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Original Article

The mutual effect of progesterone and vitamin D in an animal model of peripheral nerve injury

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Abstract

Background and purpose: Experimental and clinical studies have shown the potential role of progesterone in relieving neural injury. In addition, emerging data on vitamin D, a steroid hormone, have shown its neuroprotective properties. This study was designed to evaluate the mutual effect of vitamin D and progesterone on neuropathic pain (NP) in male rats.

Experimental approach: Chronic constriction injury (CCI) was induced by inserting four ligatures around the sciatic nerve. Hyperalgesia and allodynia (cold and mechanical) were considered positive behavioral scores of NP. After surgery, Sprague Dawley male rats (weighing 200-250 g) were assigned into 7 groups. Vitamin D (250 and 500 units/kg/day, i.p.) and progesterone (4 and 6 mg/kg/day, i.p.) were injected from the 1st day after CCI which continued for 21 days. Moreover, one group received the co-administration of vitamin D (500 units/kg/day, i.p.) and progesterone (6 mg/kg/day, i.p.) from the 1st day until the 21st post-CCI day. Behavioral tests were performed on the 7th, 14th, and 21st days.

Findings/Results: Daily supplementation with vitamin D (250 and 500 units/kg) did not alter nociception. Progesterone (4 and 6 mg/kg/day) was ineffective on thermal hyperalgesia. In the allodynia test, progesterone significantly decreased pain-related behaviors. The co-administration of vitamin D (500 units/kg/day) with progesterone (6 mg/kg/day) significantly relieved thermal hyperalgesia. Finally, the combination significantly decreased cold and mechanical allodynia.

Conclusion and implications: This study showed the mutual effect of progesterone and vitamin D on NP for the first time. Hyperalgesia and allodynia were significantly relieved following co-administration of vitamin D and progesterone.

Keywords: Allodynia; Hyperalgesia; Neuropathic pain; Progesterone; Vitamin D.

INTRODUCTION

Pain is an unpleasant sensory response to intense or detrimental stimuli. Neuropathic pain (NP) is defined as events that are occurred following neural injury (1). NP may result from a broad range of neurological complications affecting the peripheral or central nervous system. Infections, cord injuries, cancer, trauma, and metabolic disorders are the most important causes of NP. Most neuropathies have sensory, motor, and autonomic involvement, singly or in combination (2). Patients may experience some complications such as significant autonomic nervous system dysfunction, abnormal sensations, and positive sensory symptoms (3). Most peripheral neuropathies are insidious and have slow progressive periods. However, sudden appearance may occur following trauma, toxic agent exposure, and inflammation (4).

Despite extensive research in this area, most patients may experience treatment failure (5). Some pieces of evidence have demonstrated that neuroactive steroids are useful in treating neural injuries (6,7). Neuroactive steroids can reduce neuropathy in animal models (8).

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Traditionally, progesterone, a steroidal hormone, was used for hormone replacement therapy (9). Moreover, progesterone was promising as a neuroactive steroid (10). Progesterone can stimulate the myelination process and relieve undesirable events following nerve injury (11). The anti-inflammatory and antioxidant effects of progesterone are well-known (12). Glutamate is one of the critical neurotransmitters involved in pain development. The inhibitory effect of progesterone on N-methyl-D-aspartate glutamate signaling has been revealed (13). Gamma-aminobutyric acid (GABA) is one of the most critical neurotransmitters in pain suppression, and progesterone potentiates the inhibitory effects of GABA through its metabolite, allopregnanolone (14). An experimental study has revealed that the active metabolites of progesterone can reduce hippocampal injury in rats (15). Also, another study has reported that progesterone protects neurons against cerebral ischemia (16).

Vitamin D is a neurosteroid hormone with neurotrophic properties (17). Vitamin D modulates neuronal excitability like other neuroactive steroids. Furthermore, vitamin D improves brain neurotransmitter levels and up-regulates neural growth factor synthesis (18). Recently, it has been shown that vitamin D is a potent regulator for steroid hormone synthesis, and vitamin D deficiency results in inappropriate progesterone production (19). In an animal model of middle cerebral artery occlusion, pretreatment with vitamin D significantly increased glial-derived neurotrophic factor levels and attenuated cortical infarction (20). Vitamin D was able to restore dopamine levels and prevent lipid peroxidation in the substantia nigra in an experimental model of Parkinson's disease (21). Vitamin D significantly inhibits inducible nitric oxide synthase expression and attenuates oxidative damage in the central nervous system (22). The anti-inflammatory effects of vitamin D have been shown (23), and its long-term deficiency leads to inflammatory conditions (24). Experimental and clinical studies have shown the mutual effect of progesterone and vitamin D in traumatic brain injury (25,26). Accordingly, it was assumed that the compounds with the neuroprotective effects could effectively inhibit the expression and development of NP. So, this study was planned to investigate the mutual effects of progesterone and vitamin D on NP in male rats.

