

Repeated activation of delta opioid receptors counteracts nerve injury-induced TNF- α up-regulation in the sciatic nerve of rats with neuropathic pain: A possible correlation with delta opioid receptors-mediated antiallodynic effect

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Abstract

Despite mu opioid receptor agonists are the cornerstones of moderate-to-severe acute pain treatment, their effectiveness in chronic pain conditions is controversial. In contrast to mu opioid receptor agonists, a number of studies have reported the effectiveness of delta opioid receptor agonists on neuropathic pain strengthening the idea that delta opioid receptors gain importance when chronic pain develops. Among other effects, it has been shown that delta opioid receptor activation in optic nerve astrocytes inhibits tumor necrosis factor- α -mediated inflammation in response to severe hypoxia. Considering the involvement of tumor necrosis factor- α in the development and maintenance of neuropathic pain, with this study we sought to correlate the effect of delta opioid receptor agonist on the development of mechanical allodynia to tumor necrosis factor- α expression at the site of nerve injury in rats subjected to chronic constriction injury of the sciatic nerve. To this aim, we measured the levels of tumor necrosis factor- α in the sciatic nerve of rats with neuropathic pain after repeated injections with a delta opioid receptor agonist. Results obtained demonstrated that repeated administrations of the delta opioid receptor agonist SNC80 (10 mg/kg, i.p. for seven consecutive days) significantly inhibited the development of mechanical allodynia in rats with neuropathic pain and that the improvement of neuropathic symptom was timely related to the reduced expression of tumor necrosis factor- α in the rat sciatic nerve. We demonstrated also that when treatment with the delta opioid receptor agonist was suspended both allodynia and tumor necrosis factor- α up-regulation in the sciatic nerve of rats with neuropathic pain were restored. These results show that persistent delta opioid receptor activation significantly attenuates neuropathic pain and negatively regulates sciatic nerve tumor necrosis factor- α expression in chronic constriction injury rats.

Keywords

Neuropathic pain, delta opioid receptor, tumor necrosis factor- α , mechanical allodynia

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Background

Opioid receptors, known as MOR, DOR, and KOR (mu, delta and kappa opioid receptor), play a key role in pain control.^{1–3} They are expressed along nociceptive pathways from the first-order primary afferent neurons to descending inhibitory system. Each opioid receptor constitutes a distinct target for pain treatment and



selectively controls nociceptive transmission.⁴ Despite MOR opioid agonists are the cornerstones of treatment of moderate-to-severe acute pain, their effectiveness for chronic pain management is controversial.^{5,6} MOR activation indeed produces not only analgesic effects but also serious side effects, including constipation, nausea, and sedation. Also, the development of tolerance and dependence might occur.^{2,4,7} Lately, due to the availability of highly selective non-peptidic agonist, DOR has become an attractive target for pain treatment^{8–10} and a number of studies indicate a promising role of DOR in chronic pain conditions.^{11–14} In contrast to MOR agonists, DOR activation weakly influences acute pain perception but efficiently decreases persistent pain.¹⁵ Also, in animals with neuropathic pain subjected to peripheral nerve injury (PNI), DOR protein levels increase within the ipsilateral sciatic nerve indicating the occurrence of DOR trafficking in the site of injury.^{11,12,16} Interestingly, DOR knockout animals exhibit enhanced neuropathic and inflammatory nociceptive response, suggesting the existence of an endogenous DOR tone under inflammatory and neuropathic pain conditions.^{8,12}

The pathogenic role of neuroinflammation in the development of neuropathic pain has recently gained more attention.¹⁷ Pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), are considered key modulators in the cross-talk among immune cells, neurons, and glia, and their involvement in the development and maintenance of inflammatory and neuropathic pain conditions^{18,19} has been clearly demonstrated. At the site of nerve injury, TNF- α protein levels are rapidly up-regulated and increased levels are detected until day 14 after nerve injury.²⁰ Also, microinjections of TNF- α directly into normal (uninjured) nerves produces a reduction of pain threshold with development of both thermal and mechanical hyperalgesia,²¹ whereas TNF- α -neutralizing antibodies attenuate thermal hyperalgesia and mechanical allodynia in animal models of neuropathic pain.^{22,23}

Literature data supports a correlation between DOR and TNF- α .^{24–26} In a sepsis rat model, DOR activation was associated with a significant decrease in the serum levels of early and late pro-inflammatory cytokines.²⁴ Wang et al.²⁵ demonstrated that DOR activation inhibits TNF- α -mediated inflammation in response to severe hypoxia in both glial and neuron-like cells.

