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Crocin suppressed cold allodynia and anxiety through α_2-adrenoceptors in the anterior cingulate cortex following chronic constriction injury of sciatic nerve in rats

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ABSTRACT: It is believed that α-adrenoceptors have critical contribution in the process of pain information. Anterior cingulate of cortex (ACC) is a key area of brain associated with pain perception. Pharmacological studies demonstrated that crocin, as a potent antioxidant, has analgesic effects. The underlying analgesic mechanism of the crocin is far from clear. Therefore, the present study was design to examine the interaction of anti-nociceptive effects of crocin with α_1- and α_2-adrenoceptors of ACC in chronic constriction injury (CCI) model of neuropathic pain. Intra-ACC injection of crocin significantly decreased cold allodynia (using acetone test) and anxiety (using elevated plus maze test) in neuropathic rats from 2 days to 6 days' post-surgery. Co-injection of crocin and prazosin (α_1-adrenoceptors antagonist, 30 µg/5µl) had no effect on the allodynia. However, co-injection of crocin and yohimbine (α_2-adrenoceptors antagonist, 30 µg/5µl) significantly increased the allodynia on days 4 and 6 post-surgery as compared with CCI+crocin rats. Moreover, our data identified that neuropathy decreased open arm entries and locomotor activity. Additionally, crocin increased entries to open arms; but this increase was not significant as compared to CCI group. There was no significant difference between CCI+Crocin and CCI+crocin+prazosin groups. However, co-injection of crocin and yohimbine significantly decreased entries to open arms as compared with CCI+crocin group. Furthermore, co-injection of crocin with prazosin or yohimbine did not cause significant changes in locomotor activity. The present study suggested that the anti-nociceptive and anxiolytic effects of crocin appear to be mediated through α_2-, and not α_1-adrenoceptors in the ACC.

KEYWORDS: Neuropathic pain; anterior cingulate cortex; crocin; prazosin; yohimbine.

1. INTRODUCTION

Neuropathic pain is complex multidimensional experience and can arises from lesion or disease of the peripheral or central nervous system [1,2]. Patients with neuropathic pain demonstrate paraesthesia, hyperalgesia (exaggerated pain perception in response to noxious stimuli), allodynia (pain perception in response to non-noxious stimuli) and psychological disorders, such as anxiety [3,4]. The underlying mechanism of neuropathic pain is far from clear. The α-adrenoceptors have critical contribution in the process of pain information [5]. Nakai and colleagues revealed that intrathecal injection of guanfacine (an α_{2A}-adrenoceptors agonist) and nitrobiphenyline (an α_{2C}-adrenoceptor agonist) increased mechanical thresholds in a rat model of trigeminal neuropathic pain [6]. However, there are conflicting studies in this field. For example, Sodu and colleagues identified that administration of PT-31 (a novel α_{2A}-adrenoceptor agonist) reduced thermal hyperalgesia and mechanical allodynia in spinal nerve ligated rats. These effects reduced following administration of attenuated by yohimbine (α_2-adrenoceptor antagonist) [7]. Therefore, it is suggested that α-adrenoceptors have an important role in the processing of pain information in central nervous system (CNS). Accumulating evidence implicates that the anterior cingulate cortex (ACC) is

