



Oral gabapentin treatment accentuates nerve and peripheral inflammatory responses following experimental nerve constriction in Wistar rats

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HIGHLIGHTS

- A nerve pro-inflammatory effect of gabapentin treatment was identified.
- Gabapentin increased carrageenan-induced paw edema and peritoneal cell migration.
- Concern of gabapentin widespread use in systemic inflammatory diseases was raised.

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ABSTRACT

Gabapentin (GBP) is an anti-convulsive drug often used as analgesic to control neuropathic pain. This study aimed at evaluating whether oral GBP treatment could improve nerve inflammation response after sciatic nerve constriction in association with selected pain and motor spontaneous behavior assessments in Wistar rats. We evaluated nerve myeloperoxidase (MPO) and inflammatory cytokines on the 5th day post-injury, time in which nerve inflammation is ongoing. In addition, the role of GBP on carrageenan-induced paw edema and peritoneal cell migration was analyzed. GBP was given by gavage at doses of 30, 60 and 120 mg/kg, 60 min prior to chronic constriction of the sciatic nerve (CCSN) and during 5 days post-injury, 12/12 h. CCSN animals treated with saline were used as controls and for behavioral and inflammation assessments untreated sham-operated rats were also used. On the 5th day, GBP (60 and 120 mg/kg) alleviated heat-induced hyperalgesia and significantly increased delta walking scores in CCSN animals, the latter suggesting excitatory effects rather than sedation. GBP (60 mg/kg) significantly increased nerve MPO, TNF- α , and IL-1 β levels, comparing with the saline group. GBP (120 mg/kg) reduced the anti-inflammatory cytokine IL-10 nerve levels compared with the CCSN saline group. Furthermore, GBP (60 and 120 mg/kg) increased carrageenan-induced paw edema and peritoneal macrophage migration compared with the CCSN saline group. Altogether our findings suggest that GBP accentuates nerve and peripheral inflammatory response, however confirmed its analgesic effect likely due to an independent CNS-mediated mechanism, and raise some concerns about potential GBP inflammatory side effects in widespread clinical use.

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1. Introduction

Neuropathic pain is a direct consequence of injury or disease causing dysfunction of the peripheral and central nervous system at different levels, mostly affecting the middle-aged and elderly with an escalating global burden.

Treatment for neuropathic pain is still not satisfactory for most of the patients especially those with a more severe condition,

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highlighting the need for novel and more efficacious therapy for pain relief [23]. Mechanisms underlying pain relief with the use of anticonvulsants and antidepressants are a promising target for therapy improvements [11]. Previously, we have studied the beneficial effects of vigabatrin (gamma-vinyl-GABA) and other analgesic anticonvulsants (carbamazepine, phenytoin and valproic acid) in a model of neuropathic pain induced by sciatic nerve constriction in rats [1].

Gabapentin (GBP) (1-(aminomethyl)cyclohexaneacetic acid), one structural derivative of the gamma-amino butyric acid (GABA), in addition to being an anti-convulsive drug, is now further considered as an effective therapeutic drug for some forms of neuropathic and post-surgical pain [26]. Gabapentin effects of reducing allodynia may be associated with specific binding to calcium-voltage dependent $\alpha\delta$ subunits, reducing calcium cell influx with changes in neurotransmitter release [13] or activation of protein kinase-G-K⁺ channel pathways [24]. Several studies have shown gabapentin-related analgesic effects in experimentally induced neuropathic pain [7,15]. However, studies associating GBP with the inflammatory response following sciatic nerve constriction are still scarce. This is an important issue since nerve inflammation may influence drug efficacy during neuropathic pain treatment [30].

Hurley et al. after giving a single dose of GBP (300 mg/kg) via gavage, 2.5 h following carrageenan-induced intra-plantar edema in Wistar rats, did not find significant acute edema change [16].

In this current study we explored whether prolonged GBP treatment modulated inflammatory responses following sciatic nerve constriction in rats by assessing the involvement of nerve inflammatory cytokines, associating these outcomes with selected pain-related and motor behaviors. In addition, the role of GBP on carrageenan-induced paw edema and peritoneal cell migration was assessed to evaluate a peripheral inflammatory response.

