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β-Caryophyllene causes remyelination and modifies cytokines expression in C57BL/6 mice with experimental autoimmune encephalomyelitis

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ABSTRACT

The aim of this study is to evaluate the effect of β-Caryophyllene (BCP) on the production of IL-17, transcription factors (T-bet and GATA-3), and remyellination in C57BL/6 mice induced for experimental autoimmune encephalomyelitis (EAE), the model for studying pathogenesis and new therapies for multiple sclerosis. EAE was induced in three groups of C57BL/6, with administration of BCP in two groups (25 and 50 mg/kg/day) by gavage, after the 10th day of induction. At 9 days of treatment, mice were euthanized and CNS was removed for the analysis. The profiles of IL-17, T-bet, GATA-3, and the possible remyelination properties were investigated in the central nervous system (CNS) by immunohistochemistry and Weigert–Pal–Russel's method, respectively. BCP group (50 mg/kg/day) showed a reduction of IL-17 in brain, cerebellum, and medulla ($p < 0.05$) and a decrease of T-bet ($p < 0.05$) in medulla and cerebellum, while GATA-3 was increased ($p < 0.05$) in cerebellum. In both BCP-treated groups were observed remyelination and better organization of myelin. In conclusion, BCP possesses markedly *in vivo* anti-inflammatory and neuroprotective activities and remyelination properties in EAE-mice.

INTRODUCTION

Multiple sclerosis (MS) is a progressive autoimmune disease of the central nervous system (CNS), characterized by focal inflammation, demyelination, and axonal damage. Among autoimmune diseases, MS is the most prevalent, affecting about 2.5 million people worldwide, especially women. The MS symptoms are varied, showing progressive neuronal functional loss, including muscle and extremity weakness, ataxia, spasticity, fatigue, paresthesia, dizziness, visual and cognitive impairment, bowel and bladder dysfunction, emotional and sexual problems, leading to death (Elliott *et al*., 2012; Dias *et al*., 2014; Fontes *et al*., 2014).

Experimental autoimmune encephalomyelitis (EAE) is the established and widely accepted animal model for studying and understanding MS, since both EAE and MS present similar clinical and pathophysiological evolution. The study of immunopathogenesis of EAE and MS demonstrates that both are mediated by the processes orchestrated by T helper cells Th1 and Th17. Therefore, cytokines originating from Th1 cells, such as interferon gamma (INF-γ) and produced by Th17, such as IL-17, are triggers for inflammatory processes and lead to the production of inflammatory mediators, such as TNF-α and free radicals of oxygen, such as nitric oxide (NO) and hydrogen peroxide (H_2O_2), which are closely involved in the immunopathological process and tissue damage (Fontes *et al*., 2014; 2017; Murta and Ferrari, 2013).

β-caryophyllene (BCP) is a natural sesquiterpene found in several medicinal plants, being the main constituent of copaiba oil, a resin extracted from the native Brazilian tree (*Copaifera landsdorfi*), which is popularly use as antirheumatic, analgesic,

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and anti-inflammatory (Fernandes *et al*., 2007; Fontes *et al*., 2017; Galucio *et al*., 2016; Veiga Junior *et al*., 2007; Yamaguchi and Garcia, 2012). These observations have been corroborated experimentally, in which BCP demonstrated antioxidant and neuroprotective activities, suppressing neuroinflammatory responses, such as the secretion of cytokines by inhibiting the activation of nuclear factor kappa β (NF-κβ) by CNS cells, such as microglia (Gelmini *et al*., 2013; Guimarães-Santos *et al*., 2012; Paula-Freire *et al*., 2014).

Studies have also shown that BCP binds selectively with agonist activity to the cannabinoid receptor type 2 (CB_2), but it did not demonstrate affinity with the CB_1 receptors, presenting, therefore, a strong anti-inflammatory activity. Inhibition of NF-κβ by BCP in microglia leads to the reduction of inflammatory cytokines, such as TNF-α and IFN-γ, also significantly inhibiting the generation of reactive oxygen species in the mitochondria (Al Mansouri *et al*., 2014; Bahi *et al*., 2014; Gertsch, 2008; Guo *et al*., 2014; Klauke *et al*., 2014).

