Video Article

A Protocol for Transcranial Photobiomodulation Therapy in Mice

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Abstract

Transcranial photobiomodulation is a potential innovative noninvasive therapeutic approach for improving brain bioenergetics, brain function in a wide range of neurological and psychiatric disorders, and memory enhancement in age-related cognitive decline and neurodegenerative diseases. We describe a laboratory protocol for transcranial photobiomodulation therapy (PBMT) in mice. Aged BALB/c mice (18 months old) are treated with a 660 nm laser transcranially, once daily for 2 weeks. Laser transmittance data shows that approximately 1% of the incident red light on the scalp reaches a 1 mm depth from the cortical surface, penetrating the dorsal hippocampus. Treatment outcomes are assessed by two methods: a Barnes maze test, which is a hippocampus-dependent spatial learning and memory task evaluation, and measuring hippocampal ATP levels, which is used as a bioenergetics index. The results from the Barnes task show an enhancement of the spatial memory in laser-treated aged mice when compared with age-matched controls. Biochemical analysis after laser treatment indicates increased hippocampal ATP levels. We postulate that the enhancement of memory performance is potentially due to an improvement in hippocampal energy metabolism induced by the red laser treatment. The observations in mice could be extended to other animal models since this protocol could potentially be adapted to other species frequently used in translational neuroscience, such as rabbit, cat, dog, or monkey. Transcranial photobiomodulation is a safe and cost-effective modality which may be a promising therapeutic approach in age-related cognitive impairment.

Video Link

The video component of this article can be found at https://www.jove.com/video/59076/

Introduction

PBMT, or low-level laser light therapy (LLLT), is a general term which refers to therapeutic methods based on the stimulation of biological tissues by light energy from lasers or light-emitting diodes (LEDs). Almost all PBMT treatments are applied with red to near-infrared (NIR) light at wavelengths from 600 to 1100 nm, an output power ranging from 1 to 500 mW, and a fluence ranging from <1 to >20 J/cm^2 (see Chung et al.[1]).

Transcranial PBMT is a noninvasive light delivery method that is conducted by irradiation of the head using an external light source (laser or LEDs)[2]. For animal applications, this method includes contact or noncontact placement of the LED or laser probe on the animal's head. Depending on the therapeutic region of interest, a light probe can be placed either over the entire head (for covering all the brain areas) or over a specific portion of the head, such as the prefrontal, frontal, or parietal region. The partial transmission of red/NIR light through the scalp, skull, and dura mater can reach the cortical surface level and provide an amount of photon energy sufficient to produce therapeutic benefits. Subsequently, the delivered light fluence at the cortical level would be propagated into the gray and white brain matter until it reaches the deeper structures of the brain[3].

Light in the spectral bands at the red to far-red region (600-680 nm) and early NIR region (800-870 nm) corresponds to the absorption spectrum of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain[4]. It is hypothesized that PBMT in the red/NIR spectrum causes photodissociation of nitric oxide (NO) from cytochrome c oxidase, resulting in increases in mitochondrial electron transport and,

ultimately, increased ATP generation[5]. With respect to neuronal applications, the potential neurostimulatory benefits of brain PBMT using transcranial irradiation methods have been reported in a variety of preclinical studies, including rodent models of traumatic brain injury (TBI)[6], acute stroke[7], Alzheimer's disease (AD)[8], Parkinson's disease (PD)[9], depression[10], and aging[11].

Brain aging is considered a neuropsychological condition that negatively affects some cognitive functions, such as learning and memory[12]. Mitochondria are the primary organelles responsible for ATP production and neuronal bioenergetics. Mitochondrial dysfunction is known to be associated with age-related deficits in brain areas that are linked to spatial navigation memory, such as the hippocampus[13]. Because cranial treatment with red/NIR light primarily acts by modulation of mitochondrial bioenergetics, sufficient delivered light dosage to the hippocampus can result in the improvement of spatial memory outcomes[14].

The aim of the current protocol is to demonstrate the transcranial PBMT procedure in mice, using low levels of red light. The required laser light transmission measurements through the head tissues of aged mice are described. Additionally, Barnes maze, as a hippocampus-dependent spatial learning and memory task, and hippocampal ATP levels, as a bioenergetics index, are used for an evaluation of the treatment impact in animals.

Protocol

All of the procedures were carried out in conformity with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH; Publication No. 85-23, revised 1985) and approved by the regional ethics committee of Tabriz University of Medical Sciences.