MATERIALS AND METHODS

Animals

This study was done on adult male Sprague Dawley rats (200 – 250 g). Rats were purchased from the Physiology Research Center of Kashan University of Medical Sciences (Kashan, Iran), and housed in the animal room of the Physiology Research Center. The animals were individually kept in the cage with food and water available *ad libitum*, temperature of 23 ± 2 °C, 50% humidity, and a 12 h light/12 h dark cycle. All experimental protocols were certified by the Ethical Committee of Kashan University of Medical Sciences (IR.KAUMS.MEDNT.REC.1398.054).

Drugs

Vitamin D and progesterone were purchsed from Caspian Pharmaceutical Co., Iran. Almond oil obtained from Kimia Daru Sepehr (Karaj, Iran) was used to dilute progesterone and vitamin D. Ketamine and xylazine were provided by Alfasan Co., Nederland.

Experimental groups

Initially, 56 male rats were assigned into 7 groups (n = 8) (Fig. 1). Progesterone and vitamin D were injected (intraperitoneally, i.p.) quickly after chronic constriction injury (CCI) for 21 days. Dosage selection was based on previous studies (25,26). The sham group had the same surgery, but the sciatic nerve remained intact. In the CCI group, the nerve was ligated, and rats received only almond oil as a placebo during the study. CCI + Vit D 250 and CCI + Vit D 500 groups received vitamin D at the doses of 250 and 500 units/kg/day, respectively. The groups of CCI + Prog 4 and CCI + Prog 6 received progesterone at the doses of 4 and 6 mg/kg/day, respectively. The CCI + Vit D 500 + Prog 6 group received the combination of vitamin D (500 units/kg/day) and progesterone (6 mg/kg/day).

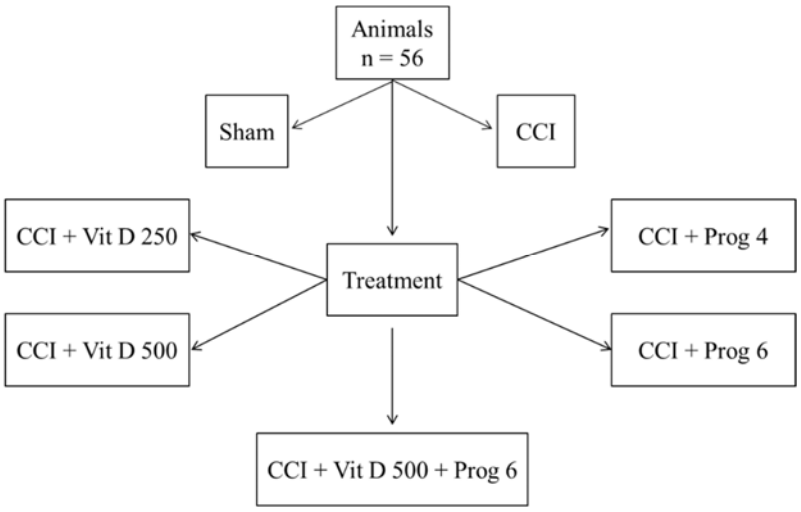

Fig. 1. Schematic presentation of animal grouping, 7 groups, n = 8 in each group. The sham group had a similar surgery to other groups, but the sciatic nerve remained intact; the CCI group received only almond oil as a placebo for 21 days. Treatment groups received the daily doses of progesterone and vitamin D for 21 days. CCI, Chronic constriction injury; Vit D 250, vitamin D 250 unit/kg/day; Vit D 500, vitamin D 500 unit/kg/day; Prog 4, progesterone 4 mg/kg/day; Prog 6, progesterone 6 mg/kg/day, Vit D 500 + Prog 6, vitamin D 500 unit/kg/day + progesterone 6 mg/kg/day.