In our previous work (Fontes *et al*., 2017), we showed that the oral treatment with BCP inhibited cytokine production, such as TNF-α, IFN-γ , IL-17, and attenuated neurological damages in the CNS of EAE-mice, leading to clinical improvement of animals. In addition, the recent approval of BCP by the FDA, after demonstrating its safety for clinical uses, makes this substance even more promising and an excellent candidate for the treatment of inflammatory neurodegenerative diseases, such as MS (Gertsch, 2008; Varga *et al*., 2018).

To further explore the potential mechanisms of BCP on the animal model of MS, at the first time, the *in situ* effects on remyelinization, inflammatory infiltrate, IL-17 production, and the presence of Th1 and Th2 cells by expression of T-bet and GATA-3, C57BL/6 mice were induced for EAE and treated by gavage with different concentrations of BCP.

MATERIAL AND METHODS

Chemicals

Pertussis toxin, $MOG_{35–55}$, *β*-caryophyllene, complete Freund's adjuvant were acquired from Sigma-Aldrich (St. Louis, MO) and *Mycobacterium tuberculosis* H37RA was from Difco (Detroit, MI, USA). Imunohistochemistry assay kits were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while all other compounds were acquired from Sigma-Aldrich.

Animals

Female C57BL/6 mice (8–12 weeks old; 21–23 g of weight) were obtained from the biotereum of the Federal University of Juiz de Fora (CBR/UFJF) and kept in micro isolator cages. All the animal experimental protocols and care were approved by the Ethical Committee for Animal Care of the Federal University of Juiz de Fora (Protocol nº. 039/2010).

Induction of EAE

Mice were subcutaneously (s.c.) injected at the both tail base with 100 µg of myelin oligodendrocyte glycoprotein peptide ($MOG_{35–55}$), emulsified with complete Freund's adjuvant (CFA) (v/v), and supplemented with 400 µg of attenuated *M. tuberculosis* H37RA. *Pertussis* toxin at 300 ng/mice was injected intraperitoneally (i.p.) on the first day of immunization and 48 hours later (Alves *et al*., 2012; Corrêa *et al*., 2010). Non-immunized mice were used as myelin control.

In vivo treatment with BCP

After the induction of EAE, mice were divided into three groups (n = 5): $MOG_{35–55}$ immunized group that received only vehicle (EAE group); $MOG_{35–55}$ immunized group that were treated by gavage with BCP (Fig. 1), at the oral doses of 25 mg/kg/day (BCP 25 group); and $MOG_{35–55}$ immunized group that were treated by gavage at doses of 50 mg/Kg/day (BCP 50 group). These doses were based on previous studies where BCP presented *in vivo* anti-inflammatory results (Fernandes *et al*., 2007). After the 10th day of immunization, when 80% of the animals demonstrated clinical aspects of EAE, the treatments were performed daily, for 9 days. At this point, on the day 19 after the induction (peak of EAE), mice were euthanized under deepening anesthesia (i.p.), brain and spinal cord were removed.

Histological assessment of CNS tissues

For histological analysis of CNS tissues, brain and spinal cord were obtained and dissected from the mice and fixed in 10% formalin. Following dissection, the CNS samples were dehydrated in three different concentrations of ethanol (70%, 90%, and 100%) in baths of 1 hour each, with another three additional baths in absolute alcohol. After this, the samples were then bleached in three baths of xylol (1 hour each) and finally underwent impregnation in paraffin in an autoclave at 58°C and inclusion in paraffin was made at room temperature. The blocks were then cut into 5 µm of thickness and, to allow the study of the inflammatory process and axonal damage, were stained by HE, as previously described (Fontes *et al*., 2017). Histopathological and immunohistochemistry examination were performed out by two different pathologists. Photomicrographs were taken with Axiostar plus (Camera Sony DCR-PC 100 e Software Axiovision