2. Materials and methods

Protocols from this study were approved by the Animal Care and Use Committee of the Federal University of Ceara and were in accordance with the Brazilian College for Animal Experimentation (COBEA) and the International Association for the Study of Pain (IASP) guidelines.

2.1. Animals

160 male Wistar rats weighing between 250 and 300 g from the Department of Physiology and Pharmacology vivarium at the Federal University of Ceara were used in this study. Rats were housed in a temperature-controlled room ($26 \pm 2^\circ\text{C}$) with free access to water and chow diet in a 12 h/12 h light/dark cycles. All surgical procedures were performed in the laboratory of Experimental Neurology in the Physiology and Pharmacology department at the Federal University of Ceara.

2.2. Sciatic nerve chronic constriction

We used the chronic constriction of the sciatic nerve (CCSN) model to induce the experimental neuropathy, described by Bennett and Xie, 1988 [2] and modified by Sommer and Myers [32]. Animals were anesthetized with intraperitoneal injection of tribromoethylene (25 mg/kg), following trichotomy and anti-sepsis of the surgery field. A 15-mm longitudinal incision of the right thigh, at the level of the femoral trochanter of the posterior limb, was used to access and expose the sciatic nerve after gluteus and femoral biceps dissection. We used three 4-0 cat-gut loose ligatures on the right paw sciatic nerve, distanced approximately 1 mm between the ligatures and proximal to the sciatic trifurcation inducing a slight nerve ischemia. In the left thigh, the sciatic nerve was exposed,

but remained untouched and surgery closed afterwards. Skin and muscular layers were sutured with a 5-0 mononylon thread.

2.3. Drugs and treatment regimens

Gabapentin (GBP) (1-(aminomethyl)cyclohexaneacetic acid, C9H17NO2) (Neurontin[®], Pfizer) capsules were dissolved in 0.9% saline solution and then given orally by gavage every 12 h during a 5 day-treatment course. GBP doses of 30, 60, and 120 mg/kg were used based on a previous study showing its safety use and analgesic effect in a model of neuropathic pain [18]. As the maximum GBP effect occurs 60 min after oral administration [17], the first dose was given 60 min prior to the nerve surgery and 60 min before each behavior test. Following the last behavior test on the 5th day post-injury the animals were euthanized and the sciatic nerve was removed to conduct histological and molecular biology studies.

2.4. Spontaneous motor behaviors

To find out whether GBP could ameliorate motor behaviors after sciatic nerve injury and rule-out a possible sedation effect, cohort animals were kept in a wooden cage (100 cm \times 50 cm \times 50 cm) for a 5-min acclimation time. The observing cage (with Plexiglas) with the floor covered with wood shavings was placed in a slightly illuminated and silent room for behavioral testing. Positioned in front of the cage, the observer could identify each behavioral component and record it using a computer software (Comporta[®]) designed by Prof. Marcus Vale (Federal University of Ceara, Fortaleza, CE, Brazil). Each animal was observed for resting-sleeping and walking behavior and observed during a 30 min-time. The first observation was 60 m before the surgery (baseline) and then on the 5th day post-surgery and 60 m after the last treatment. A delta mean behavior value was derived from baseline (prior to surgery) and 5th day post-surgery time points. Cumulative resting-sleeping behavior was measured (in seconds) if the animals were found lying immobile on the bedding. Cumulative walking behavior was also measured (in seconds). Experimenters were unaware of the identity of the experimental groups.

2.5. Thermal test

In order to confirm the GBP analgesic effect on the CCSN, we evaluated pain-induced responses to thermal stimuli. Tests using noxious (46°C) stimulation were performed involving immersion of the rat's hind paw in a bath until the withdrawal or struggle was observed. After 15 s of exposure (cut-off), the thermal stimulus was removed and the withdrawal latency was determined. The tests were performed on day zero (preoperative day) and on the 5th postoperative day 60 min after GBP-treatment. A delta score was calculated for further analyses.

