Modulatory effects of photobiomodulation in the anterior cingulate cortex of diabetic rats

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Abstract

Anterior Cingulate Cortex (ACC) has a crucial contribution to higher order pain processing. Photobiomodulation (PBM) has been used as integrative medicine for pain treatment and for a variety of nervous system disorders. This study evaluated the effects of PBM in the ACC of diabetic rats. Type 1 diabetes was induced by a single dose of streptozotocin (85 mg/Kg). A total of ten sessions of PBM (pulsed gallium-arsenide laser, 904 nm, 9500 Hz, 6.23 J/cm²) was applied to the rat peripheral nervous system. Glial fibrillary acidic protein (GFAP), mu-opioid receptor (MOR), glutamate receptor 1 (GluR1), and glutamic acid decarboxylase (GAD65/67) protein level expression were analyzed in the ACC of diabetic rats treated with PBM. Our data revealed that PBM decreased 79.5% of GFAP protein levels in the ACC of STZ rats. Moreover, STZ + PBM rats had protein levels of MOR increased 14.7% in the ACC. Interestingly, STZ + PBM rats had a decrease in 70.7% of GluR1 protein level in the ACC. Additionally, PBM decreased 45.5% of GAD65/67 protein levels in the ACC of STZ rats.

Keywords Diabetes · Hyperglycemia · Neuropathy · Phototherapy · Streptozotocin · Western blot

Abbreviations

ACC Anterior cingulate cortex
i.p Intraperitoneal
PBM Photobiomodulation
STZ Streptozotocin

1 Introduction

The anterior cingulate cortex (ACC) plays relevant role in pain perception [1], and despite its involvement in chronic pain development following peripheral nerve injury [2], however, the contribution of ACC to the experience of chronic pain in diabetic peripheral neuropathy (DPN) remains unclear. The ACC along with thalamus, insular cortex, prefrontal cortex, and somatosensory cortex is one of the main components of the pain matrix [3, 4]. Pain-related studies with animals have shown that ACC mediates

responses to inflammatory pain, neuropathic pain, spontaneous pain in the formalin test, and formalin-induced conditioned place avoidance [5–8].

According to the International Diabetes Federation (IDF), there are approximately 463 million adults (20–79 years) living with diabetes worldwide, and by 2045, this will rise to 700 million (IDF ATLAS). This increase in the number of people with diabetes will be accompanied by an increase in the prevalence of diabetes complications [9]. Half of people living with diabetes develop DPN [10, 11], one of the leading causes of neuropathy worldwide [12]. DPN is characterized by the progressive loss of peripheral nerve fibers [13], and painful neuropathy is the main clinical consequences of this complex syndrome related to diabetes [14, 15]. Additionally, there is no effective treatment that prevents peripheral nerve fiber degeneration in DPN [16], except tight glycaemic control [17].

Painful DPN is characterized by tingling, burning, sharp, shooting, and lancinating pain [18]. Pain can be constant and cutaneous allodynia may be present [19, 20], negatively affecting diabetic people mood [21, 22]. Furthermore, people living with painful DPN may also remove themselves from social activities and eventually develop depression [23, 24]. Given ACC involvement in the affective/motivational aspects of pain [25, 26] and its involvement in the glucose-monitoring network in the brain [27], it is of great

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importance to develop research that aims to investigate the ACC participation in processing pain in DPN, which is still unknown. Recalling what has already been mentioned, no single pharmacological treatment exists to prevent pain or provide total pain relieve in painful DPN [28].

Astrocyte activation or astrogliosis in ACC has been linked with chronic or neuropathic pain [29, 30]. Astrocytes are the most numerous non-neuronal cells in the brain involved in modulating a variety of neuronal activities [31, 32], including glucose metabolism [33, 34]. Astrocytes were reported to highly express μ -opioid receptor (MOR) [35], one of the three subtypes of opioid receptors that play important role in modulating pain behavior and antinociception [36]. Moreover, MOR are also efficacious mood enhancers and are involved in the activation of dopamine reward pathway [37, 38].