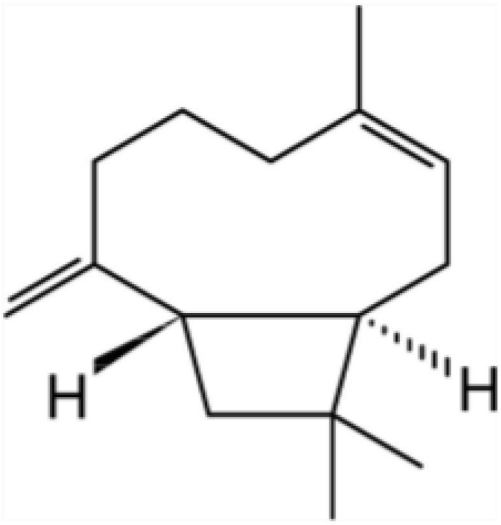

Figure 1. Chemical structure of *β*-caryophyllene.

release® version 4.8). After careful observation, the same significant anatomic were selected for digital capture in all the samples.

Weigert–Pal–Russel's Iron Hematoxylin

After obtaining the histological sections and deparaffinization procedures, the Weigert–Pal–Russel staining was done for general visualization of the CNS, as well as for the distribution and organization of the myelin layer (Behmer, 2003). A digitalized capture of four fields per sample was made in anatomical areas pre-established by Axiostar plus (Camera Sony DCR-PC 100 e Software Axiovision release® version 4.8) (Fontes *et al*., 2017). The automatic morphometry was performed by software Zen2012, from the same company. The results were initially expressed in square micrometers (myelin area per field increasing by 200×). Subsequently, simple arithmetic averages were obtained per group. The mean myelinated area of the healthy group was considered as standard (100%) and from there the other mean values (Group EAE, BCP 25, and BCP 50) were evaluated comparatively.

Immunohistochemistry

Slices with 5 µm from paraffin blocks were placed on silanized slides (3-aminopropyltriethoxysilane; Sigma-Aldrich Inc., St. Louis, MO), deparaffinized in an autoclave at 60°C for 60 minutes, hydrated in sequence by baths in xylol, absolute alcohol, 80% alcohol, 70% alcohol, and distilled water for 5 minutes at room temperature. Tissue samples were immersed in citrate buffer (1 mM; pH = 6.0), blocked with 3% hydrogen peroxide for 30 minutes and incubated with primary antibodies (polyclonal rabbit anti-IL17, anti-T-bet, and anti-GATA-3, diluted 1:100—Santa Cruz, CA) for 1 hour. A second incubation with biotinylated antibodies for 30 minutes was performed, followed by another 30 minutes incubation with the avidin-biotin peroxidase-anti-peroxidase complex. The substrate for staining used was diaminobenzidine chromogen for 1 minute and the omission of the primary antibodies allowed the obtention of the negative control.

Tissue sections were examined by light microscopy (400×), and 10 photomicrographs were collected per section (Zeiss Axiostar, Zeiss, Germany). Five out of ten representative anatomical and digitalized microscopic fields were selected and digitalized (Camera Sony DCR-PC 100 e Software Axiovision release® version 4.8; 400×). All the cells were counted (100%) and, subsequently, only cells considered positive (brown coloration in cytoplasm) were quantified to obtain the percentage. The results were expressed by simple arithmetic mean (± SD) per group.

Statistical analysis

For non-continuous data, we used the Kruskal–Wallis test followed by the Dunn's post doc test. The analysis of significance level was set at 5% ($p < 0,05$). Analyses were obtained from program GraphPad Prism version 5:00 for Windows (GraphPad instat software, version 3, San Diego, CA).

RESULTS AND DISCUSSION

The medicinal application of copaiba oil is extensive, including for industries, because it shows diuretic, healing, anti-inflammatory, and anti-tumor effects. However, the anti-inflammatory activity of copaiba oil is undoubtedly the most widespread use in folk medicine and has been extensively investigated. In Brazil, mainly in Amazon region, it has already been used as an anti-inflammatory (Veiga Junior *et al*., 2007; Yamaguchi and Garcia, 2012).

