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RETRACTED: Ultramicronized Palmitoylethanolamide and Paracetamol, a New Association to Relieve Hyperalgesia and Pain in a Sciatic Nerve Injury Model in Rat

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Abstract: Inflammation is known to be an essential trigger of the pathological changes that have a critical impact on nerve repair and regeneration; moreover, damage to peripheral nerves can cause a loss of sensory function and produces persistent neuropathic pain. To date, various potential approaches for neuropathic pain have focused on controlling neuroinflammation. The aim of this study was to investigate the neuroprotective effects of a new association of ultramicronized Palmitoylethanolamide (PEAum), an Autacoid Local Injury Antagonist Amide (ALIAmide) with analgesic and anti-inflammatory properties, with Paracetamol, a common analgesic, in a rat model of sciatic nerve injury (SNI). The association of PEAum–Paracetamol, in a low dose (5 mg/kg + 30 mg/kg), was given by oral gavage daily for 14 days after SNI. PEAum–Paracetamol association was able to reduce hyperalgesia, mast cell activation, c-Fos and nerve growth factor (NGF) expression, neural histological damage, cytokine release, and apoptosis. Furthermore, the analgesic action of PEAum–Paracetamol could act in a synergistic manner through the inhibition of the NF-κB pathway, which leads to a decrease of cyclooxygenase 2-dependent prostaglandin E_2 (COX-2/PGE_2) release. In conclusion, we demonstrated that PEAum associated with Paracetamol was able to relieve pain and neuroinflammation after SNI in a synergistic manner, and this therapeutic approach could be relevant to decrease the demand of analgesic drugs.

Keywords: Palmitoylethanolamide; inflammation; paracetamol; sciatic nerve injury

1. Introduction

Injury to peripheral nerve tissue is often due to compression, various forms of trauma, or ischemic and metabolic disorders. Loss of sensory function following persistent neuropathic pain are two causes

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of peripheral nerve damage: the injury to nerves can lead to a cascade of events, including increased infiltration of inflammatory cells (for example, macrophages), degeneration of the distal part of the nerve, and other events that are part of a complex mechanism known as Wallerian degeneration [1]. In the pathogenesis of chronic pain, the immune response and consequent inflammatory process play a key role. In some cases, pain, which is one of the five cardinal signs of inflammation, can become persistent and transform in chronic. Persistent pain for the patient can lead to decreased quality of life, especially with consequent failure of various therapeutic approaches [2]. Among the various pharmacological treatments for chronic neuropathic pain, paracetamol is often used for its anti-inflammatory and antinociceptive effect, both individually and in association with other molecules [3]. Paracetamol is widely used for its analgesic properties; however, its mechanism is still poorly understood. A potential mechanism of the antinociception activity of Paracetamol can block prostaglandin synthesis by inhibiting the cyclooxygenase (COX) enzymes. Furthermore, other mechanisms have been suggested that link Paracetamol with the opioid system, the serotonergic neurotransmitter system, and cannabinoid receptors 1 and 2 (CB1 and CB2) [4–6]. CB receptors are the target of a class of endogenous lipids known as endocannabinoids (ECS), such as 2-arachidonoylglycerol (2-AG) and anandamide (AEA). In addition to the classic ECS, the group extends to other endogenous lipids, which, despite showing low affinity for these two receptors, share the enzymes of biosynthesis and degradation with the canonical ECS, one of which is palmitoylethanolamide (PEA) [7]. It is an endogenous lipid belonging to the ALIAmide family that has been identified as regulator of mast cell reactivity [8]. PEA not only acts on mast cell hyper-reactivity [9,10], but also on microglial cells by modulating their activation and therefore playing a role in neuroinflammation events (ALIA mechanism: antagonism of local autacoid injuries) [11,12]. Abnormal control of mast cell activation can provoke a massive release of several factors, such as cytokines, resulting in a reduction of algogenic threshold (peripheral sensitization) and finally neurogenic inflammation [11,13]. To improve the dissolution rate of PEA, two new micronized and ultramicronized formulations of PEA are currently being used as a treatment, showing a lot of benefit [14]. In particular, PEA showed a relevant effect at the dose of 10 mg/kg in a neuropathic pain model obtained by sciatic nerve ligation in the mouse [11]. Interestingly, a previous clinical study showed no effect of PEAum as an add-on therapy for neuropathic pain following Spinal Cord Injury (SCI), with the exception of rescue drug use (such as paracetamol), which was decreased in the PEAum-treated patient group [15]. Thus, the decreased demand of analgesic drugs reported in this trial [15] is very relevant to the scope of our study.

In the current work, we evaluated the potential synergistic effects of the association of PEAum and Paracetamol at low doses of 5 and 30 mg/kg on pain inhibition and pathological processes after crushing of the sciatic nerve, a common model for studying cellular and molecular mechanisms in the peripheral nerve [16].

2. Results

2.1. Effect of PEAum–Paracetamol Association on Histological Changes after SNI

Histological analysis by staining with H&E showed different areas of edema with abundant presence of infiltrated and degraded myelin layers at 14 days after the lesion in the sciatic nerve of SNI + vehicle rats compared to sham groups (Figure 1a,b). Oral treatment with PEAum 5 mg/kg alone or Paracetamol at dose of 30 mg/kg was not able to significantly reduce histological damage (Figure 1c). On the contrary, oral treatment with PEAum–Paracetamol (5 mg/kg + 30 m/kg) significantly reduced the presence of edema and infiltrates compared to the SNI + vehicle group as shown by histological score (Figure 1d,e).

