The influence of the cannabinoid receptor CB1 on the periaqueductal gray in mice treated with photobiomodulation after chronic constriction injury of the sciatic nerve: a placebo-controlled trial

Influência do receptor canabinóide CB1 na substância cinzenta periaquedutal em camundongos tratados por fotobiomodulação após constrição crônica do nervo ciático: ensaio controlado por placebo

Gabriela Xavier Santos[1], Giovane Galdino de-Souza[1], Suélen Santos Alves[1], Gabriela Nagai Ocamoto[1], Nivaldo Antônio Parizotto[2], Luciana Maria dos-Reis[1]

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ABSTRACT

BACKGROUND AND OBJECTIVES: Studies have demonstrated that the cannabinoid CB1 receptor is involved in the modulation of pain, mainly by activating the descending pain control pathway. However, the role of photobiomodulation in this process is not well elucidated. Thus, the present study aimed to investigate the involvement of the CB1 receptor in the supraspinal photobiomodulation-induced antinociception.

METHODS: Male albino swiss mice were submitted to chronic constriction injury and treated with photobiomodulation. To evaluate the supraspinal involvement of the CB1 receptor in the photobiomodulation-induced antinociception, the cannabinoid CB1 receptor antagonist AM251 (0.1µg/vol 0.2µL) was injected 5 minutes before the photobiomodulation treatment. The photobiomodulation treatment was performed on the fifth day after the stereotactic surgery and chronic constriction injury at a dose of 50J/cm^2 in acute condition. The hot plate and von Frey monofilaments tests were performed to evaluate the thermal and mechanical pain sensitivity, respectively.

RESULTS: The thermal and mechanical nociceptive threshold was higher in mice with chronic constriction injury, injected with saline and treated with photobiomodulation at the dose of 50J/cm^2 in both the hot plate ($p<0.001$) and von Frey ($p>0.001$) tests. These antinociceptive effects were not detected in mice with chronic constriction injury pre-treated with AM251.

CONCLUSION: The present study suggests that CB1 receptors located in Supraspinal structures, participate in the control of neuropathic pain following photobiomodulation treatment in animals undergoing chronic constriction injury.

Keywords: Cannabinoid, Lasers, Pain, Receptors, Rehabilitation.

RESUMO

JUSTIFICATIVA E OBJETIVOS: Estudos demonstraram que o receptor canabinóide CB1 está envolvido na modulação da dor, principalmente pela ativação da via descendente de controle da dor, porém o papel da fotobiomodulação nesse processo não é bem elucidado. Assim, o presente estudo teve como objetivo investigar o envolvimento do receptor CB1 na antinocicepção induzida pela fotobiomodulação a nível supraespinhal.

MÉTODOS: Camundongos machos suíço albinos foram submetidos à lesão por constrição crônica e tratados com fotobiomodulação. Para avaliar o envolvimento supraespinhal do receptor CB1 na antinocicepção induzida por fotobiomodulação foi injetado o antagonista do receptor canabinóide CB1, AM251 (0,1µg/vol 0,2µL) 5 minutos antes do tratamento com fotobiomodulação. O tratamento de fotobiomodulação foi realizado no quinto dia após cirurgia estereotática e lesão por constrição crônica, na dose de 50J/cm^2 em estado agudo. Os testes de placa quente e monofilamentos de *von Frey* foram realizados para avaliar a sensibilidade térmica e mecânica à dor, respectivamente.

RESULTADOS: O limiar térmico e mecânico nociceptivo foi maior nos camundongos com lesão por constrição crônica, injetados com solução salina e tratados com fotobiomodulação na dose de 50J/cm^2 nos testes de placa quente ($p<0,001$) e von Frey ($p>0,001$). Esses efeitos antinociceptivos não foram detectados em camundongos com lesão por constrição crônica tratados com AM251.

