Transcranial low-level laser therapy improves brain mitochondrial function and cognitive impairment in D-galactose–induced aging mice

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A B S T R A C T

Mitochondrial function plays a key role in the aging-related cognitive impairment, and photoneuromodulation of mitochondria by transcranial low-level laser therapy (LLLT) may contribute to its improvement. This study focused on the transcranial LLLT effects on the D-galactose (DG)–induced mitochondrial dysfunction, apoptosis, and cognitive impairment in mice. For this purpose, red and near-infrared (NIR) laser wavelengths (660 and 810 nm) at 2 different fluencies (4 and 8 J/cm²) at 10-Hz pulsed wave mode were administrated transcranially 3 d/wk in DG-received (500 mg/kg/subcutaneous) mice model of aging for 6 weeks. Spatial and episodic-like memories were assessed by the Barnes maze and What-Where-Which (WWfWhich) tasks. Brain tissues were analyzed for mitochondrial function including active mitochondria, adenosine triphosphate, and reactive oxygen species levels, as well as membrane potential and cytochrome c oxidase activity. Apoptosis-related biomarkers, namely, Bax, Bcl-2, and caspase-3 were evaluated by Western blotting method. Laser treatments at wavelengths of 660 and 810 nm at 8 J/cm² attenuated DG-impaired spatial and episodic-like memories. Also, results showed an obvious improvement in the mitochondrial function aspects and modulatory effects on apoptotic markers in aged mice. However, same wavelengths at the fluency of 4 J/cm² had poor effect on the behavioral and molecular indexes in aging model. This data indicates that transcranial LLLT at both of red and NIR wavelengths at the fluency of 8 J/cm² has a potential to ameliorate aging-induced mitochondrial dysfunction, apoptosis, and cognitive impairment.

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1. Introduction

Aging is a complicated and multifactorial biological process that is characterized by a gradual decline in many organ functions. Brain aging is one of the important aspects of the aging progression which is associated with behavioral deficits, memory loss, and cognitive impairment (Grady, 2012). Mitochondria are a main apparatus in the cell respiratory chain, the dysfunction of which during aging is associated with cognitive impairment (Bishop et al., 2010). The evidence also suggests that perturbation in mitochondrial markers such as adenosine triphosphate (ATP) and membrane potential reduction or reactive oxygen species (ROS) generation, play the active role in the brain aging and age-related cognitive impairments (Jang and Van Remmen, 2009; Navarro et al., 2002). Furthermore, an imbalance between ROS synthesis and antioxidant activities during aging, triggers mitochondrial apoptotic pathways via Bax-, Bcl-2—, and caspase-3—dependent mechanisms in the brain which finally leads to neuronal death (Niu et al., 2010; Pollack et al., 2002).

D-galactose (DG) is a reducing sugar in the body that can be metabolized at the normal physiological concentrations. Nevertheless, at high concentration, it is able to convert into hydrogen peroxide and aldose which results in the generation of ROS and causes oxidative stress (Ho et al., 2003). Chronic administration of DG has been used to induce brain oxidative stress and cognitive dysfunction to mimic natural aging in rodent (Lei et al., 2008; Pourmemar et al., 2017; Tsai and Yin, 2012; Zhang et al., 2008). Moreover, long-term administration of DG triggers mitochondrial...
dysfunction and cytochrome C release which eventually cause neuronal apoptosis (Cui et al., 2006; Lan et al., 2012). Many studies showed that mitochondria-protective components are able to improve the functioning of the nervous system and delay brain aging (Liu and Ames, 2005; Liu et al., 2002). Furthermore, nonpharmaceutical therapies for treatment or prevention of age-related disorders have also aroused significant interest among researchers in the recent years (Gonzalez-Lima et al., 2014; Salgado et al., 2015).

