Photobiomodulation Improves the Inflammatory Response and Intracellular Signaling Proteins Linked to Vascular Function and Cell Survival in the Brain of Aged Rats

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Abstract
Photobiomodulation is a non-pharmacological tool widely used to reduce inflammation in many tissues. However, little is known about its effects on the inflammatory response in the aged brain. We conducted the study to examine anti-inflammatory effects of photobiomodulation in aging brains. We used aged rats (20 months old) with control (handled, laser off) or transcranial laser (660 nm wavelength, 100 mW power) treatments for 10 consecutive days and evaluated the level of inflammatory cytokines and chemokines, and the expression and activation of intracellular signaling proteins in the cerebral cortex and the hippocampus. Inflammatory analysis showed that aged rats submitted to transcranial laser treatment had increased levels of IL-1alpha and decreased levels of IL-5 in the cerebral cortex. In the hippocampus, the laser treatment increased the levels of IL-1alpha and decreased levels of IL-5, IL-18, and fractalkine. Regarding the intracellular signaling proteins, a reduction in the ERK and p38 expression and an increase in the STAT3 and ERK activation were observed in the cerebral cortex of aged rats from the laser group. In addition, the laser treatment increased the hippocampal expression of p70S6K, STAT3, and p38 of aged rats. Taken together, our data indicate that transcranial photobiomodulation can improve the inflammatory response and the activation of intracellular signaling proteins linked to vascular function and cell survival in the aged brain.

Keywords Laser · Photobiomodulation · Brain · Aging · Inflammation · Intracellular signaling proteins

Introduction
Photobiomodulation (PBM), also called low-level laser therapy [1], has emerged as a non-pharmacological, non-invasive tool capable of stimulating wound healing, reducing pain and inflammation in several diseases [2]. PBM mechanisms on the aging brain have been recently reviewed [3]. A primary mechanism of action of PBM involves the capacity of red-to-near-infrared photons of light to photo-oxidize mitochondrial cytochrome c oxidase (CCO) and promote a vascular response for oxygenation of the aging brain in vivo [4]. This action may lead to cerebral upregulation of CCO activity [5] and mitochondrial respiration for ATP production [6]. Also, PBM can release nitric oxide bound to CCO [7] and increase CCO-catalyzed nitric oxide synthesis to facilitate vasodilation and blood flow to the brain [8]. These and other well-documented PBM effects on the brain may induce anti-inflammatory and antioxidant properties [9–11].

Many human studies have also used transcranial PBM to improve brain functions in several conditions (e.g., [12–24].
For example, Vargas et al. [23] submitted healthy elderly people to PBMT and observed an improvement in cognitive functions in the psychomotor vigilance task (PVT), the test of sustained attention and the delayed match-to-sample (DMS), a test of visual working memory. Many studies using animal models have also shown interesting results of PBM on the brain (e.g., [11, 25–28]). For instance, Lu et al. [11] injected beta amyloid (Aβ) in the hippocampus of rats that were treated with laser PBM for 5 days. They noted that laser treatment restored spatial memory and object recognition memory. In addition, they observed an increase in the antioxidant capacity of hippocampal CA1 neurons and a decrease of Aβ-induced reactive gliosis and inflammation.

Recently, some authors have shown promising results of PBM in the aged brain [4, 23, 29–32]. However, there is little evidence to explain such effects, other than improved mitochondrial respiration and vascular function. It is known that brain aging is characterized by local inflammation with glial cells releasing increasing amount of pro-inflammatory cytokines such as IL-1beta, IL-6, and TNF-alpha [33, 34]. This process is directly involved with cellular dysfunctions characteristic of aging and Alzheimer’s disease [35, 36], which may result in an increase in the activation of signaling pathways linked to inflammation and cellular death such as c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38) [37].

Based on the well-documented anti-inflammatory effects of PBM in other tissues [38–40]: [41], we evaluated whether a transcranial treatment with a laser diode of 660 nm wavelength and 100 mW power can modulate the inflammatory response and expression and activation of intracellular signaling proteins in the cortex and hippocampus of aged rats.