CAUTION: This protocol includes the application of Class 3B laser instruments and will require proper training and adherence to safety guidelines. Class 3B lasers can seriously damage the eyes and can heat the skin. Class 3B lasers are not considered a burn hazard. Eye protection goggles must be worn at all times when operating the laser device.

1. Laser light transmission experiments

NOTE: Used here were three 18-month-old male BALB/c mice obtained from the animal facility of Tabriz University of Medical Sciences. A 60 mW laser (660 nm) with a circular beam shape of 2.5 mm in diameter is used as the light source. The laser source produces a circularly polarized light with a Gaussian intensity profile and is operated in continuous wave mode. A commercial photodiode power meter with a 10 nW resolution, a square 1 cm^2 photodiode active area, and a spectral response range from 400 to 1100 nm is used to measure the transmitted light power through the samples.

1. **Sample preparation**
    1. In order to obtain fresh samples, deeply anesthetize the mouse with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg).
    2. Dissect the mouse's head with regular scissors, starting from the point located just above the shoulders.
    3. Rotate the head so that the ventral side of the jaw faces up. Slide angled dissection scissors smoothly through the oral cavity until the resistance of the mandibular junction is noticed. Cut all large muscles linking the mandible bone to the skull and discard them.
    4. Remove the palatine bones, using angled dissection scissors.
    5. Discard all flesh surrounding the skull, using curved forceps.
    6. Dissect the lower portion of the skull, and then, carefully take the brain out of the remaining skull bone, with a curved spatula.
    7. Fix the intact brain tissue in a 2% agarose gel so the tissue will be suitable for slicing.
       NOTE: In order to obtain an intact skull plus scalp sample, the brain tissue should be removed from the ventral side of the animal's head without any damage to the dorsal portion of the head.

2. **Brain slicing procedure**
    1. Spread a drop of superglue (~0.05 mL) on the surface of the vibratome mounting block.
    2. Carefully attach the agarose block to the vibratome mounting block so that the ventral surface of the brain is facedown, and adjust its position.
    3. Slightly match the vibratome blade to the upper surface of the agarose block and record the cutter value as the primary level.
    4. Fill the vibratome tank with ice-cold normal saline solution.
    5. Adjust the vibratome parameters (e.g., the slice thickness [1 mm], speed [5 of 5 on device unit], and vibration frequency [5 of 5 on device unit]) to obtain satisfactory slicing.
    6. Cut the brain transversely into a slice with a 1,000 µm thickness.
       NOTE: The slice is the portion of the brain tissue delimited by the cortical surface and a plane positioned 1,000 µm inferior to the cortical surface (the dorsal hippocampus).
    7. Add a drop of water (~0.05 mL) on the optical glass surface and put the brain slice on top of it. Then, add a drop of water on the brain slice and carefully place the second optical glass on top of it.
       NOTE: A drop of water should be added to the sample glass boundaries in order to prevent tissue drying and light scattering from rough surfaces.

3. **Measurement of light transmission through the head tissues**
    1. Set up the optical equipment, including the laser device, reflecting mirrors, and power meter unit.
       CAUTION: Put on protective eye goggles prior to turning on the laser.
    2. In the absence of a sample on the power meter, turn on the laser device and focus the laser beam on the mirror that is located at the proper distance for guiding the beam perpendicular to the photodiode's active area.
       NOTE: Light transmission measurements must be performed in a darkroom at room temperature (23-25 °C), within 30 min after the head tissues have been extracted.
    3. Perform measurements on the sliced brain tissue.
        1. Place two blank optical glasses on the surface of the power meter.

2. Read the transmitted light power (I_0) from the power meter's display screen and record the value.
3. Gently place the brain sample, which is encompassed by two optical glasses, on the surface of the power meter, focus the beam on the tissue's respective area, read the transmitted power, and record the value.

4. Perform measurements on the skull plus the scalp.
    1. Place a blank optical glass on the surface of the power meter.
    2. Read the transmitted light power (I_0) from the power meter's display screen and record the value.
    3. Lightly place an optical glass with fresh skull plus scalp tissue on the surface of the power meter, match the light beam on the bregma zone, read the transmitted power, and record the value.
    4. In order to maximize the signal-to-noise ratio, repeat the light transmission measurement at least 3x for all samples.
    NOTE: The bregma zone is placed in an approximately 3 mm rostral to a line drawn through the anterior base of the ears. The thickness of the skull plus scalp tissue is measured by a standard caliper.