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particularly associated with chronic pain information processing in the CNS [8]. Moon and colleagues reported that optical inhibition of the ACC suppressed pain-associated facial cold allodynia in the trigeminal neuropathic rat model [9]. Additionally, using an in vivo electrophysiological recordings, Sellmeijer and colleagues reported increased firing rate and bursting activity of ACC during neuropathic pain. Moreover, they identified that optogenetic suppression of activity of ACC relived the averse complication of neuropathic pain [10]. Todays, therapeutic options for treatment of neuropathic pain produce only partial relief and treatment of neuropathic pain are still lacking. In the recent years, the development of folk medicine, as therapeutic agents, is in progress [11, 12, 13, 14]. In many research, a great interest of plant therapies is Saffron. *Crocus sativus L.*, known as saffron, is used in folk medicine for various therapeutic purpose [15]. Saffron is widely cultivated in Middle East and Mediterranean countries [16, 17]. Crocin is one of the major biologically active ingredients of saffron [18]. Pharmacological studies demonstrated that saffron and its components have anti-oxidative [19, 20], anti-depressive [21], neuroprotective [22], anti-nociceptive [23], anti-inflammatory [24], and anti-anxiety effects [25]. Hence, ample studies have been done on the analgesic effects of crocin in recent years [26, 27]. However, the underlying analgesic mechanism of the crocin is far from clear. Since, α-adrenoceptors have critical role in pain information process and also crocin has analgesic activity. Therefore, the present study was undertaken to examine the interaction of analgesic and anxiolytic effects of crocin with α_1- and α_2-adrenoceptors of ACC in chronic constriction injury (CCI) model of the sciatic nerve. It is shown that CCI model is an animal model of neuropathic pain, which is similar to human peripheral nerve injury and its sign and symptoms [28].

2. RESULTS

2.1. Analgesic Effects of crocin and its interaction with $\alpha_{1,2}$-adrenoceptors on cold allodynia

As shown in the figure 1, following CCI, the frequency of paw withdrawal is significantly higher than sham rats or day -1 (40±8.94) (before CCI surgery) in CCI rats (painful behavior). It is significantly increased from 2 days (93.33±6.66, *p=0.02) up to 6 days' (95±5.00, **p=0.003) post-surgery of CCI model (Figure 1). Additionally, intra-ACC injection of crocin significantly decreased the frequency of paw withdrawal in CCI+crocin group (45±9.57, 46.66±13.13, 42.50±16.52 on days 2, 4, and 6, respectively) as compared with CCI group [Figure 1, (#p=0.03 on day 2 and #p=0.02 on day 4) and (##p=0.004 on day 6)] (analgesic effects). In addition, there was no significant difference between days 2, 4 and 6 after CCI surgery and before surgery (day -1) in CCI+crocin group. Co-injection of crocin and prazosin had no significant effect on the frequency of paw withdrawal in CCI+crocin+prazosin group (46.66±8.43, 48±4.89, 28±4.89 on days 2, 4, and 6, respectively) as compared with CCI+crocin group (intact analgesic effect of crocin). However, there was significant decrease in CCI+crocin+prazosin group as compared with CCI group in days 2, 4 and 6 after CCI (Figure 1, #p=0.02, ##p=0.008, ###p=0.001; respectively). Furthermore, our analysis of data identified that there is significant difference between in CCI+crocin+prazosin (28±4.89) and sham group (70±5.77) day 6 post surgery (p=0.03). Surprisingly, co-injection of crocin and yohimbine significantly increased the frequency of paw withdrawal from 4 days (93.33±4.21) to 6 days' (96.66±3.33) post-surgery in CCI+crocin+ yohimbine group as compared with CCI+crocin group (Figure 1, ^p=0.02 and ^^^p=0.001; respectively) (inhibition of analgesic effect of crocin). Additionally, there was no significant difference between CCI group and CCI+crocin+yohimbine group in all experimental days (inhibition of analgesic effect of Crocin). Moreover, there was no significant difference between days 2, 4 and 6 after CCI surgery and before surgery (day -1) in CCI+crocin+ yohimbine group (Figure 1).