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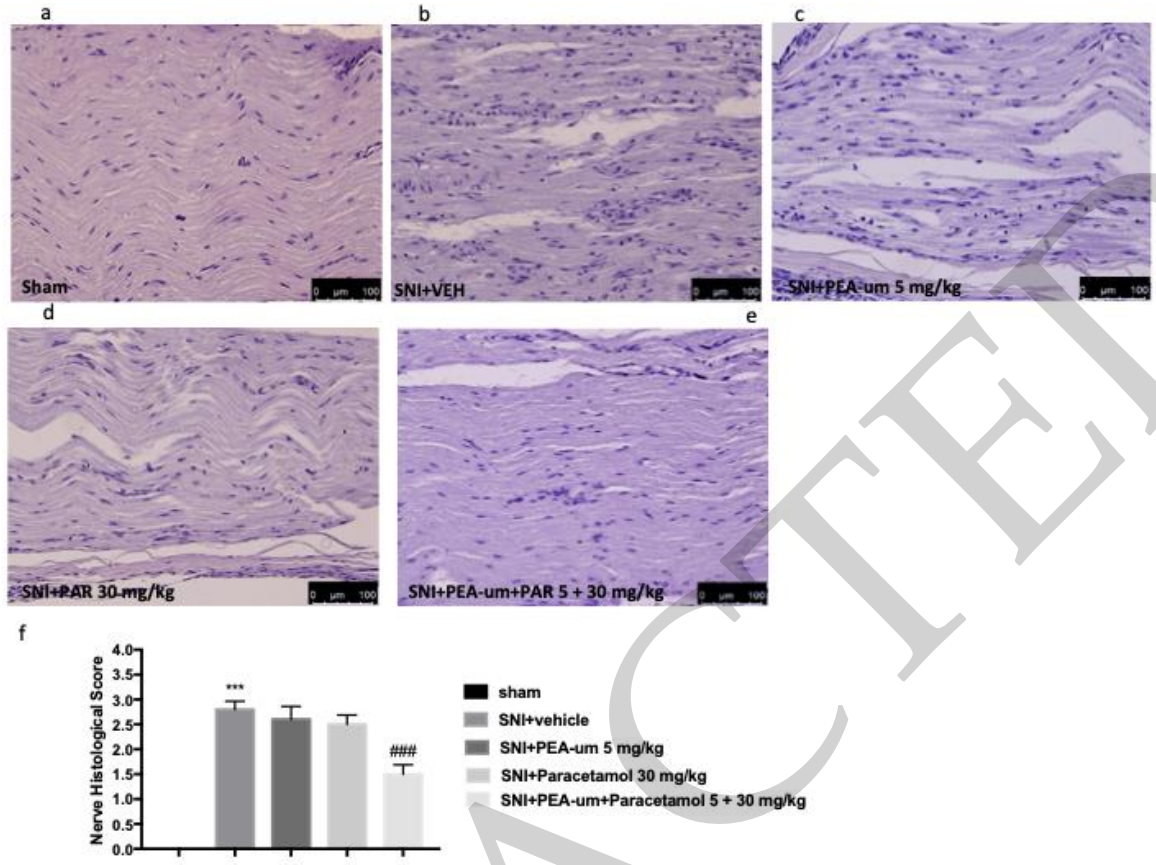


Figure 1. Effect of ultramicronized palmitoylethanolamide (PEAum)–Paracetamol on histological damage. (**a**–**e**) Treatment with PEAum + Paracetamol reduces nerve damage following injury. (**f**) Histological score. The results are expressed as means ± SEM of 8 animals for each group. *** $p < 0.001$ against sham, ### $p < 0.001$ vs. sciatic nerve injury (SNI) + vehicle group; F value 40.58; Degrees of Freedom (DF): 4.

2.2. Effect of PEAum–Paracetamol on Mast Cell Degranulation

We evaluated the action of PEAum–Paracetamol (5 mg/kg + 30 mg/kg) on the number of mast cells, which play a key role in the inflammatory process. As shown in Figure 2, 14 days after SNI, there was a significant increase in the number of mast cells in sciatic nerves of SNI + vehicle rats compared to the sham group (Figure 2a,b). The oral treatment with Paracetamol at the low dose of 30 mg/kg, as well as 5 mg/kg PEAum alone, did not significantly reduce the number of mast cells. On the contrary, the treatment with PEAum–Paracetamol (5 mg/kg + 30 mg/kg) significantly reduced the presence of mast cells compared to the SNI + vehicle group (Figure 2c,d), also shown by mast cell degranulation score (Figure 2e).

2.3. Effect of PEAum–Paracetamol on Behavior after SNI

According to the results obtained by histological analyses, we also evaluated the effects of PEAum–Paracetamol association on the development of hyperalgesia and allodynia related to tissue injury. As show in Figure 3a, rats from the SNI group presented a significant increase in the response to thermal stimuli from days 7 to 14 after nerve crush when compared with the sham animal response (Figure 3a). Single oral treatment with Paracetamol at dose of 30 mg/kg, as well as 5 mg/kg PEAum, did not show significant reduction of thermal hyperalgesia. However, rats treated with PEAum–Paracetamol (5 mg/kg + 30 mg/kg), showed a significant reduction of thermal pain compared to the SNI + vehicle group. In addition, the sciatic nerve crush produced a reduction in the nociceptive threshold to mechanical stimuli from days 7 to 14 in the operated paw (Figure 3b). Single oral treatment with both Paracetamol (30 mg/kg) and PEAum (5 mg/kg) did not show a significant decrease

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of mechanical allodynia, while PEAum–Paracetamol association showed a significant reduction of mechanical pain threshold compared to the SNI + vehicle group (Figure 3b).

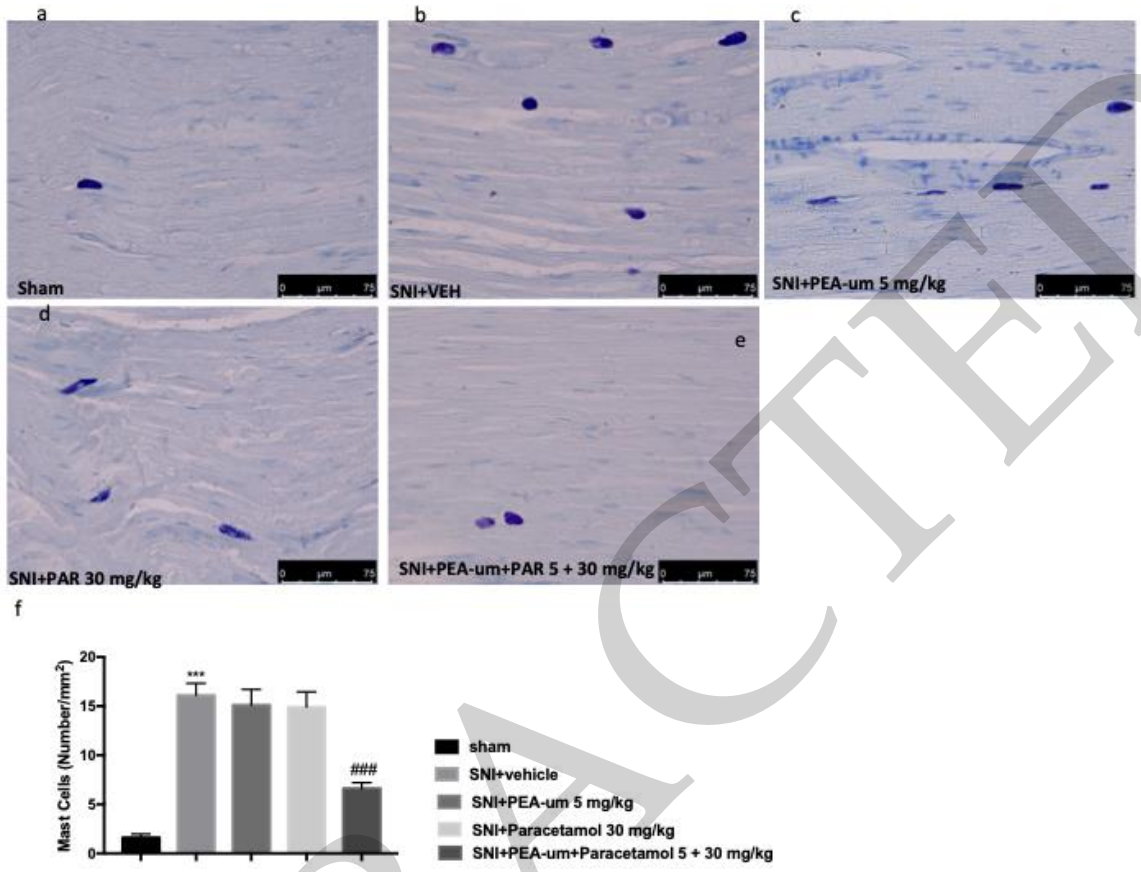


Figure 2. PEAum–Paracetamol effect on mast cell degranulation. (**a**–**e**) Treatment with PEAum + Paracetamol reduces the number of increased mast cells following SNI. Mast cell degranulation score (**f**) The results are expressed as means ± SEM of 8 animals for each group. *** $p < 0.001$ against sham, ### $p < 0.001$ vs. SNI + veh group; F value 29.34; DF: 4.