Neuropathic pain model

CCI was performed to induce NP in the animals. At first, the animals were anesthetized by ketamine (50 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). After anesthesia, an incision was made on the skin above the femoral bone. The superficial connective tissue was separated from the biceps femoris muscles. The common sciatic nerve was exposed and dissected from surrounding tissue. Four ligatures (4.0 chromic gut) were tied loosely around the sciatic nerve at 1 mm intervals (27,28). Finally, the wound was closed with a monofilament 4.0 suture. In the sham group, the sciatic nerve remained intact.

Behavioral tests

Behavioral tests were performed on the 7th, 14th, and 21st days after CCI.

Mechanical allodynia

The primary manifestation of NP is cold and mechanical allodynia. Noxious stimuli that do not usually irritate were considered allodynia. To examine mechanical allodynia, von Frey filaments (steeling, Wood Dale, IL, USA) in the order including 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, 26.0, and 60 g were used. At first, rats were placed on a mesh floor and allowed to adapt for approximately 15 min. Then, a series of von Frey filament stimuli were pushed to the plantar surface of the hind paw. The stimulation was repeated 3 times, pressing down on the hind paw until the rat drew its paw or the fiber bent. The smallest filament that induced at least 3 drawing responses during 5 repetitions was recorded as the withdrawal threshold. Each filament was applied for nearly 1 s, and the inter-stimulus intervals were about 5 s (29,30).

Thermal hyperalgesia

To investigate the mechanisms involved in chronic pain, animal models of NP have been developed (31). The reaction of the operated paw to the thermal stimulus was recorded as thermal hyperalgesia. This reaction occurred at a normally non-nocuous temperature. A plantar test apparatus (Ugo Basile, Varese, Italy) was used to determine sensitivity to the thermal hyperalgesia. Paw withdrawal latency was shown as second. To avoid paw injury, the interval between the start of radiation and its interruption was set to 22 S (32). This test was repeated 3 times for each rat with a time interval of 5 min.

Cold allodynia

An acetone test was performed to measure cold allodynia. The test was carried out 5 times (at 5-min intervals). Cold allodynia was considered a percentage of paw withdrawal frequency (33,34).

Statistical analysis

All data were expressed as mean ± SEM, and analyzed by GraphPad Prism 9.0 software. Two-way repeated measures ANOVA followed by Tukey *post hoc* test was used to compare the results. $P < 0.05$ was considered a significant difference.

RESULTS

Behavioral tests of neuropathic pain

After CCI, the signs of autotomy were not observed. Following CCI, paw withdrawal latency was significantly reduced compared to sham group (Fig. 2). The pain threshold was not changed in the sham group (Fig. 3). Also, the paw withdrawal threshold (Fig. 3) and paw withdrawal frequency (Fig. 4) significantly were changed by nerve ligation.

Mutual effects of vitamin D and progesterone supplementation on heat hyperalgesia

As shown in Fig. 2, supplementation with vitamin D (250 and 500 units/kg/day) from the first day after surgery until the 21st day did not attenuate paw withdrawal latency. In addition, the administration of progesterone (4 and 6 mg/kg/day) did not reverse heat hyperalgesia (Fig. 2). On the other hand, the co-administration of an ineffective dose of vitamin D (500 unit/kg), as a neuroactive steroid, with progesterone (6 mg/kg) significantly reduced thermal hyperalgesia compared to CCI group ($F_{treatment}$ (6,49) = 78.13, $F_{time \times treatment}$ (12,98) = 1.503) (Fig. 2).