2.2. Anxiolytic Effects of Crocin and its interaction with $\alpha_{1,2}$-adrenoceptors on anxiety-like behavior

In the EPM test, CCI rats decreased entries to open arms of EPM on day 6 post-surgery (9.08±8.08) (displayed increased anxiety), when compared to day -1 (before CCI surgery) (41.80±2.90) or sham group (58.92±2.79) (Figure 2A, *p=0.03). Additionally, intra-ACC injection of crocin increased entries to open arms of EPM in CCI+crocin group (39.83±15.51); but this increase was not significant as compared to CCI group. However, there was no significant difference between days -1 (37.76±3.37) and 6 (39.83±15.51) post-CCI surgery in CCI+crocin group (anxiolytic effects of crocin) (Figure 2A). Moreover, there was no significant difference between CCI+crocin and CCI+crocin+prazosin groups on day 6 (18.55±8.51) (Figure 2A). However, co-injection of crocin and yohimbine significantly decreased entries to open arms of EPM (3.65±2.60) (increased anxiety) as compared with CCI+crocin group on day 6 post-surgery (Figure 2A, #p=0.014). This decreased pattern also was significant as compared with day -1 in CCI+crocin+yohimbine group (Figure 2A,

**p=0.005). Furthermore, we identified that correlation between allodynia and open arm entries was observed in the CCI rats (Figure 3, R Square= -1, n=6 in each group). Our EPM data also identified that, locomotor activity significantly decreased in CCI group (2.00±0.25, *p=0.02, Figure 2B) (displayed increased anxiety or increased pain perception), when compared to day -1 (7.85±1.68) (before CCI surgery) or sham group (before: 8.66±1.25; day 6: 7.00±1.84) (Figure 2B). However, there was no significant difference between CCI (2.00±0.25) and CCI+crocin (2.33±0.61) groups on day 6 post-CCI surgery (anxiolytic effects of crocin) (Figure 2B). Furthermore, co-injection of crocin with prazosin (2.00±0.51) or yohimbine (2.66±0.98) did not cause significant changes in locomotor activity as compared to the CCI+crocin (2.33±0.61) or CCI (2.00±0.25) groups.

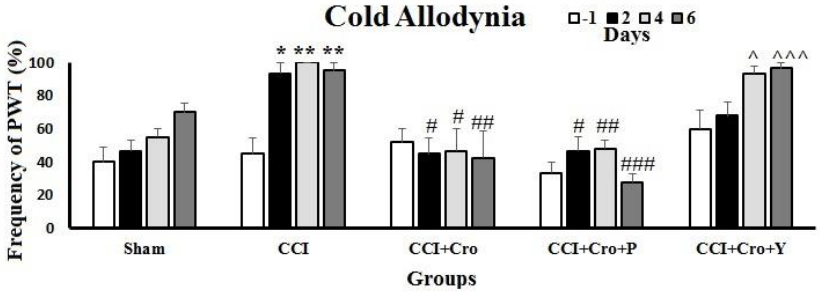

Figure 1. Effects of Crocin and its interaction with α-adrenoceptors on cold allodynia evaluated. Frequency of hind paw to acetone stimulation was assessed on ipsilateral hind paws of experimental groups, at day -1 (baseline), and days 2, 4, and 6 post-neuropathy. Differences in measured parameters among 4 groups analyzed by using Two-way analysis of variance (ANOVA), followed by the Tukey post hoc test. * denote a significant difference with sham animals or day -1 (baseline) in each group; # denote a significant difference with CCI animals. ^ denote a significant difference with CCI+Crocin animals. CCI; chronic constriction injury, CCI+Cro; chronic constriction injury+Crocin, CCI+Cro+P; chronic constriction injury+Crocin+ Prazosin, CCI+CroY; chronic constriction injury+Crocin+ yohimbine; PWT: paw withdrawal threshold.