ACC is composed of both excitatory and inhibitory neurons [39, 40], and GABAergic transmission in the ACC plays a critical role in modulating nociception and chronic pain in humans and animal [41]. Glutamatergic AMPA (α amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors (GluR1, GluR2, GluR3, and GluR4) involved in a variety of central nervous system functions [42–44], including pain modulation [45], mediate the majority of excitatory synaptic transmission in the brain [46]. However, the roles of glutamatergic receptors in ACC during pain receive few attentions [6]. Additionally, changes in GABA content have recently been described in the central nervous system [47], including ACC of chronic pain patients [48, 49].

Based on the information presented here, the present study aimed to assess whether photobiomodulation (PBM) applied to the peripheral nervous system would have modulatory effects on the ACC in animal model of streptozotocin-induced DPN. PBM has been used as complementary medicine for chronic and neuropathic pain treatment [50, 51], nerve fibre regeneration [52], and a variety of diseases [53–57]. Of note, it is well known the beneficial effects of PBM on the central nervous system [58–61].

2 Materials and methods

2.1 Animals

The experiments were carried out in accordance with the CONCEA guidelines (CONCEA, Brazil), a constituent body of the Ministry of Science, Technology, and Innovation (MCTI, Brazil). All protocols, including STZ-induction type 1 diabetes and photobiomodulation therapy, were approved by the Animal Research Ethics Committee (CEUA) of the Biomedical Sciences Institute of the University Sao Paulo (protocol number: CEUA 2269190619). All animals were handled according to the guidelines for

the use of laboratory animals [62]. The study used a total of 30 adult male Wistar rats (250–300 g) supplied by the central animal facility of the Biomedical Sciences Institute of University of Sao Paulo. Rats were kept at room temperature of 22 ± 2 °C with light/dark cycle (12:12 h). Food and water were provided ad libitum. The rats were allowed to acclimatize for at least 5 days prior to the onset of the study. They were randomly divided into three groups: (1) Naive rats were used as control animals (CTRL); (2) STZ animals, which were intraperitoneally injected with streptozotocin (STZ, 85 mg/kg), and (3) STZ + PBM animals, which were injected with streptozotocin (STZ, 85 mg/kg) and treated with photobiomodulation (PBM). After STZ i.p injection, three rats died along the experimental timeline. At the end of the study, we had nine rats per group.

2.1.1 Streptozotocin-induced type 1 diabetes

For the STZ-induced type 1 diabetes, a single dose of streptozotocin (85 mg/kg; S0130-1G, Sigma-Aldrich) diluted in 500 μ L of 0.9% Saline were injected in the peritoneal cavity [63]. Blood glucose levels were assessed 48H after STZ intraperitoneal injection to confirm hyperglycemia. Plasma glucose levels higher than 300 mg/dl were considered indicative of diabetes [64]. Glycemic control was assessed once a week using an ULTRAMINI® I ONETOUCH® blood glucose-monitoring system. A single administration of streptozotocin induced insulin-dependent diabetes mellitus within 48H by the destruction of pancreatic islet cells [65].

2.2 Photobiomodulation

Rats in the STZ + PBM group were irradiated with a GaAs laser (Gallium Arsenide, Laserpulse-Laser, Ibramed, Brazil) emitting a wavelength of 904 nm, output power of 45 mW, 0.13 cm² beam area, 9500 Hz frequency, duty cycle (DC) 0.0617%, pulse time of 65 ns, and 6.23 J/cm² fluence (Table 1). CTRL and STZ groups were not submitted to PBM. PBM initiated 60 days after diabetes induction. This time point was chosen, because we showed in a previous study that pain-related behavior (allodynia) and degeneration of peripheral nerve fibers were increased during persistent hyperglycaemia in the long term (60 days) [63]. The PBM treatment was performed under anesthesia with isoflurane (Cristalia, MG, Brazil) every other day totalling ten sessions. After sterilization, the laser probe was lightly placed on the unshaved skin surface of the rat's right thigh. Nine points in the region of the sciatic nerve were irradiated for 18 s each (Fig. 1). Points were irradiated between intervals of 30 s.