Regarding the activity of copaiba oil in its various immunomodulatory activities, its effects were investigated *in vitro* in C57BL/6 mice induced for EAE. It was demonstrated that mice spleen cells, after stimulation with $MOG_{35–55}$, showed an inhibition of the production of IFN-γ, TNF-α, IL-17, NO, and H_2O_2 when measured by ELISA (Dias *et al*., 2014).

Chemically, BCP is a sesquiterpene, the major constituent (approximately 50%) of the copaiba oil-resin. It is present in several plants and its concentration in this oil varies with the species and the region of its extraction (Fernandes *et al*., 2007; Veiga Junior *et al*., 2007; Yamaguchi and Garcia, 2012).

The anti-inflammatory effects of sesquiterpenes are known, for example, α-humulene, which leads to a significant increase in the release of anti-inflammatory mediators in mice's allergic airways inflammation models (Rogerio *et al*., 2009).

According to Medeiros *et al*. (2007), BCP inhibits the migration of neutrophils induced by LPS in paw edema in rats. Another experiment demonstrated that in dextran-induced colitis, BCP reduced the inflammatory infiltrate in the colon, decreasing myeloperoxidase and IL-6 levels, leading to a significant improvement in the disease (Cho *et al*., 2007).

In relation to the inflammatory development of atherosclerosis, BCP presented an ability to inhibit the process by reducing the expression of vascular cell adhesion molecule 1 (VCAM-1), which are crucial for the migration of cells to the inflamed site (Zhang *et al*., 2017).

In addition, the anti-inflammatory and neuroprotective activities of BCP on EAE have been discussed in the literature. These effects were recently shown by Fontes *et al*. (2017), which showed that BCP *in vitro* (at concentrations of 20 and 40 µg/ml) is able to reduce NO production and some inflammatory cytokines in cultured splenocytes of C57BL/6 EAE-mice. Also, when orally administered *in vivo*, BCP (25 and 50 mg/kg/day) caused a reduction in the production of IFN-γ, TNF-α, IL-17, NO, and H_2O_2, as well as a decrease in inflammatory areas in the CNS. According to Fontes *et al*. (2017), the reduction in these mediators was correlated with the significant reduction of the clinical score and mean weight of the BCP-treated animals and, consequently, the severity of the symptoms.

Many authors reported that the anti-inflammatory activity of BCP is related to its capacity to selectively stimulate the type 2 cannabinoid receptor (CB_2), which exhibits, after activation, antioxidant, and anti-inflammatory activities. Thus, there is a significant inhibition of the generation of reactive oxygen species and inactivation of NF-κappa β transcriptional factor in the microglia, which implies in the reduction of pro-inflammatory cytokines, such as TNF and IFN- (Cho *et al*., 2007; Fontes *et al*., 2017; Rogerio *et al*., 2009; Zhang *et al*., 2017).

Regarding the mechanism of BCP in EAE-mice, together with our previous work (Fontes *et al*., 2017), in this present study we analyzed, by immunohistochemistry, the *in situ* effects of BCP in EAE-mice.

The tissue expression of IL-17, T-bet, and GATA-3 transcriptional factors was investigated in EAE-mice orally treated with BCP (25 and 50 mg/kg/day) in comparison with untreated EAE-mice. These effects were evaluated in the separate regions of the CNS (brain, cerebellum, and medulla) for a better understanding of their histopathological aspects.

In EAE model, the literature indicates TCD4 + cells with a self-responsive Th17 profile as the major responsible for the immunopathogenic development. Also, demyelinating lesions are triggered, among other factors, by cytokines, such as IL-17 and chemokines, with high levels of IFN-γ and, therefore, potent activation of macrophages, with high production of NO and H_2O_2 (Ivanov *et al*., 2006; Machado, 2012; Teniente-Serra *et al*., 2017; Yang *et al*., 2008).

After immunohistochemistry, the tissue aspects for IL-17, T-bet, and GATA-3 present in Figure 2, while Figure 3 shows the remyelination results. As described in Material and Methods, these stains are from brain, medulla, and cerebellum, and the statistical analyzes that will be described for each marker represent the mean number of labeled cells/field representative of five fields/animal in each group.

We found a statistically significant ($p < 0.05$) dose-dependent decrease of IL-17 tissue expression in the BCP-treated groups in all the regions of the CNS (brain, cerebellum, and medulla) (Fig. 4).

