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Effects of intracerebroventricular injection of vitamin B_{12} on formalin-induced muscle pain in rats: Role of cyclooxygenase pathway and opioid receptors

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Abstract

Vitamin B_{12} modulates pain at the local and peripheral levels. This study has investigated the effects of intracerebroventricular (ICV) injection of vitamin B_{12} on themuscle pain. We used diclofenac (cyclooxygenase inhibitor) and naloxone (opioid receptors antagonist) to clarify the possible mechanisms. For ICV injections, a guide cannula was implanted in the left lateral ventricle of the brain. Muscle pain was induced by intramuscular injection of formalin (2.50%; 50 µl) in the right gastrocnemius muscle and the number of paw flinching was recorded at 5-min blocks for 60 min. Locomotor activity was performed using an open-field test. Formalin induced a biphasic pain. Vitamin B_{12} (1.25, 2.50, 5.00 and 10.00 µg per rat) and diclofenac (12.50 and 25.00 µg per rat) significantly reduced both phases pain intensity. Significant antinociceptive effects were observed after combined treatments of diclofenac (6.25 and 12.50 µg per rat) with vitamin B_{12} (0.63 and 2.50 µg per rat), respectively. Prior ICV injection of naloxone (10.00 µg per rat) prevented vitamin B_{12} (10.00 µg per rat) and diclofenac (25.00 µg per rat) induced antinociceptive effects. All the above-mentioned chemicals did not alter locomotor behavior in an open-field test. The present results showed that the cyclooxygenase pathway and opioid receptors may be involved in the central antinociceptive effect of vitamin B_{12}. In addition, opioid receptors might be involved in diclofenac-induced antinociception.

آثار تزریق داخل بطنی مغزی ویتامین B_{12} بر درد ماهیچه ای ناشی از فرمالین در موش های صحرایی: نقش مسیر سیکواکسیژناز وگیرنده های اپیوئیدی

چکیده

ویتامین B_{12} درد را در سطوح موضعی و محیطی تنظیم می کند. این مطالعه آثار تزریق داخل بطنی مغزی ویتامین B_{12} را بر درد ماهیچه ای بررسی کرده است. برای مشخص شدن مکانیسم های احتمالی، ما از دیکلوفناک (مهار کننده سیکلواکسیژناز) ونالوکسان (آنتاگونیست گیرنده های اپیوئیدی) استفاده کردیم. برای تزریقات داخل بطنی مغزی، یک کانول راهنما در بطن جانبی چپ مغز قرار داده شد. درد ماهیچه ای با تزریق داخل ماهیچه ای فرمالین (۲/۵۰ درصد؛ ۵۰ میکرولیتر) در ماهیچه گاستروکنمیوس ایجاد و تعداد تلنگرهای پنجه پا در بلوک های های پنج دقیقه ای به مدت ۶۰ دقیقه ثبت گردید. فعالیت حرکتی با استفاده از آزمون میدان باز انجام شد. فرمالین یک درد دو مرحله ای ایجاد کرد. ویتامین B_{12} (۱/۲۵، ۲/۵۰، ۵/۰۰ و ۱۰/۰۰ میکروگرم به ازای هر موش صحرایی) و دیکلو فناک (۱۲/۵۰ و ۲۵/۰۰ میکروگرم به ازای هر موش صحرایی) شدت هر دو مرحله درد را به طور معنی داری کاهش دادند. متعاقب استفاده های توأم ویتامین B_{12} در مقادیر۰/۶۳ و ۲/۵۰ میکروگرم به ازای هر موش صحرایی به ترتیب با مقادیر ۶/۲۵ و ۱۲/۵۰ میکروگرم دیکلوفناک به ازای هر موش صحرایی آثار کاهش دهنده درد معنی داری مشاهده گردید. پیش تزریق نالوکسان (۱۰/۰۰ میکروگرم به ازای هر موش صحرایی) از آثار ضد درد ایجاد شده توسط ویتامین B_{12} (۱۰/۰۰ میکروگرم به ازای هر موش صحرایی) و دیکلوفناک (۲۵/۰۰ میکروگرم به ازای هر موش صحرایی) جلوگیری کرد. تمام مواد شیمیائی مذکور فعالیت حرکتی در آزمون میدان باز را تغییر ندادند. نتایج حاضر نشان دادند مسیر سیکلواکسیژناز وگیرنده های اپیوئیدی ممکن است در اثر ضد درد مرکزی ویتامین B_{12} دخالت داشته باشند. همچنین ، گیرنده های اپیوئیدی ممکن است در ضد دردی دیکلوفناک نقش داشته باشند.