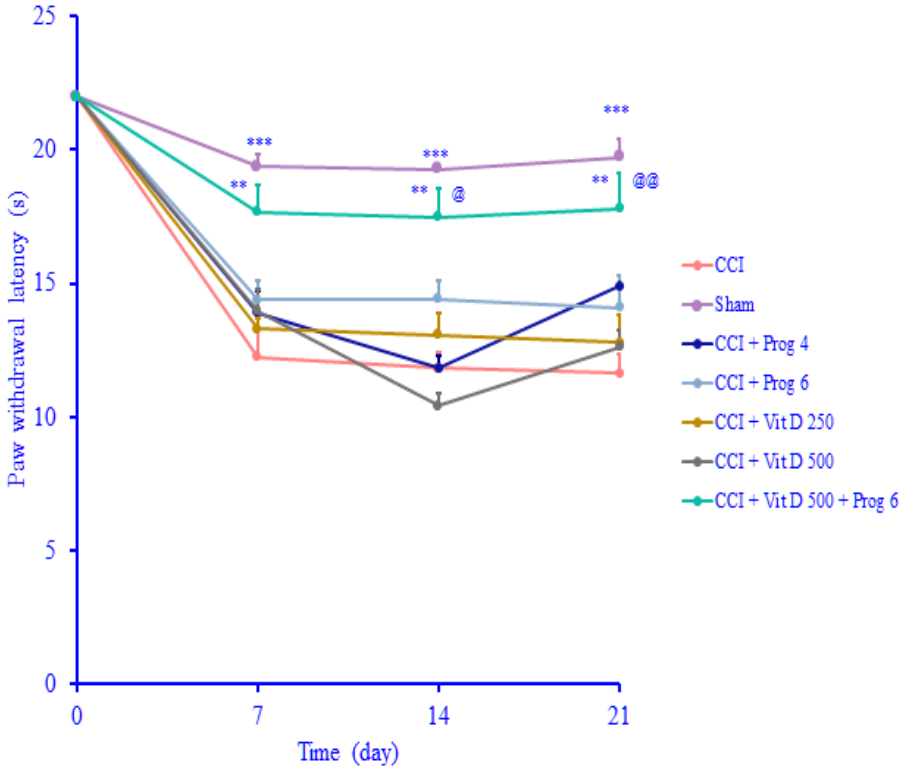

Fig. 2. Mutual effect of vitamin D and progesterone on heat hyperalgesia. The data were expressed as mean ± SEM, n = 8 in each group. $^{**}P < 0.01$ and $^{***}P < 0.001$ show significant differences compared to the CCI group; $^{@}P < 0.05$ and $^{@@}P < 0.01$ versus the CCI + Vit D 500 group. CCI, Chronic constriction injury; Vit D 250, vitamin D 250 unit/kg/day; Vit D 500, vitamin D 500 unit/kg/day; Prog 4, progesterone 4 mg/kg/day; Prog 6, progesterone 6 mg/kg/day, Vit D 500 + Prog 6, vitamin D 500 unit/kg/day + progesterone 6 mg/kg/day.

Mutual effects of vitamin D and progesterone supplementation on mechanical allodynia

As shown in Fig. 3, mechanical allodynia was significantly improved by progesterone (4 and 6 mg/kg/day) compared to the CCI group. However, vitamin D did not alter mechanical allodynia (Fig 3). The co-administration of vitamin D (500 unit/kg/day) with progesterone (6 mg/kg/day) significantly reduced the paw withdrawal threshold (Fig.3, $F_{treatment}$ (6,49) = 25.92, $F_{time \times treatment}$ (12,98) = 5.350).

Mutual effects of vitamin D and progesterone supplementation on cold allodynia

In the cold allodynia test, paw withdrawal frequency was significantly attenuated following the administration of progesterone (4 and 6 mg/kg/day) compared to the CCI group (Fig. 4). However, vitamin D (250 and 500 unit/kg/day) was ineffective in the test. On the other hand, the co-administration of vitamin D (500 unit/kg/day) with progesterone (6 mg/kg/day) significantly reduced paw withdrawal frequency in comparison to the CCI group (Fig. 4) ($F_{treatment}$ (6,49) = 60.72, $F_{time \times treatment}$ (12,98) = 1.68).