However, to date no studies indicate whether TNF- α expression is under the control of DOR activation in neuropathic pain; thus, the aim of this study was to investigate whether the effect of the DOR agonist as an analgesic agent in rats with neuropathic pain could be related to the TNF- α expression at the site of nerve injury. To address this issue, we evaluated the effect of repeated administrations of the DOR agonist, SNC80, for seven consecutive days starting from the day of

injury, on (a) the development of mechanical allodynia in rats underwent to chronic constriction injury (CCI) of the sciatic nerve and (b) changes in the expression of TNF- α protein level in the rat sciatic nerve at different time points from CCI by using Western blot analysis.

Materials and methods

Animals

Experiments were performed on male Sprague–Dawley rats (Harlan Laboratories, S.Pietro al Natisone (UD)) weighing 180–200 g. Animals were kept at a constant room temperature ($25 \pm 1^\circ\text{C}$) under a 12:12 h light and dark cycle with free access to food and water. Each rat was used for only one experiment. All tests were performed at room temperature ($22–24^\circ\text{C}$) between 08:00 and 15:00. Experimental procedures were approved by the Local Ethical Committee and the Institutional Animal Care And Use Committee (IACUC), and all experiments were conducted in accordance with International Guidelines as well as European Communities Council Directive and National Regulations (EEC Council 86/609 and DL 116/92).

CCI model of neuropathic pain

The CCI model was used to induce neuropathic pain in rats. CCI was performed according to Bennett and Xie²⁷ with minor modifications.⁵ Briefly, animals were anaesthetized with 2–4% isoflurane inhalation anesthesia, and an incision was made just below the hipbone, parallel to the left common sciatic nerve. The sciatic nerve was exposed and four ligatures (4/0 chromic silk, Ethicon) were loosely tied around the nerve at the level of the mid-thigh and proximal to the trifurcation of the nerve at about 1 mm spacing, until a brief twitch in the respective hind limb was observed. For sham operation, the sciatic nerve was exposed but not manipulated. The rat body temperature was maintained by using a warm blanket kept at constant temperature during surgical procedures until the rats recovered from anesthesia.

Assessment of mechanical allodynia

The assessment of tactile allodynia was performed by measuring the withdrawal threshold of the hind paw in response to a series of calibrated von Frey's filaments.²⁸ Rats were placed in a clear plastic testing chamber with a wire mesh bottom and allowed to acclimatize for 20 min. The ventral surface of the hind paw was mechanically stimulated from below with an ascending series of graded von Frey's filaments with bending forces ranging from 0.02 to 30 g. The paw withdrawal threshold (PWT) was determined by the "up-down" method²⁹

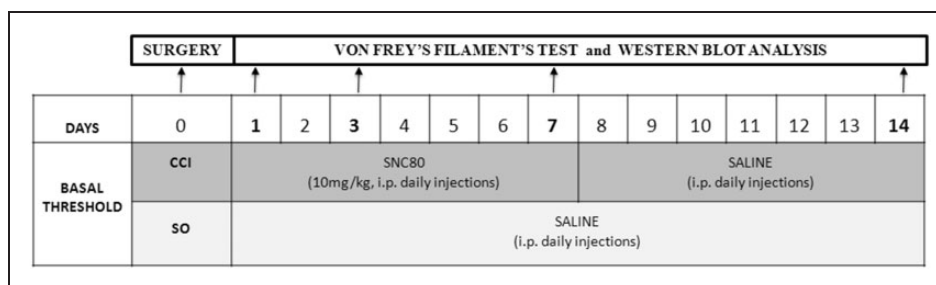


Figure 1. Experimental design.

of sequentially increasing and decreasing the stimulus strength and was expressed as the mean withdrawal threshold.

