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Low-Level Laser Therapy for Closed-Head Traumatic Brain Injury in Mice: Effect of Different Wavelengths

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Abstract

Background and Objectives—Traumatic brain injury (TBI) affects millions worldwide and is without effective treatment. One area that is attracting growing interest is the use of transcranial low-level laser therapy (LLLT) to treat TBI. The fact that near-infrared light can penetrate into the brain would allow non-invasive treatment to be carried out with a low likelihood of treatment-related adverse events. LLLT may treat TBI by increasing respiration in the mitochondria, causing activation of transcription factors, reducing inflammatory mediators and oxidative stress, and inhibiting apoptosis.

Study Design/Materials and Methods—We tested LLLT in a mouse model of closed-head TBI produced by a controlled weight drop onto the skull. Mice received a single treatment with continuous-wave 665, 730, 810, or 980 nm lasers (36 J/cm² delivered at 150 mW/cm²) 4-hour post-TBI and were followed up by neurological performance testing for 4 weeks.

Results—Mice with moderate-to-severe TBI treated with 665 and 810 nm laser (but not with 730 or 980 nm) had a significant improvement in Neurological Severity Score that increased over the course of the follow-up compared to sham-treated controls. Morphometry of brain sections showed a reduction in small deficits in 665 and 810 nm laser treated mouse brains at 28 days.

Conclusions—The effectiveness of 810 nm agrees with previous publications, and together with the effectiveness of 660 nm and non-effectiveness of 730 and 980 nm can be explained by the absorption spectrum of cytochrome oxidase, the candidate mitochondrial chromophore in transcranial LLLT.

Keywords

photobiomodulation; low-level laser therapy; traumatic brain injury; mouse model; Neurological Severity Score

INTRODUCTION

Head injury in humans is the most common neurological disorder under the age of 50. Victims of traumatic brain injury (TBI) suffer short- and long-term physical, cognitive, behavioral, and emotional impairments that depend on the severity of the injury [1,2]. Moderate and severe TBI, accidental or inflicted, is a major health and socio-economic problem throughout the world. In the United States alone, approximately 2 million injuries occur each year resulting in 56,000 deaths and 18,000 survivors suffering from permanent neurological impairment [3–5]. The consequent direct and indirect annual costs in the United States are estimated at \$56 billion [6]. The World Health Organization (WHO) has projected that by 2020, road traffic accidents, a major cause of TBI, will rank third as a cause of the global burden of disease and disablement, behind only ischemic heart disease and unipolar major depression [7]. Moreover, military conflicts in recent years have given rise to a large numbers of cases of TBI caused by over-pressure blast injuries from improvised explosive devices [8,9]. Efforts to improve treatment and outcome of TBI must therefore remain the priority for clinicians and researchers [10].

The pathophysiology of TBI is very complex and still poorly understood [11]. Immediately following the primary impact, activation of several different pathways begins, resulting in secondary brain injury. These include inflammation, oxidative stress, ionic imbalance, increased vascular permeability, mitochondrial dysfunction, and excitotoxic damage [12,13]. These processes result in brain edema, increased intracranial pressure (ICP), and impaired cerebral perfusion [14]. This combination of cellular and physiologic disturbances causes increased neuronal cell death, enlargement of infarct size, and neurological, motor, and cognitive impairment. Since there are no approved specific pharmacological agents that block the progression of the secondary injury, the current management of TBI is mainly supportive and aims at treating brain edema, reducing ICP, and combating complications, such as hypoxia and shock [15]. Despite promising preclinical data, most of the clinical trials that have been performed in recent years have failed to demonstrate any significant improvement in outcome [16].