واژه های کلیدی: درد عضلانی ناشی از فرمالین، دیکلوفناک، گیرنده های اپیوئیدی، موش های صحرایی، ویتامین B_{12}

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Introduction

Muscle pain is a major clinical problem affecting nearly most of the world's population.[1] Little is known about muscle pain because of the complexity of muscle nociception.[2] To better understand the muscle pain mechanisms, different experimental pain models are used including intramuscular (IM) injection of carrageenan, acidic saline and formalin.[2,3] In addition to peripheral modulation, supra-spinal pathways of pain such as descending pain modulation centers are involved in muscle pain mechanisms.[4]

Vitamin B_{12} is an essential water-soluble vitamin[5] and has fundamental roles in the brain function at all ages and also in the prevention of central nervous system developmental disorders, mood disorders and dementia.[6] Recently, vitamin B_{12} has been demonstrated to have potential effects on inflammatory and neuropathic pains in experimental and clinical studies.[7,8] The cyclooxygenase pathway inhibition, the availability and effectiveness of noradrenaline and 5-hydroxytriptamine in the descending inhibitory nociceptive system may involve in analgesic effects of vitamin B_{12}.[7]

Diclofenac, an acetic acid-type, non-steroidal anti-inflammatory drug (NSAID), is commonly used as a potent pain killer exhibiting inhibitory actions on cyclooxygenase-2 pathway.[9] In addition to a local peripheral action on inflamed tissue; diclofenac also affects pain processing at the spinal and supra-spinal levels.[10-13] Vitamin B_{12} and diclofenac affect each other function in producing analgesic effects. Accordingly, it has been reported that local co-administration of vitamin B_{12} and diclofenac enhances diclofenac-induced antinociception and intra-peritoneal (IP) co-injection of these chemicals increases vitamin B_{12}-induced antinociception.[10]

It is well known that mu-, delta- and kappa-opioid receptors are widely distributed in the peripheral and nervous systems[14] and play a central role in local, peripheral and central processings of pain.[15] It has been reported that a non-specific antagonist of these receptors, naloxone, reverses the antinociceptive effect of diclofenac in acetic acid-induced visceral nociception in rats.[12,13] Only in one study, it has been reported that prior microinjection of naloxone into the dorsal hippocampus prevents vitamin B_{12}-induced antinociception in the formalin-induced orofacial pain.[16] However, prior IP injection of naloxone did not prevent vitamin B complex (thiamine/pyridoxine/cyanocobalamin)-induced antinociception in visceral and somatic pain models in rats.[17]

Regarding the fact that central effects of vitamin B_{12} on muscle pain and contribution of cyclooxygenase pathway and opioid receptors in the effect of vitamin B_{12} have not been reported previously, this study was aimed to investigate the central effects of vitamin B_{12} on muscle pain. To clarify the possible mechanisms, we used diclofenac (a cyclooxygenase inhibitor) and naloxone (an opioid receptors antagonist). To explore the influence of the above-mentioned compounds on locomotor activity, an open-field test was employed in rats.