2.6. Nerve inflammation markers

In order to evaluate GBP effect on nerve inflammation, we obtained snap-frozen sciatic nerves following CCSN for nerve cytokine measurements by immunoenzymatic assays and by immunohistochemistry.

2.6.1. ELISA assays for nerve cytokines

CCSN animals treated with GBP (60 and 120 mg) or saline were sacrificed on the 5th day post-nerve injury and a 20-mm-long sciatic nerve segment, proximal to the ligatures, was harvested and stored in a -80°C freezer until the assay was performed. The tissue collected was homogenized and processed. The detection of TNF- α , IL-1 β , and IL-10 concentrations was determined by ELISA, as described previously [20]. Values are expressed as picograms/milliliter (pg/ml).

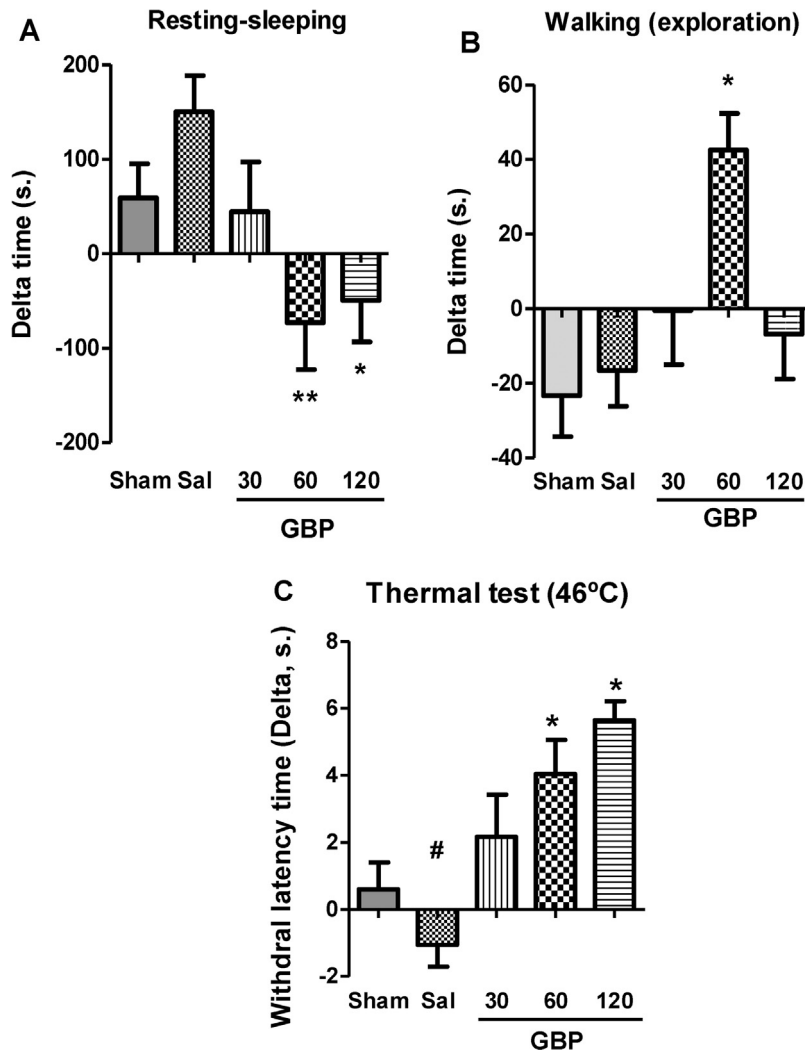


Fig. 1. Gabapentin (GBP) treatment (30, 60 and 120 mg/kg) effect on the spontaneous behaviors resting-sleeping (A) and walking (B) and on the thermal test (C). At least $n = 10$ animals/group. Data (mean \pm SEM) are expressed as delta scores. For the thermal test cut-off: 15 s. * $p < 0.05$ vs. saline group by ANOVA.