2. Photobiomodulation therapy (PBMT)

NOTE: Forty-five male BALB/c mice assigned to three groups of 15 mice each were used. The groups were composed of young-control mice (2 months old) that received sham-PBMT, aged-control mice (18 months old) that received sham-PBMT, and aged-PBMT mice (18 months old) that received PBMT. The sham-PBMT treatment consisted of treatment identical to the PBMT group but with the laser inactive. Mice were obtained from the animal facility of Tabriz University of Medical Sciences and were housed in the animal holding unit of the Neurosciences Research Center (NSRC) at 24-25 °C and 55% relative humidity, with a 12 h light and 12 h dark photoperiod. Food and water were provided *ad libitum*. All mice were acclimatized for at least 1 week prior to treatment.

1. **Laser treatment procedure**
NOTE: A diode GaAlAs laser with continuous wave mode at 660 nm wavelength was used for transcranial PBMT treatment. The laser device was operated at an output power of 200 ± 2 mW and an irradiance of 6.66 W/cm^2, with a spot size of 0.03 cm^2. An average fluence of 99.9 J/cm^2 per each session was delivered to the scalp surface for 15 s of irradiation. The irradiation was administered 1x daily for 2 consecutive weeks.
    1. Bring the mice in their home cages to the therapy room, approximately 20 min prior to beginning the treatment.
    2. Connect an electric protector to the wall outlet.
    3. Insert the laser device plug into an electric protector.
    4. Cover the tip of the laser probe with a transparent nylon film in order to prevent any scratching to the surface.
    5. Carefully connect the probe to the channel of the laser device.
    6. Turn on the laser device and wait a few seconds for it to warm up.
    7. Adjust the laser/treatment parameters, including the irradiation time and operation mode.
    8. In the absence of any samples, determine the laser average power by contacting the tip of the probe to the active area of the power meter on the laser device. Record the value.
    9. Repeat the calibration process (step 2.1.8) at least 5x, read the incident powers from the power meter's display screen, and record the values.
    10. Gently hold a mouse by the dorsal skin of the animal's neck in the palm of a hand and immobilize its head.
    NOTE: In the current protocol, the laser probe is placed on the bregma zone, which is ~3 mm rostral to a line drawn between the internal base of the ears.
    11. Lightly place the tip of the probe directly on the scalp at the midline, approximately 3 mm rostral to a line drawn through the anterior base of the ears.
    NOTE: Hold the probe at an approximately 45° angle to the plane of the abdomen.
    12. In order to avoid direct irradiation to the animal's eyes, first contact the tip of the probe on the head and, then, turn on the laser device.
    13. Turn on the laser and stably hold the probe until the completion of the irradiation.
    14. After the end of the therapy, withdraw the laser probe from the head and gently return the mouse to its cage.
    15. Turn off the laser device and disconnect the probe from the device.
    16. Clean the laser probe with an appropriate optical cleaner.
    17. Transfer the mice to the animal facility.

3. Behavioral Tasks

1. **Open-field test**
    1. Assess the locomotor activity of each mouse by the total distance traveled during an open-field test, as described previously[15].

2. **Barnes maze task**
    1. **Apparatus**
    NOTE: The spatial learning and memory task is performed in a Barnes maze[16]. The apparatus used for this neurobehavioral task consists of a circular platform made of black wood (95 cm in diameter) with 20 equidistant, 5 cm-diameter circular holes that are located on the platform, 3 cm from the perimeter. The apparatus is elevated 50 cm from the floor to prevent the animal from climbing down. A movable black plastic escape box (20 cm x 15 cm x 5 cm) is placed under the escape hole. A black maze is used for testing white mice, and a black mat should be placed under the maze when a software tracking system is used.
        1. Place the maze apparatus in the center of a quiet room with bright overhead lighting.
        2. Place a "Do Not Enter" sign on the outside of the task room door.
        3. Attach visual-spatial cues to the perimeter walls.

4. Position a digital video camera above the maze platform.
5. Clean the surface of the maze platform with 70% ethanol to remove unwanted olfactory cues.
6. Add a small amount of bedding from the animal's home cage to the inside of the escape box to serve as an olfactory cue.

2. **Adaptation session**
   1. Bring each mouse to the task room approximately 30 min prior to beginning the experiment, in order for the mouse to become habituated.
   2. Remove the mouse from its cage and gently place the animal in the escape box for 1 min.