Low-level laser (or light) therapy (LLLT) has been clinically applied for many indications in medicine that require the following processes: protection from cell and tissue death, stimulation of healing and repair of injuries, and reduction of pain, swelling, and inflammation [17,18]. One area that is attracting growing interest [19] is the use of LLLT to treat stroke [20], TBI [21], neurodegenerative diseases [22], and spinal cord injuries [23]. The notable lack of any effective drug-based therapies for most of these diseases has motivated researchers to consider the use of light as an effective approach to mitigating what

is considered to be a group of serious diseases. The fact that near-infrared (NIR) light can penetrate into the brain and spinal cord could allow non-invasive treatment to be carried out with a low likelihood of treatment-related adverse events. Although in the past it was generally accepted that the central nervous system could not repair itself, recent discoveries in the area of neuronal stem cells and adult neurogenesis have brought this dogma into question [24]. LLLT may have beneficial effects in the acute treatment of TBI by improving mitochondrial function, stimulating signaling pathways, and transcription factors, inhibiting neuronal apoptosis and upregulating adult neurogenesis (Fig. 1). In this study, the effects LLLT mediated by different wavelengths of laser-light were tested in a mouse model of closed-head TBI and by measuring the neurobehavioral outcome of the traumatized mice.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital (protocol #2009N000006) and met the guidelines of the National Institutes of Health. Adult male BALB/c mice (weight 20–25 g; Charles River Laboratories, Wilmington, MA) were used in this study. The animals were housed at one mouse per cage and were maintained on a 12-hour light–12-hour dark cycle with access to food and water ad libitum.

Traumatic Brain Injury Model

Closed-head injury (CHI) was induced under isoflurane anesthesia using a weight drop device. The weight drop apparatus included a vertical 15-cm-long plastic tube (diameter 32 mm) with a 69 g weight (diameter 25 mm) dropped through the upper end of the tube. Briefly, following anesthesia, a midline longitudinal incision in the scalp was performed and the skull was exposed. The mouse was placed on a foam-pad and a Teflon-tipped cone (3 mm diameter) was placed 1 mm lateral to the midline in the mid-coronal plane. The head was manually held in place and a 69 g weight was dropped on the cone from a height that was adjusted to yield a trauma that could be categorized as moderate to severe [Neurological Severity Score (NSS) 6–8] to the left hemisphere. After brain trauma, the skin was sutured. Mice with depressed skull fracture or visible hemorrhage were excluded from the study. The severity of the trauma was confirmed 1 hour later by assessing the NSS score. After recovery from anesthesia, the mice were returned to their cages with post-operative care and ad libitum access to food and water.

Neurobehavioral Evaluation

The neurological status of the traumatized mice was evaluated at different time intervals after CHI according to a NSS. The neurological tests are based on the ability of the mice to perform 10 different tasks (Table 1) that evaluate the balancing, motor ability, and alertness of the mouse. One point is given for failure to perform a task; thus, a normal, uninjured mouse scores 0. The severity of injury is defined by the initial NSS, evaluated 1-hour post-CHI, and is a reliable predictor of the late outcome. Thus, the higher the score, the more severe is the injury; mild injury in mice with an NSS of <5; moderate injury with NSS of 5–6; severe injury in mice with an NSS of 7–8; and fatal or near-fatal injury is defined in mice having an NSS of 9–10. Only mice with an NSS of 6–8 at 1 hour were included in the study.

Laser Treatment

All mice were subjected to CHI and assessment of NSS was performed 1-hour post-TBI. The NSS scores of the mice ranged from 6 to 8, indicating a moderately severe trauma. The mice were then divided into five groups of 8–12 mice per group, so that the mean NSS in

each group were similar, to ensure similar average severity of injury in all groups. One group of mice served as a sham-treated TBI control, which received brain injury and then underwent the same procedures as the laser-treated group, but did not receive actual laser-irradiation. The lasers that used were: 665 nm, model BWF-665-1 from B&W-Tek (Newark, DE); 730 nm, model 730/6 from Diomed Inc (Andover, MA); 810 nm, model DioDent Micro 810, ConBio (Fremont, CA); 980 nm, model V-Raser, ConBio. Mice were immobilized in a plastic holder so their head was exposed. The lasers were coupled into a fiber-optic that was arranged to deliver a 1-cm diameter spot centered on the top of the mouse head over the sutured incision. The power densities generated were measured with a power meter (Model DMM 199 with 201 Standard head, Coherent, Santa Clara, CA) and were adjusted to be 150 mW/cm². Four laser-treated groups of mice received a single treatment with 665, 730, 810, or 980 nm laser (36 J/cm² over 4-min) 4-hour post-injury.