Western blot analysis

Both right and left sciatic nerves were rapidly removed, frozen in liquid nitrogen, and stored at 80°C until proteins extraction. Tissue samples were homogenized in lysis buffer (Tris-HCl pH = 7.4, 1% Triton-X100, NaCl 150 mmol/L, and EDTA 1 mmol/L), 10 µL buffer/1 mg tissue, and a cocktail of protease inhibitors (1:100, Sigma Aldrich). The homogenate was centrifuged at 15,000 r/min for 15 min at 4°C and supernatant was collected. For Western blotting, protein samples containing an equal amount of protein (50 µg) were electrophoresed on 12% SDS-PAGE gels and transferred to nitrocellulose membranes blocked with 5% non-fat milk powder in TBST buffer. Membranes were incubated overnight at 4°C with primary antibodies, mouse TNF-α (1:1000, Novex) and rabbit β-tubulin (1:1000, Cell signalling) used 1:1000, for proteins detection. After three washing in TBST (Tris 50 mM, NaCl 150 mM, 0.1% tween 20, adjust pH with HCl to pH 7.6), membranes were incubated with anti-mouse (1:20000, Jackson) and anti-rabbit HRP-conjugated (1:50000, Jackson) secondary antibody, for 1 h at room temperature. Proteins bands were visualized with Luminata Forte Western HRP substrate according to the manufacturer's instructions and revealed with Uvitec Cambridge Imaging System. The density of each band was quantified using ImageJ analysis software. All values are shown as the means, and each value represents the average of three independent experiments. Western blot quantification data were analyzed by one-way ANOVA and post hoc test (Bonferroni test). The level of significance was set at $p < 0.05$.

Experimental protocol

Study protocol. Animals were randomly assigned into three groups: (1) rats underwent to CCI (CCI animals); (2) sham operated mice underwent to similar surgery but with no ligatures placed around the sciatic nerve

(SO animals); and (3) un-manipulated mice (naïve animals). Animals were treated as following:

- CCI animals treated with normal saline (NS) (CCI-NS) (i.p. for 14 consecutive days);
- SO animals treated with NS (SO-NS) (i.p. for 14 consecutive days);
- CCI animals treated with SNC80 (CCI-SNC80) (10 mg/kg, i.p., for seven consecutive days plus NS for seven more consecutive days).

The behavioral measurements were carried out 1 day before, and 3, 7, 14 days after CCI. For Western blotting analysis, four rats per group were sacrificed on days 1, 3, 7 and 14 (Figure 1).

Drugs

SNC80 was purchased by Santa Cruz Biotechnology and was dissolved in normal saline before the administration.

Results

Effects of SNC80 on the development of mechanical allodynia

To investigate the effect of persistent DOR activation on CCI-induced mechanical allodynia, rats were injected with the DOR agonist, SNC80 (10 mg/kg, i.p.) for seven consecutive days after the induction of CCI. Basal mechanical threshold was evaluated one day before surgery and then on day 1, 3, 7, and 14 after CCI and compared to vehicle injected CCI and SO rats (Figure 2).

After surgery, CCI rats developed progressive behavioral signs of mechanical sensitization, quantized as a decrease in the PWT in response to stimulation with von Frey's filaments starting from postoperative day 3. No changes in mechanical threshold were observed in SO rats. In CCI rats, the decrease in PWT persisted up to day 14 in comparison to SO animals (Figure 2). I.p. administration of the DOR agonist, SNC80, immediately after the ligature, and for seven consecutive days,

