively. It was also recently demonstrated that transcranial infrared laser therapy applied 6 hours postembolic stroke in rabbits\textsuperscript{10} and 24 hours postischemic stroke in rats\textsuperscript{11} caused a significant improvement of neurological score over sham-operated experimental animals. Light-emitting diodes were also shown to regulate cytochrome C oxidase, leading to increased energy metabolism in vitro in visual neurons functionally inactivated by toxin (KCN).\textsuperscript{12} Given the therapeutic benefits demonstrated for LLLT in the myocardial infarction setting and its promising results after application in stroke-induced rabbits,\textsuperscript{13} we investigated in the present study the effects of treatment of stroke with LLLT initiated 4 and 24 hours after stroke onset in rats on their functional neurological outcome. The possible mechanisms associated with this phenomenon were explored using immunochemical localization of several markers.

## Materials and Methods

### Animal Models and Experimental Protocol

The institutional animal care committees of the Henry Ford Health Sciences Center and Assaf Harofeh Medical Center approved all experimental procedures.

Two sets of experiments were performed. In experiment 1, stroke was induced in 43 adult (280–320 g) male Sprague-Dawley rats (Harlan Inc, Rehovot, Israel) by permanent middle cerebral artery occlusion (MCAO) through a craniotomy. Experiment 1 was designed to determine the appropriate timing of laser application. Rats were killed 21 days poststroke and mortality rate was 16% (seven of 43 rats) up to 3 weeks follow up. Experiment 2 was performed using delayed (24 hours poststroke) laser application (based on results from experiment 1) with more sensitive behavioral neurological tests and immunohistochemistry with a longer follow-up time (30 days after induction of stroke) for indication of enhanced neurogenesis. In experiment 2, permanent MCAO was induced in 18 male (270–300 g) Wistar rats (The Jackson Laboratory, Bar Harbor, Maine) by means of a filament inserted through the carotid artery, as previously described.\textsuperscript{13,14} Mortality rate was 17% (three of 18 rats) for the 4-week follow up. In both experiments, rats were initially anesthetized with 3.5% halothane and maintained with 1 to 2% halothane in 70% N\textsubscript{2}O and 30% O\textsubscript{2}. All rats in experiment 2 received a daily intraperitoneal injection of 50 mg/kg bromodeoxyuridine (BrdU) (Sigma) starting at 24 hours poststroke initiation for 13 days. A catheter (DE-50) was inserted to the right femoral artery for continuous (up to 7 days) monitoring of mean arterial blood pressure and obtaining blood samples to measure glucose, pH, pO\textsubscript{2}, and pCO\textsubscript{2}. Rectal temperature was measured during stroke induction by a thermocouple. These measures were in normal range in both control and laser-treated groups in both experiments (data not provided).

### Neurological Functional Tests

Rats were scored for normal neurological function (score=0) before the induction of stroke. In experiment 1, all rats were scored for cumulative neurological deficit at 3 hours poststroke using the following criteria: extension of forelimb, body twisting when lifted by the tail, beam walking, restoration of the forelimb to the original position when displaced, grasping and
balancing on a 2-cm wide beam, tendency to lean to the contralateral side, and walking behavior.13 The rats (37 of 43 rats) that survived after stroke induction were then randomly divided into three groups with similar neurological score. One group served as the control (n=12) and the other two as laser-irradiated groups (laser irradiated at 4 [n=12] or 24 hours [n=13] poststroke). Neurological scoring was performed every alternate day until killing at 3 weeks poststroke. In experiment 2, all rats (n=18) were scored 24 hours poststroke for neurological deficit according to the modified Neurological Severity Score (mNSS) test.13 mNSS is a composite of motor, sensory, reflex, and balance tests.11 Neurological function is graded on a scale of 0 to 18 (normal score is 0; maximal deficit score 18). One score point is awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher the score, the more severe the injury. These rats were then divided into three groups (five rats in each) so that the sum of mNSS scores of all rats in each group were similar. The mNSS for the rats ranged from 8 to 10 in each group 24 hours poststroke. One group served as control and the other two as laser-irradiated groups (continuous wave [CW] and pulsed mode [Pu]). Both mNSS and full and half adhesive removal tests14 were performed weekly poststroke for 4 weeks. In the adhesive removal tests, two small pieces of adhesive-backed paper dots (of equal size, 113.1 mm²) were used as bilateral tactile stimuli occupying the distal–radial region of each forelimb. The time taken by the rat to remove each stimulus from the forelimbs was recorded for five trials per day. Before surgery for stroke induction, the animals were trained for 3 days. If the rats were able to remove the dots within 10 seconds, they were subjected to middle cerebral artery occlusion. To increase the sensitivity of the adhesive removal test during the recovery, at 21 plus days, adhesive tabs were cut in half to 66 mm². Measurements were performed until all target tabs were removed. All neurological scoring was conducted using a double-blind experimental design.