Table 1 Photobiomodulation parameters

Irradiation parameters	
Wavelength (nm)	904 nm
Operating mode	Pulsed
Frequency (Hz)	9500 Hz
Pulse on duration (nanoseconds)	65 ns
Beam shape	Circular
Treatment parameters	
Beam spot size at target (cm ²)	0.13 cm ²
Exposure duration (seconds)	18 s/point
Radiant exposure (J/cm ²)	6.23 J/cm ²
Radiant energy (J)	0.81/point
Number of points irradiated	9
Irradiated area (cm ²)	1.17 cm ²
Application technique	Skin contact
Number and frequency of treatment	10 sessions performed every other day
Total radiant energy (J)	7.29 J/ session. 72.9 J total sessions

(GluR1), and Glutamic Acid Decarboxylase (GAD65/67) protein levels were quantified in the rat ACC. For total protein extraction, ACC was immersed in lysis buffer (90 mM KCl, 10 mM Hepes, 3 mM MgCl₂, 5 mM EDTA, 1% glycerol, 1 mM DTT, 0.04% SDS, 20 mM Aprotinin, 20 mM Pepstatin, 20 mM Leupeptin, 40 μ M PMSF, 100 mM Orthovanadate). The total protein concentration was obtained by Bradford method [66]. Subsequently, 40 μ g (ACC) of total protein underwent polyacrylamide gradient gel (4 and 20%) [67] electrophoresis and transferred to nitrocellulose membrane (Bio-Rad). The membrane was subsequently stained with Ponceau solution to evaluate the similarity of protein concentration between the samples and for data normalization. Following Ponceau washing with TBST (20 mM TRIS, 148 mM NaCl, Tween 20 0.1%), membrane was incubated with primary antibodies shown in Table 2 under constant agitation at 4 °C overnight. After washing with TBST, the membrane was incubated with the peroxidase-conjugated anti-rabbit (1:5000) secondary antibodies for MOR, GluR1, and GAD65/67 and with the anti-mouse (1:5000) secondary antibodies for GFAP and β -Actin, at room temperature for

2.3 Protein expression analysis: Western blotting

On the next day after the last PBM session (10th session), all the rats were anesthetized with 5% isoflurane and decapitated when fully sedated. The animals' brains (anterior cingulate cortex) were removed for analysis of protein levels by Western blot assay. ACC were pooled from three rats of each group. Glial fibrillary acidic protein (GFAP), μ -Opioid receptor (MOR), Glutamate Receptor 1

Table 2 List of primary antibodies used for Western Blotting

Antibody	Host species	Molecular weight	Dilution	RRID
GFAP	Mouse	50 KDa	1:1000	AB_477010
MOR	Rabbit	50 KDa	1:1000	AB_2156520
GluR1	Rabbit	106 KDa	1:1000	AB_2113602
GAD65/67	Rabbit	65/67 KDa	1:1000	AB_90715
Beta-Actin	Mouse	42 KDa	1:5000	AB_476743

Fig. 1 Photobiomodulation session **a** Represents laser device and laser probe (904 nm). PBM sessions were applied to the rat peripheral nerve fibers by superficial skin contact—rats were anesthetized

(O₂/Isoflurane) to receive PBM therapy. **b** Nine points were irradiated with PBM (904 nm) on the rat right leg

90 min. The membrane was then washed again with TBST and submitted to chemiluminescence detector (UviTec Gel Doc Systems). The bands corresponding to the protein of interest were quantified by optical densitometry using the ImageJ software (NIH; USA), and the values were expressed as percentage [68]. For GFAP, MOR, GluR1, and GAD65/67, protein expression in the ACC, the same nitrocellulose membrane was used after stripping process for each antibody, and then, β -actin data were the same for all antibodies.