Corroborating with our results that showed lower expression of IL-17 *in situ*, previous reports also showed lower levels of IL-17 by splenocytes both with copaiba oil (which presents BCP as the main constituent) (Dias *et al*., 2014) and BCP (Fontes *et al*., 2017). Also, Fontes *et al*. (2017) suggested a positive correlation of low levels of IL-17 with the reduction of inflammatory infiltrate in the CNS in BCP-treated mice.

In addition, the literature confirms that these data may be associated with the best clinical performance of the animals and the decrease of the inflammatory process demonstrated by HE staining.

IL-17 presents well-established encephalopathogenic actions, such as BBB breaking, as well as direct action on various cell types (microglia, endothelial cells, epithelial cells, synoviocytes, fibroblasts, and myeloid cells), leading to the production of inflammatory cytokines and stimulation of neutrophil infiltration (Guo *et al*., 2014; Ivanov *et al*., 2006; Kroenke *et al*., 2008).

The Th1 population profile is historically recognized as a stimulator of the inflammatory process in EAE and MS, especially at the spinal cord (Goverman, 2009). In our research, this was investigated by immunohistochemistry through its transcriptional factor, T-bet. Our results showed a significant reduction ($p < 0.05$) in the expression of T-bet cells in medulla and cerebellum in the groups orally treated with 50 mg/kg/day of BCP (Fig. 5). Although the brain was not found statistically significant differences, in our experiments, there is a clear tendency in reduction of the T-bet expression for all BCP-treated groups. In this way, considering the pathophysiology of MS, the literature reports the minor importance of the Th1 profile in the brain in comparison with Th17 response profile (Goverman, 2009).

Th1 cells is known to produce cytokines, such as TNF-α, IFN-γ, and IL-2, which can directly attack myelin layer and promote demyelination by activation of macrophages, astrocytes, and glia cells. When Th1 and Th17 profiles are inhibited, the literature shows the reduction in levels of TNF-α and oxygen radicals, such as NO and H_2O_2. These radicals are closely

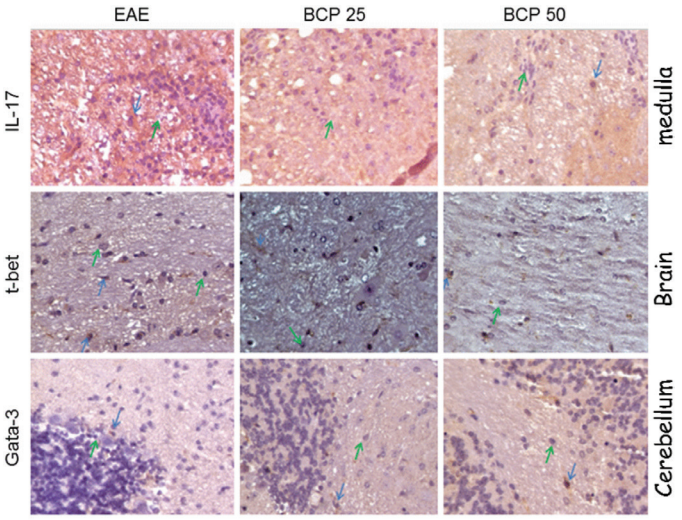

Figure 2. Tissue expression of IL-17 (spinal cord), T-bet (brain), and GATA-3 (cerebellum) in the CNS of C57BL/6 mice induced for EAE, treated with BCP at concentrations of 25 and 50mg/ kg/day. The photos represent immunohistochemical expression of IL-17, T-bet, and GATA-3 in the CNS of the vehicle-treated EAE group, BCP 25 and 50 mg/kg/day groups. Green arrows: negative control of the reaction; Blue arrows: positive reaction control (Camera Sony DCR-PC 100 e Software Axiovision release® version 4.8).