Laser Treatment
A Ga-As diode laser wavelength 808 nm (Photothera Inc) was used in the study. The laser was used transcranially on the exposed (shaved skin) skull by placing the tip of the fiber optic (4-mm diameter) onto the skin at two locations on the head (3 mm dorsal to the eye and 2 mm anterior to the ear) on the contralateral hemisphere to the stroke. These locations had been determined from prior measurements to be sufficient to illuminate one brain hemisphere as a result of dispersion of the laser beam by the skin and the skull. LLLT of the contralateral or both hemispheres demonstrated no difference in functional outcome.17 The laser irradiation power at the tip of the fiber optic was set to give a power density of 7.5 mW/cm² at the brain tissue level. This optimal power density was determined based on our previous studies on the effect of LLLT on the ischemic heart2 and preliminary experiments in stroke-induced rats. The laser power density at the brain tissue level was determined in preliminary experiments with fresh skull using a laser power meter (Ophir Inc) to measure transmission through the skull and an infrared viewer to measure the laser beam diameter after dispersion through the skull. The duration of laser irradiation was 2 minutes (energy density of 0.9 J/cm²) at each point on the skull. In experiment 1, the laser was set on CW, whereas in experiment 2, both CW and pulsed mode (70 Hz) were applied at a power density of 7.5 mW/cm². Control rats were sham-operated but the laser was not turned on. Possible heating effect by LLLT was measured by thermocouple probes inserted into subdural region. Results indicated minimal heating of approximately 1°C at the subdural region when laser light was delivered at four times (3.6 J/cm²) the optimal energy delivered to the stroked rats (0.9 J/cm²). Furthermore, no pathologic changes up to 3 months were noticed in rat brains lased at a 10x dose of that previously mentioned (75 mW/cm² for 2 minutes).16

Histology and Measurement of Infarct Volume
Rat brain tissue was fixed by transcardial perfusion with saline followed by perfusion and
immersion in 4% paraformaldehyde. The brains were then sliced into 2-mm thick cross-sections throughout the brain (experiment 1) or coronal sections from bregma –1 to 1 mm (corresponding to center of the ischemic lesion) were taken (experiment 2). Sections were embedded in paraffin. Lesion volume was measured by calculation from a series of seven hematoxylin and eosin-stained sections as previously described. The lesion volume is presented as percentage of the lesion volume compared with the contralateral hemisphere volume.

**Immunohistochemistry**

Immunohistochemical staining was used for localization of bromodeoxyuridine (BrdU, a marker for proliferating cells), tubulin isotype III (TUJ1, an early neuron marker), and doublecortin (DCX, a microtubule protein expressed in migrating neuroblast in embryonic and adult subventricular zone [SVZ]). These markers were traced in cells in the ipsilateral hemispheres’ SVZ. SVZ area is defined as shown in Figure 1. Briefly, standard paraffin blocks were obtained from the center of the lesion corresponding to coronal coordinates for bregma –1 to 1 mm. A series of 6-µm thick sections at various levels (100-µm interval) were cut from this block and analyzed using light and fluorescent microscopy (Olympus, BH-2). BrdU immunohistochemical analysis (a mouse monoclonal antibody [mAb] against BrdU, 1:100, Boehringer Mannheim, and TUJ1 monoclonal anti-B-tubulin isotype III [1:400 dilution, Sigma]) were used. In addition, DCX (C-18, goat polyclonal IgG antibody, 1:200 dilution) were performed. BrdU-positive cells were measured in the SVZ of the ipsilateral hemisphere from an average of five equally spaced slides (approximately 100-µm interval). For quantitative measurements, stained cells in the SVZ were digitized under a 20x objective (Olympus BX40) using a 3-CCD color video camera (Sony DXC-970MD) interfaced with an MCID image analysis system (Imaging Research). All digitalized images were then contrast-enhanced to clearly differentiate positivity from background, and a thresholding procedure was established to determine the proportion of immunoreactive area within each fixed field of view. For BrdU, the results were expressed as number of positive cells per unit area SVZ. For TUJ1 and DCX, data are presented as a percentage of positive immunoreactive area within the total area of the ipsilateral SVZ. Cells that immunoreacted both to BrdU and TUJ1 in the SVZ were expressed as percent of the total BrdU-positive cells in the SVZ.