Transcranial low-level therapy (LLLT) with far-red to near-infrared (NIR) light is a novel noninvasive modality used to modulate neuronal activity in animal and human studies (Barrett and Gonzalez-Lima, 2013; Chung et al., 2012; Salehpour and Rasta, 2017; Xuan et al., 2014). Over the past decade, transcranial LLLT has been suggested to treat different neurodegenerative (Lapchak, 2012; Purushothuman et al., 2014) and psychiatric disorders (Salehpour et al., 2016; Schiffer et al., 2009), given its good safety profile and efficiency. Light at specific wavelengths between 600 and 1100 nm has maximum penetration depth in skull (Bashkatov et al., 2006; Firbank et al., 1993). In addition, this spectral region corresponds to light absorption by mitochondrial chromophores such as cytochrome c oxidase (CCO), which is the terminal enzyme of the respiratory chain (KarU, 2014). This photoabsorption process causes more electron transfer through the mitochondrial respiratory chain and leading to increasing of ATP production (Mochizuki-Oda et al., 2002; Oron et al., 2007). Consequently, further secondary cellular effects such as modulation of ROS and increase in the mitochondrial membrane potential (MMP) could occur (de Freitas and Hamblin, 2016). There is some evidence that shows photoneuromodulation can exert neuroprotective effects via reduction of neuronal cells apoptosis in the brain cognition-related compartments (De Taboada et al., 2011; Grillo et al., 2013; Lu et al., 2016; Uozumi et al., 2010). Also, a clinical study has showed preognitive effects of photoneuromodulation by red/NIR transcranial LLLT in traumatic brain injury (TBI) patients (Naeser et al., 2011). An improvement in the neurologic condition of patients in a persistent vegetative state following NIR light treatment was also reported (Nawashiro et al., 2012). Furthermore, NIR transcranial LLLT enhanced some aspects of cognition in healthy subjects (Barrett and Gonzalez-Lima, 2013).

The aim of this study is to evaluate the effects of transcranial LLLT using red and NIR lights on the DG-induced learning and memory impairment and possible impact of mitochondrial dysfunction and apoptosis.

2. Materials and methods

2.1. Animals

Seventy-two adult male BALB/c mice from the animal center of Tabriz University of Medical Sciences (TUOMS), weighing 25–30 g were used for this study. Before and during the study, mice were socially housed in standard cages (5 in each cage) and kept on a 12/12-h light/dark cycle at a temperature of 23 °C ± 2 °C, with free access to tap water and standard pellet food ad libitum. All animals were weighed weekly during the experiment. All of the procedures were conducted 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 TUOMS (No: IR.TBZMED.REC.1395.445).

2.2. Experimental design

After a week of adaptation, mice were randomized into the control (n = 12) and aging-induced groups (n = 60). The control group received normal saline (0.9% NaCl, 0.2 mL/mice). Aging was induced by injection of 500 mg/kg of DG (Sigma, St. Louis, MO, USA) through the subcutaneous (s.c.) route once daily for 6 weeks. Separate groups of DG-injected mice received sham, red, or NIR laser irradiation transcranially at 2 fluencies of 4 or 8 J/cm², 3 d/wk, which was named as following: (1) DG+sham (DG injection with sham laser irradiation); (2) DG+red4 (DG injection with 4 J/cm² of red laser irradiation); (3) DG+NIR4 (DG injection with 4 J/cm² of NIR laser irradiation); (4) DG+red8 (DG injection with 8 J/cm² of red laser irradiation); and (5) DG+NIR8 (DG injection with 8 J/cm² of NIR laser irradiation).

All tests and analysis were performed by an experimenter who was unaware of the entity of experiments. Fig. 1 shows the study design, including procedures and time periods.

2.3. Laser treatment

A commercially available therapeutic Diode laser (Thor Photomedicine, Chesham, UK) consisting of a red probe (AlGaInP) with 660 nm wavelength and an NIR probe (GaAlAs) with 810 nm wavelength were used for irradiation sessions. The laser was operated at the maximum output power of 200 mW, a power density of 4.75 W/cm², and 10-Hz pulsed wave mode with 88% duty cycle for both probes. For laser irradiation, each mouse was held firmly by the dorsal skin of the neck, and its head was carefully located. The laser probe was positioned over the head in contact with the scalp that covered the entire mouse brain. To determine the delivered dose on the cortex, transmission values of red and NIR light through the fresh postmortem scalp and skull were measured by a 7-mm diameter power meter sensor of the laser device. Average energy densities (fluencies) of 4 and 8 J/cm² per each session were delivered to the animal’s cortical surface with both red and NIR lasers. Transcranial LLLT was administered 3 times a week, with a minimum of 48 hours between the sessions, over a period of 6 weeks. Laser treatment was done at least 5 hours after DG injections. Sham-treated mice (DG+sham laser group) were undergone identical to laser treatment procedure except that the laser device was not turned on.