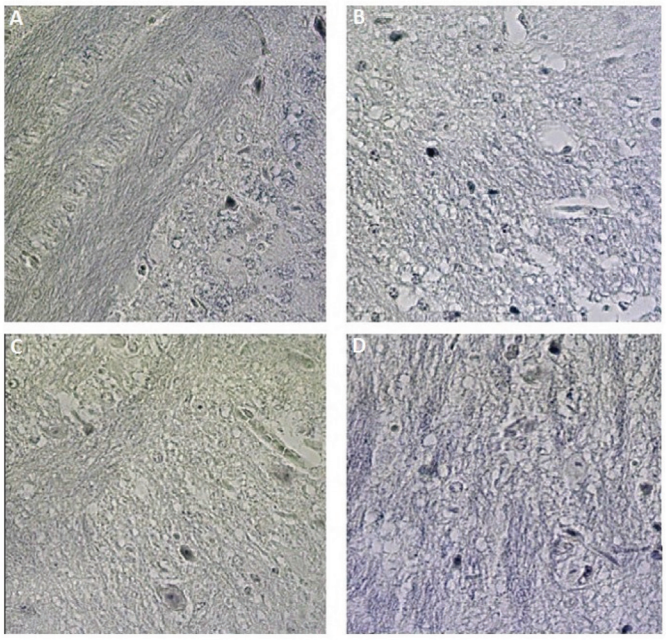

Figure 3. Distribution of myelin stained by Weigert–Pal–Russel Hematoxylin in the CNS (brain) of normal C57BL/6 mice (A), induced for EAE with administration of vehicle (B), treated with BCP at concentrations of 25 mg/kg/day (C), and 50 mg/kg/day (D) (Camera Sony DCR-PC 100 e Software Axiovision release® version 4.8).

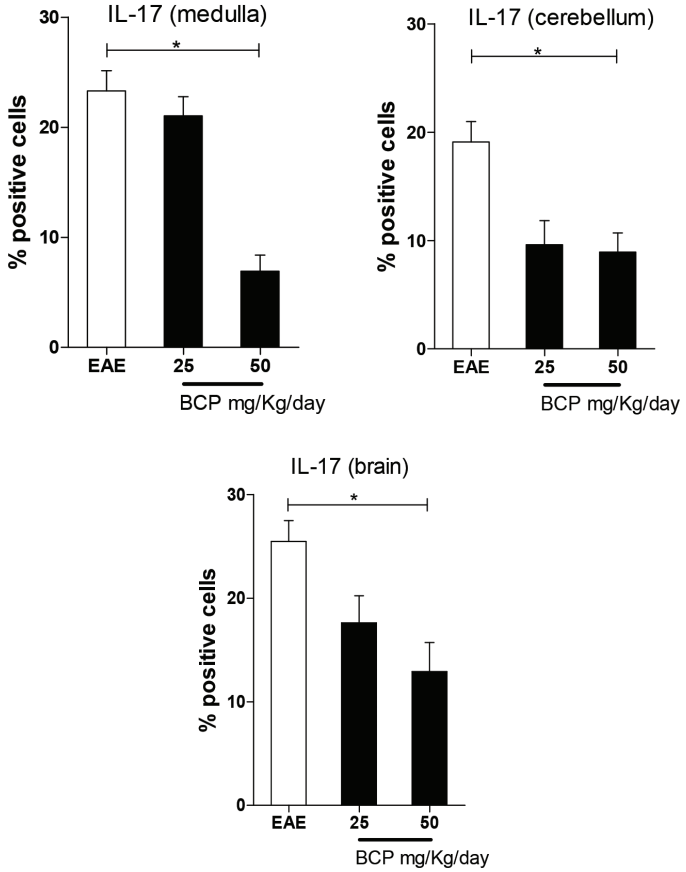

Figure 4. Percentage of positive cells in labeling for IL-17 expression in medulla, cerebellum, and brain (p <0.005).