2.4. Effect of PEAum–Paracetamol on Spinal c-Fos Activation and NGF Expression after SNI

As a marker of neuronal activity linked to algesic stimuli, the expression of c-Fos was evaluated by western blot analysis on the lumbar portion of the spinal cord. At 14 days after the lesion of the sciatic nerve, a significant increase in c-Fos expression in rats of the SNI + vehicle group was found. In contrast, treatment with Paracetamol alone and with PEAum–Paracetamol significantly reduced the expression of c-Fos as shown in the panels of Figure 3c. NGF seems to be involved in tissue inflammation associated with hyperalgesia. In that regard, NGF expression was evaluated by western blot analysis. After 14 days, increased expression of NGF was found in the spinal cord extract in SNI + vehicle rats compared to controls (Figure 3d). PEAum–Paracetamol (5 mg/kg + 30 mg/kg), but not Paracetamol alone (30 mg/kg), was able to reduce these expressions (Figure 3d).

2.5. Effect of PEAum–Paracetamol on Cytokine Levels and Oxidative Stress after SNI

In this study, we also analyzed whether proinflammatory cytokines are implicated in the initiation of neuropathic pain after sciatic nerve injury. Elevated serum levels of TNF-α (Figure 4a) and IL-1β (Figure 4b) were found in the serum of vehicle rats compared to sham. PEAum–Paracetamol significantly reduced these proinflammatory cytokine levels, more than by Paracetamol alone at a dose of 30 mg/kg, showing a possible synergistic effect (Figure 4a,b).

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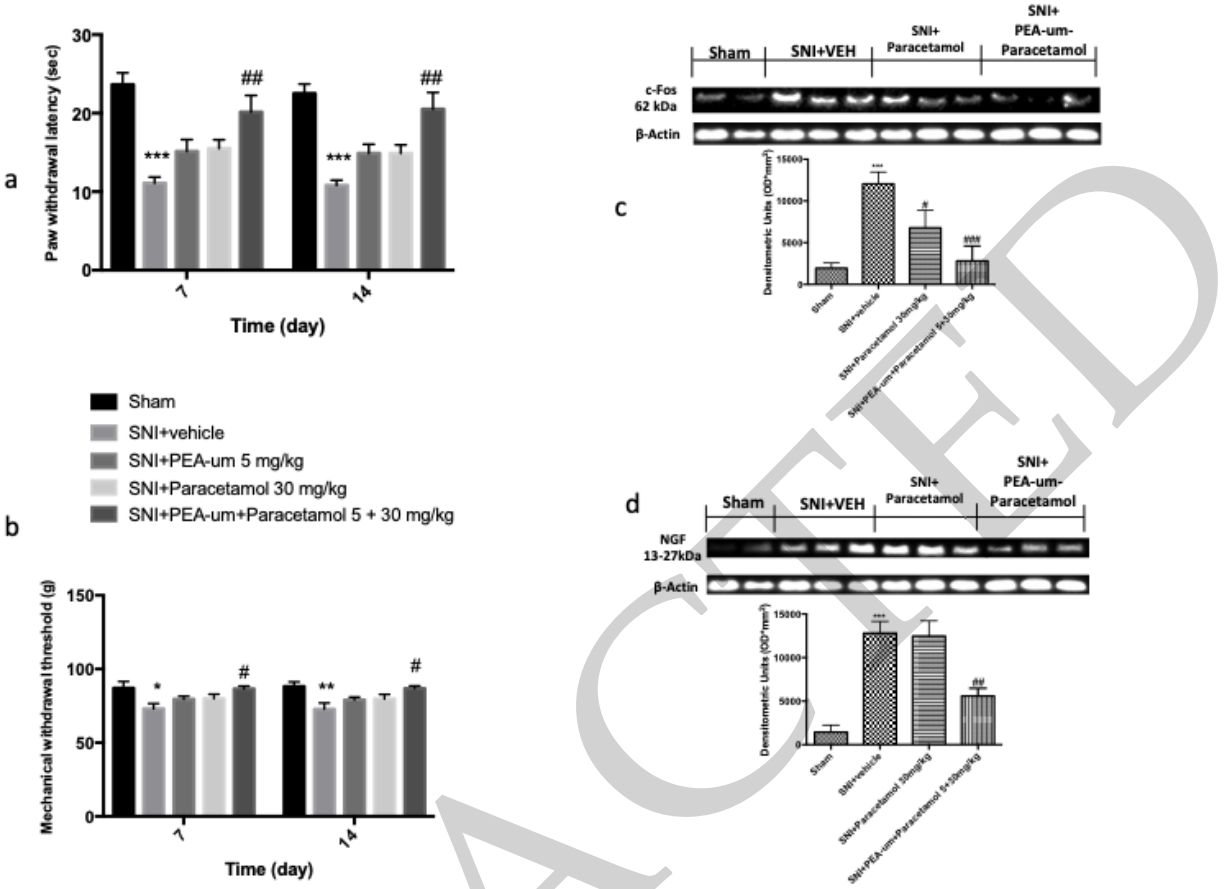


Figure 3. The analgesic effect of daily treatment of PEAum + Paracetamol 5 mg/kg + 30 mg/kg on thermal hyperalgesia (Hot Plate test) (**a**) and mechanical allodynia (**b**). c-Fos (**c**) expression and nerve growth factor (NGF) (**d**) by western blot analysis in the lumbar portion of the spinal cord. Data are expressed as mean ± SEM from $n = 8$ rats for each group. * $p < 0.05$ vs. sham group, *** $p < 0.001$ vs. sham group. # $p < 0.05$ vs. SNI + veh group, ## $p < 0.01$ vs. SNI + veh group, ### $p < 0.001$ vs. SNI + veh group. F value 25.46; DF: 3 (**c**); F value 44.5; DF: 3 (**d**).