2.4. Barnes maze test

2.4.1. Apparatus

The spatial learning and memory test was performed in a Barnes maze (Sunyer et al., 2007). The maze was made of wood and consisted of a circular platform 100 cm in diameter raised 50 cm from the ground. Twenty circular holes, 5 cm in diameter, were located through the platform 3 cm from the perimeter. A movable plastic escape box (20 × 15 × 5 cm) was placed under the escape hole. The maze was placed in a room where distinct spatial cues were located on the walls. An 80-dB white noise was used as negative stimuli during the test.

![Fig. 1. Timescale of D-galactose or normal saline administration, laser treatments, behavioral tests, and sampling.](www.mindthegraph.com) Abbreviation: LLLT, low-level laser therapy.
2.4.2. Task sessions

The task consisted of 3 sessions including adaptation, training, and probe sessions which lasted for 5 days. The first day consisted of an adaptation session and 4 trials and 3 subsequent days consisting of 4 trials per day, each separated by a 3-minute interval. In the adaptation session, a mouse was located in the center of the maze in a start chamber. After 10 seconds, simultaneous with white noise turning on, the start chamber was removed and the mouse was set free to explore the maze for 3 minutes to enter into the escape box. When the mouse entered the escape box, the white noise was turned off and the animal was allowed to remain in the box for 1 minute. On the fifth day, a probe trial was carried out without the escape box. Between all test sessions, the maze and escape box were cleaned with 70% alcohol to remove odor cues. A digital video camera was secured above the maze. The latency time (the period it takes the animal to find the escape box), and time spent in error holes (the sum of periods that animals spent around all of the holes except target hole) during the training session, and time spent in the target quadrant (where the escape box was located during training session), and relative error time (a ratio of time spending in target hole to time spending in error holes) during the probe session were extracted using a video tracking program Etho Vision (Noldus, The Netherlands).

2.5. What-Where-Which (WWWhich) task

2.5.1. Apparatus

The episodic-like memory was evaluated by WWWhich task (Davis et al., 2013). The experimental apparatus consisted of 2 open-field arena made of Plexiglas (30 x 30 x 25 cm). The arenas were altered into two different contexts. In context 1, the floor was attached to a LEGO base plate and the walls were painted matte black. While context 2 consisted of a smooth matte black floor and walls were painted with black and white vertical stripes. All objects for testing were assembled from LEGO.

2.5.2. Task sessions

After 24 hours of the last treatments, the WWWhich task was performed. The task consisted of 3 sessions including habituation, exposure, and test sessions (Davis et al., 2013). In the habituation session, mice were habituated singly to each context for 5 minutes for 1 day, and locomotor activity was recorded through the session in one of the contexts (same context for all animals). During the test sessions, mice were faced with one of the A, B, C, or D situations (object/contextual combination) each day.
exposure sessions, 2 different objects (A and B) were first placed in the context 1 and mice were given 3 minutes to explore it. Next, mouse was placed in the holding cage for 30 seconds and the objects reversely (B and A) placed in context 2. Next, mice were allowed to explore it for 3 minutes and then mice were returned to the holding cage. The test session was performed after 5 minutes interval. Mice were encountered with 2 copies of one of the objects (A or B) were placed in one of the 2 contexts (1 or 2) for 3 minutes. Exposure and test sessions were performed on one trial for 4 consequence days with new objects for each day (Fig. 2). Exploration was defined as time spent with the nose oriented toward and within 1 cm of the object. Circling or sitting on the object were not considered as exploratory behavior. The episodic-like memory (object/contextual combination) was evaluated by displacement index (DI). The DI was calculated for each group as follow: DI = (N – F)/(N + F), where N is the exploration time of the novel object and F is the exploration time of the familiar object. After each trial, the contexts and subjects were cleaned thoroughly using 70% ethanol to eliminate the presence of any olfactory cues. All experimental sessions were videotaped from above and behavioral data were extracted from the videos using a video-tracking program Etho Vision (Noldus, The Netherlands).