Methods

Animals

Twenty-month-old male Wistar rats (n = 10) were used in this study. The colony room was maintained at 21 ± 2 °C with a 12 h light/dark schedule (light: 7 am until 7 pm), and food and water were provided ad libitum throughout the experimental period. All experimental protocols were approved by the ethics committee of the Universidade de Mogi das Cruzes (UMC) (#003/2020) and all efforts were made to minimize animal suffering in accordance with the proposals of the International Ethical Guideline for Biomedical Research (CIOMS 1985).

Laser and Control Protocols

Figure 1 shows a photo of the red laser used. The aged rats were randomly distributed into two groups: laser (n = 5) and control (n = 5). The animals of the laser group were manually immobilized and received the treatment with a laser diode of 660 nm wavelength and 100 mW power, with beam area of 0.03 cm², and irradiance at aperture of 3.33 W/cm² for 30 s at each of 5 irradiation points on the head, totalizing 15 J of energy, and scalp fluence of approximately 100.0 J/cm² at scalp surface, for 10 consecutive days. These PBM therapy were chosen based on our previous publications, showing that these parameters had anti-inflammatory effects in other tissues (Table 1) [38–41]. The target coordinates on the scalp were as follows: point 1 = AP + 4.20 mm and ML 0.00 mm; point 2 = AP − 3.00 mm and ML − 6.60 mm; point 3 = AP − 3.00 mm and ML + 6.60 mm; point 4 = AP 0.00 mm and ML 0.00 mm; and point 5 = AP − 5.52 mm and ML 0.00 mm. Therefore, the target brain regions were sensory-motor and limbic areas: secondary motor cortex (M2), anterior cingulate cortex (Cga), primary somatosensory cortex-upper limb (S1ULp), secondary somatosensory cortex (S2), posterior cingulate cortex (Cgp), retrosplenial dysgranular cortex (RSD), and retrosplenial granular cortex (RSCg) [42], as in our previous metabolomics study in the rat [25]. The animals of the control group were handled the same way, except that the laser was not turned on.
Tissue Preparation

One hour after the final laser or control session, aged rats from the laser (n = 5) and control (n = 5) groups were euthanized by decapitation and their cerebral cortex and hippocampus were immediately collected and frozen. The whole cerebral cortex and the hippocampus were homogenized in ice-cold RIPA lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS) with freshly added protease (Cat# M222–1 ml; Lot# 1295C056; Amresco) and phosphatase (Cat# B15001-A and B; Lot# 510,011; Biotool) inhibitors. Homogenates were centrifuged at 10,000 × g for 10 min at 4 °C and supernatants were collected for cytokine/chemokine quantification.

Methods for the Protein Detection and Analysis

Milliplex® MAP rat cytokine/chemokine magnetic bead panel assay (RECYMAG65K) was used to quantify the levels G-CSF, eotaxin, GM-CSF, IL-1alpha, leptin, MIP-1alpha, IL-4, IL-1beta, IL-2, IL-6, IL-13, IL-10, IL-5, IL-17alpha, IL-18, MCP-1, IP-10, VEGF, fractalkine, MIP-2, TNF-alpha, and RANTES in the brain samples of the studied groups. Milliplex® MAP kits 48-681MAG and 48-680MAG were used to evaluate the expression and brain activation of signaling proteins Akt, p70S6K, STAT3, STAT5, ERK, JNK, NF-kB, and p38. The plates were run on a Luminex™ MagpixTM instrument and results were analyzed with the Milliplex Analyst 5.1 software using a logistic 5P weighted regression formula to calculate sample concentrations from the standard curves.

Statistical Analyses

Statistical procedures were conducted using the Mann–Whitney U test that allows comparison of non-parametric data. All analyses were performed using the Statistical Package for the Social Science (SPSS Inc., IBM, version 221.0, Chicago, IL, USA). A statistical difference was considered significant when the p value was lower than 0.05. All plots were acquired using the GraphPad Prism (6.0).