3 Statistical analysis

Statistical analyses of data were generated using GraphPad Prism, version 8 (GraphPad Software Inc., San Diego, CA). For Western blot analysis statistical comparison among groups was performed using one-way analysis of variance; differences between means were tested by Bonferroni's multiple comparison test. In all cases, $p < 0.05$ was considered statistically significant.

4 Results

4.1 Type 1 diabetes onset through a single dose of STZ evaluated by blood glucose levels

One single intraperitoneal injection of STZ (85 mg/kg) diluted in 500 μ L of Saline 0.9% was used to induce type 1 diabetes in male Wistar rats. The difference between means (STZ and CTRL) was 292.3 md/dl in ad libitum blood glucose level. STZ group had a mean blood glucose level of 387.7 mg/dl compared to CTRL, 95.40 mg/dl. STZ revealed a significant blood glucose level increase compared to CTRL [$F(3,16) = 313.6$; $p < 0.0001$] (Fig. 2).

4.2 Effect of photobiomodulation on Glial Fibrillary Acidic Protein (GFAP) in the Anterior Cingulate Cortex (ACC)

STZ group revealed an increase in GFAP protein expression in the ACC of diabetic rats, compared to CTRL and STZ + PBM groups (Fig. 3) [$F(2,6) = 2859$; $p = 0.0001$]. Western blotting data also revealed that PBM significantly decreased GFAP protein expression in the ACC in STZ + PBM group compared to STZ and CTRL groups ($p < 0.0001$). PBM decreased 138.52% of GFAP protein expression in the ACC of STZ + PBM group compared to STZ and 79.49% compared to CTRL (Fig. 3).

Fig. 2 Blood Glucose Effect of STZ on ad libitum blood glucose levels. & $p < 0.0001$ between Control and STZ. * $p < 0.0001$ among 15, 45, 75 and day 0 in STZ. £ $p < 0.0001$ among 75 and 45, 15 days in STZ

Fig. 3 Glial fibrillary acidic protein. Western blotting analysis of PBM on GFAP in the rat ACC. * $p < 0.0001$ STZ/CTRL; ** $p < 0.0001$ STZ + PBM/STZ; *** $p < 0.0001$ STZ + PBM/CTRL. Each black point in the graph represents a pool of three rats

4.3 Effect of photobiomodulation on μ -Opioid Receptor (MOR) in the Anterior Cingulate Cortex (ACC)

STZ group revealed a decrease in MOR protein expression in the ACC of diabetic rats, compared to CTRL and STZ + PBM groups (Fig. 4) [$F(2,6) = 39.05$; $p = 0.0004$]. Western blotting data also revealed that PBM increased MOR protein expression in the ACC of STZ + PBM group compared to STZ ($p < 0.0049$) group. PBM increased 14.77%

Fig. 4 Mu-opioid receptor. Western blotting analysis of PBM on MOR in the rat ACC. * $p < 0.0004$ STZ/CTRL; ** $p < 0.0049$ STZ+PBM/STZ; *** $p < 0.0467$ STZ+PBM/CTRL. Each black point in the graph represents a pool of three rats

of MOR protein expression in the ACC of STZ+PBM group compared to STZ group (Fig. 4).

4.4 Effect of photobiomodulation on Glutamate Receptor 1 (GluR1) in the Anterior Cingulate Cortex (ACC)

STZ group revealed a decrease in GluR1 protein expression in the ACC of diabetic rats, compared to CTRL group (Fig. 5) [$F(2,6) = 724.4$; $p = 0.0001$]. Western blotting data also revealed an even greater decrease in STZ+PBM group compared to both groups. PBM decreased 10.48% of GluR1 protein expression in the ACC of STZ+PBM group compared to STZ and 70.7% compared to CTRL (Fig. 5). STZ group had a decrease of 60.22% of GluR1 protein expression compared to CTRL.