Sacrifice and Histomorphometry

At day 28 mice were sacrificed by deep anesthesia (1.5% isoflurane) and transcardial perfusion with phosphate-buffered saline (PBS) followed by fixation by perfusion and immersion in 4% paraformaldehyde. After the brains were removed and further fixed overnight, they were embedded in OCT compound (Sakura Finetek USA Inc., Torrance, CA), frozen in liquid nitrogen, cut into 15- μ m-thick axial sections, and stained with hematoxylin and eosin (HE). Because there were no focal lesions visible in this CHI model of TBI but rather a diffuse axonal injury, the following morphometric technique was employed. Brains were imaged with a light microscope (BH-2, Olympus America Inc, Center Valley, PA), with a magnification objective of 4 $\times$ and photographed using a digital camera (FD400, Sony USA, San Diego, CA). Outlines of the brains were drawn manually to calculate the total pixel area occupied by the brain assuming no small defects, and the actual area of H&E stained pixels was calculated using Image J software (NIH, Bethesda, MD).

Statistical Analysis

Data are presented as mean $\pm$ SD. In a two-dimensional coordinate system, the area under the curve (AUC) data, which represent the time courses of NSS in the various groups of mice, were calculated using numerical integration [25]. Differences in the AUC between the untreated TBI and the laser-treated groups and between different laser-treated groups were compared for statistical significance using one-way ANOVA. Significance was defined as $P < 0.05$. SPSS statistics V17.0 software (IBM Corp, Armonk, NY) was used for statistical analyses.

RESULTS

Neurobehavioral Evaluation After Laser Treatment to the Head

The NSS measurements in control sham-treated TBI mice displayed a gradual improvement from 7.3 at 1-hour post-TBI to 4.5 at 5 days; to 3.4 at 14 days; and to 2 at 28 days. Figure 2A shows that mice treated with 665 nm laser at 4-hour post-injury treatment started to show a significantly greater improvement than sham-treated control mice at day 5 (2.78 vs. 4.5, $P < 0.05$) and this improvement increased at day 9 (1.55 vs. 3.75, $P < 0.001$) and remained at this significance level at day 14 (1.22 vs. 3.37, $P < 0.001$). At day 21 the improvement was a little less significant (0.89 vs. 2.62, $P < 0.01$) and at day 28 (0.78 vs. 2.0, $P < 0.05$).

Figure 2B shows that 730 nm laser had absolutely no beneficial effect on the NSS of the mice, and laser-treated mice improved at the same rate as the sham-treated control mice throughout the entire course of the follow-up.

Figure 2C shows the effect of laser-irradiation at 810 nm on the NSS of mice compared to sham-treated control mice. In a similar fashion to the scores of the 665 nm treated mice, the NSS scores of the 810 nm laser-treated mice showed a significant improvement over sham-treated control mice at day 5 (3.14 vs. 4.5, $P < 0.01$), and this continued to increase at day 9 (2.8 vs. 3.75, $P < 0.001$). The improvement remained at this significance level throughout the rest of the follow-up; on day 14 (1.64 vs. 3.37, $P < 0.001$), on day 21 (0.71 vs. 2.62, $P < 0.001$), and day 28 (0.35 vs. 2.0, $P < 0.001$).

Figure 2D shows the effect of laser irradiation at 980 nm on the NSS of mice. In a similar fashion to the response seen with 730 nm, there was no significant difference between 980 nm laser and sham-treated control at any time point.

We integrated the NSS values for each mouse over the 28-day time course of the experiment to give an AUC value for each mouse. The means of these AUC values for each group of mice was compared in Figure 3. There was not significant difference between mean AUC values in sham-treated controls and mice treated with 730 nm laser or with 980 nm laser. The mean AUC for 665 nm laser treated mice was approximately half that seen in the sham, 730 nm, and 980 nm groups ($P = 0.002$ – 0.038). The mean AUC for 810 nm laser-treated mice was also about half of that seen in sham, 730 nm, and 980 nm groups ($P = 0.002$ – 0.032). There was no difference between mice treated with 665 and 810 nm lasers.

