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/cm^2.

***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.[1–3] 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.[4–7] 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.[4–6]

LLLT has also been shown **to** biomodulate processes in the nervous system. Anders et al[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[9] have shown that LLLT in stroked **rats** causes suppression of nitric oxide synthase activity and upregulation of TGF-ß1, which are considered neuro**to**xic and neuroprotective, respectively. It was also recently demonstrated that transcranial infrared **laser therapy applied** 6 hours postembolic stroke in rabbits[10] and 24 hours postischemic stroke in **rats**[11] caused a significant improvement of neurological score over sham-operated experimental animals. Light-emitting diodes were also shown **to** regulate cy**to**chrome C oxidase, leading **to** increased energy metabolism in vitro in visual neurons functionally inactivated by **to**xin (KCN).[12] Given the therapeutic benefits demonstrated for LLLT in the myocardial infarction setting and its promising results after application in stroke-induced rabbits,[10] 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 cranio**to**my. 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 fol**low** 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 immunohis**to**chemistry with a longer fol**low**-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 Labora**to**ry, Bar Harbor, Maine) by means of a filament inserted through the carotid artery, as previously described.[13,14] Mortality rate was 17% (three of 18 **rats**) for the 4-week fol**low** up. In both experiments, **rats** were initially anesthetized with 3.5% halothane and maintained with 1 **to** 2% halothane in 70% N_2O and 30% O_2. All **rats** in experiment 2 received a daily intraperi**to**neal 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) moni**to**ring of mean arterial blood pressure and obtaining blood samples **to** measure glucose, pH, p_{O_2}, and p_{CO_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 fol**low**ing criteria: extension of forelimb, body twisting when lifted by the tail, beam walking, res**to**ration 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.[15] The **rats** (37 of 43 **rats** that survived after stroke induction) were then randomly divided in**to** 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 mo**to**r, sensory, reflex, and balance tests.[13] 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 in**to** 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 tests[14] 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 mm^2) 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 mm^2. 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 (Pho**to**thera 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) on**to** 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/cm^2 at the brain tissue **level**. This optimal power density was determined based on our previous studies on the effect of LLLT on the ischemic heart[5] 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/cm^2) 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/cm^2. Control **rats** were sham-operated but the **laser** was not turned on. Possible heating effect by LLLT was measured by thermocouple probes inserted in**to** 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/cm^2) the optimal energy delivered **to** the stroked **rats** (0.9 J/cm^2). Furthermore, no pathologic changes up **to** 3 months were noticed in rat brains lased at a 10x dose of that previously mentioned (75 mW/cm^2 for 2 minutes).[16]

Histology and Measurement of Infarct Volume

Rat brain tissue was fixed by transcardial perfusion with saline fol**low**ed 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.[17] 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][18]). 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 TUJI 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.

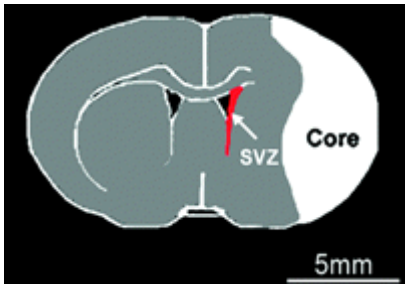

Figure 1. Representative schematic cross section of rat brain indicating lesion area in the stroked rat (core) and subventricular zone (red-colored area).

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 cranio**to**my; 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**.

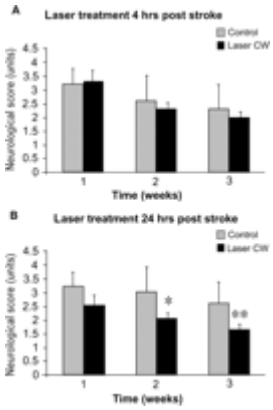

Figure 2. Neurological score of control and **laser**-irradiated **rats** (A) 4 hours and (B) 24 hours poststroke (middle cerebral artery occlusion by cranio**to**my, experiment 1) up **to** 21 days poststroke. Statistical significance **level**s are: *P<0.05 and **P<0.01.

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.

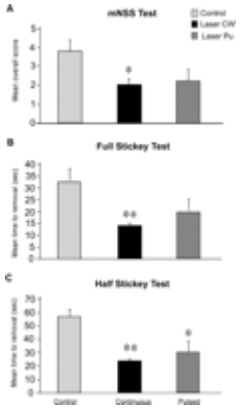

Figure 3. (A) Mean overall score and time **to** removal of (B) full and (C) half adhesive tab of control and **laser**-irradiated (24 hours poststroke) **rats** at 28 days poststroke (filament, middle cerebral artery occlusion, experiment 2). Statistical significance **level**s are: *P<0.05 and **P<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 TUJI 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 fol**low**ed 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** non**laser**-treated **rats**.

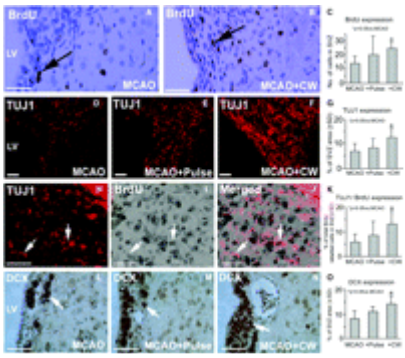

Figure 4. Representative micrographs and quantitative immunoreactivity of (A–C) bromodeoxyuridine (BrdU), (D–G) tubulin isotype III (TUJI), double staining of (H–K) TUJ1 and BrdU and (L–O) doublecortin (DCX) of control and **laser**-irradiated rat brain sections of the SVZ of ipsilateral hemisphere. 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 cranio**to**my) 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 cranio**to**my 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 cranio**to**my in **rats** versus injection of microbeads through the carotid artery in rabbits).

The biostimula**to**ry 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 cranio**to**my 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,[2,4] and angiogenic activity[3,6] as well as antiapop**to**tic activity.[19] 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.[9]

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

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