4.5 Effect of photobiomodulation on Glutamic Acid Decarboxylase (GAD65/67) in the Anterior Cingulate Cortex (ACC)

STZ group revealed a decrease in GAD65/67 protein expression in the ACC compared to CTRL group (Fig. 6) [$F(2,6) = 103.6$; $p = 0.0001$]. After PBM treatment, our western blotting data revealed that PBM also decreased GAD65/67 protein expression in STZ+PBM group compared CTRL ($p < 0.0001$). STZ group had a decreased of 47.51% of GAD65/67 protein expression compared to CTRL and STZ+PBM had a decreased of 45.51% compared to CTRL (Fig. 6). No statistical difference was found

Fig. 5 Glutamate receptor 1. Western blotting analysis of PBM on GluR1 in the rat ACC. * $p < 0.0001$ STZ/CTRL; ** $p < 0.0003$ STZ+PBM/STZ; *** $p < 0.0001$ STZ+PBM/CTRL. Each black point in the graph represents a pool of three rats

Fig. 6 Glutamic acid decarboxylase. Western blotting analysis of PBM on GAD65/67 in the rat ACC. * $p < 0.0001$ STZ/CTRL; ** $p < 0.0001$ STZ+PBM/CTRL. No statistical significance was found between STZ+PBM and STZ. Each black point in the graph represents a pool of three rats

between STZ + PBM and STZ regarding protein expression of GAD65/67 in the rat ACC.

5 Discussion

In the present study, we investigated the effects of PBM on the supraspinal brain region involved in the cognitive and emotional processing of peripheral painful sensation, ACC. In our study, we used rat model of diabetic peripheral neuropathy induced by a single dose of streptozotocin (STZ, 85 mg/kg) injected in the peritoneal cavity (intraperitoneal, i.p). Rats that were injected with STZ developed hyperglycemia 24 h after STZ i.p injection. Furthermore, STZ-induced diabetic rats developed the most common signs of diabetic neuropathy. It includes severe weight loss, polyuria, polyphagia and, in some cases, cataracts (data not shown). Additionally, we have previously shown that one single dose of STZ (85 mg/kg) induced allodynia in rat model of diabetic peripheral neuropathy and that PBM restore pain threshold in those rats [63]. Streptozotocin-induced diabetic rats had an average blood glucose level of 387.7 mg/dl. Control (CTRL) rats developed no signs of diabetic neuropathy and had an average blood glucose level of 95.40 mg/dl throughout the experimental timeline (12 weeks).

Currently, there is an increasing body of evidence showing the involvement of ACC in the perception of physical and affective components of pain [69–73]. Of note, little is known about the direct effects of hyperglycemia (diabetes) on ACC. Moreover, there is a limited but growing body of evidence concerning the effects of PBM on the brain [58, 60, 61, 74]. Furthermore, less is known about how PBM that was applied to the peripheral nervous system has the potential to modulate protein levels in supraspinal segments in the central nervous system. We present here the modulatory effects of photobiomodulation on (1) glial fibrillary acidic protein (GFAP), (2) μ -opioid receptor (MOR), and (3) glutamate receptor (GluR1) protein levels in the ACC of diabetic rats in streptozotocin-induced diabetic peripheral neuropathy.

Initially, our results showed that hyperglycemia increases GFAP protein levels in the ACC of diabetic rats (STZ) when compared to control (CTRL) group. It must be mentioned that one of the major roles played by astrocytes is their direct involvement in energy storage and supply for the brain [33, 75, 76]. Moreover, Coleman et al. [77] and Saraiva et al. [78] demonstrated changes in GFAP expression in the central nervous system (CNS) in rodent diabetes model. However, the effects of high glucose on astrocytes metabolism and function remains unclear [75]. Of note, we showed here that diabetic rats treated with PBM (STZ + PBM) had a significant decrease of GFAP protein levels in the ACC when

compared to STZ group. There was also statistical difference between STZ + PBM and CTRL group.