2.6. Brain mitochondrial isolation

Mice from all experimental groups were decapitated after deep anesthesia with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), at 24 hours after last behavioral test. The whole brain (excluding cerebellum) were quickly taken out from the skull and placed in ice-cold isolation buffer containing 200-mM mannitol, 70-mM sucrose, 10-mM HEPES, and 2-mM EDTA, pH 7.5. Then, entire brain tissues were homogenized in ice-cold extraction buffer containing 2 mg/mL albumin (10% w/v). The samples were centrifuged at 600 g in 4 °C for 15 minutes. The pellet was resuspended in storage buffer containing 10-mM HEPES, pH 7.4, 250-mM sucrose, 1-mM ATP, 0.08-mM ADP, 5-mM sodium succinate, 2-mM K_2HPO_4, and 1-mM DTT. Protein in tissue or mitochondrial suspensions was determined by Bradford method (Bradford, 1976).

2.7. Active mitochondria levels measurement

Mitochondria labeling were done using MitoTracker probe (Cell signaling, USA) in the brain cells. The probe passively diffused across the plasma membrane and accumulates in active mitochondria. A 100 mg of entire brain tissue was cut into 2–4 mm pieces and an appropriate amount of trypsin (0.1%) was added. Tissue fragments were washed 3 times with PBS containing 25% bovine serum albumin. A single cell suspension was obtained by trituration of fragments in DMEM (Life technologies, Germany) containing 10% fetal calf serum (Integro, The Netherlands), 25,000 IU/L penicillin, and 25 mg/L streptomycin (Sigma, Germany). The MitoTracker Green was directly added into growth media at a concentration of 200 nM and incubated for 15 minutes. The pellet was resuspended in storage buffer containing 200-mM mannitol, 70-mM sucrose, 10-mM HEPES, and 2-mM EDTA, pH 7.5. Then, entire brain tissues were homogenized in ice-cold extraction buffer containing 2 mg/mL albumin (10% w/v). The samples were centrifuged at 600 g in 4 °C for 15 minutes. The pellet was resuspended in storage buffer containing 10-mM HEPES, pH 7.4, 250-mM sucrose, 1-mM ATP, 0.08-mM ADP, 5-mM sodium succinate, 2-mM K_2HPO_4, and 1-mM DTT. Protein in tissue or mitochondrial suspensions was determined by Bradford method (Bradford, 1976).

2.8. Mitochondrial membrane potential assay

Change of MMP (∆Ψ_m) in brain cells was evaluated using JC-1 dye (Mitochondria Staining Kit [Sigma-Aldrich, St. Louis, MO, USA]). In normal condition, JC-1 concentrates in the mitochondrial matrix, where it forms red fluorescent aggregates. Any event that dissipates the MMP leads to shifting from red to green fluorescence (JC-1 monomers). Stain and buffers were prepared based on the kit instruction. Mitochondria were suspended in JC-1 stain in the final concentration of 0.6 μM. Fluorescence intensity in samples was measured by fluorimetry method. The ∆Ψ_m calculated by the ratio of red (λ_ex = 490 nm, λ_em = 590 nm) to green (λ_ex = 485 nm, λ_em = 530 nm) fluorescence intensity in the mitochondria suspension and normalized to samples protein.

2.9. Mitochondrial cytochrome c oxidase activity

The CCO activity was determined using the commercial kit (CYTOCOX1, Sigma, USA). The assay in this kit is based on Lemberg method (Lemberg, 1969) in which spectrophotometrically followed the decrease in absorbance at 550 nm of ferricytochrome c caused by its oxidation to ferricytochrome c by CCO. Cytochrome c was mixed with a 0.1-M DTT solution to prepare its reduced form. The reduction was confirmed by the change of color from dark orange-red to pale purple-red. Change in absorbance per min was measured and results were expressed as nmol per min per mg protein.

2.10. ATP colorimetric assay

The colorimetric assay kit (MAK190, Sigma, USA) was used for measuring of ATP. Briefly, 10–mg tissue was homogenized in 100 μL of ATP assay buffer (provided in the kit). After adding ATP probe in the presence of the developer (provided in the kit), the absorbance at 570 nm were measured. The standard curve was used to obtain the ATP concentration and it was presented in nmol per mg protein.