2.6.2. Nerve myeloperoxidase (MPO) assay

The MPO enzyme is found primarily in azurophilic granules from inflammatory cells and has been extensively used as a biochemical marker for inflammatory cell infiltration into various tissues. After 5 days of GBP (60 and 120 mg/kg) treatment (12/12 h), the sciatic nerve MPO activity assay was measured as described elsewhere [20]. Results were reported as MPO units/mg of tissue. CCSN and sham operated rats receiving saline were used as controls.

2.6.3. Immunohistochemistry of nerve cytokines

Under deep anesthesia, rats were transcardially perfused with saline and then the distal sciatic nerve segment was harvested 5 mm-distal to the ligatures, and immediately immersed in buffered formaline (pH 7.4) for 24 h and sent to the histology core for immunohistochemistry processing. Immunohistochemistry for TNF- α , IL-1 β , and the macrophage marker-Iba-1 was performed using the streptavidin–biotin–peroxidase method in formalin-fixed, paraffin-embedded tissue sections (4 μ m thick), using polyclonal rabbit anti-mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), as described elsewhere [20]. Negative control sections were processed simultaneously with the primary antibody being replaced by PBS-BSA 5%. None of the negative controls showed immunoreactivity. Slides were counterstained with Harry's haematoxylin, dehydrated in a graded alcohol series, cleared in xylene, and cover slipped.

2.7. Peripheral inflammation models

In order to evaluate a possible GBP effect on other peripheral inflammatory conditions, we subjected CCSN-free rats to well-known models of inflammation.

2.7.1. Carrageenan-induced paw edema

In order to study peripheral GBP inflammatory effects in rats without sciatic nerve injury, paw edema was induced by intra-plantar injection of carrageenan (100 μ g/paw, 0.1 ml) in experimental rats receiving a 5-day GBP treatment (60 and 120 mg/kg, 12/12 h). Rats receiving saline were used as controls. Carrageenan was injected in the ipsilateral hind paw 60 min after last dose of GBP. Paw volume was measured by plethysmometry (Ugo-Basile 7140 Plethysmometer) immediately before (basal volume) and at 1, 2, 3, 4 and 5 h after carrageenan administration. Results were expressed as paw volume variation (ml), calculated by subtracting paw basal volume from the specific volume at times 1, 2, 3 or 4 h.

Furthermore, paw skin samples were removed in a subset of experimental rats to measure skin MPO activity 5 h after carrageenan injection.

2.7.2. Inflammatory cell migration into peritoneal cavity

In order to study the GBP inflammatory effects without sciatic nerve injury, animals without CCSN were challenged with