Materials and Methods

Animals. Healthy adult male Wistar rats (250-270 g) were used in this study (n = 96). The animals were maintained in laboratory under controlled 12 hr light-dark cycle and ambient temperature (22.00 ± 0.50 ˚C) with *ad libitum* food and water. All experiments were performed between 13:00-17:00 hr. The research and animal care procedures used here were approved by Animal Ethics Committee of Faculty of Veterinary Medicine, Urmia University, Urmia, Iran (Ref No. AECVU-146-2017) and performed in accordance with the National Research Council Guide for Care and Use of Laboratory animals.[18]

Chemicals. The following chemicals were used: vitamin B_{12}, diclofenac monosodium and naloxone hydrochloride (Sigma-Aldrich Chemical Co., St. Louis, USA). Chemicals were dissolved in sterile normal saline 30 min before ICV administration.

Surgical procedure. To deliver the chemical agents into the brain, a permanent guide cannula was implanted in the lateral ventricle of the brain. In brief, each rat was anaesthetized with IP injection of a mixture of 80 mg kg^{-1} ketamine (Alfasan, Woerden, Netherlands) and 10 mg kg^{-1} xylazine (Alfasan) and a 23-gauge, 13-mm stainless-steel guide cannula was stereotaxically (Stoelting Stereotaxic Apparatus, Wood Dale, USA) placed in the lateral ventricle of the brain. The stereotaxic coordinates according to Paxinos and Watson were 1.40 mm posterior to the bregma, 2 mm lateral to the midline and 4 mm below the top of the skull.[19] The guide cannula was anchored with two screws and dental acrylic. A 13-mm stylet was inserted into the guide cannula to keep it patent prior to injection. All animals were allowed to recover from surgery for 10 days.

Animal groups. In the present study, 96 rats were divided into 16 groups each containing six rats. Groups 1 and 2 received IM injection of normal saline and formalin, respectively. Groups 3-8 were ICV injected with normal saline (control) and vitamin B_{12} at doses of 0.63, 1.25, 2.50, 5.00 and 10.00 µg per rat, respectively. Groups 9-11 were ICV injected with 6.25, 12.50 and 25.00 µg per rat diclofenac, respectively. Groups 12 and 13 were received ICV administration of diclofenac (6.25 µg per rat) plus vitamin B_{12} (0.63 µg per rat) and diclofenac (12.50 µg per rat) plus vitamin B_{12} (1.25 µg per rat), respectively. Groups 14-16 were ICV administered naloxone alone (10.00 µg per rat) and prior to vitamin B_{12} (10.00 µg per rat) and diclofenac (25.00 µg per rat) administration, respectively.

The ICV injection. The ICV injections of normal saline (control), vitamin B_{12}, diclofenac and naloxone were

performed using a 5-µL Hamilton syringe over a period of 30 sec with a constant volume of 2.00 µL per rat. After completion of each ICV injection, the injection needle was left in place for further 30 sec to facilitate the drug solution diffusion. The ICV injections of diclofenac and naloxone were performed 10 min before the induction of muscle pain, whereas vitamin B_{12} was administered 5 min before the induction of muscle pain.

Muscle pain. We used formalin-induced muscle pain in the present study. For this purpose, a diluted formalin solution (50 µL; 2.50%) was IM injected into the belly of gastrocnemius muscle and pain-related behavior including paw flinching number was measured at 5-min blocks for 1 hr.[20] As well as the paw formalin test, a biphasic pain behavior was produced. In the present study, data collected between 0 and 5 min and between 20 and 45 min after IM injection of formalin represented the first and the second phases, respectively.

Locomotor activity. The decrease in nociceptive responses induced by most, but not all analgesics may be related to the reduced locomotor activity, rather than a strict analgesic effect.[21] To explore the influence of the above-mentioned compounds on locomotor activity, an open-field test was employed in rats.[22] Our test apparatus consisted of a 120 cm × 120 cm × 50 cm wooden box. The floor of the arena was divided into 16 equal squares. The numbers of locomotor behavior including number of horizontal movements (crossing squares with all paws) and vertical movements (rearing) were counted for 5 min.