Brain Sections and Morphometry

Representative images of the brain sections removed at day 28 are shown in Figure 4A. Because of the absence of focal brain lesions in this closed-head model of TBI it was impossible to distinguish any gross differences between the sham-treated control and the 665 nm or 810 laser-treated groups. There were small defects visible (arrows in Fig. 4A) in the brain parenchyma but it was supposed that these were tissue-processing artifacts. In further studies, these defects should be investigated at higher magnification to establish their exact pathological features. However, when we calculated the pixel area occupied by the entire shape of the brain according to the black outline visible in Figure 4A, and subtracted from this value, the pixel area of the brain stained by H&E using Image J, we arrived at the total area of the small lesions marked by arrows in Figure 4A. This area of deficits was expressed as a fraction of the total area to normalize for the varying sizes of the whole brain images. The values obtained for these parameters in sham, 665 nm, and 810 nm laser-treated mice are shown in Table 2. To our surprise when we compared the means of these fractional lesion areas we found significant differences between groups. Figure 4D shows the mean fractional defect areas for 660 nm laser-treated mice (0.065 ± 0.024 , $P < 0.05$) and for 810 nm laser-treated mice (0.053 ± 0.029 , $P < 0.01$) were significantly lower than that for untreated TBI mice (0.12 ± 0.029).

DISCUSSION

This study has shown that both red laser (665 nm) and NIR laser (810 nm) can significantly improve the neurobehavioral performance of mice after CHI TBI. Interestingly however, NIR lasers at 730 or 980 nm did not produce the same beneficial positive effects on NSS improvement. In addition, the error bars were higher for the 980 nm laser-treated mice than for the other wavelengths and this may lessen the reliability of the finding.

The principal tissue chromophore that is proposed to be responsible for photobiomodulation effects is cytochrome c oxidase (CCO). CCO has distinct absorption bands in the red (around 665 nm) and in the NIR (around 810 nm) [26]. There is a minimum in the CCO absorption spectrum at 730 nm, and there have been reports that this wavelength is ineffective in preserving cultured cortical neurons from cyanide toxicity [27], while 670 and

830 nm were highly active in this application. In our hands 980 nm was ineffective. However, it should be mentioned that several reports show that 980 nm is an active wavelength in LLLT applications [28–30]. It is entirely possible that our finding that 980 nm was ineffective only meant that we used the wrong parameters. In other words, if the fluence or irradiance of 980 nm laser was lower than we used (or possibly higher) the effect on TBI might have been positive.

LLLT is increasingly being used for tissue preservation and functional stimulation at the cellular level, after various forms of injury. Stimulated wound healing, reduction of edema and inflammation, pain relief, and prevention of tissue loss are the main applications. During LLLT, absorption of red or NIR photons by CCO in the mitochondrial respiratory chain causes an increase in cellular respiration that continues for much longer than the light is present when delivered at appropriate fluences, irradiances, and exposure durations [31]. Primary cellular effects include increases in mitochondrial activity and ATP levels [32], modulation of reactive oxygen species [33], induction of transcription factors (including the pro-survival NF- κ B) [34], and inhibition of apoptosis [35].

Secondary injuries after TBI are attributable to spreading cellular damage that can grow to encompass a much greater area of the brain than was originally damaged and results from excitotoxic cell death after glutamate release and sequelae of inflammation [36]. Secondary injury cascades can affect neurological function unrelated to specific tissue damage. Glutamate depolarizes neurons that suffer a huge influx of sodium and calcium but could survive if they had sufficient ATP to power the Na⁺/K⁺-ATPase pumps. ATP is increased by LLLT and could prevent excitotoxicity.

LLLT modulates many biological and disease processes increasing fibroblast migration, proliferation and differentiation, angiogenesis, and healing in ischemic heart and skeletal muscles, tendons, and bones. There are reports of beneficial effects of LLLT on functional recovery of injured peripheral nerves (780 nm) [37,38] and also of injured central neurons in the spinal cord (810 nm) [23,39].