2.11. Brain mitochondrial ROS production

The fluorescent dye dichlorodihydrofluorescein diacetate (DCFDA) was used to determine the ROS production level in brain mitochondria (Novalija et al., 2003). The mitochondria were incubated with 2–μM DCFDA at 37 °C for 20 minutes. The fluorescence intensity was determined (λ_ex = 485 nm, λ_em = 530 nm) in a fluorescence microplate reader. The ROS levels were represented as fluorescence intensity and normalized to samples protein.

2.12. Western blot

Expression of proteins in the brain tissue was analyzed by Western blot method as described previously (Sadigh-Etehadt et al., 2015). Briefly, brain tissue was homogenized in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail. Proteins were separated using 12.5% polyacrylamide gel and transferred onto a polyvinylidenedifluoride membrane (Roche, UK). Then, the membranes were incubated with anti-Bax (1:500, sc-493), Bcl-2 (1:500, sc-492), and caspase-3 (1:500, sc-7148) antibodies. Next, the membrane was incubated with the horseradish peroxidase–conjugated secondary antibody (1:5000, sc-2004). Finally, membranes were placed in ECL prime Western blotting detection reagent (Amersham, UK) and the signals visualized by exposure to autoradiography film (Kodak, USA). For internal control, anti–β-actin (1:500, sc-130656) antibody was used. All antibodies were purchased from Santa Cruz (USA). The signal intensity of each band was quantitated using ImageJ 1.62 software (National Institutes of Health, USA).
2.13. Statistical analysis

Descriptive data were expressed as mean ± SEM. Comparison of different groups was carried out by a one-way ANOVA followed by the post hoc Tukey test. All analyses were performed using IBM SPSS statistics software (version 22 for Windows; SPSS Inc, USA). p-values <0.05 were considered significant in all comparisons.

3. Results

3.1. Barnes test

3.1.1. Training session

The one-way ANOVA followed by Tukey test showed that DG-treated sham mice were delayed in finding the escape box compared with control group on the third and fourth days (p < 0.05, and p < 0.01, respectively) of the training session. Both the red and NIR laser treatments (at a fluency of 8 J/cm²) significantly reduced latency time on the third day of training compared with DG-sham mice (p < 0.01). On the fourth day of the training session, significant differences were also observed in 4 J/cm² (p < 0.05) and 8 J/cm² (p < 0.01) of red and NIR laser groups compared with DG-sham group (Fig. 3A).

In addition, as shown in Fig. 3B, chronic administration of DG in sham group significantly increased time spending in error holes over the last 2 days of the training session (p < 0.05, and p < 0.01, respectively). Time spent in error holes significantly diminished on days 3 (p < 0.05 for 4 J/cm²; and p < 0.01 for 8 J/cm²) and 4 (p < 0.01 for both doses) of the training session in red and NIR laser groups compared with DG-sham group.

3.1.2. Probe session

As shown in Fig. 4A, over the course of probe testing, DG-treated sham mice spent the significantly shorter period of time in the target quadrant compared with control mice (p < 0.01). Both red and NIR lasers at a fluency of 8 J/cm² significantly increased time spending in the target quadrant compared with DG-sham group (p < 0.05).

Data from mean relative error time showed a significant difference between DG-sham and control groups (p < 0.01). However, laser treatments using red and NIR light (8 J/cm²) significantly decreased relative error time during probe session (p < 0.01) (Fig. 4B and C).

3.2. What-Where-Which test

There was no significant difference in the total observation time (sum of time spent exploring both novel and familiar objects) among groups (p > 0.05) (Fig. 5A). Further, no significant difference was observed for locomotor activity among groups (p > 0.05; Fig. 5B). Chronic administration of DG significantly impaired the DI compared with control mice (p < 0.01). However, laser treatment using red and NIR light (8 J/cm²) significantly rescued the performance of DG-treated mice (p < 0.01) (Fig. 5C and D).

![Fig. 4.](image-url)
3.3. Active mitochondria levels

MitoTracker Green staining was used to determine active mitochondria levels. There was lower active mitochondria in DG-treated sham mice than the control group \((p < 0.05)\). Both red and NIR laser treatments \((8 \, \text{J/cm}^2)\) significantly augmented the abundance of active mitochondria in DG-treated mice \((p < 0.05)\). Despite the increase in the amount of active mitochondria following NIR laser treatment \((4 \, \text{J/cm}^2)\), this enhancement was not statistically significant \((p > 0.05)\) (Fig. 6A and B).