Cannula verification. The brains of euthanized rats were removed and placed in a formalin solution (10.00%). After 24 hr, coronal sections of the brains were viewed to observe the cannula tip placement in the lateral ventricle according to the atlas of Paxinos and Watson.[19]

Statistical analysis. Statistical comparisons were performed using GraphPad Prism (version 5.30; GraphPad software Inc., San Diego, USA). The data obtained from 5-min blocks were analyzed by one-way repeated measure analysis of variance (ANOVA) followed by the Tukey's post hoc test. The first and the second phases of pain were analyzed using one-way ANOVA followed by Tukey's post hoc test. In figures, data are expressed as the mean ± SEM. A value of $p < 0.05$ was considered statistically significant.

Results

After IM injection of normal saline, paw flinching number was 0.60 ± 0.25 only at 0-5 min block. The numbers of paw flinching at first and 4th-9th 5-min blocks were significantly ($p < 0.05$) more than those of 2nd and 3rd and 10th - 12th 5-min blocks (Fig. 1A). The numbers of paw flinching at the first (0-5 min) and second (20-45 min) phases were 28.80 ± 2.20 and 128.00 ± 6.40, respectively (Fig. 1B).

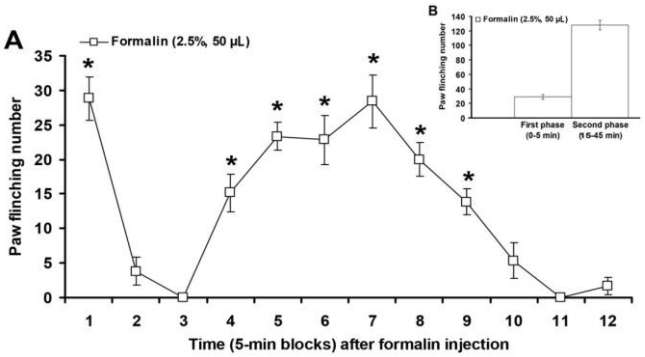

Fig. 1. The number of paw flinching at 5-min blocks (A) and at the first and second phases (B) after intramuscular (IM) injection of formalin. The number of paw flinching was recorded immediately after IM injection of formalin. Data are the means ± SEM obtained from six rats.
* $p < 0.05$ compared to 2nd, 3rd and 10th – 12th five-min blocks.

Injection of vitamin B_{12} at a dose of 0.63 µg per rat did not alter the intensity of the first and second phases of muscle pain. The ICV injection of vitamin B_{12} at doses of 1.25, 2.50, 5.00 and 10.00 µg per rat significantly ($p < 0.05$) reduced the number of paw flinching at the first and second phases of formalin-induced muscle pain (Fig. 2A).