CONCLUSÃO: O presente estudo sugere que os receptores CB1 localizados nas estruturas supraespinhais participam do controle da dor neuropática, após tratamento com fotobiomodulação em animais submetidos à lesão por constrição crônica.

Descritores: Canabinóide, Dor, Lasers, Reabilitação, Receptores.

Gabriela Xavier Santos – https://orcid.org/0000-0002-6366-0909;
Giovane Galdino de Souza – https://orcid.org/0000-0002-1898-1973;
Suélen Santos Alves – https://orcid.org/0000-0001-6705-233X;
Gabriela Nagai Ocamoto – https://orcid.org/0000-0001-5496-3548;
Nivaldo Antonio Parizzoto – http://orcid.org/0000-0003-1774-9053;
Luciana Maria dos Reis – https://orcid.org/0000-0002-0672-7804.

1. Universidade Federal de Alfenas, Departamento de Fisioterapia, Alfenas, MG, Brasil.
2. Universidade Federal de São Carlos, Departamento de Fisioterapia, São Carlos, SP, Brasil.

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Correspondence to:
Universidade Federal de Alfenas
Av. Jovino Fernandes Salles, 2600 – Santa Clara
37133-840 Alfenas, MG, Brasil.
E-mail: reislucianamaria@gmail.com

INTRODUCTION

Neuropathic pain is defined as the pain arising as a direct consequence of a lesion or disease affecting the somatosensory system either at the peripheral or central level, even in the absence of nociception[1,2].

The neuropathic pain is maladaptive and does not have biological importance. It is a significant cause of permanent incapacity, mainly when it becomes chronic. Patients usually present spontaneous pain, allodynia, and hyperalgesia and do not respond well to many types of treatment. They may also present comorbidities, such as depression, anxiety, sleep disorders, and, consequently, lower quality of life[3-5].

Pain transmission in the spinal cord is modulated by supraspinal structures, such as periaqueductal gray (PAG), which makes the neuronal connection with the locus coeruleus (LC). The LC, in its turn, is connected to the rostral ventromedial medulla (RVM), which sends projections to the dorsal horn of the spinal cord through the dorsolateral spinal funiculus. This pathway results in the inhibition of the nociceptive information[6,7]. Moreover, the pain caused by lesions in the peripheral nervous system and modulated by the PAG are more likely related to different systems, such as the endocannabinoid system[8,9].

The endocannabinoid system consists of cannabinoid receptors 1 (CB1) and 2 (CB2), their endogenous binders, and enzymes that catalyze their biosynthesis and degradation[10]. The CB1 receptors are present mainly in the central nervous system, especially in regions related to transmission and pain modulation, such as PAG, RVM, the dorsal horn of the spinal cord, and other motor and limbic structures. On the other hand, the CB2 receptors are located mainly in the peripheral nervous system, but not exclusively[11].

Among the most widely used therapies to treat neuropathic pain are the pharmacological approaches. However, these treatments are not efficient all the time in many patients[12]. Therefore, numerous studies have focused on the search for new therapeutic strategies for the treatment of neuropathic pain. Photobiomodulation (PBM) using low-level laser therapy (LLLT) has been investigated as an alternative treatment for treating this chronic condition.

PBM is a low-cost and non-invasive approach with few contraindications and side effects[13]. Studies have demonstrated positive effects of PBM on neuropathic pain relief in both humans[14-16] and animal models[17,18].

The peripheral attenuation of pain by PBM occurs through two distinct mechanisms: (1) the light interacts directly with neuron promoting the temporary inhibition of the axonal transport in small nerve fibers (A δ and C)[19] and (2) the light may induce anti-inflammatory effects that reduce the oxidative stress and increase the synthesis of ATP by the activation of a cascade of metabolic effects, reducing proinflammatory cytokines, such as prostaglandins and interleukins leading to decrease the activation of nociceptors[20].