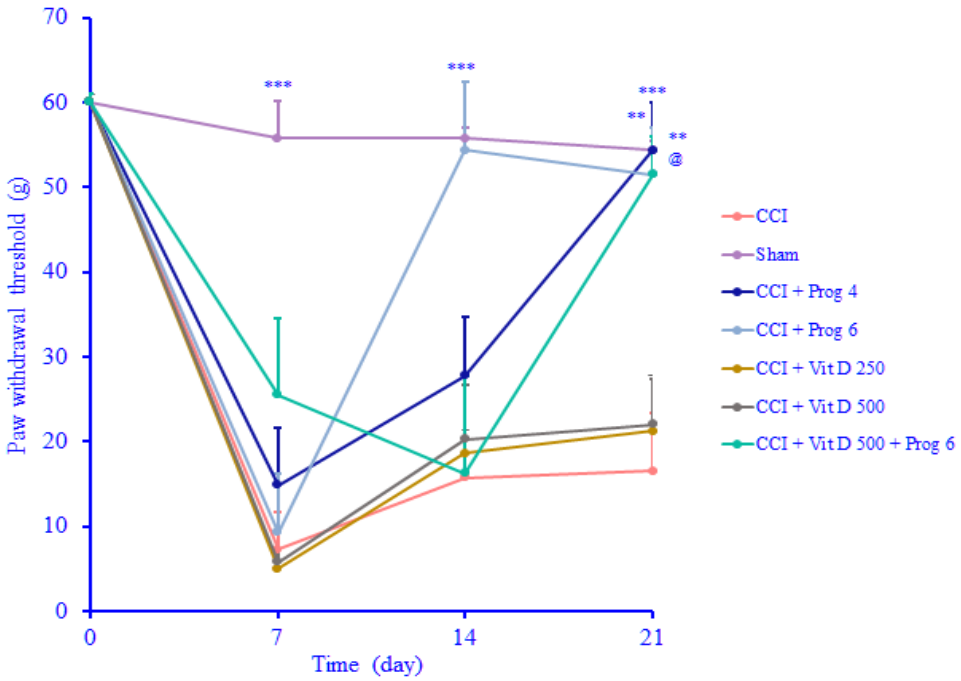

Fig. 3. Mutual effect of vitamin D and progesterone on the mechanical allodynia. The data were expressed as mean ± SEM, n = 8 in each group. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to the CCI group; $^{@}P < 0.05$ versus the CCI + Vit D 250 group. CCI, Chronic constriction injury; Vit D 250, vitamin D 250 unit/kg/day; Vit D 500, vitamin D 500 unit/kg/day; Prog 4, progesterone 4 mg/kg/day; Prog 6, progesterone 6 mg/kg/day, Vit D 500 + Prog 6, vitamin D 500 unit/kg/day + progesterone 6 mg/kg/day.

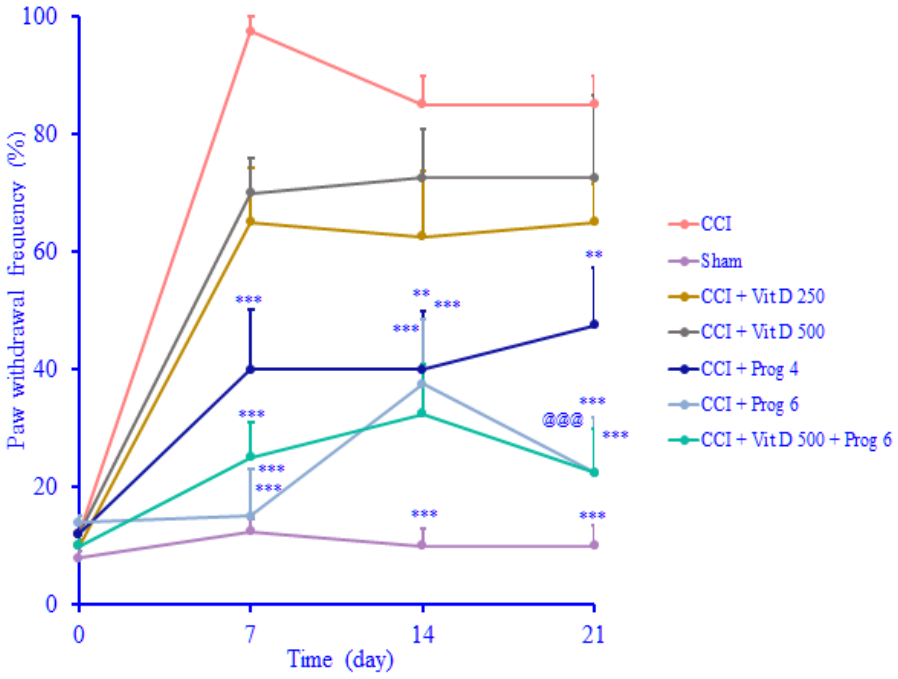

Fig. 4. Mutual effect of vitamin D and progesterone on the cold allodynia. The data were expressed as mean ± SEM, n = 8 in each group. $^{**}P < 0.01$ and $^{***}P < 0.001$ show significant differences compared to the CCI group; $^{@@@}P < 0.001$ versus the CCI + Vit D 250 group. CCI, Chronic constriction injury; Vit D 250, vitamin D 250 unit/kg/day; Vit D 500, vitamin D 500 unit/kg/day; Prog 4, progesterone 4 mg/kg/day; Prog 6, progesterone 6 mg/kg/day, Vit D 500 + Prog 6, vitamin D 500 unit/kg/day + progesterone 6 mg/kg/day.