3.4. Mitochondrial membrane potential determination

An obvious reduction in MMP was observed in the DG-treated sham group \((p < 0.01)\), whereas the MMP was significantly higher in the red and NIR laser treatments \((8 \, \text{J/cm}^2)\) compared with the DG-sham group \((p < 0.01)\) (Fig 7A).

3.5. Cytochrome c oxidase activity

The CCO activity significantly reduced in the DG-treated sham mice \((p < 0.01)\). A significant increase in CCO activity was detected in DG-treated mice exposed to red and NIR laser \((8 \, \text{J/cm}^2)\) \((p < 0.01)\). Irradiation with 4 J/cm\(^2\) of red and NIR laser did not increase the activity of CCO as compared with the DG-treated sham group \((p > 0.05)\) (Fig. 7B).

3.6. ATP levels

Fig. 7C shows that the DG-treated mice had a significant decrease in ATP levels compared with control mice \((p < 0.05)\). Red and NIR laser treatments \((8 \, \text{J/cm}^2)\) significantly rescued ATP to normal levels \((p < 0.05)\), whereas both the lasers at 4 J/cm\(^2\) were least effective and showed a minimal benefit in reversing the ATP content to control level \((p > 0.05)\).

3.7. ROS assessment

A strong production of ROS was observed in DG-treated sham mice as compared with the control group \((p < 0.01)\). Data in Fig. 7D show that the laser treatment using red and NIR lights at both 4 and 8 J/cm\(^2\) significantly reduced ROS production compared with the DG-sham group \((p < 0.01)\).

3.8. Western blotting

3.8.1. Bax/Bcl-2 levels

As shown in Fig. 8A and C, chronic administration of DG increased Bax levels and decreased Bcl-2 levels, so that the ratio of Bax to Bcl-2 was significantly increased compared with the control group \((p < 0.01)\). The remarkable reduction in Bax to Bcl-2 ratio following laser treatment using both red and NIR lights \((8 \, \text{J/cm}^2)\) was observed \((p < 0.01)\). However, both red and NIR laser irradiation at a fluency of 4 J/cm\(^2\) was not shown significant effects on attenuating the DG-induced increase in the ratio of Bax to Bcl-2 \((p > 0.05)\).

3.8.2. Caspase-3 levels

Chronic administration of DG significantly increased caspase-3 levels in DG-sham group compared with the control group \((p < 0.01)\). The notable drop in the caspase-3 protein levels following red and NIR laser treatments \((8 \, \text{J/cm}^2)\) was observed \((p < 0.01)\). On the other hand, red and NIR laser treatments at a fluency of 4 J/cm\(^2\) did not significantly decrease the caspase-3 levels compared with the DG-treated mice \((p > 0.05)\) (Fig. 8B and C).
4. Discussion

Since the role of mitochondria as a main target of bioenergetics and oxidative damage in the neuronal aging process and consequent cognitive impairment (Sastre et al., 2002), the present study focused on the photoneuromodulation response of mitochondrial function, apoptosis, and different learning and memory aspects in DG-induced mice aging model.

Brain bioenergetics impairment, active mitochondria reduction, CCO dysregulation, and ATP decline have been suggested as mechanisms underlying memory and cognitive impairment during aging (Navarro et al., 2002; Reddy, 2009). Recently, regulation of neuronal function following LLLT have been shown in cell cultures (Huang et al., 2013), animal models (Lu et al., 2016), and clinical studies (Barrett and Gonzalez-Lima, 2013; Blanco et al., 2017). To investigate the neuroenhancement effects of laser in terms of wavelengths, 2 different wavelengths of 660 nm (red) and 810 nm (NIR) were applied, which matches to the peak absorption spectrum of CCO (Karu and Kolyakov, 2005).