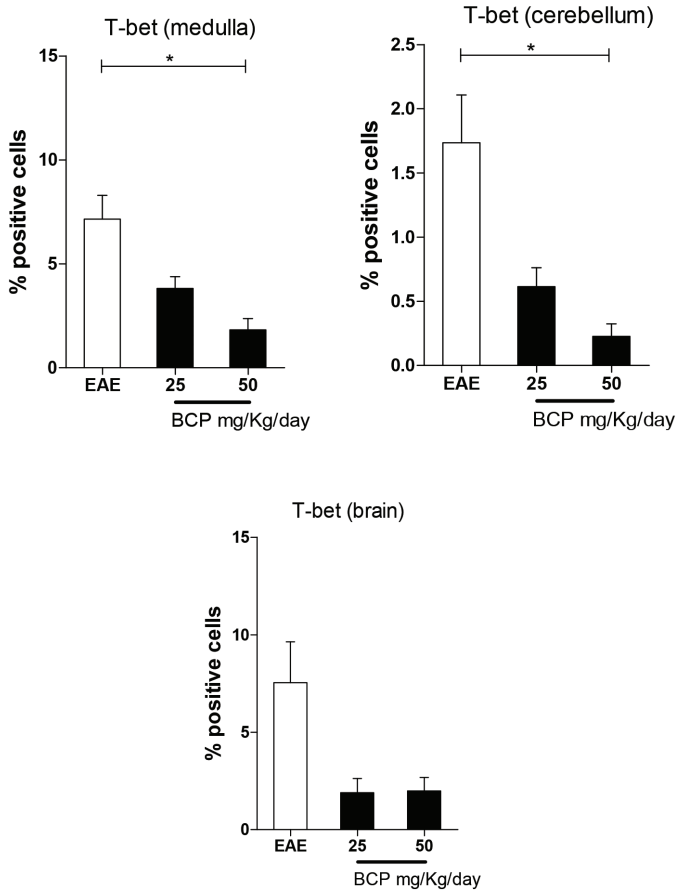

Figure 5. Percentage of positive cells in labeling for T-bet expression in medulla, cerebellum, and brain (p <0.005).

associated with oxidative stress, the inflammatory process, and demyelination (Goverman, 2009; Machado, 2012).

Therefore, our results (Figs. 4 and 5) showed a clear *in situ* reduction in the expression of the Th1 (T-bet) and Th17 (IL-17) profiles. These findings corroborate with the literature, which showed the lower production of cytokines associated with these markers in splenocytes culture (Dias *et al*., 2014; Fontes *et al*., 2017). It is also important to note that the present work was innovative in showing the effect of BCP directly at the CNS level.

The Th2 profile was also investigated by the expression of transcriptional factor GATA-3 (Fig. 6). The literature correlates the regulation of the inflammatory process in the EAE with the increase of Treg and Th2's cytokines in the CNS (Balabanov *et al*., 2006; Fontes *et al*., 2017; Machado, 2012; Yang *et al*., 2008). Our results showed a significant (p < 0.05) increase in GATA-3 expression in animals treated with the highest dose of BCP in cerebellum (Fig. 6).

CD4 + T cells are belonging to the Th2 population and pointed out by several authors as important modulator of Th2 response and, consequently, as of great relevance for decreasing the inflammatory process in EAE and MS. Recent data have shown that the animals treated with glatiramer acetate and dimethylfumarate demonstrated an elevation of Th2 expression in tissues. However, some drugs to treat MS, such as interferon, natalizumab, fingolimod, alemtuzumab, and teriflunomide did not demonstrated this profile (Bar-Or *et al*., 2014; Chiarini *et al*., 2015; Machado, 2012; Pinschewer *et al*., 2011).

In addition to our results for the Th17, Th1, and Th2 profiles, our research verified the effect of BCP on the myelin slayer in animals induced with EAE (Fig. 7). After stained by Weigert–Pal–Russel Iron Hematoxylin, our findings showed an intense remyelination (Fig. 7) along with an evident better organization in CNS for BCP-treated groups. Significant areas of remyelination were found in both treated groups (p < 0.001), without a dose-dependence response.

The remyelination process often occurs due to the action of cells from the innate and adaptive immune response (Dittmer *et al*., 2018; Dombrowski *et al*., 2017). Among these cells, T cells, which due to their phenotypic and functional variations, are shown to be effective in myelin damage but are also necessaries for the successful of the remyelination process. The regeneration of myelin in the CNS involves the differentiation of oligodendrocyte progenitor cells (OPC). During MS, despite the large amount of OPC, the remyelination process may fail, suggesting a compromise during the process of oligodendrocyte differentiation. Recent work has discussed stimuli for remyelination and proliferation of oligodendrocytes in EAE, demonstrating the importance of this process in the clinical recovery of diseased animals and prognostic evolution (Dittmer *et al*., 2018; Dombrowski *et al*., 2017).