However, the involvement of supraspinal structures in pain control after peripheral PBM application has not been clearly elucidated in the published studies. Therefore, the objective of our study is to identify the influence of the CB1 receptor on the dorsolateral column of the periaqueductal gray (dlPAG) in mice treated with photobiomodulation after chronic constriction injury (CCI) of the sciatic nerve.

METHODS

The study was designed as a placebo-controlled trial. The protocols for animal studies were performed in accordance with the IASP and the Brazilian College of Animal Experimentation (COBEA).

Initially, 35 male swiss albino mice (35-40g) were used in this study. However, seventeen animals were excluded based on the following criteria: the cannula did not hit the dlPAG; the mice removed its cannula, and there was no reduction in the nociceptive threshold after CCI. Therefore, eighteen animals were included and divided into 3 groups: (1) CCI+PBM 0J/cm^2, (2) CCI+PBM 50J/cm^{2,} and (3) CCI+SALINE+50J/cm^2 with 6 animals in each group. They were kept under controlled conditions (on a 12h light-dark cycle and temperature at 23±2°C). Mice were given *ad libitum* access to food and water and they were transferred to the habituation room at least one hour before the experimentation.

Surgical procedures

First, the mice were anesthetized intraperitoneally (i.p.) with ketamine (0.5mL, Dopalen® Brasil), xylazine (0.25mL, Anasedan® Brasil), and saline (3.0mL), to a total volume of 0.1mL/kg. Posteriorly, the mouse head and right hind leg (region close to the sciatic nerve anatomical course) were shaved and cleaned with iodine. Then, the animals were positioned in a digital stereotaxic apparatus (Stoelting Co, wood dale, United States). After a 1cm long incision, we removed all soft tissue from the surface of the skull for the cranium implantation of a stainless-steel 7mm 26G guide cannulas leading to the dlPAG. We also placed a dummy into the guide cannula to reduce the risk of occlusion and infection.

The stereotaxic coordinates used for cannula implantation were established as per Franklin and Paxinos: - 4.1mm posterior to bregma; - 1.4mm lateral to the midline, and – 2.3mm ventral to skull surface[21].

After the cannula implantation, we induced the neuropathic pain through CCI[22]. With the mouse still anesthetized in the stereotaxic apparatus and lying on its chest, an incision was made 3-4mm below the femur, and the connective tissue between the gluteus superficialis and the biceps femoris muscles was cut, enabling clear visualization of the sciatic nerve. Then, we performed the constriction of the right sciatic nerve, tying four ligatures with a double knot, using a non-inflammable sterile mononylon 6.0 with stereotaxic angle at 26°.

Next, the animals were placed in their own cages and monitored until they recovered from anesthesia. A period of 5 days was considered for surgery recovery, and the animals were monitored daily until the end of the experiment for signs of infection.

Drug preparation and injection

The influence of the CB1 receptor on the dlPAG was analyzed through the injection of the cannabinoid CB1 receptor antag-

onist AM251 (0.1µg – TOCRIS® USA) (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) diluted in saline solution (0.9%) with 2% of Dimethyl Sulfoxide (DMSO) or saline.

The injection of AM251 in dlPAG was given 5 days after the surgical procedure. An 8mm 33G injection needle (1.0mm beyond the tip of the guide cannula) was connected to a 5µL Hamilton microsyringe (Hamilton Company®) via a polyethylene tube (PE-10). The needle was introduced into the cannula for the injection of 0.2µL for 45 seconds. The movement of a small bubble air in the PE-10 was observed to confirm the successful injection of the drug[23].

Application of photobiomodulation with low-level laser therapy

The device used for PBM was the LASER HTM COMPACT® (HTM indústria de Equipamento Eletroeletrônico Ltda, Amparo, São Paulo, Brasil). The mice were subject to irradiation with infrared aluminum gallium arsenide (AsALGa) laser with 830nm, continuous wavelength, fluence of 50J/cm^{2,} and output power of 30mW, following a standard protocol[24].