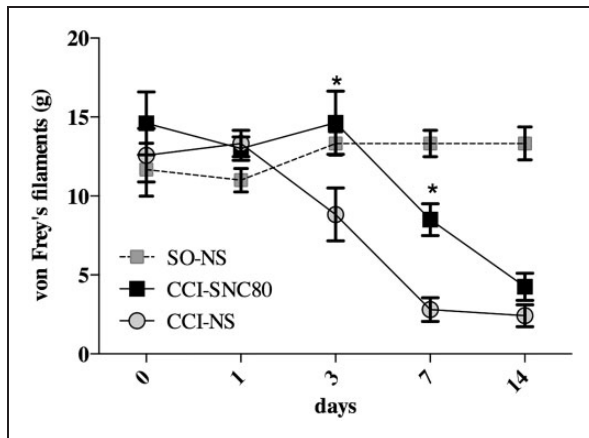


Figure 2. Effect of the DOR agonist SNC80 on CCI-induced mechanical allodynia. CCI rats were treated either with SNC80 (10 mg/kg, i.p.) (CCI-SNC80 group) or normal saline (CCI-NS group), respectively, from 1 to 7 and 8 to 14 days after surgery. SO rats (SO-NS group) received normal saline for 14 consecutive days. Mechanical thresholds, measured with von Frey's filaments, were evaluated before (day 0) and 1, 3, 7, and 14 days after surgery. Results are expressed in grams (g) and represent the means \pm SEM from 8 to 10 rats. * $p < 0.05$ vs. NS-treated CCI rats (CCI-SNC80 vs. CCI-NS) (Two-way ANOVA + Bonferroni post hoc test).

significantly reduced the development of mechanical allodynia as measured at day 3 and 7 after CCI when compared to vehicle-treated CCI rats (CCI-NS). However, the antiallodynic effect disappeared when the treatment with SNC80 was stopped.

Effect of SNC80 on sciatic nerve TNF- α protein level

To test the hypothesis that repeated systemic injections of the DOR agonist, SNC80, might regulate TNF- α expression in the sciatic nerve, we performed Western blot analysis in both ipsi- and contralateral sciatic nerves of CCI and SO rats. Also, a naïve group of rats was included in this experiment. Ipsi- and contralateral sciatic nerves of all rats were removed on day 1, 3, 7, and 14 after surgery and processed for TNF- α analysis.

Western blotting analysis shows no significant differences in TNF- α between groups at day 1 after surgery (Figure 3(a)). On the contrary, on day 3 after surgery, a significant increase of TNF- α expression was observed in the sciatic nerve of CCI rats ipsilateral to the nerve ligature compared to both contralateral sciatic nerve and SO or naïve animals (Figure 3(b)). Interestingly enough, a three-day treatment with the DOR agonist, SNC80, was able to significantly reduce the ipsilateral TNF- α up-regulation in CCI rats, suggesting that DOR activation is able to reduce CCI-induced TNF- α up-regulation in the sciatic nerve. Strikingly, SNC80 completely blocked CCI-induced TNF- α expression in the ligated sciatic nerve after a seven-day treatment (Figure 3(c)).

In a different set of experiments, we investigated whether the effect of SNC80, administered for seven days after surgery, on TNF- α persists until day 14 from CCI. With this in mind, CCI rats were injected for seven consecutive days with SNC80 from surgery and for seven more consecutive days with NS. The sciatic nerve was removed at day 14 from the ligature and compared to both SO and naïve rats. Western blotting analysis clearly shows that SNC80 effect on TNF- α disappeared at day 14 from surgery (seven days from last administration) (Figure 3(d)) suggesting that a continuous DOR activation is needed to block TNF- α up-regulation in the sciatic nerve induced by CCI.

Discussion

Results obtained in this study demonstrated that a continuous administration of the DOR agonist SNC80 significantly inhibited the development (onset and maintenance) of mechanical allodynia in rats with neuropathic pain underwent to CCI. We have shown that the improvement of neuropathic pain symptoms by repeated DOR agonist administration is strictly related to the reduced expression of the pro-inflammatory cytokine TNF- α in the sciatic nerve. In addition, we demonstrated that when DOR agonist treatment was suspended, both allodynia and TNF- α up-regulation were restored.

Neuropathic pain is characterized by an altered transmission and modulation of nociception.^{3,30} Unfortunately its pharmacological treatment remains challenging due to ineffective therapies and resistance to classical analgesics including opioids.^{5,6} A number of studies have reported the effectiveness of DOR agonists on the inhibition of inflammatory³¹ and neuropathic pain induced by diabetes¹⁴ and sciatic nerve injury,^{11,18} strengthening the idea that DORs gain importance when chronic pain develops. DOR density and activity are up-regulated in chronic pain models and enhancement of thermal hyperalgesia, mechanical allodynia, and thermal allodynia was observed in male DOR knockout mice exposed to a partial sciatic nerve ligation.^{8,12} These data indicate the possible existence of a tonic activation of DOR during inflammatory and neuropathic pain that could counteract the manifestations of these pathological processes.