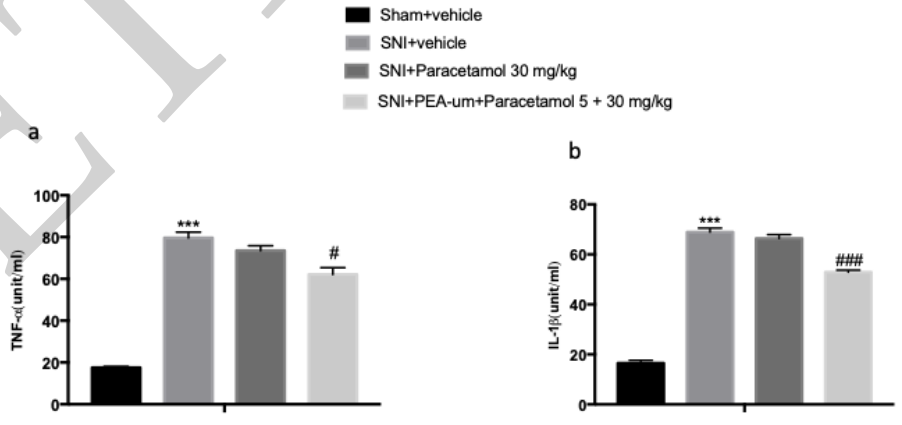


Figure 4. Effect of PEAum–Paracetamol on cytokine release. Serum TNF-α (**a**) and IL-1β (**b**) proinflammatory cytokine levels were detected. PEAum + Paracetamol was able to reduce proinflammatory cytokines. The results are expressed as means ± SEM of 8 animals for each group. *** $p < 0.001$ vs. sham; # $p < 0.05$ vs. SNI + veh, ### $p < 0.001$ vs. SNI + veh group; F value 130.3; DF: 3 (**a**); F value 333.7; DF: 3 (**b**).

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2.6. Effect of PEAum–Paracetamol on Astrocyte and Microglial Activation after SNI

The activation of astrocytes plays a fundamental role in neuroinflammation and the state of neuropathic pain. Astrocyte and microglial cell activation was assessed by immunofluorescence staining for GFAP and Iba-1 markers. GFAP and Iba-1 levels were very low in the sham group (Figure 5a,e), but significantly elevated after sciatic nerve crush (Figure 5b,f). Oral treatment with Paracetamol was not able to reduce these markers of neuroinflammation. On the other hand, PEAum–Paracetamol (5 mg/kg + 30 mg/kg) significantly reduced the increased expression of GFAP and Iba-1 (Figure 5c,d,g,h), showing a possible synergistic action.

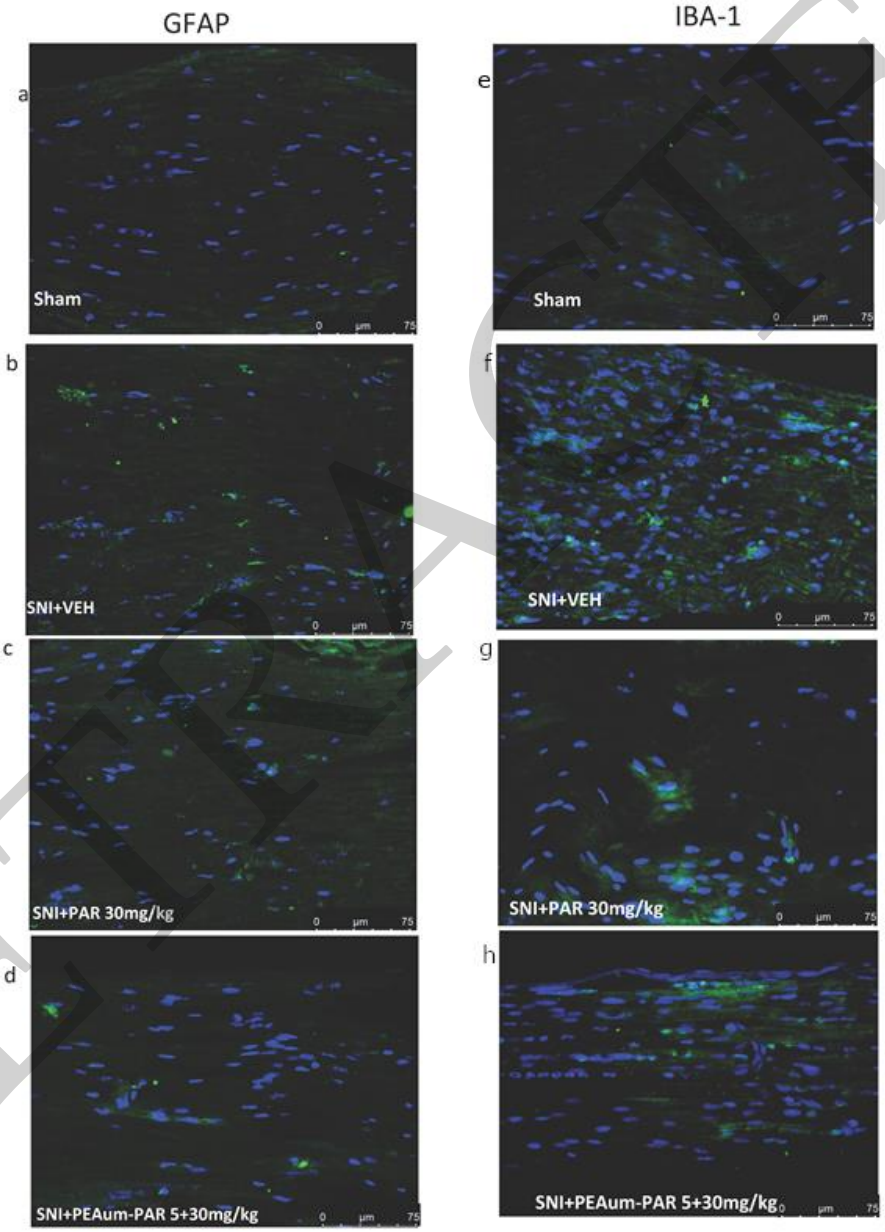


Figure 5. Effect of PEAum–Paracetamol on glial cell activation. The figure shows the immunofluorescence analysis for the activation of microglia (**a**–**d**) and astrocytes (**e**–**h**) following sciatic nerve injury. Treatment with PEAum + Paracetamol reduced the number of astrocytes and microglia, which had increased following sciatic nerve injury.

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2.7. Effect of PEAum–Paracetamol on the Inflammatory Response after SNI

To study the possible mechanisms of the anti-inflammatory effects of PEAum–Paracetamol association, the pathway of NF-κB, a transcription factor involved in the production of proinflammatory cytokines, was evaluated by western blot analysis. In the sham group, NF-κB is stabilized by IkB-α at the cytoplasmic level. After SNI, degradation of IkB-α begins to allow translocation of NF-κB into the nucleus. Our results showed that, following the damage, the level of the IkB-α protein was decreased in the cytoplasm (Figure 6a), while high levels of NF-κB p65 were seen in the nucleus compared to the sham group (Figure 6b). However, PEAum–Paracetamol (5 mg/kg + 30 mg/kg), more than treatment with Paracetamol alone (30 mg/kg), significantly prevented the degradation of IkB-α and the nuclear translocation of NF-κB compared to the SNI + vehicle group (Figure 6a,b). In addition, to better investigate the mechanism of PEAum–Paracetamol association, we also evaluated the effect on COX-2 inhibition and Prostaglandin 2 release (PGE_2) by western blot analysis and ELISA assay in the lumbar portion of spinal cord tissues. After 14 days of SNI, the results showed an increase in both COX-2 and PGE_2 in SNI + vehicle rats. Sole Paracetamol treatment showed a slight significant effect on COX-2 expression, but did not have an on PGE2 release at 14 days after damage (Figure 6c,d). The increase in COX-2 expression and PGE_2 release was reverted by PEAum–Paracetamol (Figure 6c,d).