The PBM was applied immediately after the injection of the drug in dlPAG. For this procedure, the animals were gently handled to avoid stress and the laser pointer was positioned perpendicular to the skin over the CCI area. We performed a single radiation with a dose of 50J/cm^2 for 300 seconds using the punctual technique. The total size of the radiated area was 1cm^2.

The mice were randomly divided into 3 groups with 6 animals per group: (1) PBM radiation at the dose of 0J/cm^2, laser OFF, and injection of AM251 (CCI + PBM 0J/cm^2 + AM251); (2) PBM radiation at the dose of 50J/cm^2, laser ON, and injection of AM251 (CCI + PBM 50/cm^2 + AM251). The control group, (3) PBM radiation at dose of 50J/cm^2 with laser ON, and injection of saline (CCI + PBM 50J/cm^2 + SALINE).

After the PBM radiation, the animals were allocated in the von Frey apparatus for acclimatization. After 30 minutes of acclimatization, the hot plate test and von Frey testing were initiated.

Nociceptive tests

The hot plate test[24] was used for the evaluation of thermal hyperalgesia. The animals were placed on a 48ºC (47.8-49.4ºC) hot plate (Insight®, Brasil). The latency, time necessary, for the response to the pain stimulus (hind-paw lick, jump etc.) was recorded. In the absence of a reaction, the mice were removed from the hot plate at 30 seconds to avoid tissue injury, and 30 seconds latency was recorded as the response.

The von Frey[5] monofilaments were used for the evaluation of mechanical hyperalgesia. Mice were placed in a plastic cage suspended above a wire mesh grid and allowed to move freely and acclimatize to the testing apparatus for 30 minutes before the experiments. The von Frey monofilaments (Aesthesia®, EUA) were pressed against the plantar surface of the right paw. A positive response was noted if the paw was sharply withdrawn upon the application of the monofilament. We performed three measures of the nociceptive threshold for each animal, separated by 3-minute intervals. The mean of the three measurements was recorded as the mechanical paw withdrawal threshold.

The hot plate test and von Frey testing were performed at three time points: (1) before the surgical procedure (baseline), (2) on the fifth day, prior to the drug infusion and PBM radiation, and (3) also on the fifth day, 30 minutes after the drug injection and PBM radiation (Figure 1). Animals that did not demonstrate a significant reduction in the sensory threshold (compared with the values obtained at baseline) were excluded.

Histological verification of cannula placements

After the completion of all the procedures, the cannula placement was histologically examined. To this end, the mice were anesthetized intraperitoneally with ketamine (0.5mL), xylazine (0.25mL), and saline (3.0mL), to a total volume of 0.1mL/kg. Next, the mice received injections of polyethylene blue, following the same protocol described previously. Then, the mice were euthanized, and their brains were removed and immersed in 10% formalin for fixation. The brains were frozen, and sections were cut at 40µm on a freezing microtome (Lupetec®, Brasil). The samples were analyzed through a microscope (Biolab®, Brasil). The visualization of the methylene blue dispersion indicated the cannula placement. The animals whose cannula did not reach the dlPAG were excluded.

This study was also submitted and approved by the Ethical Committee for the Use of Animals of the Federal University of Alfenas (CEUA- UNIFAL- MG/Brasil – protocol number 09/2016).

Statistical analysis

The data are presented as mean±S.E.M. the statistical analysis of behavioral experiments, we performed the two-way variance analysis (ANOVA) followed by the Bonferroni post hoc test for multiple comparisons, being considered statistically significant values of $p<0.01$. Statistical analysis and preparation of figures were performed using GraphPad Prism Software, Version 5 (GraphPad Software, La Jolla, CA).