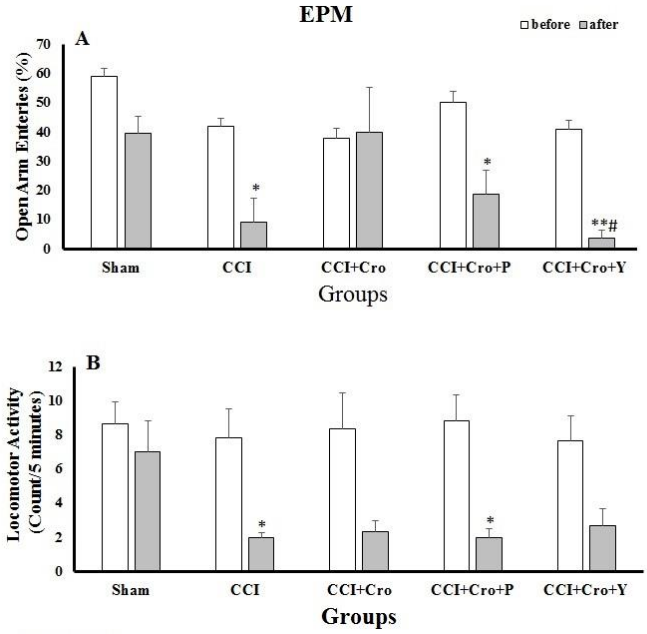

Figure 2. Effects of Crocin and its interaction with α-adrenoceptors evaluated on anxiety-like behaviors at days -1 (baseline) and 6 post-neuropathy. Percentage of open arms entries (A) and number of locomotor activity, total movement, (B) was evaluated as an anxiety index. Differences in measured parameters analyzed by using one-way analysis of variance (ANOVA), followed by the Tukey post hoc test. * denote a significant difference with sham animals or day -1 (baseline) in each group; # denote a significant difference with CCI+Cro animals. CCI; chronic constriction injury, CCI+Cro; chronic constriction injury+Crocin, CCI+Cro+P; chronic constriction injury+Crocin+ Prazosin, CCI+Cro+Y; chronic constriction injury+Crocin+ yohimbine.