Results

Cortical and Hippocampal Levels of Cytokines and Chemokines

To evaluate whether the PBM had anti-inflammatory effects on the aged brain, we quantified the cortical and hippocampal levels of several chemokines and cytokines in aged rats submitted to 10 consecutive days of laser treatment or control treatment. The detailed results of Mann–Whitney tests are presented in Supplementary Tables 1 and 2. The laser treatment increased the cortical level of IL-1alpha (p = 0.008) and reduced the IL-5 level (p = 0.046) (Fig. 2).

In the hippocampus, an increase in the IL-1alpha (p = 0.035) level was observed in the laser group. However, the laser treatment was able to reduce the levels of IL5 (p = 0.027), IL-18 (p = 0.049), and fractalkine (p = 0.037) in aged rats (Fig. 3). Taken together, these data showed that PBM changed the levels of neuroinflammatory markers in aged rats.

Cortical and Hippocampal Expression and Activation of Signaling Proteins

We investigated the cortical and hippocampal expression and activation of signaling proteins in aged rats submitted to laser treatment vs. control treatment. The detailed results of Mann–Whitney tests are presented in Supplementary Tables 3 and 4. The laser treatment decreased the cortical expression of ERK (p = 0.028) and p38 (p = 0.009). Interestingly, the laser treatment was able to increase the cortical activation of ERK (p = 0.014) and STAT3 (p = 0.0016) (Fig. 4).

In the hippocampus, the laser treatment increased the expression of p70S6K (p = 0.016) and STAT5 (p = 0.050) and decreased the expression of p38 (p = 0.028) in aged rats (Fig. 5).
**Discussion**

The aim of our study was to investigate levels of pro- and anti-inflammatory cytokines and chemokines and the expression and activation of signaling proteins in the brain of aged rats submitted to repeated treatment with a laser diode of 660 nm wavelength and 100 mW power. Our results indicate that transcranial PBM was able to modulate the expression and activation of signaling proteins and the inflammatory profile in the brain of aged rats.

**Anti-inflammatory Effects of PBM on the Aged Brain**

The laser treatment increased the levels of IL-1alpha and decreased the levels of IL-5 in both the cortex and hippocampus of aged rats. Interestingly, IL-1alpha has been shown to promote angiogenesis and to increase proliferation and migration of endothelial cells [43, 44]. The IL-1alpha findings are consistent with improving vascular function and oxygenation of the aged brain [4], which may help explain the facilitation of neurocognitive functions.
shown by transcranial PBM studies in older humans [23]. In contrast, a reduction of IL-5 may be beneficial to the aged brain because IL-5 induces proliferation and activation of microglia, which is characteristic of inflammatory reactions [45].

Moreover, PBM promoted an anti-inflammatory effect by reducing the hippocampal levels of IL-18 and fractalkine. IL-18 is a pro-inflammatory cytokine that inhibits cell differentiation and reduces neurogenesis and induces neuronal death in cultured neural progenitors [46, 47]. Further evidence suggests that IL-18 can activate the p38 signaling pathway [48]. Also, high levels of this cytokine are observed during aging and in neurodegenerative diseases [49, 50]. Fractalkine, in turn, is a chemokine regulated by pro-inflammatory cytokines such as TNFalpha and IL-1beta, which is involved in the communication between neurons and microglia [51]. High levels of fractalkine were observed in the cortex and hippocampus of a rat model of Alzheimer’s disease [52]. In this sense, reducing the levels of both IL-18 and fractalkine by laser treatment may contribute to improving the inflammatory response in the aging brain and in aging-related neurodegenerative diseases.

**Effects of PBM on Signaling Proteins in the Aged Brain**

PBM reduced the expression of ERK and p38 and increased the activation of STAT3 and ERK in the cortex of aged rats. In the hippocampus, PBM increased the expression of p70S6K and STAT5 and decreased the expression of p38. p38 pathway is stimulated by oxidative stress and pro-inflammatory cytokines which is related to cell proliferation, differentiation, and apoptosis [53, 54]. Also, p38 levels are elevated in the aging brain [37]. However, PBM treatment decreased p38 expression in the cortex and hippocampus of aged rats. Our data corroborate the findings of Salehpour...
et al. [55]. They noted that PBM reduced cortical and hippocampal levels of p38 in a mouse model of restraint stress. It is possible that these changes are linked to the anti-inflammatory and antioxidant properties of PBM in the brain of aged rats.