Consistently, our results demonstrated that a continuous DOR activation inhibited the development of mechanical allodynia and that the DOR antiallodynic effect disappeared when the treatment with the DOR agonist, SNC80, was stopped. Moreover, results from this study confirm our hypothesis that DOR agonists could have a critical role in the development of neuropathic pain by reducing TNF- α up-regulation in the injured nerve. Several studies showed the TNF- α overexpression in different states of chronic pain.^{20,32} Consistent with data

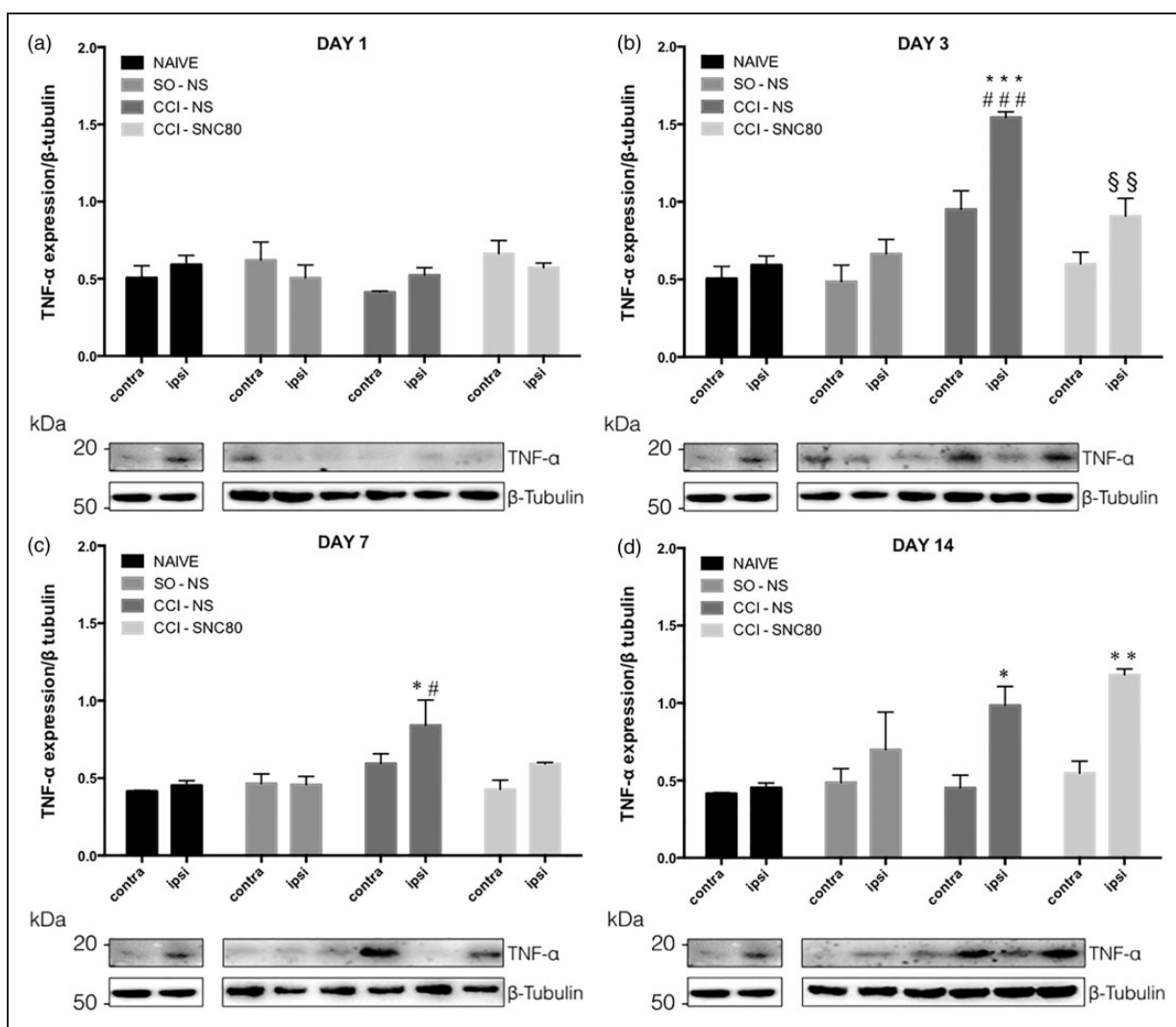


Figure 3. Effect of SNC80 on TNF- α expression in the sciatic nerve of CCI rats. Quantification of TNF- α levels was performed in the ipsilateral and contralateral sciatic nerve (ipsi and contra, respectively) at day 1 (a), day 3 (b), day 7 (c), and day 14 (d) from surgery of naive, SO-NS, CCI-NS, and CCI-SNC80-treated rats. A representative Western blot analysis is shown in lower panels. Data are means \pm SEM of three different analysis. * $p < 0.05$ versus ipsi naive; ** $p < 0.01$ versus ipsi naive; *** $p < 0.001$ versus ipsi naive; # $p < 0.05$ versus ipsi sham; #### $p < 0.001$ versus ipsi sham; §§ $p < 0.01$ versus ipsi vehicle. One-way ANOVA + Bonferroni post hoc test.

from literature,²⁰ in this study we observed a significant up-regulation of TNF- α in the ligated sciatic nerve on day 3, 7, 14 after surgery, in vehicle-treated CCI rats (Figure 3). The correlation between TNF- α overexpression and CCI symptoms is slightly different from what observed by other authors that described a fast TNF recovery in sciatic nerves after CCI.³² However, differences in the species (mouse vs. rat) and in the experimental procedures might account for these differences in the timing of TNF- α expression. In parallel with antiallodynic effect, SNC80 reduced TNF- α expression until the suspension of DOR agonist chronic treatment providing an explanation of the possible mechanism underlying the reduction of allodynia. Thus, in addition to its role in

persistent pain relief,^{8,11–15} a chronic DOR activation may regulate TNF- α to inhibit the development of mechanical allodynia.