![Figure 1. Representative schematic cross section of rat brain indicating lesion area in the stroked rat (core) and subventricular zone (red-colored area).](image)

**Statistical Analysis**

One-way analysis of variance followed by Student Neuman Keuls test was used (using Sigma stat [Sigma] software). Data are presented as mean±SD. A value of $P<0.05$ was regarded as statistically significant.
Results

There were no statistically significant differences in stroke lesion volume between control (10.8±2.5%) and laser-treated (8.8±3.7%) rats in experiment 1.

The neurological scores of the control rats and laser-treated rats 4 or 24 hours postinduction of stroke and control rats (by craniotomy; experiment 1) are presented in Figure 2. The neurological score was significantly reduced from 2 weeks onward after induction of stroke in the rats irradiated with laser 24 hours after stroke induction. At 3 weeks poststroke, there was a highly significant (P<0.01) reduction of 32% in the neurological deficit in the laser-treated rats relative to control ones (Figure 1). At the same time intervals, the rats that had received the laser treatment 4 hours poststroke did not show significant reduction in neurological deficit relative to the control nontreated rats.

Figure 2. Neurological score of control and laser-irradiated rats (A) 4 hours and (B) 24 hours poststroke (middle cerebral artery occlusion by craniotomy, experiment 1) up to 21 days poststroke. Statistical significance levels are: *P<0.05 and **P<0.01.

Figure 3 presents the results of neurological scores from experiment 2 (mNSS and time taken to remove the adhesive tab from the forelimb) at 28 days poststroke. The sham-operated rats recovered at this time interval from a score of 8 to 10 at 24 hours poststroke to a score of 3.8±0.7. At the same time interval, the score of the LLLT rats was 2.0±0.2. Thus, there was a significant (P<0.05) 47% improvement in the overall mNSS score of CW laser-treated rats versus controls. A trend of improvement was also observed in the pulsed laser but was not significant. The time to remove the full and half adhesive tab, at 28 days poststroke, showed a highly significant (P<0.01) 58% improvement in the CW laser-treated versus control rats (Figure 3). When rats were irradiated with pulsed laser, a 40 and 46% (P<0.05) improvement in full and half sticker removal time, respectively, was observed at 28 days poststroke.

Figure 3. (A) Mean overall score and time to removal of (B) full and (C) half adhesive tab of control and laser-irradiated (24 hours poststroke) rats at 28 days poststroke (filament, middle cerebral artery occlusion, experiment 2). Statistical significance levels are: *P<0.05 and **P<0.01.
Quantitative and qualitative results of immunoreactivity to TUJ1, BrdU, DCX as well as colocalization of BrdU/TUJ1 in the SVZ (ipsilateral side) in control and laser-treated rats in experiment 2 are presented in Figure 4. The number of BrdU immunoreactivity cells as well as percentage area of TUJ1 reactivity in the ipsilateral SVZ was approximately twofold significantly higher in the CW laser-treated brains as compared with controls (Figure 4). Percentages of DCX immunoreactivity of the SVZ area was significantly ($P<0.05$) elevated (75%) by the laser treatment relative to control. The results also indicate that application of the CW laser was followed by a twofold, significant ($P<0.05$) increase in the percent of cells expressing BrdU/TUJ1-positive double immunoreactivity. The pulsed laser application did not cause a significant increase in these parameters relative to nonlaser-treated rats.