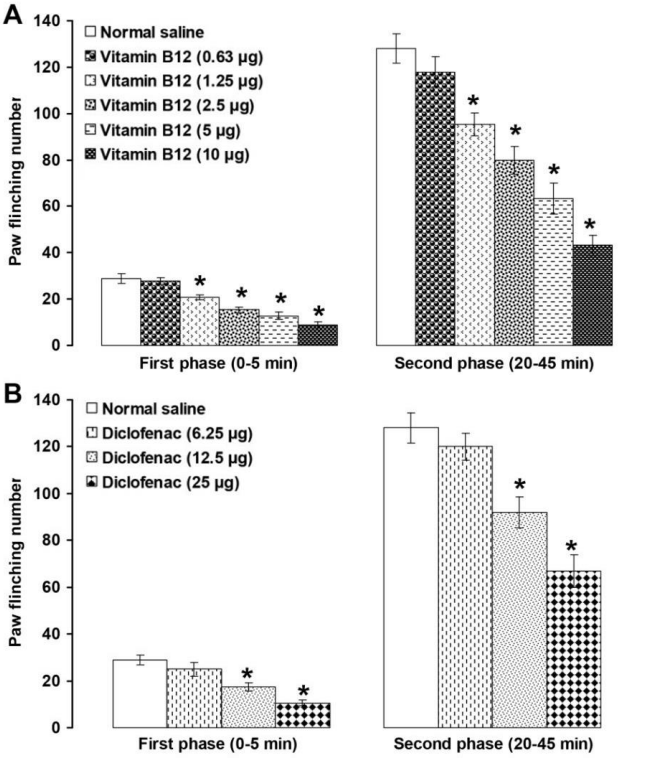

Fig. 2. The effects of intracerebroventricular (ICV) administration of vitamin B_{12} (A) and diclofenac (B) on muscle pain behavior induced by intramuscular (IM) injection of formalin. Vitamin B_{12} and diclofenac were ICV administered 5 and 10 min before IM injection of formalin, respectively. Data are the means ± SEM obtained from six rats.
* $p < 0.05$ compared to normal saline treated group.

The ICV injection of diclofenac at a dose of 6.25 µg per rat had no effect, whereas at doses of 12.50 and 25.00 µg per rat it significantly ($p < 0.05$) decreased the number of paw flinching at the first and second phases of muscle pain induced by IM injection of formalin (Fig. 2B).

After a combination treatment with 6.25 µg per rat diclofenac and 0.63 µg per rat vitamin B_{12}, a significant ($p < 0.05$) antinociceptive effect was observed compared to normal saline treated group (Fig. 3A). By increasing the combined doses of diclofenac and vitamin B_{12} to 12.50 µg per rat and 2.50 µg per rat respectively, the induced antinociceptive effect showed significant ($p < 0.05$) differences with the alone used of them (Fig. 3B).

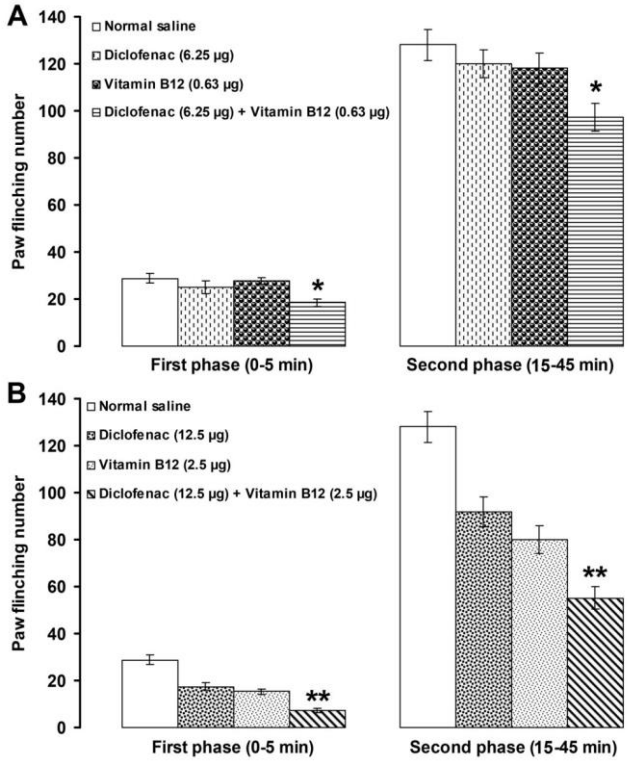

Fig. 3. The effects of intracerebroventricular (ICV) combined treatments with lower (A) and medium (B) doses of vitamin B_{12} and diclofenac on muscle pain behavior induced by intramuscular (IM) injection of formalin. Diclofenac and vitamin B_{12} were ICV administered 10 and 5 min before IM injection of formalin, respectively. Data are the means ± SEM obtained from six rats. * $p < 0.05$ compared to normal saline treated group. ** $p < 0.05$ compared to normal saline, diclofenac (12.50 µg) and vitamin B_{12} (2.50 µg) treated groups.