Results showed chronic administration of DG significantly reduced the active mitochondria marker. Previous studies in Alzheimer’s disease (AD) had also shown the loss of brain mitochondrial number (Hirai et al., 2001; Xie et al., 2013). Respiration and ATP production in brain is correlated with the numbers of active mitochondria (Moyes and Battersby, 1998; Perkins and Ellisman, 2011) and its diminution could lead to a decline in energy metabolism in aged conditions (Stoll et al., 2011). Red and NIR lights (8 J/cm²) increased the abundance of active mitochondria in aged mice. Higher concentration of active mitochondria in ischemic fibroblast cells irradiated with 660 nm light (5 J/cm²) has been also reported (Houreld et al., 2012).

Mitochondria are the primary site of photon absorption in cellular level, and CCO is the key molecule which is responsible for this photoreaction (Karu and Afanaseva, 1995). Photo-neuromodulation is based on light absorption by the metal centers of CCO, which results in acceleration of mitochondrial electron transfer and, consequently, ATP production (Karu, 2010).

In this study, administration of DG resulted in deficits in CCO activity, which was in agreement with previous reports (Prakash and Kumar, 2013; Zhang et al., 2010). On the other hand, red and NIR lasers (8 J/cm²) caused a significant increase in CCO activity. Also, augmentation of CCO activity in neuronal mitochondria following light irradiation has been shown (Lu et al., 2016; Purushothuman et al., 2014; Rojas et al., 2012).

The reduced CCO activity and subsequent low ATP production in the hippocampus of the normal aged brain were demonstrated (Bertoni-Freddari et al., 2004). Our data showed that red and NIR lights (8 J/cm²) could reverse the ATP levels to normal and compensate energy deficiency. This is consistent with the previous studies showing that LLLT increase ATP production in AD (De Taboada et al., 2011; Lu et al., 2016; Sommer et al., 2012) and TBI (Dong et al., 2015) models. It should be stated that the effectiveness of both the 660 and 810 nm lights in the improvement of CCO activity and ATP content in this study is in good agreement with Wong-Riley et al. study which demonstrated a direct relationship between light irradiation at 670 and 830 nm and CCO absorption.
spectrum in primary neurons (Wong-Riley et al., 2005). For normal memory and cognitive functions, synaptic assembly and activity, ATP supply has a crucial role (Reddy, 2009). Due to the high concentration of mitochondria in the neuronal tissue (Schwarz, 2013), it seems that mitochondrial CCO could be a potential mediator in photoneuromodulation of brain bioenergetics and cognitive improvement.

Administration of DG decreased brain MMP levels which were consistent with the previous in vitro (Shen et al., 2014) and in vivo (Du et al., 2015; Zhu et al., 2014) reports. The age-related oxidative stress is believed to be the main cause of MMP collapse (Hagen et al., 1997) and it causes cell respiratory deficiency and decline of ATP synthesis (Beal et al., 1993). In this study, red and NIR lights (8 J/cm²) increased the MMP levels. This is in line with other studies.
showing that NIR (~810 nm) rescue MMP decline that is induced by beta-amyloid (Aβ) (Lu et al., 2016), oxidative stresses (Huang et al., 2013), and oxygen-glucose deprivation (Yu et al., 2015a). Absorption of light by mitochondrial inner membrane enzymes thereby enhancing the MMP would be a possible explanation for this beneficial effect (Gavish et al., 2004).

Mitochondria are pacemakers of cellular aging due to the ongoing generation of free radicals such as ROS (Leutner et al., 2001). Increased ROS production affects neurons in part by damaging mitochondrial components, which ultimately results in senescence (Loeb et al., 2005). Augmentation of mitochondrial ROS production following administration of D G has been shown (Hao et al., 2014; Yu et al., 2015a; Zhang et al., 2010). Our data from ROS levels in the brain of DG-treated mice also showed a remarkable increase. In contrast, NIR and red lights suppressed mitochondrial ROS production. Our findings are in line with previous studies reporting that NIR laser (810 nm, 3 J/cm²) (Huang et al., 2013) and red LEDs (635 nm, 18 J/cm²) (Lim et al., 2011) reduced brain ROS levels. Although the biphasic dose response for LLLT-induced regulation of ROS levels has been reported (Huang et al., 2009; Sharma et al., 2011), exact correspond cellular mechanism is unknown. While, reduction in ROS levels may occur via enhancement of total antioxidant capacity in neuronal cells (Lu et al., 2016), studies showed that apoptosis initiate by releasing cytochrome c from mitochondria into the cytoplasm via a caspase-3 activity following a reduction in ATP levels and MMP (Green and Kroemer, 2004; Hengartner, 2000). The increased Bax/Bcl-2 ratio also promotes the activation of caspases and results in cell apoptosis (Qian et al., 2008). In this study, administration of DG increased Bax/Bcl-2 ratio and caspase-3 level, which is in consistent with previous findings (Gao et al., 2015; Lu et al., 2010; Ullah et al., 2015; Zhu et al., 2014).