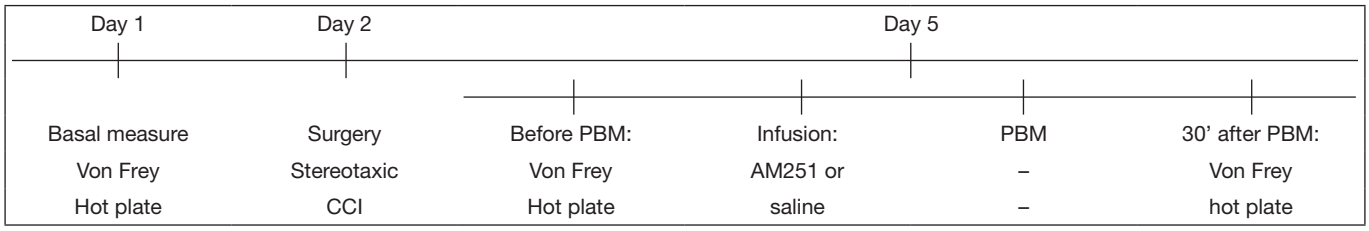

Figure 1. Timeline representation of the experimental protocol
CCI = chronic constriction injury; PBM = photobiomodulation using low-level laser therapy; AM251 = (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide)- Cannabinoid CB1 receptor antagonist.

RESULTS

Figure 2 shows the results concerning the thermal nociceptive threshold assessed by the hot plate test. We observed that after the CCI there was a reduction of the nociceptive thermal threshold in all the evaluated groups ($p<0.001$) compared to baseline. After irradiation with PBM, a significant increase of the nociceptive thermal threshold in the (3) CCI + PBM 50J/cm^2 + saline group (23.5±1.87) was observed when compared to the (1) CCI + PBM 0J/cm^2 + AM251 group (14.08±1.07) ($p<0.001$), showing antinociceptive effect of 50J/cm^2 PBM and there was no effect of AM251 on the nociceptive threshold. The AM251 reversed the analgesic effect of the PBM as observed in the comparison between the groups (3) CCI + PBM 50J/cm^2 + saline (23.5±1.87) and (2) CCI + PBM 50J/cm^2 + AM251 (12.76±0.87) ($p<0.001$).

Figure 3 shows the results concerning the mechanical nociceptive threshold assessed by the von Frey test. We observed that after the CCI there was a reduction of the mechanical nociceptive threshold in all the evaluated groups ($p<0.001$) compared to baseline. After irradiation with PBM, a significant increase of the nociceptive thermal threshold in the (1) CCI + PBM 50J/cm^2 + saline group (1.065±0.071) was observed when compared to the (2) CCI + PBM 0J/cm^2 + AM251 group (0.49±0.06) ($p<0.001$), showing antinociceptive effect of 50J/cm^2 PBM and no effect of AM251 on the nociceptive threshold. The AM251 reversed the analgesic effect of PBM as observed in the comparison between the groups (1) CCI + PBM 50J/cm^2 + saline (1.065±0.071) and (2) CCI + PBM 50J/cm^2 + AM251 (0.50±0.07) ($p<0.001$).