The ICV injection of naloxone (10.00 µg per rat) alone non-significantly increased the first and second phases of pain intensity (Figs. 4A and 4B). Prior ICV injection of naloxone (10.00 µg per rat) significantly ($p < 0.05$) inhibited the suppressive effects of vitamin B_{12} (10.00 µg; Fig. 4A) and diclofenac (25 µg; Fig. 4B) on both the first and second phases of muscle pain behavior.

The numbers of cage crossing and rearing were 34.80 ± 3.20 and 14.30 ± 2.30, respectively, after ICV injection of normal saline. These locomotor activities were not influenced by separate and combined ICV injections of vitamin B_{12}, diclofenac and naloxone (Data not shown).

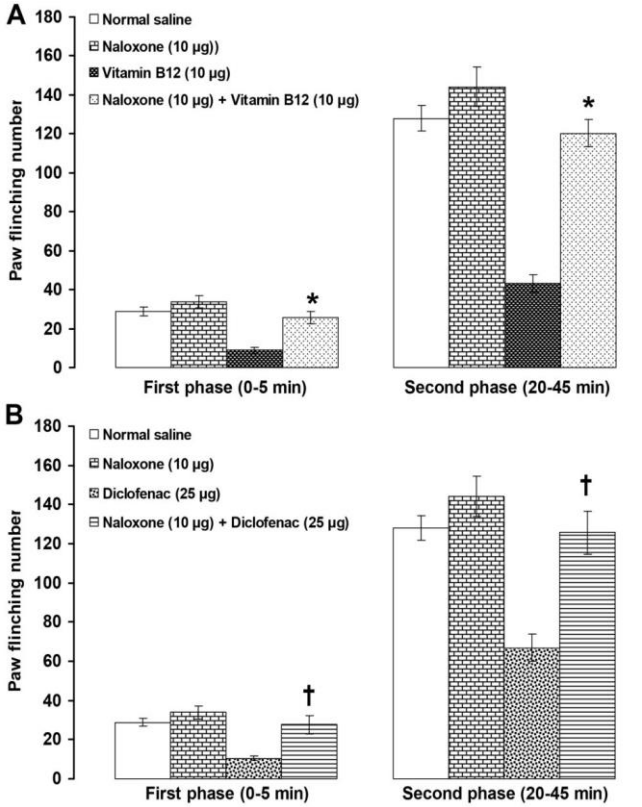

Fig. 4. The effects of intracerebroventricular (ICV) injection of naloxone on vitamin B_{12} (A)- and diclofenac(B)-induced antinociception. Naloxone and diclofenac were ICV injected 10 min before induction of pain. Vitamin B_{12} was ICV administered 5 min before intramuscular injection of formalin. Data are the means ± SEM obtained from six rats.
* $p < 0.05$ compared to vitamin B_{12} (10.00 µg) treated group. † $p < 0.05$ compared to diclofenac (25.00 µg) treated group.

Discussion

In the present study, IM injection of a diluted formalin solution (2.50%; 50 µL) into the gastrocnemius muscle produced a biphasic pattern of paw flinching behavior. This is in consistent with other studies in which a biphasic pattern of paw flinching has been reported after IM injection of formalin into the gastrocnemius muscle.[3,20] Formalin, a nociceptive stimulus, has been used frequently to study pain mechanisms in laboratory animals and according to these studies, a biphasic pattern of pain related behaviors were produced by injection of small amounts (20-50 µL) of dilute solutions (0.10-5.00%) of formalin into the various parts of body such as hind paw, upper lip, vibrissa pad and gastrocnemius muscle.[3,17,22-24]

The first phase is due to direct effect of formalin on nociceptors and the second phase is attributed to the release of local inflammatory mediators responsible for sensitization of primary and spinal sensory neurons and the subsequent signal transduction into the brain.[25] Muscle pain is produced by activation of muscle nociceptors which are connected to the central nervous system through non-myelinated (group IV) or thinly myelinated (group III) nerve fibers.[26] Some populations of these nociceptors can be activated by strong mechanical stimuli as well as endogenous inflammatory mediators such as bradykinin, serotonin and prostaglandine E_2.[27] Taken together, it seems that IM injection of formalin produces biphasic patterns of pain-related behaviors similar to formalin injection in other parts of body.