3. **Training session**
   NOTE: The training session is repeated for each mouse on 4 consecutive days.
   1. Gently remove the mouse from the escape box.
   2. Place the mouse in the center of the arena; then, place the start chamber on top of the mouse.
   3. Remove the start chamber after 10 s, and allow the mouse to explore the arena for 3 min.
   4. Quietly move to the computer area and put on noise-canceling headphones.
   5. Trigger a negative auditory stimulus consisting of a loud white noise of approximately 80 dB at the platform level and begin videotaping the mouse.
   6. Turn off the white noise and stop videotaping when the mouse enters the escape box. Allow the animal to remain undisturbed in the box for 1 min.
   7. Remove the mouse from the escape box and place it back into its cage.
   8. Repeat steps 3.4.2 through 3.4.7 4x per day, with 3 min intervals between repeated trials.
      NOTE: Between all trials, remove any urine or feces from the arena surface and clean the maze with 70% ethanol.

4. **Probe trial session**
   1. Following the last training trial, 24 h later, remove the escape box from the maze platform and repeat steps 3.4.2 through 3.4.5.
   2. After 3 min, turn off the white noise and stop videotaping. Remove the mouse from the maze arena and place it back into its cage.
   3. After all the animals have been tested, clean the maze platform and the start chamber. Turn off the room lights and remove the "Do Not Enter" sign from the door.
   4. Store the video recordings from the testing sessions to an external hard drive for further analysis.
   5. Set up the video-tracking software program and extract the parameters of interest from the recorded videos, including the latency time to find the target hole during 4 days of training sessions and the time spent in the target quadrant during the probe trial session.

4. Biochemical assessment

1. **ATP levels in the hippocampus**
   1. Deeply anesthetize each mouse with an intraperitoneal injection of a mixture of ketamine (100 mg per gram of body weight) and xylazine (10 mg per gram of body weight).
   2. Decapitate the animal and rapidly remove the brain tissue from the skull.
   3. Dissect out the hippocampus and homogenize the tissue in ice-cold sample buffer (provided by the kit) with a tissue homogenizer.
   4. Immediately centrifuge the homogenate at 2,000 x *g* for 3 min at 4 °C.
   5. Transfer the supernatant to a clean tube.
   6. Assess the hippocampal ATP levels, using the spectrophotometric method as described previously[11].

Representative Results

Statistical analyses

The statistical analysis of data obtained from the Barnes training sessions was analyzed by two-way ANOVA; the other behavioral tests and analysis of hippocampal ATP levels among groups were carried out by one-way ANOVA, followed by Tukey's post hoc test. All data are expressed as means ± the standard error of the mean (SEM), except for the laser transmission data, which are shown as means ± the standard deviation (SD). The significance level was set at $p < 0.05$.

Laser light transmission

The laser light (660 nm) transmission through the skull plus scalp tissue (with a sample thickness of 0.85 ± 0.09 mm) of the aged mice was 15.67% ± 0.87% when a laser beam was focused on the bregma (**Figure 1**). Based on this light transmission, since the initial fluence on the scalp surface was 99.9 J/cm^2 (6.66 [W/cm^2] x 15 [s]), it could be estimated that an approximate fluence of 16 J/cm^2 reached the cortical surface.

The laser transmittance, through a 1 mm slice of aged brain tissue, was 10.10% ± 0.95% (**Figure 1**). From these values, it could be estimated that the light fluence decreased from 16 J/cm^2 at the cerebral cortex tissue level to approximately 1.6 J/cm^2 at a 1 mm depth from the cortical surface.

Open field test

There were no statistically significant differences in locomotor activity in the open-field test among all experimental groups (**Figure 2**).

Barnes maze task

When the escape latency was analyzed during the 4 days of training and with experimental groups during the Barnes maze task, a two-way ANOVA revealed significant effects of day ($p < 0.001$) and group ($p < 0.001$), but not group x day ($p = 0.47$). An intergroup analysis of the data showed that the latency times of the aged-control animals were significantly longer than those of the young-control group on the third ($p < 0.01$) and fourth ($p < 0.001$) days of the training session. However, the latency times of the PBMT-treated aged mice were significantly shorter on the fourth day ($p < 0.05$), compared with the aged-control mice ($p < 0.01$) (**Figure 3**). In the probe trial session, aged-control mice spent significantly shorter times in the target quadrant, compared with the young-control mice ($p < 0.01$). However, the PBMT-treated aged mice spent significantly longer times in the target quadrant as compared with aged-control mice ($p < 0.05$) (**Figure 4**).