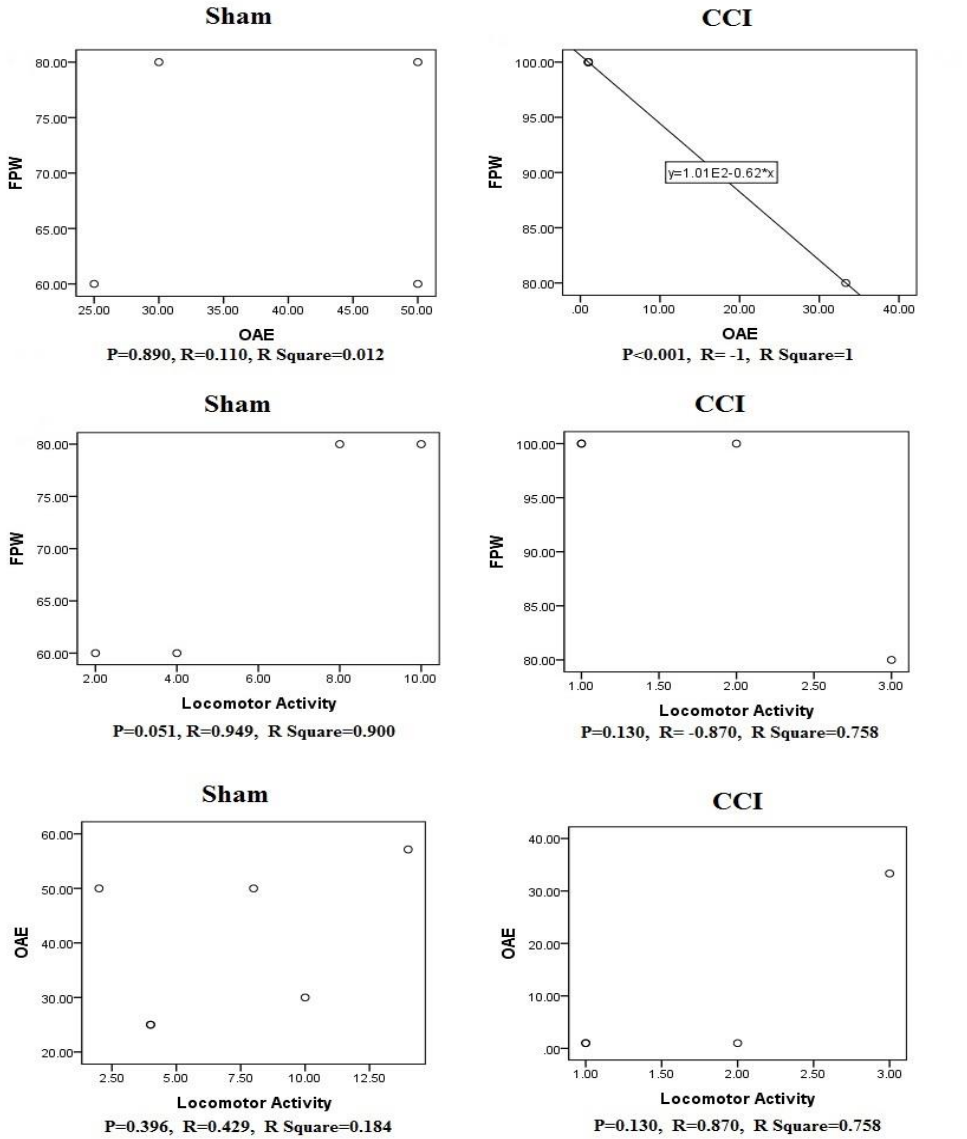

Figure 3. Correlation between FPW, expressed as percent, and locomotor activity, expressed as total movement count, and also between open arm entries, expressed as percent, and locomotor activity (total movement)] evaluated in the sham (n=6) and CCI (n=6) (neuropathy animals) groups. Data pooled from 6 days after CCI injury. Only significant correlation between allodynia (FPW) and open arm entries was observed in CCI rats. No significant correlation between these parameters was observed in sham-operated rats. The corresponding Pearson correlation (R), R Square, and P values as determined by regression analysis are indicated below each corresponding panel. FPW; frequency of paw withdrawal, OAE; open arm entries.

3. DISCUSSION

In the present study, we identified that CCI model leads to the development of cold allodynia (from 2 days up to 6 days' post-surgery) and anxiety-like behaviors (only 2 days' post-surgery, EPM results for days 2 and 4 are not shown). Cold allodynia and anxiety-like behaviors were identified by an increase in paw withdrawal frequency and a decrease in the percentage of open arms entries of the EPM, respectively. No painful behavior was observed in the sham rats. Ample documents reported that CCI model induced cold allodynia and anxiety [3,29,30]. Additionally, we identified that significant correlation observed between cold allodynia and open arm entries in the CCI rats (R Square= -1). However, there was no statistical difference between cold allodynia and open arm entries in the sham rats. Similarly, there was no statistical difference between [open arm entries and locomotor activity] and [allodynia and locomotor activity] in both sham and