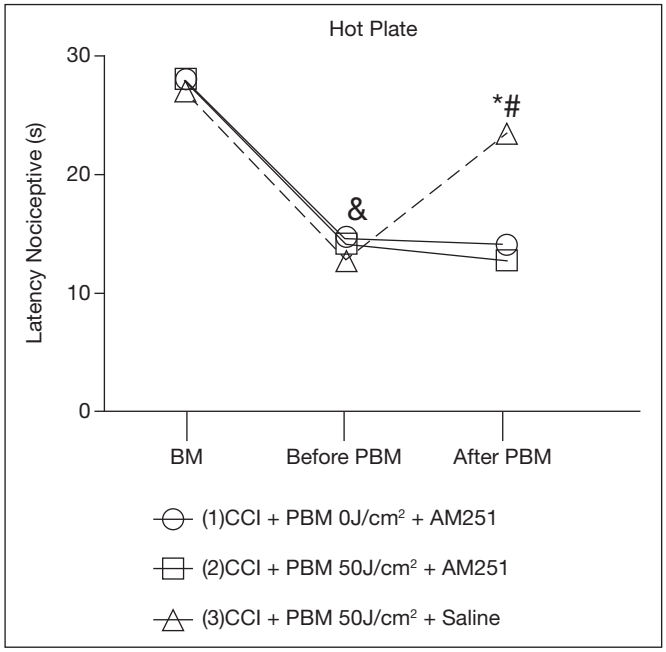

Figure 2. Effect of PBM 50J/cm^2 or 0J/cm^2 with intra-lateral periaqueductal gray side injection (I.dlPAG) of CB1 receptor antagonist AM251 or saline on thermal nociceptive latency of paw withdrawal, by the test of the hot plate.
CCI = chronic constriction injury; Moments of measurement: BM = baseline measurement of nociceptive threshold; Before PBM = threshold measurement before application of PBM, After PBM = measurement of threshold after PBM application. The data represent the mean±SD of the mean; values of $p<0.01$ were considered significant.

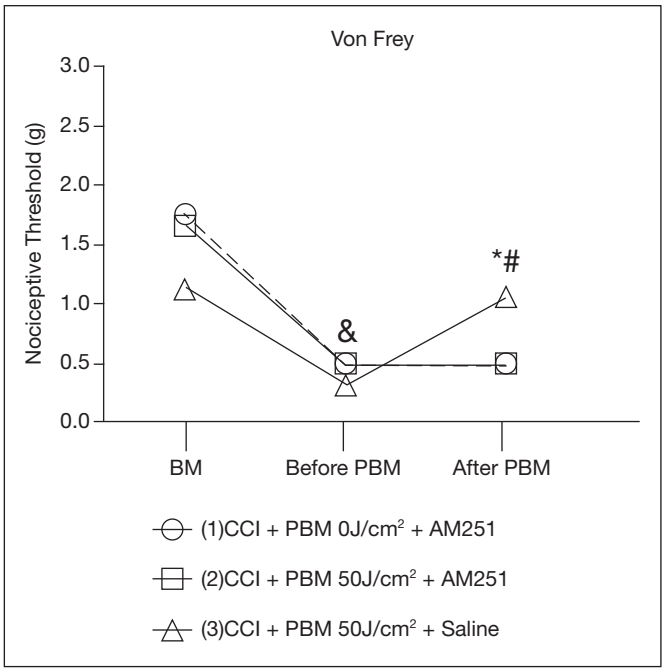

Figure 3. Effect of the PBM 50J/cm^2 or 0J/cm^2 associated with intra-lateral periaqueductal gray matter infusion (I.dlPAG) of CB1 receptor antagonist AM251 or saline on the mechanical nociceptive threshold of paw withdrawal, by the test of filaments of Von Frey.
CCI = chronic constriction injury; Moments of measurement: MB = baseline measurement of the nociceptive threshold; Before PBM = threshold measurement before application of PBM; After PBM = measurement of threshold after PBM application. The data represent the mean±SD of the mean; values of $p<0.01$ were considered significant.

DISCUSSION

In the present study, all groups showed a reduction in the nociceptive threshold, when compared to the baseline, evaluated by the hot plate test and the von Frey test, aligning with the previous studies[22,24,25]. An increase in nociceptive threshold was observed in the group (2) CCI+PBM 50J/cm^2 + saline when compared with baseline values after application of 50J/cm^2 PBM, showing good results for the treatment of neuropathic pain. Most of the previous studies showed beneficial effects even with a large variation in creep, from 1J/cm^2 to 1312J/cm^2 [14]. A recent study[24] used the PBM with wavelength 808nm and varied fluences of 10, 20, and 40J/cm^2 in order to establish a therapeutic window. The results showed that only higher fluences 20 and 40J/cm^2 were able to produce β-endorphin increase and effectively reduce neuropathic pain, corroborating with the results of the present study.