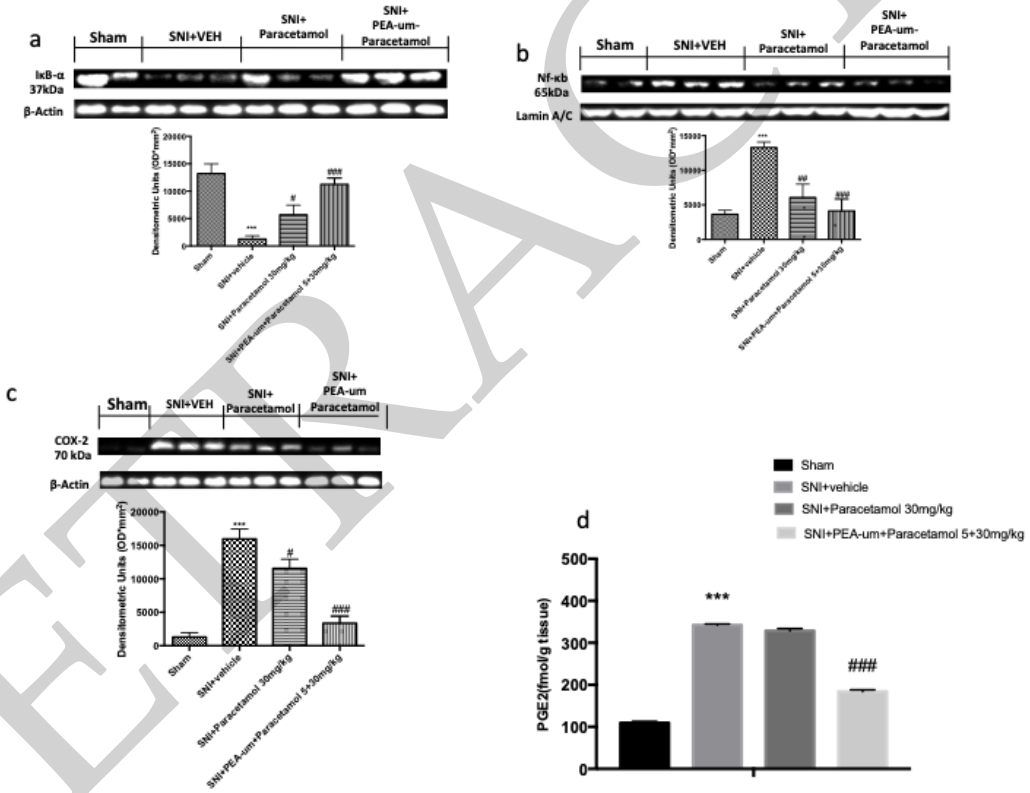


Figure 6. Effect of PEAum–Paracetamol on inflammatory pathways. Treatment with PEAum + Paracetamol, when compared with Paracetamol alone, reduces degradation and consequently the expression of Ikb-α (**b**) shown in the veh group following SNI. The COX-2 expressions by western blot analysis in the lumbar portion of the spinal cord (**c**). PGE2 level in the lumbar portion of the spinal cord determined by ELISA (**d**). Each data are expressed as mean ± SEM from $n = 8$ rats for each group. *** $p < 0.001$ vs. sham group. # $p < 0.05$ vs. SNI + veh group, ## $p < 0.01$ vs. SNI + veh group, ### $p < 0.001$ vs. SNI + veh group; F value 44.5; DF (degree of freedom) 3 (**a**); F value 31.14; DF 3 (**b**); F value 100.2; DF: 3 (**c**); F value 764; DF: 3 (**d**).

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2.8. Effect of PEAum–Paracetamol on the Apoptotic Pathway after SNI

Apoptosis is involved in cell death in numerous neurological disorders. Because of this, the effect of PEAum–Paracetamol on the apoptotic process was also evaluated. TUNEL staining showed a greater number of apoptotic cells in nerve tissues following lesion (Figure 7a,b) compared to sham groups. PEAum–Paracetamol (5 mg/kg + 30 mg/kg) more significantly reduced the number of apoptotic cells (Figure 7c,d) than treatment with Paracetamol alone (30 mg/kg). At the same time, the expression of proteins involved in the apoptotic pathway, such as caspase-3, was evaluated by western blot analysis. High levels of Caspase-3 were found in the vehicle groups 14 days after lesion of the sciatic nerve (Figure 7e). Treatment with PEAum–Paracetamol reduced caspase levels further than Paracetamol alone (at a low dose of 30 mg/kg) 14 days after SNI induction, confirming the previous results obtained by TUNEL assay. Moreover, crush of the sciatic nerve produced an alteration of the expression of the proapoptotic protein Bax and the antiapoptotic protein Bcl-2 (Figure 7f,g). In fact, we noticed a significant increase in Bax expression and consequent reduction of Bcl-2 following SNI, whereas PEAum–Paracetamol treatment caused a significant reduction of the expression of BAX and, at the same time, increased Bcl-2 expression in a more significant manner compared to Paracetamol alone at low dose of 30 mg/kg, demonstrating a possible synergistic effect (Figure 7h).