Hippocampal ATP levels

The aged-control mice had a significant decrease in hippocampal ATP levels, compared with young-control mice ($p < 0.05$). However, the mean ATP contents in the hippocampus of the aged-PBMT mice were significantly greater than those in the aged-control mice ($p < 0.05$) (**Figure 5**).

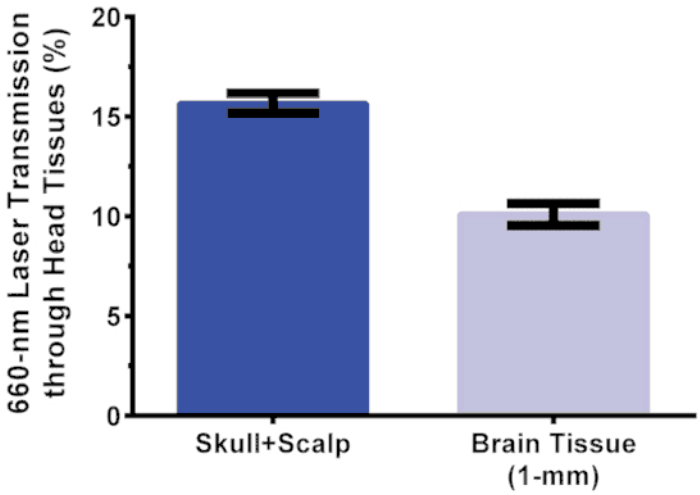

Figure 1: Laser light transmission data through the skull plus scalp and the brain tissue. Data are expressed as mean ± SD. SD = standard deviation. Please click here to view a larger version of this figure.

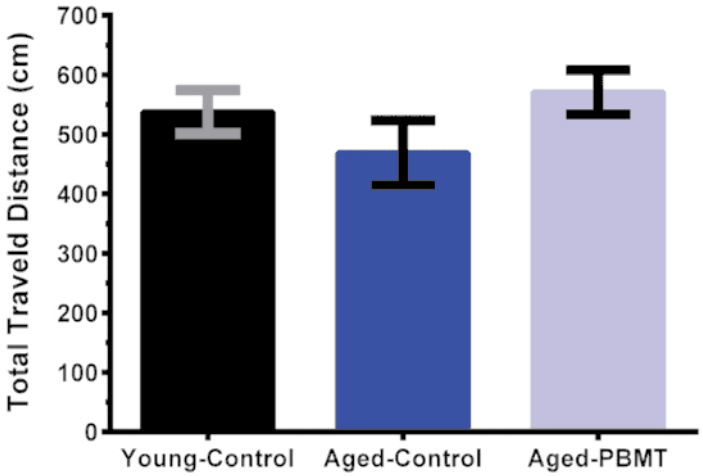

Figure 2: Locomotor activity data from the open-field test. Data are expressed as the mean ± SEM. PBMT = photobiomodulation therapy; SEM = standard error of the mean. Please click here to view a larger version of this figure.

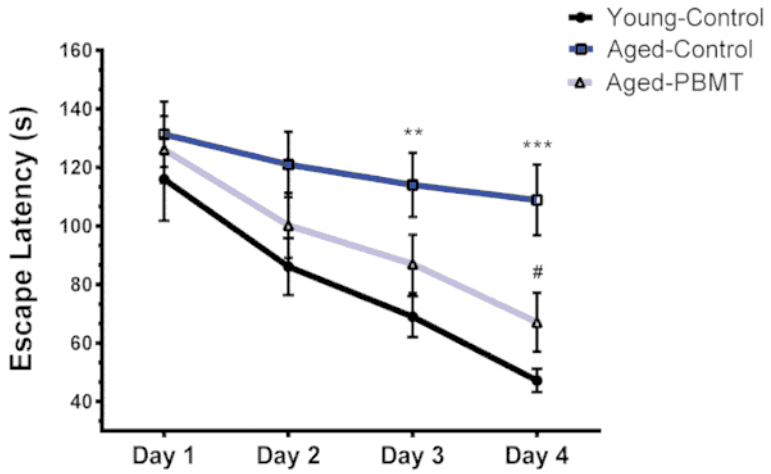

Figure 3: Escape latency for mice groups during the 4 days of training sessions. Values represent the mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$, compared with the young-control mice. $^{\#}p < 0.05$, compared with the aged-control mice. PBMT = photobiomodulation therapy; SEM = standard error of the mean. Please click here to view a larger version of this figure.