**Figure 4.** Representative micrographs and quantitative immunoreactivity of (A–C) bromodeoxyuridine (BrdU), (D–G) tubulin isotype III (TUJ1), double staining of (H–K) TUJ1 and BrdU and (L–O) doublecortin (DCX) of control and laser-irradiated rat brain sections of the SVZ of ipsilateral hemisphere. Note higher number of cells per area positively reacting to BrdU, TUJ1, or DCX in the section derived from laser-treated brains vs control ones. Arrows indicate cells that are positively stained for (A and B) BrdU or colocalization of (H–J) TUJ1 and BrdU or (L–N) DCX. Digitized images were contrast-enhanced to clearly differentiate positivity from background (see Methods). LV, lateral ventricle. Bar=50 µm. *$P<0.05$.

**Discussion**

The results of the present study clearly indicate that transcranial LLLT applied at a delay of 24 hours but not at 4 hours (MCAO through craniotomy) after induction of stroke significantly improves the neurological function of rats after the insult. The possibility that LLLT applied at 4 hours poststroke in the MCAO through craniotomy in a rat model did not change neurological outcome, but could demonstrate a different effect in the MCAO through a filament model cannot be ruled out.

These results reinforce a previous study also demonstrating significant neurological outcome improvement by means of transcranial LLLT (CW and similar parameters to the present study) given up to 6 hours after embolic stroke in rabbits$^{10}$ and 24 hours poststroke in rats.$^{11}$ At 24 hours poststroke, the LLLT did not improve neurological outcome in the rabbit model.$^{10}$ It may be postulated that the different findings are a consequence of the models used, which differ from each other both in animal species and the method of induction of stroke (through filament or craniotomy in rats versus injection of microbeads through the carotid artery in rabbits).

The biostimulatory effects of LLLT have been investigated over the years to a much greater extent in the CW mode than the pulsed mode.$^1$ Furthermore, there are no publications on attenuation of ischemic tissues by pulsed laser. The present study demonstrated that using the specific frequency of 70 Hz in the pulsed mode (and same power density as the CW mode),
the beneficial effect of the CW mode was superior to the pulsed mode for the stroke-induced rats. Yet, it should be noted that a beneficial effect was observed in the adhesive removal tests in the pulsed mode. Thus, the therapeutic effects of frequencies other than 70 Hz require further investigation.

Significant improvement in neurological outcome was not evident before 2 and 4 weeks poststroke in the rat model in which stroke was induced through craniotomy and filament insertion, respectively. It is therefore reasonable to assume that the laser irradiation induces a cascade of processes that result in part from induction of neurogenesis and migration of neurons and neuron-supporting cells. This hypothesis is supported by the absence of a reduction in lesion volume by the laser irradiation. Furthermore, the significant increase in cells that are double stained for BrdU and TUJ1 in the SVZ of the LLLT rats lie credence to enhanced induction of neurogenesis by LLLT. Also, the presence of large numbers of cells that positively immunoreact to DCX in the SVZ of laser-treated rats suggests that increased numbers cells that migrated from SVZ to the infarcted area in the ipsilateral hemisphere contribute to the improved functional performance of the laser-treated rats. However, mechanisms other than neurogenesis may be associated with attenuation of the behavioral deficits in stroke-induced rats by LLLT. Indeed, it has been demonstrated that the mechanism of laser action on the ischemic heart tissue and myogenic cells is associated with upregulation of heat shock proteins, increase in antioxidants, and angiogenic activity as well as antiapoptotic activity. Thus, the beneficial effects of the LLLT on stroke-induced animals may be achieved by attenuation of several processes in concert. It was previously shown that LLLT upregulated TGF-ß1 in the ischemic brain, which is considered neuroprotective, while concomitantly suppressing nitric oxide synthase.

In conclusion, our data demonstrate that CW laser irradiation of rat brain initiated 24 hours poststroke promotes restoration of neurological function. These data suggest that LLLT warrants further study as a potential therapy for stroke.

**References**