It was also presented here that PBM increased μ -opioid receptor (MOR) in the ACC of diabetic rats (STZ + PBM) when applied to the peripheral nervous system. STZ rats had a decrease in MOR protein levels when compared to CTRL group. Opioid receptors comprise four members, the classical μ (MOR), δ (DOR), and κ (KOR) receptors, and the non-classical nociceptin/orphanin FQ (NOP) receptor [79]. MOR receptors are the most important class of receptors clinically, playing an important role in pain treatment [36, 80, 81]. Interestingly, MOR was reported to be highly expressed in the central nervous system astrocytes [82]. However, the role of astrocytic MOR has not been investigated [82, 83]. Furthermore, little is known about the role of astrocytes and MOR in diabetic peripheral neuropathy, especially regarding ACC.

In the present study, we also showed that hyperglycemia decreased glutamate receptor (GluR1) protein levels in the ACC of diabetic rats (STZ) compared to control (CTRL). Surprisingly, PBM decreased even further GluR1 protein levels in the diabetic rats (STZ) that were treated with PBM. Glutamate is the main excitatory neurotransmitter in the mammalian brain where astrocytes play an important role in removing 90% of such neurotransmitter from the synapse in the central nervous system [84, 85]. Bolo et al. [86] and Wiegers et al. [87] demonstrated elevated brain glutamate levels in people with type 1 diabetes, but they did not correlate such results with ionotropic glutamate receptor 1 (GluR1). Moreover, Andersen et al. [84] showed impaired glutamate and glutamine metabolism in the hippocampus of mouse model of type 2 diabetes but did not make any correlation between this finding and GluR1.

Additionally, we evaluated the effects of PBM on the modulation of protein levels of glutamic acid decarboxylase (GAD65/67) on the ACC of diabetic rats (STZ). GAD65/67 plays an important role in the synthesis of gamma-aminobutyric acid (GABA) in the central nervous system (CNS) [88]. GABA is the principal inhibitory transmitter in the CNS, including the spinal dorsal horn and ACC [88, 89]. Our results demonstrated that diabetic rats (STZ) had a significant decrease in the levels of GAD65/67 in the ACC compared to control rats. Interestingly, PBM had no effect in GAD65/67 protein levels of diabetic rats. The effects of hyperglycemia on GAD65/67 protein modulation should be further investigated.

Regarding the abscopal effects of PBM our data summarized here corroborates the findings presented by Mitrofanis et al. [90]. Mitrofanis and colleagues showed that ten consecutive days of remote PBM had neuroprotective effects in mice model of Parkinson's disease (PD) [90]. According to their study, remote PBM produces modest but widespread changes in the brain transcriptome [90]. With respect

to working memory and cognitive functions in which ACC plays an important role, Salehpour et al. [91] demonstrated in a case report that an individual affected by Alzheimer's disease (AD) treated with transcranial e intranasal PBM had improvements in cognitive abilities, working memory, as well as in quality of life [91]. Another interesting finding with respect to indirect effects of PBM was demonstrated by Kern et al. [92]. In STZ-induced diabetes in mice, PBM attenuated diabetes-induced retinopathy by improving diabetic-changes in superoxide generation, leukostasis, and expression of ICAM-1 [92]. Kern and colleagues showed that such significant results were achieved with the mice's head protected from the light therapy, the retina itself did not receive PBM directly [92].

6 Conclusion

In conclusion, the present study demonstrated the abscopal effects of PBM on the ACC of diabetic rats. Interestingly, hyperglycemia had expressive modulatory effect on the protein levels of GFAP, MOR, GluR1, and GAD65/67 on the ACC of diabetic rats. Surprisingly, PBM applied to the peripheral nervous system of these diabetic animals modulated the protein levels of GFAP, MOR, and GluR1 on the ACC. PBM had no effect on the modulation of GAD65/67. Our results suggest that PBM applied to the periphery may have interesting effects on supraspinal brain regions, especially on the ACC. We must further investigate the effects of hyperglycemia in this brain region and why and how PBM that was applied to the peripheral nervous system elicits its effect on supraspinal brain region.

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Declarations

Conflict of interest The authors declare no financial or commercial conflict of interest.

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