TNF- α is known to be one of the most prominent pro-inflammatory cytokines whose overexpression is involved in the inflammatory response and the onset of neuropathic pain consequent to PNI.³³ Neuropathic pain induces broad molecular and cellular adaptations, including neuronal and glial changes that together contribute to consolidate persistent pain.¹² Activated Schwann cells up-regulate TNF- α , which at the site of nerve injury begins the cascade of chemotropic and macrophage-mediated pathologic events associated with Wallerian degeneration.^{17,34,35}

It was shown that TNF- α by itself produced hyperalgesia when injected into nerve.²¹ Vogel et al.³⁶ indicate that mechanical allodynia is a complex phenomenon based on TNF- α -induced sensitization of several ion channels; this TNF- α modulation of allodynia is mediated by the activation of TNF- α receptor 1 and 2. Also, TNF- α inhibition causes significant reduction in degenerative tissue remodeling, as well as CCI-induced nociceptive behaviors^{17,37} and application of neutralizing antibodies to TNF- α attenuates pain-related behavior in CCI mice.²³

The correlation between DOR activation and sciatic nerve TNF- α expression in CCI rats is supported by some literature data.^{25,26,38–40} DOR activation inhibits TNF- α -mediated inflammatory processes following exposure to severe hypoxia,²⁵ and it has been demonstrated that stimulation of the DOR receptor suppresses activation of p38 MAPK and reduces the production of pro-inflammatory mediators including TNF- α in macrophages providing new clues to the potential mechanisms by which DOR agonists may protect against post-ischemic tissue injury. DOR decreases TNF- α levels in the hypoxic cortex.²⁶ Moreover, SNC-121, a DOR agonist, attenuated TNF- α -induced MMP-2 secretion from human optic nerve head astrocytes³⁹ and DOR activation opposes the production of TNF- α during early phases of glaucomatous injury.⁴⁰

In summary, our data further support the hypothesis that DOR activation plays a key role in neuropathic pain, and provide novel information that the expression of sciatic nerve TNF- α is negatively regulated by this opioid receptor in neuropathic pain conditions.

Authors' Contributions

CP, NV, RP, and SC designed and performed in vitro experiments. CP and GA designed and performed in vivo experiments. CP, SC, RT, GA, and GMS participated in the interpretation of data and wrote the manuscript. All the authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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