DISCUSSION

Animal models of NP have been developed to find effective treatments for NP (35). One of the standard models of NP is CCI in the sciatic nerve in rats. Hyperalgesia and allodynia are the main characteristics of this type of pain, and they are well-established in animal models. Positive responses to the thermal stimulus after CCI have been defined as thermal hyperalgesia (36).

The current study showed the mutual effect of vitamin D and progesterone on NP, especially in thermal hyperalgesia. Neither vitamin D nor progesterone influenced paw withdrawal latency. However, the co-administration of vitamin D with progesterone for 21 days significantly decreased the thermal hyperalgesia. The response to stimuli that are generally not painful is defined as allodynia. Cold and mechanical allodynia are positive symptoms in peripheral neuropathies (37). According to the present results and a similar study (28), the administration of progesterone (4 and 6 mg/kg) increased the pain threshold in the acetone and von Frey tests. Moreover, the co-administration of vitamin D and progesterone also decreased pain sensitivity in the acetone and von Frey tests. Therefore, the mutual effects of progesterone and vitamin D for inhibiting allodynia were observed, significantly.

Distinct mechanisms are involved in nociception. The low threshold, large diameter, myelinated Aβ fibers are responsible for mechanical sense conduction (38). While thin unmyelinated primary C fibers transmit cold stimuli to the spinal cord (39). Several mechanisms participate in the mutual effect of progesterone and vitamin D exhibited in the current study. Progesterone has several properties such as neuroprotective and anti-inflammatory effects (40), and can modify brain-derived neurotrophic factor release and modulate neuronal survival and axonal regeneration (41). Within the nervous system, microglia are responsible for progesterone synthesis (42). The neuroprotective properties

of progesterone have been described in animal studies (43). Progesterone reduces lipid peroxidation and suppresses oxidative stress and inflammation (44). Allopregnanolone, an active progesterone metabolite, has positive modulatory effects on GABAA receptors and decreases nociception in animal models of NP (45,46).

Vitamin D, similar to other neurosteroids, modifies neuronal firing, intrinsic excitability, and neural apoptosis and increases neural growth factor synthesis (47-50). Furthermore, vitamin D suppresses cyclooxygenase-2 expression and inhibits macrophage colony-stimulating factors in astrocytes and microglia (51). It has been suggested that vitamin D may interact with other neurosteroids such as progesterone in various tissues. Vitamin D is a potent regulator of steroid hormone production and its deficiency results in altered progesterone synthesis (52). Vitamin D receptors have been found in microglia, astrocytes, and Schwann cells, which are involved in inflammation and directly affected by progesterone (53). Vitamin D possesses progesterone-like activity, and its receptor is induced in T cells by progesterone. The findings reveal the link between the function of progesterone and vitamin D and demonstrate the cooperation of them to regulate the immune system (54). Atif *et al*. have reported that vitamin D significantly enhances the neuroprotective effects of progesterone, and the co-administration of vitamin D and progesterone stimulates the neurotrophic and regenerative cascade necessary for tissue repair (55). Hua *et al.* have shown the mutual effect of progesterone and vitamin D in maintaining spatial memory (56). Progesterone and its metabolites by enhancing the inhibitory effects of the GABAergic system as well as vitamin D by increasing intracellular Ca^{2+}-binding proteins have protective effects after nerve injury. Both progesterone and vitamin D inhibit inflammation, induce trophic factors, and reduce lipid peroxidation (55,57). Vitamin D potentiates axon regeneration following nerve injury (58). Progesterone also can attenuate myelin loss, modify inflammation, partially accelerate remyelination, and stimulate myelin regeneration (59). Accordingly, both progesterone and vitamin D are neurosteroid hormones acting on neural repairer pathway mechanisms to reduce nerve injury and enhance nerve repair, and able to intensify their mutual effects following simultaneous administration.

CONCLUSION

The present study concluded that the co-administration of vitamin D and progesterone could inhibit the progression of NP. In summary, vitamin D and progesterone have mutual neural repairment ability and anti-nociceptive effects in NP. However, further randomized clinical trials are required to confirm the effect and explore the safety of progesterone and vitamin D co-administration in NP.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

A. Abed and S. Nasirzadeh contributed to the conception, design, statistical analysis, and the drafting of manuscript; H.R. Banafshe, G.A. Hamidi, M.N. Tehrani, and M. Shabani contributed to the conception, data collection, and manuscript drafting. All authors read and approved the finalized article.

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