Transcranial LLLT has been used to significantly improve recovery in a number of animal models of stroke [20]. LLLT at 808–810 nm can penetrate the brain and was shown to lead to enhanced production of ATP in the rat cerebral cortex [40]. Findings of increased neurogenesis in the subventricular zone (SVZ) were reported in an ischemic stroke animal model treated with TLT [41]. Based on these findings, it is thought that TLT may have multiple mechanisms of action and could be beneficial in acute ischemic stroke [20]. In a rat model of cerebral ischemia induced by a 1-hour occlusion of middle cerebral artery, LLLT (660 nm laser) delivered immediately after reperfusion through a craniotomy, reduced nitric oxide synthase activity, and upregulated TGF- β 1 expression simultaneously [42]. The success of transcranial LLLT in these animal studies of stroke encouraged a clinical trial (NEST-1) in human stroke patients [43] in which only one transcranial NIR (808 nm) laser treatment at ~18 hours after stroke led to significant improvement. A second larger clinical trial (NEST-2) failed to meet its primary endpoint [44], but a subset of patients (moderate strokes) showed significant benefit [45].

Oron et al. [46] carried out a study of transcranial LLLT in a mouse model of closed-head TBI. They used 810 nm laser delivered to the skull (skin removed) 4-hour post-TBI with a total power of 200 mW and a calculated irradiance to the cortical surface of either 10 or 20 mW/cm² giving a calculated fluence of 1.2–2.4 J/cm² to the brain. They found an improvement in NSS in the laser-treated mice compared to untreated TBI mice starting at 5 days and increasing as time went on in a similar manner to the improvement in NSS we reported in the present study. However, the extent of improvement was higher in our study

than in the Oron study [46]. The model of CHI employed in the Oron study [46] was also somewhat different to ours in that it resulted in pronounced focal brain lesions in the area of brain under the skull impact as described in the protocol by Shapira et al. [47].

Recently, Khuman et al. [48] carried out a study of transcranial LLLT in a mouse model of open skull controlled cortical impact (CCI). They used 800 nm laser applied directly to the contused parenchyma or delivered transcranially beginning 60–80 min after CCI. Injured mice treated with 60 J/cm² (500 mW/cm² over 2 min) either transcranially or via an open craniotomy had significantly improved latency to the hidden platform in the Morris Water Maze and probe trial performance compared to non-treated TBI controls. The beneficial effects of LLLT in open craniotomy mice were associated with reduced microgliosis at 48 hours. Little or no effect of LLLT on post-injury cognitive function was observed using other doses, a 4-hour administration time point and 7-day administration of 60 J/cm². No effect of LLLT (60 J/cm² open craniotomy) was observed on post-injury motor function (days 1–7), brain edema (24 hours), nitrosative stress (24 hours), or lesion volume (14 days).

We very recently [49] completed a study of transcranial LLLT in mice with CCI-TBI. We used 810 nm laser (36 J/cm² at 50 mW/cm²) delivered to the head at 4-hour post-TBI and compared CW-laser with pulsed-laser (10 or 100 Hz both at 50% duty cycle). Surprisingly, while all three-laser regimens gave significantly improved NSS scores, the 10 Hz pulse structure was significantly better than the other two laser regimens. Furthermore, significant improvements in mouse performance in the forced swim test and the tail suspension test suggested that psychological problems, such as depression and anxiety associated with TBI might also be helped by LLLT. There was significantly reduced lesion area at 28 days in the 10 Hz laser group.

The fact that three separate groups have now obtained positive results using transcranial LLLT in mouse models of TBI suggests that this is an approach that could rapidly advance to clinical trials. As further preclinical studies are reported the optimum laser parameters and optimum treatment timing will become clearer. The total lack of reports of adverse effects after delivery of LLLT to the head encourages us to believe that transcranial laser could be an extremely promising therapy for brain injury in humans.

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Fig. 1.