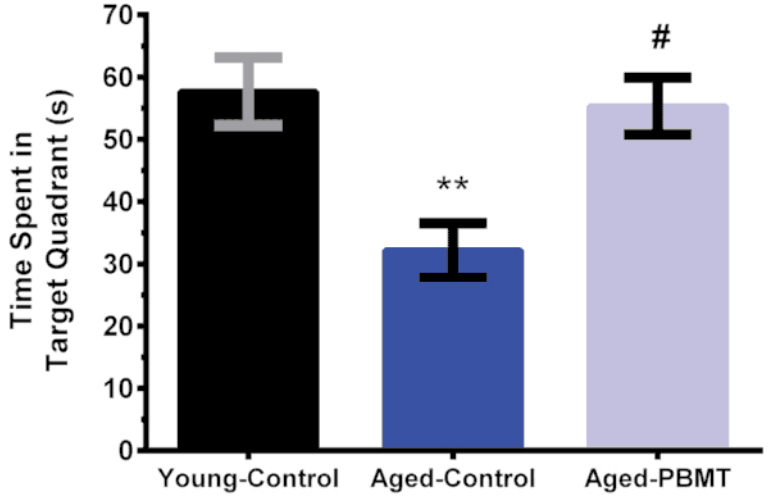

Figure 4: Time spent in the target quadrant in the probe session, in different groups. Values represent the mean ± SEM. $^{**}p < 0.01$, compared with the young-control mice. $^{\#}p < 0.05$, compared with the aged-control mice. PBMT = photobiomodulation therapy; SEM = standard error of the mean. Please click here to view a larger version of this figure.

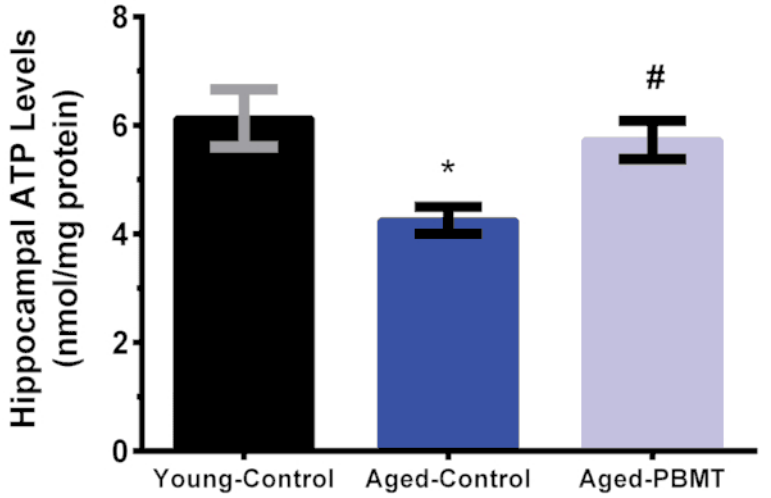

Figure 5: ATP contents in the hippocampus tissue. Values represent the mean ± SEM. $^{*}p < 0.05$, compared with the young-control mice. $^{\#}p < 0.05$, compared with the aged-control mice. PBMT = photobiomodulation therapy; SEM = standard error of the mean. Please click here to view a larger version of this figure.

Discussion

We describe a protocol for conducting a transcranial PBMT procedure in mice. This protocol is specifically targeted to neuroscience laboratories that perform photobiomodulation research focused on rodents. However, this protocol can be adapted to other laboratory animals that are frequently used in the neuroscience field, such as rabbit, cat, dog, or monkey.

Currently, there is increased interest in investigating transcranial PBMT with red/NIR lasers and LEDs. In order to successfully carry out the entire treatment procedure in rodents, there are a few essential steps to consider.

First, it is critical that, before attempting any treatments in live animals, the light penetration is precisely measured through the animal head tissues in order to deliver an optimum photon dosage (J/cm^2).