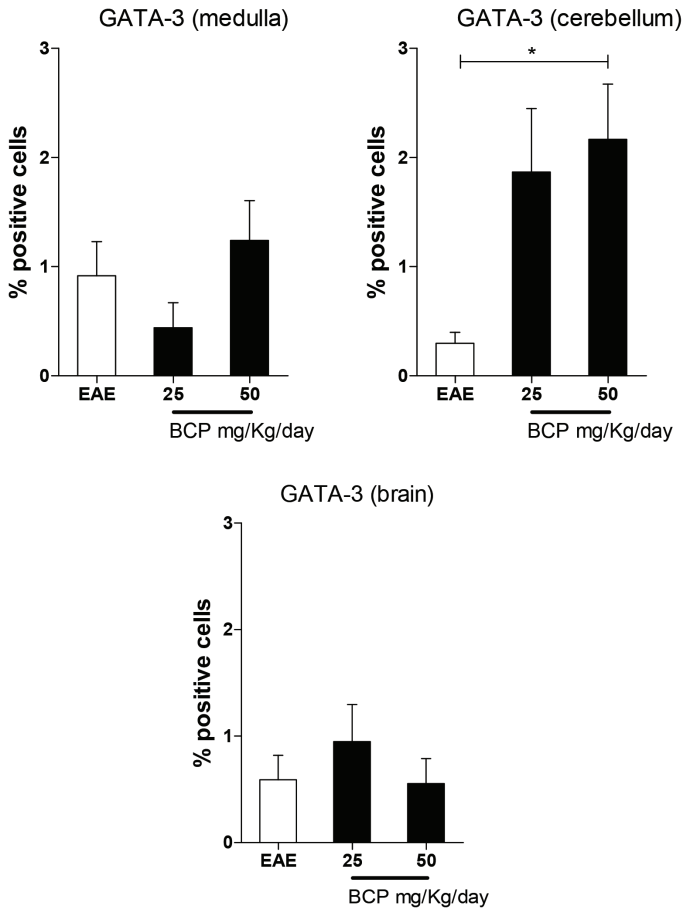

Figure 6. Percentage of positive cells in labeling for GATA-3 expression in medulla, cerebellum, and brain (p <0.005).

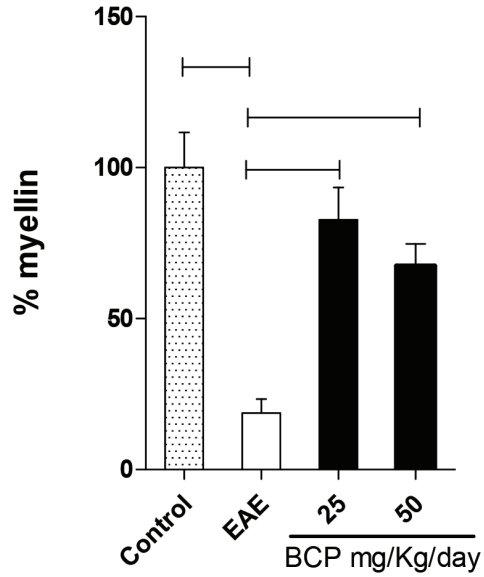

Figure 7. Percentage of remyelination of the treated groups and EAE in comparison to the normal control group (100% myelin).

CONCLUSION

The present work showed the immunomodulatory effect of BCP on the inflammatory aspects of EAE in C57BL/6 mice *in situ*. The effects on decreasing IL-17 cytokine expression, T-bet transcription factor associated with increasing in GATA-3 (cerebellum), and the strong remyelination observed demonstrated, at tissue level, the unquestionable therapeutic potential of BCP for the treatment of the inflammatory and demyelinating diseases, such as EAE and MS.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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