The red and NIR lights at 8 J/cm², but not 4 J/cm² notably regulated Bax, Bcl-2, and caspase-3 activity and rescue neurons from apoptosis. Thus, it suggests that red and NIR LLLT at the optimum dose of 8 J/cm² has a potential anti-apoptotic effect. The possible reason for this anti-apoptotic effect may be associated with the increasing effect of LLLT on the MMP, which avoid the opening of the mitochondrial permeability, cytochrome c release, and finally, caspase-3 activation (Xuan et al., 2014). Further, the ability of LLLT to reduce the ROS production and consequent decrease in apoptotic signaling may be key to realizing the neuroprotective potential of photoneuromodulation. The anti-apoptotic effects of light were also reported in Aβ (Duan et al., 2003; Zhang et al., 2012) and other neurotoxins in vitro (Liang et al., 2006; Yu et al., 2015b) or transient cerebral ischemia (Yip et al., 2011) and TBI (Xuan et al., 2014) in vivo models.

Impaired spatial learning and memory have been reported in animal DG brain aging model (Kumar et al., 2010; Lu et al., 2010; Wei et al., 2005). In the present study, administration of DG impaired spatial and episodic-like memories. Michalikova et al. showed improved working memory of normal middle-aged mice in a 3D maze following NIR LED irradiation (Michalikova et al., 2008). The present study showed that red and NIR lights (8 J/cm²) significantly reversed spatial learning and memory impairment in Barnes test. These are in line with recent report indicating that NIR laser (808 nm, 15 J/cm²) improves performance of AD mice model in Barnes test (Lu et al., 2016). In another study, mitigated spatial memory deficit in AD mice following NIR laser treatment (808 nm, 6 J/cm²) has been shown (De Taboada et al., 2011). We also observed that red and NIR lights (8 J/cm²) notably improved the episodic-like memory in the DG-treated mice. This data is also in agreement with the work of Lu et al., which showed improvement of recognition memory of Aβ-received mice after laser treatment in novel object recognition test (Lu et al., 2016).

Data from mice showed that 2.5% and 3% of light with corresponding wavelengths of 670 and 810 nm, respectively, could reach a depth in the brain of 5 mm (Reinhart et al., 2017). Based on this, it could be assumed that in our study ~0.25 J/cm² of red or NIR lights (at cortical fluence of 8 J/cm²) reach brainstem which is somewhat in the biostimulatory range for LLLT (Moroz et al., 2014; Sharma et al., 2011). In the human cases, using high-power laser device, 2.9% of 810-nm light reach 30-mm depth of brain tissue (Henderson and Morries, 2015). This is a clear limitation in the human use of transcranial LLLT in brain conditions that are associated to deeper parts dysfunction. Given this, to deliver light to the deeper regions of the brain which are involved in AD and PD pathologies, most recently there have been attempts to develop some new approaches such as the combination of transcranial and intranasal LLLT (Saltmarche et al., 2017) as well as intracranial LLLT method (Darlot et al., 2016).

It could conclude that transcranial LLLT using red and NIR lights (8 J/cm²) rescued mice brain from DG-induced spatial and episodic-like memory impairment probably via modulation of mitochondrial function and cytochrome c as well as ROS production and neuronal apoptosis. In addition, we applied fluency of 4 J/cm², but this may not be the sufficient therapeutic regimen, in terms of fluency. Moreover, the NIR laser showed slightly, but not significantly better results than red and effects did not appear to be dependent on laser wavelength. This piece of result suggests that photoneuromodulation of brain could be a promising candidate for neuroenhancement of age-related cognitive impairments. Nevertheless, the optimal treatment parameters of transcranial LLLT with the best efficacy and safety should be determined in future studies.

Disclosure statement

The authors have no conflicts of interest to disclose.

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