Second, based on which brain regions are affected by pathology and targeted for treatment, several parameters need to be optimized, to maximize light penetration and increase the likelihood of positive outcomes. These include irradiation time, treatment interval, applied irradiance, and fluence. For example, in the aged animal models, it is crucial to deliver a sufficient radiation dose to the brain hippocampus and frontal cortex because these regions are linked to age-related pathologies[2]. An optimal fluence rate in target tissues is another important factor in PBMT. Most researchers discuss factors that affect light transmission but often neglect to consider that a biphasic response in brain target tissues exists not only for fluence (J/cm^2) but also for the rate of fluence delivery. In other words, a fluence of 1 J/cm^2 delivered over 1 min is not equivalent to 1 J/cm^2 delivered over 1 s[17,18].

There are several additional factors that should also be considered before executing transcranial PBMT studies. Transcranial PBMT in rodents is commonly applied using laser or LEDs probes with the probe tip size scaled to the animal's brain size. For application in rodents, moderate-power lasers (with a power output of ≤ 500 mW) can deliver a great amount of light energy in a short time and reduce both treatment time and treatment-related stress to the animal. Although Class 3B lasers do not have significant photothermal effects in PBMT dosage ranges (≤20 J/cm^2), cooling the scalp surface with a transparent optical substance, such as ice or gel, is recommended during transcranial application.

In some experimental transcranial PBMT studies, optical fiber is used instead of a laser or LED probe, due to its advantages for irradiation of a specific small area on the head. For example, in focal ischemic stroke, TBI, and PD models, an accurate irradiation of the damaged area is warranted. However, optical fibers generally have a small beam area, so this will affect the total amount of energy delivered in one session and will require researchers to repeat the procedure in more than one spot to compensate for the decreased area. In most experimental transcranial PBMT studies, irradiation of the head is conducted in the alert, unanesthetized animal. In order to ensure animal stability, manual head holding and the use of restraint devices are recommended. In the manually holding method, due to the fact that that animal might move suddenly and possibly moving its head away from the irradiation zone, a portion of irradiated light might be wasted. Furthermore, both methods can induce extra stress to the animal and could be a potential confounding factor. In some cases, the irradiation procedure is performed in an anesthetized animal. It should be noted that too much anesthesia can adversely affect the experimental outcomes in neuroscience studies. Therefore, a shorter irradiation interval should be carefully considered in these types of experiments.

In the present study, we first measured the transmission of light through the skull plus scalp of male BALB/c aged mice to determine the amount of 660 nm laser energy that reached the cortical surface. The results indicated that 16% of the initial light on the surface of the unshaved scalp was transmitted through to the brain. Transmission data from other laboratories in male BALB/c mice have shown that only 1.2% of 670 nm laser light was able to penetrate the intact skull[19]. It has also been reported that approximately 90% of 670 nm LED light is attenuated inside the mouse cranium[20].

The effective neurostimulatory dosage of the red laser on the cortical tissue level was confirmed in a previous study performed in our laboratory[11]. We showed that a daily cortical fluence of an 8 J/cm^2 laser at 660 nm has procognitive effects in a mouse aging model[11]. In the therapy section of the current study, to deliver approximately 16 J/cm^2 to the cortical surface, we needed to leave the laser on for 15 s, which was tolerated by the mice. In the present work, we also measured the light power received at the hippocampus surface. Based on the results of the experiment, an approximate value of 10% was measured as laser transmittance through a 1 mm slice of aged brain, corresponding to a light fluence of approximately 1.6 J/cm^2 reaching 1 mm deep from the cortical surface. Data from other studies using BALB/c mouse brain have revealed a 65% reduction of 670 nm LED light intensity across each millimeter of cerebral tissue[21]. It has also been shown that approximately 2.5% of 670 nm LED light reaches a depth in the brain tissue of 5 mm, the distance from the skull surface to the substantia nigra compacta (SNc) area[22].

The hippocampus plays a cardinal role in the consolidation of spatial memory[23]. In fact, the hippocampal bioenergetics capacity is associated with spatial navigation memory and learning. The findings presented here suggest that a light dosage of approximately 1.6 J/cm^2 at the hippocampus level could be sufficient to produce an improvement of spatial memory outcomes in aged mice. It could be presumed that an enhancement of memory performance in the cognitive-behavioral task (Barnes maze) could be due to an improvement of hippocampal energy metabolism that appears to be induced by a red laser at a specific wavelength of 660 nm.

Disclosures

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Sciences to conduct a study on transcranial photobiomodulation for generalized anxiety disorder. The other authors have no conflicts of interest to disclose.

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