The local peripheral effect of PBM is widely described in the literature. Hsieh et al.[25] demonstrated that PBM applied transcutaneous at the CCI site reduces the levels of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and hypoxia-inducible factor 1α (HIF-1α) leading to improved hypoxia/tissue ischemia. It elevates the levels of endothelial growth factor (VEGF) and nerve growth factor (NGF), resulting in improvement of functional recovery, nerve regeneration and analgesia. Another study observed the reduction of the proinflammatory cytokines Fractalkine (FKN) and IL-1β, and the reduction of the glial satellite cells in the dorsal root ganglion,

causing pain relief and reduced paw protection behavior behavior in animals with neuropathy and treated with PBM[26].

Moreover, previous studies reinforce that the PBM provides improvement in the sciatic functional index in animals with neuropathy[27,28], accelerates nerve regeneration[29-31], increases the expression of the neuronal growth marker GAP 43 related to the process of regeneration[32], increased number of myelinated fibers, improved electrophysiological function, and increased vascular network and collagen[33].

In the present study, it was possible to observe the participation of the CB1 receptor of PAG in the antinociceptive effect after PBM, since the antagonization of these receptors by AM251, a selective antagonist of CB1 receptors, in the PAG, was able to reverse the antinociceptive effect of PBM 50J/cm^2.

Although CNS structures play a crucial role in the modulation of neuropathic pain, there is a scarcity in the literature of data proving the involvement of supraspinal structures, especially regarding PAG, in the antinociceptive effect mediated by PBM. Similarly, the endocannabinoid system, especially the CB1 receptor, has been related to the modulation of neuropathic pain[8,9]. However, little is known about the participation of these receptors in supraspinal structures, such as PAG, in the modulation of neuropathic pain disorders by PBM.

A study[34] showed that the intracerebroventricular and systemic administration of selective agonist of CB1 receptors, ACEA ([N-(2-chloroethyl) 5, 8, 11, 14-eicosaetraenamide]), promotes the reduction of acute and chronic mechanical allodynia in mice submitted to brachial plexus avulsion. However, this antinociceptive effect was better observed in the activation of the central pathway.

Another study reinforces the involvement of the supraspinal structures in pain control by the endocannabinoid system when performing drug infusion CP-55.940 and WIN55,212-2 (cannabinoid receptor agonist) in the lateral ventricle, an increase in the latency in the tail-withdrawal test[35]. In a model of neuropathic pain induced by chemotherapy, the antinociceptive effect was observed, in face of the mechanical and thermal allodynia stimulus, by inhibiting endocannabinoid resorption and its Degradation (FAHH), however, after antagonizing the receptors of CB1 or CB2, the effect provided by the inhibition of FAHH[8] was completely blocked. Concerning the dlPAG, a study demonstrated that it is related to the analgesic effect induced by stress, assessed by the tail test and that this effect is mediated by the activation of the CB1 receptors, through its endogenous ligand anandamide and 2- Araquidonylglycerol (2-AG)[36] reinforcing the results found in this study.

It is possible to suggest the participation of the CB1 receptor in supraspinal structures, specifically of the dlPAG, in the effect of pain modulation after treatment with PBM in mice submitted to CCI, which makes the study pertinent since in the literature is not well Elucidated if the application of the PBM peripherical involves the participation of the CNS.

Studies aimed at understanding the mechanisms of action of resources used in clinical practice are relevant as they strengthen evidence-based practice. New studies with agonist drugs and inhibitors of endogenous endocannabinoid degradation are necessary to better elucidate the analgesia processes by the PBM.

CONCLUSION

According to the data presented, it is suggested the participation of the CB1 receptors in the dlPAG, in the antinociceptive effect promoted by the PBM by laser of gallium arsenide aluminum (AsAlGa) wavelength of 830 nanometers, continuous and with radiant power maximum and average of 30mW in the intensity of 50 J/cm^2, after chronic constriction of the sciatic nerve.

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