STAT3 modulates the expression of genes responsible for important physiological functions such as cell regulation and apoptosis control [56, 57]. STAT5 is linked to neuronal survival [58, 59]. Also, STAT5 is necessary for the neuroprotective and neurotrophic effects of growth hormone on hippocampal neurons [60]. However, STAT3 and STAT5 brain levels are decreased during aging [61]. Nevertheless, laser treatment increased STAT3 activation and the expression of STAT5 in the cortex of aged rats. These observed changes in intracellular signaling proteins may be linked to the anti-inflammatory effects of PBM in the cortex and hippocampus of aged rats.

High levels of neuroinflammatory markers are observed during aging [62, 63], indicating a pro- and anti-inflammatory imbalance [64]. In particular, it is possible that PBM of the neuroinflammatory response is related to increases in the activation of intracellular signaling proteins STAT3 and ERK in the cortical region. In this sense, STAT3 is a transcription factor that interacts with polypeptide receptors in the cell membrane, mediating extracellular signals such as growth factors and cytokines [65]. When activated by tyrosine phosphorylation, STAT3 dimerizes and translocates to the nucleus, thus activating the target genes [66]. The activation of STAT3 suppresses the expression of pro-inflammatory mediators, promoting an immune evasion and blocking the production and detection of inflammatory signals by several components of the immune system [67].

ERK can be activated by growth factors, such as the epidermal growth factor (EGF) that is associated with the attenuation of pro-inflammatory mediators (García-Ojalvo et al., 2019), activating rat sarcoma (Ras), which recruits root abundant factor (Raf) for the membrane. Raf activates Mek, which in turn activates ERK. ERK activation triggers cell proliferation, differentiation, and cell migration [53]. It is possible that the neuroinflammatory response in the cortex may trigger the activation of the ERK signaling pathways which in turn activates STAT3 [68]. In this context, it is possible that the increased ERK activation is linked to the anti-inflammatory effect of laser treatment.

Limitations

We did not evaluate functional parameters, such as cognitive functions, since our goal was to investigate whether repeated laser treatment alters the cortical and hippocampal inflammatory and signaling profiles in aged rats. Also, the use of a small number of old rats with one laser dose and the absence of a control group of younger rats are also limitations. We used only one dose due to the difficulty in obtaining aged rats. This dose was used based on our previous studies showing anti-inflammatory effects in other tissues. The inclusion of a young control group would be interesting to analyze the differences in intracellular signaling protein levels and inflammatory response between young and aged rats. More studies are necessary to investigate these issues. Here, we conducted a study to examine anti-inflammatory effects of photobiomodulation in aging brains. Our research interest was based in previous studies showing changes in protein levels and inflammatory response in the aged brain [37, 61, 69–71].

Conclusion

Taken together, our data suggest that transcranial PBM improves the inflammatory response and the activation of intracellular signaling proteins linked to vascular function and cell survival in the brain of aged rats.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12035-021-02606-4.

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Author Contribution Conceived and designed the experiments: FSC, FCBM, and SGS. Performed the experiments: FSC and FCBM. Analyzed the data: FSC and SGS. Contributed reagents/materials/analysis tools: FSC, BHSA, and SGS. Wrote the manuscript: FSC, FGL, and SGS. Approved the final version of the manuscript: FSC, FCBM, BHSA, FGL, and SGS.

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Availability of Data and Material Not applicable.

Declarations

Ethics Approval All experimental protocols were approved by the ethics committee of the Universidade de Mogi das Cruzes (UMC) (#003/2020).

Consent to Participate Not applicable.

Consent for Publication. Not applicable.

Conflict of Interest The authors declare no competing interests.

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