CCI rats. Moreover, our data revealed that intra-ACC injection of crocin, significantly attenuated both cold allodynia and anxiety-like behavior (only open arm entries parameter). Safakhah and colleagues reported that application of crocin (30 mg/kg) decreased hyperalgesia and allodynia on day 26 following neuropathy in rats, and its analgesic effects continued up to day 40 [11]. Similarly, crocin markedly suppressed cold and mechanical allodynia in sciatic nerve-crush injury in rats [31,32]. It is well documented that ACC is a cortical area responsible for process of pain perception [33]. For example, recent evidence indicates nerve injury-induced neuropathic pain induces hyperactivity of L5 pyramidal neurons in the bilateral ACC, even in the absence of pain stimuli in mice [33]. Moreover, it is reported that lesions or inactivation of the ACC suppressed nociceptive responses to noxious stimuli [33]. Since the exact underlying mechanism(s) mediating analgesic and anti-anxiety effects of crocin is far from clear. So, to the best of our knowledge, this is the first time that such study investigates the mechanism of anti-nociceptive effects of crocin. It is well documented that α-adrenoceptors are an important therapeutic target for pain [5]. In the spinal cord level, descending adrenergic projection from the brain stem nuclei to the spinal cord can suppress pain perception [34]. The locus coeruleus nucleus, as a major noradrenergic nucleus, have critical role in suppression of pain perception [34]. Indeed, the activity of α_2-adrenoceptors directly suppress pain transmission through decreasing of the release of excitatory neurotransmitters (such as glutamate and substance P) in both normal and neuropathic animals [5, 35]. It is also reported that the efficacy of G-protein coupling spinal α_2-adrenoceptors increased following neuropathic pain [36]. In contrast, in the higher centers (cortex level), pyramidal neurons in the many area of cortex such as ACC receive many adrenergic inputs from locus coeruleus [37]. Koga and colleagues in 2020 reported that application of norepinephrine induced both pre- and post-synaptic potentiation effects in ACC neurons [37]. Using optogenetic method, they also identified that activation of locus coeruleus projection to the ACC increased excitatory transmission in vitro and produced behavioral sensitization for mechanical stimulation [37. Therefore, activation of adrenergic system have complicated effects in different area of central nervous system during pain perception. Further studies are needed to clear the role of adrenoceptors in different area of the central nervous system. Todays, antidepressants, as first-line drugs, are used for management of neuropathic pain [38]. Antidepressants inhibited noradrenaline transporters, result in increased noradrenaline concentration in the synaptic space [38]. Noradrenaline reuptake inhibition enhances analgesic effects, mainly through α_2-adrenoceptors in the dorsal horn of the spinal cord [38]. Therefore, adrenoceptors play an important role in the processing of pain information. In the present study, our data identified that only concomitant application of yohimbine with crocin inhibited the analgesic and anti-anxiety effects of crocin. Indeed, crocin seems to have failed to reduce cold allodynia and anxiety in the presence of the α_2-adrenoceptor antagonist, yohimbine, in the ACC. There are two possibilities to justify the present results. The first possibility is that both crocin injection and increased α_2-adrenoceptors activity following neuropathic surgery [39] have synergistically reduced allodynia. Therefore, inhibition of the α_2-adrenoceptors reduced the analgesic effects of crocin. A second possibility is that crocin may in turn (directly or indirectly) activate α_2-adrenoceptors and through it leading to analgesic and anti-anxiety effects. This requires much more study. Therefore, our data for the first time, have shown it is likely that the anti-nociceptive and anti-anxiety effects of crocin is mediated by α_2-adrenoceptors in the ACC. However, further studies are needed to confirm this effect. The limitation of our study is the degree of variation amongst the rats subjected to CCI surgery, due to variability in the tightness of the ligation of nerve. Additionally, the type of suture agents (such as chemicals from the chromic gut) can also contribute to variability. It is likely that chemicals from the chromic gut induce some behavioral alterations [40].

4. CONCLUSION

Crocin application into the ACC suppressed cold allodynia and anxiety in neuropathic rats. The anti-nociceptive and anxiolytic effects of crocin appear to be mediated through α_2-adrenoceptors in the ACC.

5. MATERIALS AND METHODS

5.1. Animals

Adult Wistar male rats, (weight 180-200 g, n = 6/group) were obtained from breeding colony of Baqiatallah University of Medical Sciences, Tehran, Iran. Animals were housed one per cage and placed under 12 hours light/dark cycle in a room at 22 -24 ℃. Animals had free access to food and water. All experiments conducted in agreement with the National Institutes of Health Guide for Care and Use of Laboratory Animals, and was approved by the local ethical committee (Ethical code: IR.BMSU.REC.1396.750).

5.2. Chemicals

Crocin [digentiobiosyl all-tarnscrocetin (8, 8′-di-apocarotene-8,8′-dioic acid) ester] (product number: 17304) purchased from Sigma–Aldrich Inc. (St Louis, MO, USA). Prazosin and yohimbine purchased from Iran Daru Pharmaceutical company. The drug was dissolved in physiological saline (0.9%).