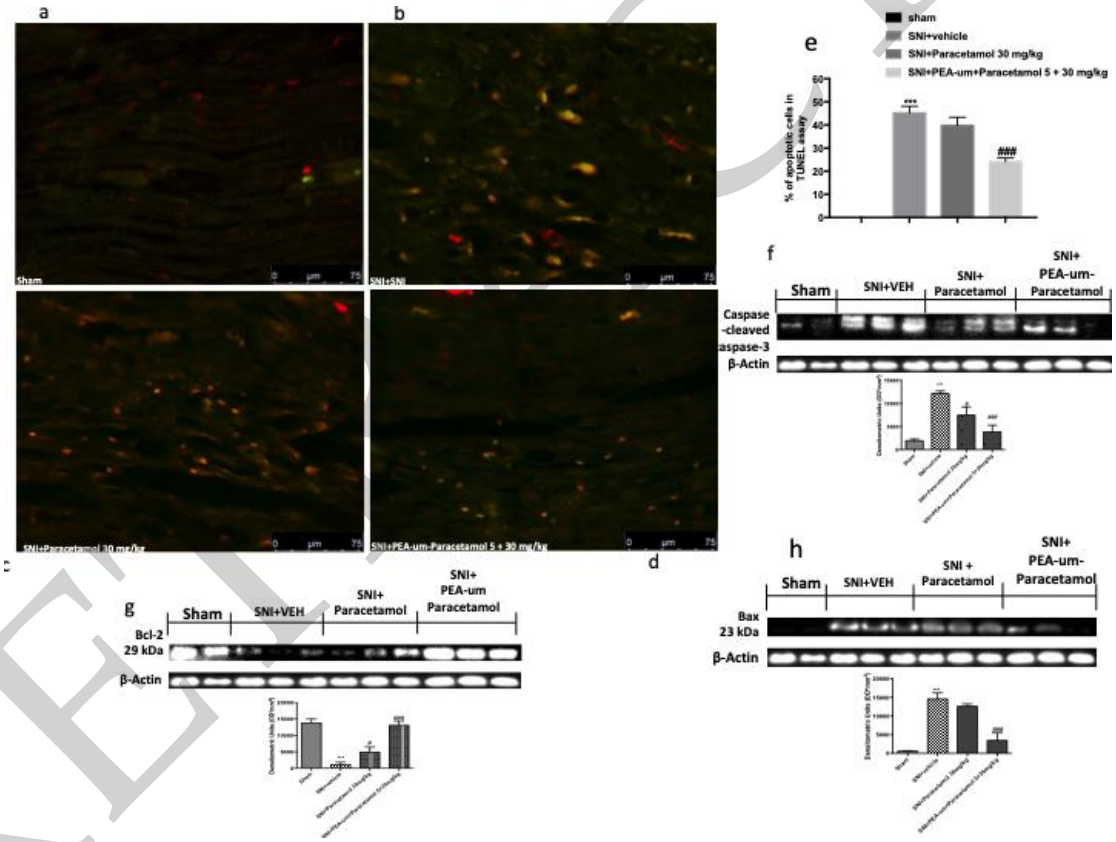


Figure 7. Effect of PEAum–Paracetamol on the apoptotic process. (**a**–**d**) show the analysis of the apoptotic pathway by TUNEL. (**e**) Cell death score. Treatment with PEAum + Paracetamol (**c**) reduces the number of apoptotic cells following injury to the sciatic nerve. Representative Western blots show the effects of PEAum + Paracetamol on the apoptosis markers Caspase-3, Bax, and Bcl-2. Oral administration of PEAum + Paracetamol significantly reduces the expression of Caspase-3 (**f**) and Bax (**h**) and increases Bcl-2 expression (**g**), more than Paracetamol alone. The results are expressed as means ± SEM of 8 animals for each group. *** $p < 0.001$ against sham; # $p < 0.05$ vs. SNI + veh group, ### $p < 0.001$ vs. SNI + veh group; F value 69.78; DF (Degree of Freedom) 3 (**e**); F value 32.79; DF: 3 (**f**); F value 76.59 (**g**); DF: 3; F value 74.89; DF: 3 (**h**).

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3. Discussion

Evidence shows that hyperalgesia is an important component in the pathogenesis of neuropathy [17,18]. A previous study showed that PEAum was able to reduce the neuropathic pain symptoms in diabetic patients [19]. In addition, it was seen that PEAum shows analgesic properties at the dose of 10 mg/kg in a rat model of inflammation and pain [20]. Recently, it has been demonstrated that PEA-OXA, the oxazoline of PEA, has a protective action following sciatic nerve crush [16]. At the same time, Paracetamol, another potential treatment for pain, is widely used to attenuate the hypersensitivity in neuropathic condition [18]. In this study, we demonstrated a possible combination of the analgesic actions of PEAum and Paracetamol at low doses, which can be used as a new approach to relieve neuropathy. In particular, daily treatment with PEAum–Paracetamol showed beneficial effects after SNI, in terms of a reduction of edema, infiltrate, and degradation of myelinated fibers, as well as of histological damage, mast cell activation, and degranulation. A key role in the development of neuropathic pain is played by mast cell degranulation after peripheral nerve injury [13]. The accumulation of mast cells in the injured nerve area [21] leads to release of various proinflammatory mediators, such as TNF-α and IL-1β [22], and these factors are responsible for the recruitment of leukocytes. We showed an increase in mast cell activation following nerve crush damage, correlated to an increase in TNF-α and IL-1β release. PEAum–Paracetamol caused a reduction in mast cell degranulation, followed by a decrease in the levels of cytokines released, in line with previous studies [16,17]. Several works have demonstrated that TNF-α and IL-1β synergistically play a role in regulating the regeneration of peripheral nerves through an indirect mechanism that stimulates NGF expression in fibroblasts after injury [23]. However, in the first stage of neuropathy, it was seen that mast cells release NGF [24], leading to the generation of neuropathic pain [16]. Several emerging studies have emphasized the key roles of NGF in neuropathic pain conditions, although it was initially classified as a trophic factor for both sympathetic and sensory neurons during its development [25]. Our findings revealed that PEAum–Paracetamol was able to decrease mast cell degranulation and the expression of NGF, in line with the decreased levels of cytokine release. Mast cell activation can also cause the release of various substances, such as histamine, that sensitize the nociceptors and contribute to hyperalgesia [13]. In that regard, SNI leads to a reduction of the nociceptive threshold after mechanical and thermal stimuli, thus increasing the sensitivity to pain. Clinical and experimental literature data reported that neuropathic pain can result from both peripheral and central nervous system alterations [26,27]. Exaggerated inflammation in the periphery could then alter the confined microenvironment in the spinal cord, causing microglia activation [17]. In turn, microglia synthesize proinflammatory cytokines and neuroactive particles that provoke hyperactivity of spinal nociceptive neurons [28]. Several studies have shown a correlation between pain and two typical signs of damage to the central nervous system, the activation of microglia and astrocytes [29,30], that were increased at the spinal level in the neuropathic pain state [31]. Our results showed that at the damaged nerve level, there was a significant increase of GFAP and Iba-1 expression, while PEAum–Paracetamol treatment appeared to reduce glial activation following damage [16,17]. Spinal microglia could contribute to pain via amplifying neuronal activity [32]. Accordingly, here, we investigated the potential involvement of c-Fos activation in the development of pain, [33], a marker of activity of neurons excited by algesic stimuli that has been seen to increase following peripheral nerves together with the mechanical allodynia and thermal hyperalgesia [34]. It was also shown that c-Fos could regulate NGF induction after sciatic nerve lesion, and this initial induction of c-Fos seems to be promoted by IL 1 [35]. In this study we reported that the increase in mechanical allodynia and thermal hyperalgesia is accompanied by an increase in the spinal levels of c-Fos. The PEAum–Paracetamol combination was able to reduce both the mechanical allodynia and thermal hyperalgesia, and at the same time reduced c-Fos expression levels, suggesting an antihyperalgesic effect of the association of these two compounds in a synergistic manner. Most of the inflammatory mediators implicated in pain sensitization are under the control of the NF-κB transcription factor [36]. NGF action on its main receptor can lead to Ikb-α degradation, consequently causing NF-κB translocation [36]. PEAum–Paracetamol caused increased cytosolic levels