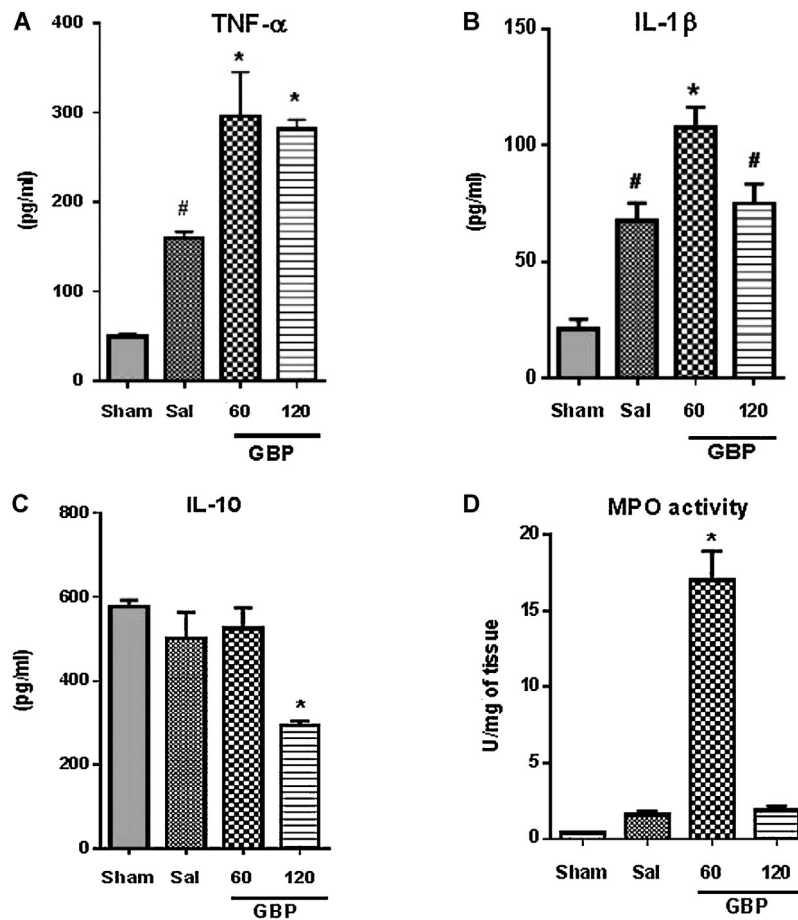


Fig. 2. Gabapentin (GBP) treatment (60 and 120 mg/kg) effect on TNF- α (A), IL-1 β (B), and IL-10 (C) levels in the injured sciatic nerve detected by ELISA. N =at least 5 for all groups. Data (mean \pm SEM) are expressed in picograms/ml. * p < 0.05 vs. saline group (Sal), by ANOVA. (D) Oral gabapentin treatment (60 and 120 mg/kg) increases myeloperoxidase (MPO) activity in the injured sciatic nerve. N =6 (all groups). Data (mean \pm SEM) are expressed as units/milligram of nerve tissue. * p < 0.05 vs. saline group (Sal) by ANOVA.

intraperitoneally injected carrageenan (300 μ g/1.0 ml) 60 min after the last dose of GBP in experimental rats receiving a 5-day GBP treatment (60 and 120 mg/kg, 12/12 h). Rats receiving saline were used as controls. Three hours after carrageenan injection, animals were euthanized and peritoneal fluid was collected. Total and differential cell counts were performed as described by Souza and Ferreira (1985) [6].

2.8. Statistics

Normal distribution was assessed using Kolmogorov–Smirnov's test. Effects amongst groups were assembled using one-way ANOVA for parametric data. In case of p < 0.05, Tukey's test was applied to draw multiple comparisons amongst groups. In some behavioral tests, independent Student's T test was used when appropriate. Values are shown as mean \pm SEM.

3. Results

3.1. GBP effect on spontaneous and induced behaviors

3.1.1. Locomotion behavior

After 5-days of GBP treatment, oral GBP (60 and 120 mg/kg) caused significant reductions in the resting-sleeping behavior ($\Delta = -72.95 \pm 49.6$; $\Delta = -49.53 \pm 43.74$ s, respectively) in comparison with the neuropathic saline group ($\Delta = 150 \pm 38.58$ s) (p < 0.01), Fig. 1A. On the other hand, GBP

(60 mg/kg) increased the walking behavior ($\Delta = 42.50 \pm 9.82$ s.), as opposed to the neuropathic saline group ($\Delta = -16.60 \pm 9.55$ s, p < 0.05) (Fig. 1B), therefore showing that GBP was not able to induce sedation but rather induced an excitatory effect.

3.2. Thermal test

In the thermal test performed to experimental rats on the 5th day post-injury, GBP (60 and 120 mg/kg) significantly increased the ipsilateral hind limb (operated)'s withdrawal latency time ($\Delta = 4.04 \pm 1.01$ s, p < 0.001 and 5.64 ± 0.57 s, p < 0.01, respectively), compared with the neuropathic saline group (-1.06 ± 0.65 s) (Fig. 1C).

3.3. GBP effect on sciatic nerve inflammation

3.3.1. ELISA assay

Sciatic nerve constriction to untreated rats induced a significant increase in the nerve TNF- α levels (p < 0.05). Both GBP doses (60 and 120 mg) significantly increased (120.9% and 92%, respectively) sciatic nerve TNF- α level, as compared to the neuropathic saline group (Fig. 2A). In addition, sciatic nerve constriction to untreated rats induced a significant rise (almost 3-fold increase) in the nerve IL-1 β levels (p < 0.05) compared to the control sham group. Nevertheless, GBP (60 mg) was able to increase nerve IL-1 β levels even higher (59.2%), as compared to the neuropathic saline group (Fig. 2B). GBP (120 mg) significantly reduced (41.6%) IL-10

nerve levels as compared to the neuropathic saline group and as compared with GBP at dose of 60 mg (Fig. 2C).