5.3. Experimental design

In the current study, animals were divided into 5 groups (n=6 per group). These groups were as follows: [Group 1: sham group]; [Group 2: neuropathy group (CCI)]; [Group 3: neuropathy+crocin (40 µg/5 µl [41]) group (CCI+crocin)]; [Group 4: neuropathy+crocin+ prazosin (30 µg/5 µl [42,43]) group (CCI+crocin+ prazocin as α1-adrenoceptor antagonist)]; and [Group 5: neuropathy+crocin+ yohimbine (30 µg/5 µl [42,43]) group (CCI+crocin+ yohimbine as α2-adrenoceptor antagonist)]. All drugs were injected intra-ACC to the animals daily from 1 day up to 6 days after induction of neuropathy, using cannula implantation. Cold sensitivity and anxiety were obtained 1 day prior to neuropathic surgery, and on days 2, 4, and 6 post-surgery, using acetone test and elevated plus maze, respectively.

5.4. Cannula implantation

Intra-ACC injection of drugs were performed using stereotaxic surgery. The anesthetized rat placed in a homemade stereotaxic frame (Borge Sanat, Tehran, Iran) and guide cannula (stainless steel 28-gauge) implanted into the right ACC (AP 1.5 mm from bregma, ML ± 0.6 mm from midline, DV 1.5 mm beneath the surface of the skull) [44]. The cannula fixed to the bone by stainless steel screws and acrylic cement. A 5.0 µL Hamilton syringe with a 33-gaugeneedle was used to inject 5 µL of drugs. The syringe was left in place for 3 min to ensure diffusion of the injected.

5.5. Induction of neuropathic pain model

Neuropathic pain (CCI model) was induce, as it was previously introduced by Bennett and Xie [45]. Briefly, after anesthetizing the animals with chloral hydrate (350 mg/kg, i.p), the left body of sciatic nerve (1 cm) was exposed and then four loss ligatures (4/0 catgut) was tied around the nerve, about 1 mm apart, until a brief twitch in the hind limb was observed. In sham animals, only the left sciatic nerve was exposed, but not ligated.

5.6. Cold allodynia (Acetone test)

To quantify cold threshold of the neuropathic hind paw, foot withdrawal (as a positive response) in response to acetone drop was evaluated [46]. Briefly, the rat was placed under a transparent Plexiglas chamber with a metal mesh floor and acetone drop was applied to the plantar surface of the hind paw, using a syringe. The acetone was applied 5 times (every 5 min) to neuropathic paw (ipsilateral to injury). The frequency of paw withdrawal was again expressed as a percent as follows: (Number of positive response × 100) / (5 trials). Withdrawal of the paw or licking/shaking of the toes are considered as a positive response.

5.7. Elevated plus maze (EPM)

The elevated plus maze consisted of 2 open and 2 closed arms, animals were placed on the center platform of the maze, facing an open arm for 5 minutes. Their movements on the maze monitored for 5 minutes period with a camera. The percent of time spent in open arms and also the percent of entrance in open arms were used as an index of anxiety-like behaviors. Also, total movements (the number of entries in open arms+ the number of entries in closed arms) was assessed as a locomotor activity index. Less time spent in the open arms and less number of entrances to open arms were in favor of anxiety [47].

5.8. Statistical analysis

The present data are presented as mean ± standard error of the mean (SEM). Data analyzed using the SPSS software (IBM. SPSS Statistic., version 24.0). Differences in measured parameters among 4 groups analyzed by using Two- and one-way analysis of variance (ANOVA), followed by the Tukey post hoc test. Linear regression analysis was also performed. The differences considered to be significant when the probability was less than 0.05.

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Conflict of interest statement: The authors declare no conflict of interest.

Ethics committee approval: This study was approved by the local ethical committee with approval number IR.BMSU.REC.1396.750 on 2018-03-12.

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