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of Ikb-α, which is important for the inhibition of NF-κB translocation, and consequent decreased nuclear levels of NF-κB, proposing a protective action of PEAum–Paracetamol in the development of neuropathic pain. The activation of the NF-κB pathway is a pivotal event in a whole series of changes, as in gene expression, and in the increase of the expression of different proteins (such as cytokines and chemokines) and neurotransmitters, and therefore in painful hypersensitivity [37], as well as gene expression levels of several proteins such as COX-2 [17]. An important mediator in the genesis of neuropathic pain is cyclooxygenase 2-dependent prostaglandin E2 (COX2/PGE_2), abundantly produced after SNI. It was seen that, both in invading macrophages [38–40] and in Schwann cells [41,42], there was an upregulation of COX-2 expression in the injured nerves of rats and humans. The COX-2 upregulation in the sciatic nerve injured caused increased levels of PGE_2 [42–44], suggesting a potential key role of COX2/PGE_2 levels in the development and maintenance of neuropathic pain. In that regard, PEAum–Paracetamol was able to inhibit the NF-κB pathway, COX-2 expression, and PGE_2 levels more effectively than paracetamol alone at a low dose of 30 mg/kg. Therefore, we can deduce that the synergistic action of PEAum with Paracetamol could lead to a potentiation of their inhibitory effect on the NF-κB pathway and consequently on COX-2 activity and the release of prostaglandins. Glial cells activation was linked not only with the neuroinflammation, but also with other mechanisms, such as the apoptotic pathway [45]. Neuronal apoptosis plays a key role, as excessive activation of this process can compromise the efficiency of the reparative processes [46]. As a marker of the programmed cell death process, we investigated the Caspase-3 levels [47] and Bax and Bcl-2, two factors involved in the regulation of this process [48]. PEAum–Paracetamol significantly reduced caspase-3 and BAX expression and appreciably increased Bcl-2 expression. Moreover, PEAum–Paracetamol considerably reduced the number of apoptotic cells detected by TUNEL assay.

4. Materials and Methods

4.1. Animals

The Sprague–Dawley rats (males 270–300 g, Envigo, Milan, Italy) were placed in a controlled place (room maintained at 22 ± 1 °C with cycles of 12 h light/dark) and supplied with water and food ad libitum. Animals became familiar with their environment over one week. The Review Board for animal care of the University of Messina approved the research (approval number 549/2018-PR). All in vivo experiments followed the new regulations, Europe (EU Directive 2010/63), Italy (D.Lgs 2014/26), and the ARRIVE guidelines.

The sample size was estimated using a priori power analysis of G*Power software for statistical testing (Die Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany).

4.2. Experimental Model

The experimental model of neuropathic pain was induced by lesion of the sciatic nerve, as previously described [49]. The rats were anesthetized with 2% isofluorane/oxygen; then, a 1 cm incision was made in the skin near the lateral femur after shaving the left lateral thigh. The muscular layers were divided with blunt scissors, the sciatic nerve was located and isolated, the lesion was induced by a clip for 30 s.

Experimental groups:

1. Sham: The rats received anesthesia but did not receive the lesion, and were administered vehicle (1% carboxymethylcellulose and saline) orally for 14 days (n = 8)
2. Sham + PEAum: The rats received anesthesia but did not receive the lesion, and were administered PEAum (5 mg/kg in 1% carboxymethylcellulose and saline o.s.) daily for 14 days (n = 8) (Data not shown)
3. Sham + Paracetamol: The rats received anesthesia but did not receive the lesion, and were administered Paracetamol (30 mg/kg in 1% carboxymethylcellulose and saline o.s.) daily for 14 days (n = 8) (Data not shown)

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4. Sham + PEA + Paracetamol: The rats received anesthesia but did not receive the lesion, and were administered PEAum + Paracetamol (5 mg/kg + 30 mg/kg in 1% carboximethylcellulose and saline o.s.) daily for 14 days (n = 8) (Data not shown)
5. Sciatic nerve injury (SNI) + Vehicle: the sciatic nerve crush was applied to the rats after anesthesia, and subsequently administered only with the vehicle (1% carboxymethylcellulose and saline) orally for 14 days (n = 8)
6. SNI + PEAum: rats were subjected to SNI and treated with PEAum (5 mg/kg in 1% carboximethylcellulose and saline o.s.) daily for 14 days after SNI. (n = 8)
7. SNI + Paracetamol rats were subjected to SNI and treated with Paracetamol (30 mg/kg in 1% carboximethylcellulose and saline o.s.) daily for 14 days after SNI. (n = 8)
8. SNI + PEAum + Paracetamol: rats were subjected to SNI and treated with PEA + Paracetamol (5 mg/kg + 30 mg/kg in 1% carboximethylcellulose and saline o.s.) daily for 14 days after SNI. (n = 8)

The low dosage of Paracetamol was decided on the basis of literature regarding animal studies on SNI [18]. The low dose of PEAum was decided based on our previous studies in which it showed a significant effect at 10 mg/kg [50] and in line with dose–response experiments conducted in our laboratory. All the experimental procedures, behavioral, biochemical, and histological analysis, were carried out under blind conditions.

4.3. Hot Plate Test

To assess hind paw heat sensitivity, Hargreaves' test was conducted using a plantar test device (plantar test; Ugo Basile, VA, Italy) [51] as seen previously [52]. Animals were allowed to freely move within an open-topped transparent plastic box on a glass floor 20 min before the test. A mobile radiant heat source was then placed under the glass floor and focused on the hind paw. Paw withdrawal latencies were measured with a cutoff time of 15 s to prevent tissue damage. The heat stimulation was repeated three times to obtain the mean latency of paw withdrawal with an interval of 10 min. All the results are expressed as paw withdrawal latency (s).

4.4. Von Frey Test

Mechanical allodynia was measured by Electronic von Frey test (BIO-EVF4, Bioseb, Vitrolles, France). The apparatus consisted of a portable force transducer fitted with a plastic tip. When applying a pressure on the tip, the maximal force applied (in grams) is automatically recorded by the electronic device and displayed on the screen. The tip was applied to the plantar area of the hind paw and it began to exert an increasing upward force until the paw was withdrawn. The withdrawal threshold was defined as the force, in grams, at which the rat withdrew its paw. Withdrawal was determined three times, and the reported value is the mean of the three evaluations [53].

4.5. Histology

At the end of the 14 days, standard hematoxylin and eosin staining (H&E) was performed for histological examination as previously described [54]. The portions of sciatic nerve tissue were included in paraffin, then the paraffin blocks were cut, obtaining sections with a thickness of 5 µm. They were then deparaffinized with xylene, stained with H&E staining, and observed under an optical microscope (Leica QWin V3, Cambridge, United Kingdom). The sciatic nerve tissue samples were then observed by light microscopy. For the score related to infiltration in the sciatic nerve, scores of 0, 1, 2, 3, and 4 were used, indicating 0%, 1–25%, 26–50%, 51–75%, and >75% infiltration, respectively [55]. The identification of mast cells was performed in sciatic nerve tissue samples, the sections were analyzed by toluidine blue staining as previously described [56].