Furthermore, GBP (60 mg) markedly increased (982.16%, $p < 0.001$) nerve MPO activity as opposed to the neuropathic saline group. However, a higher dose (120 mg) did not show differences from the control (Fig. 2D).

3.3.2. IL-1 β , TNF- α , and Iba-1 immunohistochemistry

Cross-sections from the sciatic nerve showed more diffuse immunostaining for pro-inflammatory cytokines IL-1 β and TNF- α , following chronic nerve constriction on the 5th day post-injury, as opposed to the control group (supplementary Fig. 1). In addition, increased Iba-1 immunolabeling was found especially within endoneurial Schwann cells. Oral GBP (60 mg/kg) markedly increased nerve expression of IL-1 β , TNF- α , and the macrophage marker, Iba-1. The immunolabeling was found mostly in Schwann and macrophage-type cells (supplementary Fig. 1).

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neulet.2013.10.010>.

3.4. GBP effect on peripheral inflammation

3.4.1. Carrageenan-induced paw edema

Oral GBP at doses of 60 and 120 mg increased carrageenan-induced paw edema three hours after carrageenan intraplantar injection ($\Delta = 0.46 \pm 0.04$ and 0.56 ± 0.06 ml, i.e. 53% and 87%, respectively), as compared to the saline group ($\Delta = 0.21 \pm 0.04$). GBP (120 mg/kg) also significantly elevated carrageenan-induced paw edema on the 2th and 4th hour (supplementary Fig. 2A). Furthermore, GBP (120 mg) increased by 96% (33.98 ± 6.94 MPO units/mg of tissue) MPO activity in the paw skin when compared to saline group (17.33 ± 2.34 MPO units/mg tissue), data not shown.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neulet.2013.10.010>.

3.4.2. Carrageenan-induced cell migration to the peritoneal cavity

Oral GBP (60 mg) significantly increased (262.19%) peritoneal total cell migration ($10.25 \pm 1.63 \times 10^{-3}$ cells), as compared to the challenged saline group ($2.83 \pm 0.68 \times 10^{-3}$ cells). Albeit not reaching a significant level, there was a trend of increase cell migration by the 120 mg dose ($5.93 \pm 1.7 \times 10^{-3}$ cells, 109.54%) when compared to saline group (supplementary Fig. 2B). Regarding differential cell count, we found that GBP (60 mg/kg) caused a 29.6-fold significant increase in macrophage migration, as compared to the neuropathic saline group, however GBP treatment at the dose of 120 mg/kg did not cause any significant effect. Likewise, GBP do not cause any significant effect on neutrophil migration to the peritoneal cavity (data not show).

4. Discussion

Our studies confirmed the analgesic effect of GBP using the thermal test following sciatic nerve injury, further supporting the beneficial GBP role on neuropathic pain. DE VRY et al. using the same model also demonstrate antihyperalgesic and anti-allodynic effects of GBP on the dose of 50 mg/kg, i.p. (27th day post-injury) [14].

In contrast to Gustafsson et al. who have shown reduced locomotion and rearing behaviors after acute and cumulative doses of GBP (350 μ mol/kg, \sim 60 mg/kg) [12], we found that prolonged GBP treatment increased walking and reduced rest-sleeping delta scores as compared to controls. We speculate that

this effect may be associated with increased serotonin levels, since blood serotonin was found increased in healthy young men after chronic GBP treatment [29] and since hyperactive starved animals show increased serotonin central neurotransmission [27].