Graphical illustration of the possible mechanisms of transcranial LLLT for TBI. Red or near-infrared light is absorbed in the mitochondria (possible by cytochrome c oxidase in the respiratory chain) leading to release of ATP, nitric oxide, and modulation of reactive oxygen species. These second messenger molecules can lead to activation of transcription factors that migrate to the nucleus where they alter expression levels of numerous genes. These new gene products may lead to increased survival of neurons in the damaged brain, to an increase in adult neurogenesis in the hippocampus, for example, to reduced levels of inflammation and to an overall reduced level; of apoptotic and necrotic cell death in the brain.

Fig. 2.

Time course of NSS scores of sham and laser-treated mice. **A:** Sham-treated control versus 665 nm laser. **B:** Sham-treated control versus 730 nm laser. **C:** Sham-treated control versus 810 nm laser. **D:** Sham-treated control versus 980 nm laser. Points are means of 8–12 mice and bars are SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (one-way ANOVA).

Fig. 3. Means of AUC values for individual mice in the five groups. Values are means of 8–12 mice per group and bars are SD. *P*-values shown were determined by one-way ANOVA.

Fig. 4. Morphometry of brain sections removed from mice at day 28. Micrographs of typical H&E stained brain sections showing outline to calculate total brain area and small defects marked with arrows. **A:** Sham-treated control. **B:** 665 nm laser-treated mouse. **C:** 810 nm laser-treated mouse. **D:** Mean fractional defect area in brains of sham, 665 nm, and 810 nm laser-treated mice. Values are means from 8 to 12 mice per group and bars are SD. * $P < 0.05$; ** $P < 0.01$ (one-way ANOVA).

TABLE 1

Neurological Severity Score (NSS) for TBI Mice

Task	NSS
Presence of mono- or hemiparesis	1
Inability to walk on a 3-cm-wide beam	1
Inability to walk on a 2-cm-wide beam	1
Inability to walk on a 1-cm-wide beam	1
Inability to balance on a 1-cm-wide beam	1
Inability to balance on a round stick (0.5-cm wide)	1
Failure to exit a 30-cm-diameter circle (for 2 minutes)	1
Inability to walk straight	1
Loss of startle behavior	1
Loss of seeking behavior	1
Maximum total	10

Mice are awarded 1 point for each failure to complete a task.

TABLE 2

Morphometry Measurements on Areas of Brain Sections Taken at 28 Days From Sham, 665 nm, and 810 nm Laser-Treated Mouse Groups

Mouse	Whole area (pixels) ^a	Stained area (pixels) ^b	Area of deficits (pixels) ^c	Fractional area that is deficit ^d
Sham 1	2,733	2,362	371	0.135
Sham 2	2,750	2,389	361	0.131
Sham 3	2,763	2,342	421	0.152
Sham 4	4,059	3,519	540	0.133
Sham 5	4,062	3,638	424	0.104
Sham 6	4,056	3,565	491	0.121
Sham 7	2,689	2,517	172	0.063
Sham 8	2,236	1,960	276	0.123
Sham 9	2,741	2,439	302	0.11
Mean ± SD				0.119 ± 0.029
665 nm 1	2,711	2,553	158	0.058
665 nm 2	2,726	2,545	181	0.066
665 nm 3	2,630	2,474	156	0.059
665 nm 4	2,569	2,398	171	0.067
665 nm 5	2,470	2,378	92	0.037
665 nm 6	2,448	2,303	145	0.059
665 nm 7	1,756	1,619	137	0.078
665 nm 8	1,658	1,516	142	0.086
Mean ± SD				0.064 ± 0.024
810 nm 1	1,342	1,274	68	0.051
810 nm 2	1,021	936	85	0.083
810 nm 3	1,086	997	89	0.082
810 nm 4	1,143	1,025	118	0.103
810 nm 5	1,367	1,278	89	0.065
810 nm 6	2,890	2,854	36	0.012
810 nm 7	2,743	2,654	89	0.032
810 nm 8	2,699	2,514	185	0.069
810 nm 9	3,150	3,026	124	0.039
810 nm 10	2,951	2,908	43	0.015
810 nm 11	3,008	2,912	96	0.032
Mean ± SD				0.053 ± 0.029

^aCalculated by drawing an outline around the brain section and determining pixel area.

^bCalculated by measuring H&E stained pixel area.

^cColumn 1 – column 2.

^dColumn 3/column 1.