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4.6. Western Blot

Western blot analysis was performed on the lumbar portion of the spinal cord; the samples were homogenized in lysis buffer. The cytosolic and nuclear extracts were treated as previously described [57]. The tissues taken were homogenized with the lysis buffers added according to the weight of the sample, and separated following two centrifuge cycles at 12,000 rpm, 4 min for the cytosolic fraction and 10 min for nuclear part. Protein expression was evaluated by specific antibodies: anti-NF-κB p-65 (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA), anti-IkB-α (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA), anti-Caspase-3 (1:500; Santa Cruz Biotechnology, Dallas, TX, USA), anti-c-Fos (1:500; Santa Cruz Biotechnology, Dallas, TX, USA), anti-BAX (1:500; Santa Cruz Biotechnology, Dallas, TX, USA; #sc-7480), anti-Bcl-2 (1:500; Santa Cruz Biotechnology, Dallas, TX, USA; #sc7382), anti-NGF (1:500; Santa Cruz Biotechnology, Dallas, TX, USA), anti-COX 2(1:500; Santa Cruz Biotecnology, Dallas, TX, USA), in PBS 1×, 5% fat-free milk powder and 0.1% Tween-20 at 4 °C overnight. To ascertain that the wells had been loaded with equal amounts of protein, the samples were also incubated in the presence of the β-actin control antibody (cytosolic fraction 1:500; Santa Cruz Biotechnology, Dallas, TX, USA) and Laminin A/C (nuclear fraction 1: 500 Sigma-Alldrich Corp. St. Louis, MO, USA). The signals were captured through a chemiluminescence detection system (ECL) according to the manufacturer's instructions (Thermo, NYSE USA). The expression relative to the band proteins was quantified by densitometry with BIORAD ChemiDocTM XRS + software and standardized for the levels of expression of β-actin and Laminin levels A/C. The images of the band signals (8 bit/600 dpi resolution) were imported into the analysis software (Image Quant TL, v2003). The membrane wells were finally stripped with 2% glycine so that they could be retested several times with the same membrane in order to optimize protein detection and visualize other proteins without the need for further gels and transfers.

4.7. ELISA

Sciatic nerves were harvested from rats 14 days after surgery. The tissue was snap frozen and stored at −70 °C until it was assayed for PGE2 using an enzyme-immunoassay kit (EIA, Assay Design, Ann Arbor, MI, USA) according to the manufacturer's instructions, as reported previous studies [44].

4.8. TUNEL

The TUNEL staining protocol was carried out in agreement with the manufacturer, Roche. The sciatic nerve sections included in the paraffin were deparaffinized in xylene and rehydrated by a series of alcohols at decreasing percentages of ethanol, permeabilized with 0.1 M citrate buffer, and then incubated in TUNEL reaction mixture for 60 min at 37 ° C in the dark. The tissue was then rinsed in PBS three times for 5 min and then observed using exciting wavelengths in the range of 520–560 nm (maximum 540; green) and in the range 570–620 nm (maximum 580 nm; red).

4.9. Immunofluorescence

The analysis of the expression of GFAP and IBA-1, markers for astrocytic and microglia activation, respectively, were performed after boiling the sciatic nerve sections in 0.1 M citrate buffer for 1 min as previously described with modified procedure for the sciatic nerve tissue [58,59]. The sections were incubated with monoclonal anti-GFAP (1:250; Cell Signaling Technology, Leiden, Netherlands) and monoclonal anti-Iba-1 (1:250; Santa Cruz Biotechnology), in a humidified chamber overnight at 37 °C. The sections were then incubated with secondary antibody anti-mouse fluorescein conjugated with isothiocyanate Alexa Fluor-488 (1:2000, Molecular Probes, Monza, Italy) or Texas Red-conjugated anti-rabbit antibody Alexa Fluor-594 (1:1000, Molecular probes) for 1 h at 37 °C. For nuclear staining, 2 µg/mL 4', 6'-diamidino-2-phenylindole (DAPI; Hoechst, Frankfurt, Germany) in PBS was added. The sections were observed using a Leica DM2000 microscope (Leica, Milan, Italy) using 40× magnification. Optical sections of fluorescence samples were obtained using a HeNe laser (543 nm), an ultraviolet laser (361–365 nm), and an argon laser (458 nm) at a scan speed of 1 min, 2 s with a

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maximum of eight averages; the 1.5 µm sections were obtained using a 250 mm hole. The same settings were used for all images obtained from the other samples that had been processed in parallel. Digital images were cutouts and montages of figures prepared using Adobe Photoshop 7.0 (Adobe Systems, Palo Alto, CA, USA). The colocalization of the images was examined with the ImageJ software (National Institutes of Health) as previously described [60].

4.10. Cytokines Measurement

Serum Tumor Necrosis Factor-(TNF-α) and interleukin (IL)-1β levels were evaluated using a colorimetric commercial ELISA kit (Calbiochem-Novabiochem Corporation, Milan, Italy).

4.11. Statistical Evaluation

All values are expressed as mean ± standard error of the mean (S.E.M.) of N observations. For in vivo studies, N represents the number of animals used. In the experiments concerning histological and immunofluorescence analyses the figures shown are representative of three experiments carried out on different days on tissue sections taken from all the animals in each group. The results were analyzed by one-way ANOVA followed by a post-hoc Bonferroni test for multiple comparisons. p values less than 0.05 were considered significant.

4.12. Materials

PEAum and Paracetamol were kindly offered by Epitech Group SpA (Saccolongo, Italy). Unless otherwise indicated, all compounds were obtained from Sigma-Aldrich. All solutions used for in vivo infusions were prepared using non pyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, United Kingdom).

5. Conclusions

Here, we showed the beneficial effects of PEAum and Paracetamol combined at low doses of 5 + 30 mg/kg, respectively, in a model of SNI. The anti-inflammatory action of PEAum together with the analgesic action of Paracetamol was able to cause a decrease of anhedonic behavior with inhibited inflammatory and apoptosis processes both in spinal and in sciatic nerve tissues. The synergistic effect could be related to a decrease of the NF-κB activation pathway together with a reduction of PGE-2 release and COX activity, an important target of the pharmacological action of Paracetamol. This therapeutic strategy with the use of lower doses than those commonly used of PEAum and Paracetamol could be important to combat several diseases associated to pain and inflammation. Further studies are needed to better understand the targets of this pharmacological synergy.

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Conflicts of Interest: Salvatore Cuzzocrea is a coinventor on patent WO2013121449 A8 (Epitech Group Srl), which deals with methods and compositions for the modulation of amidases capable of hydrolyzing *N*-acylethanolamines employable in the treatment of inflammatory diseases. This invention is wholly unrelated to the present study. Moreover, Cuzzocrea is also, with Epitech Group, a coinventor on the patents EP 2 821 083, MI2014 A001495, and 102015000067344, which are unrelated to the study. The remaining authors report no conflicts of interest.

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Abbreviations

PARA	paracetamol
NGF	Nerve growth factor
ICAM-1	intercellular adhesion molecules-1
IL-1β	interleukin 1β
IBA-1	Ionized calcium binding adaptor molecule 1
PGE-2	Prostaglandin E2
COX-2	Cicloxigenase-2
NF-κB	nuclear factor kappa B
PEA	Palmitoylethanolamide
PBS	Phosphate buffered saline
ALIA	Autocoid local injury antagonism
GFAP	Glial fibrillary acid protein
SNI	Sciatic nerve injury
TNF-α	tumor necrosis factor-α

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