In addition, we found increased MPO levels in the injured sciatic nerve following GBP treatment. MPO is found in the azurophilic granules of monocyte–macrophage and neutrophil inflammatory cells. Furthermore, following GBP treatment, increased immunolabeling of macrophage-type cells (including Schwann cells) was seen in the sciatic nerve stroma (Iba-1 immunolabeling). TNF- α up-regulation seen in the nerve milieu could increase extra-cellular matrix metalloproteases [31], ultimately breaking the blood–nerve barrier and driving more migrating inflammatory cells to the endoneurium stroma [22]. Although TNF- α -related hyperalgesia involving C fibers has been demonstrated following sciatic nerve injury [35], the primary blocking effect of GBP on calcium channels may overcome this effect.

GBP-induced nerve expression of pro-inflammatory cytokines would be beneficial during sciatic nerve re-myelination after injury. According to George et al. data [8], TNF- α nerve mRNA was found elevated on the first 12th hour and remains significantly elevated on the 5th day post crush injury, a period in which myelin debris removal is conspicuous and that coincides with the peak of macrophage influx to the nerve endoneurium (6 day-post nerve constriction in the rat) [3,4]. TNF- α could be released by activated Schwann cells with autocrine and paracrine effects [28] and could recruit additional extra-nerve macrophages [19], required to degrade myelin. Activated macrophages by myelin fragments could release IL-12 and more TNF- α [5]. In our study, IL-10 nerve levels were found similar to the sham group on the 5th day post-constriction, levels that were decreased by GBP treatment (120 mg/kg). George et al. found that endoneurial IL-10 immunoreactivity is acutely deprived 24 h-following sciatic nerve injury that is lately recovered around 5–7 days post-injury [9]. IL-10 is anti-inflammatory cytokine that counterbalances TNF- α and IL-1 β increased levels during Wallerian degeneration [9]. GBP may have a role to sustain the inflammatory-driven myelin clearance.

IL-1 β can promote axonal outgrowth following sciatic nerve injury, which may be mediated by NGF [33]. IL-1 β produced by macrophages can activate nerve Schwann cells and fibroblasts to release NGF [21]. In contrast, IL-1 β anti-antibodies can impair neural regeneration [10]. IL-1 β treated rats (100 ng/ml), after 2 weeks-following sciatic nerve injury, could hasten sensitive nerve fiber regeneration and promote Schwann cell proliferation [33]. Furthermore, pro-inflammatory cytokines, such as IL-1 β , could drive immune-mediated phagocytosis with lymphocyte involvement [34].

Our data support the concept that fine-regulated inflammatory responses are beneficial to nerve regeneration, likely due to removal of myelin debris by macrophage-like activated cells, as myelin debris could be inhibitory to nerve regeneration [34]. The phagocytosis of myelin debris by Schwann cells and macrophages could therefore favor axonal sprouting [33].

GBP treatment increased peritoneal macrophage migration and paw edema induced by carrageenan, suggesting that GBP *per se* is a pro-inflammatory factor, that unexpected effect highlights the need of caution for long-term GBP prescription in conditions of systemic inflammatory diseases.

Although consistently utilized for nerve injury studies, the chronic sciatic nerve constriction model used may have potentiated the GBP-induced nerve inflammatory response due to a foreign body type reaction. Studies using the partial sciatic nerve transection without ligature are being planned by our laboratory to rule-out this possible effect.

Recently, an elegant study from Nadeau et al., using IL-1R1 and TNFR1 knockout mice, further confirms the importance of TNF- α and IL-1 β signaling in improving sciatic nerve recovery following nerve constriction. In addition, corroborating with our immunoassays, these authors found significantly elevated expression of TNF- α and IL-1 β mRNA transcripts with a peak in the first day but with significant high levels until 7 days post-nerve injury, with improved neuropathic pain [25].

More studies are warranted to appreciate more in detail the mechanisms, time course and fine histology nerve changes involved in the GBP induction of pro-inflammatory cytokines and their effects on neuropathic pain.

5. Conclusion

Altogether our findings suggest that GBP accentuates nerve and peripheral inflammatory response, and confirmed its analgesic effect, that could be mainly due to an independent CNS-mediated mechanism, and raise some concerns of potential GBP inflammatory side effects in widespread clinical use.

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Conflict of interest

No conflict of interest exists for this study.

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