Our present results showed that ICV injection of vitamin B_{12} suppressed the first and second phases of muscle pain-related behavior induced by formalin. We used ICV injection because there are no reports showing the direct central effect of vitamin B_{12} on pain. Only in one study, Erfanparast *et al*.[16] have reported an antinociceptive effect of vitamin B_{12} after intra-hippocampal microinjection in the formalin-induced orofacial pain in rats. On the other hand, there are many reports demonstrating local and peripheral roles of vitamin B_{12} in modulation of pain mechanisms. Local, peripheral and IP injections of vitamin B_{12} have attenuated formalin-induced orofacial pain.[10] In addition, long-term systemic administration of vitamin B_{12} has suppressed cold and mechanical allodynia in sciatic nerve crush injury model of neuropathic pain.[28] In this context, Hosseinzadeh *et al.* have reported antinociceptive effects of vitamin B_{12} in acute and chronic models of pain in mice.[29]

Our present results showed that alone ICV injection of diclofenac suppressed both phases of formalin-induced flinching behavior. It is well known that diclofenac affects local, peripheral, spinal and supra-spinal mechanisms of pain.[10-13] The analgesic effects of diclofenac may be related to prostaglandin synthesis inhibition, voltage-gated ion channels modulation and interaction with α- and β-adrenoreceptors.[30-32] In the present study, we used diclofenac to clarify the possible mechanisms of vitamin B_{12} analgesia. In a combination of ineffective doses of diclofenac and vitamin B_{12}, a normal saline-comparing antinociception was observed. By increasing the used doses to effective doses, the induced antinociception was more than that of alone used diclofenac and vitamin B_{12}. Besides showing a cyclooxygenase inhibiting mechanism of vitamin B_{12}, these results also indicate a synergistic effect between diclofenac and vitamin B_{12} in producing analgesia. At the local and peripheral pain mechanisms, inhibition of cyclooxygenase is a dominant effect of vitamin B_{12}. Synergistic effects have been reported after local and systemic co-administrations of vitamin B_{12} and diclofenac in formalin-induced orofacial pain.[10] In addition, documented neuroprotective effects have been reported after concurrent use of vitamin B_{12} with diclofenac or celecoxib.[33] It seems that the synergistic effects between vitamin B_{12} and NSAIDs can occur at the local, peripheral and central levels of pain modulating mechanisms.

In the present study, naloxone alone non-significantly increased muscle pain intensity and prior administration of naloxone prevented antinociceptive effects of vitamin B_{12} and diclofenac. This indicates the involvement of opioid receptors in vitamin B_{12}- and diclofenac-induced antinociception at the supra-spinal level. Naloxone is a competitive antagonist of mu-, delta- and kappa-opioid receptor with a high affinity to the mu-opioid receptor[34] and has been frequently used to clarify the involvement of opioid receptors in pain and analgesia mechanisms.[23,35,36] There are no reports showing the involvement of opioid receptors in vitamin B_{12}-induced antinociception at the local and peripheral levels and the results of this study confirm the central involvement of these receptors in vitamin B_{12} analgesia reported by Erfanparast *et al*. at the level of the hippocampus.[16] On the other hand, some scholars have reported the involvement of peripheral and central opioid receptors in the antinociceptive effects induced by NSAIDs such as diclofenac.[13,37,38]

In conclusion, the results of the present study showed that IM injection of formalin produces a biphasic pain behavior. The ICV injection of vitamin B_{12} and diclofenac suppressed muscle pain. A synergistic effect was observed between vitamin B_{12} and diclofenac in producing analgesia. Central cyclooxygenase pathway as well as central opioid receptors may be involved in the antinociceptive effects induced by vitamin B_{12} and diclofenac.

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Conflict of interest

The authors declare that there is no conflict of interest.

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