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Journal of Acupuncture and Meridian Studies

journal homepage: www.jams-kpi.com

Research Article

Isoflurane and the Analgesic Effect of Acupuncture and Electroacupuncture in an Animal Model of Neuropathic Pain

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Available online 6 February 2018

Received: Aug 19, 2017
Revised: Jan 30, 2018
Accepted: Jan 31, 2018

KEYWORDS
acupuncture;
electroacupuncture;

Abstract
The present study aimed to determine whether isoflurane interferes with the analgesic effects of acupuncture (Ac) and electroacupuncture (EA), using a neuropathic pain (NP) rat model. In total, 140 male Wistar rats were used; isoflurane-induced nociceptive response was evaluated using the von Frey test, serum calcium-binding protein β (S100β) levels and nerve growth factor (NGF) levels in the left sciatic nerve. The NP model was induced by chronic constriction injury of the sciatic nerve at 14 days after surgery.

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pISSN 2005-2901 eISSN 2093-8152
https://doi.org/10.1016/j.jams.2018.01.004

isoflurane;
neuropathic pain

Treatment was initiated after NP induction with or without isoflurane anesthesia (20 min/day/8 days). The von Frey test was performed at baseline, 14 days postoperatively, and immediately, 24 h, and 48 h after the last treatment. Results of the nociceptive test and three-way analysis of variance were analyzed by generalized estimating equations, the Bonferroni test, followed by Student—Newman—Keuls or Fisher's least significant difference tests for comparing biochemical parameters (significance defined as $p \leq 0.05$). At baseline, no difference was noted in the nociceptive response threshold among all groups. Fourteen days after surgery, compared with other groups, NP groups showed a decreased pain threshold, confirming establishment of NP. Ac and EA enhanced the mechanical pain threshold immediately after the last session in the NP groups, without anesthesia. Isoflurane administration caused increased nociceptive threshold in all groups, and this effect persisted for 48 h after the last treatment. There was an interaction between the independent variables: pain, treatments, and anesthesia in serum S100β levels and NGF levels in the left sciatic nerve. Isoflurane enhanced the analgesic effects of Ac and EA and altered serum S100β and left sciatic nerve NGF levels in rats with NP.

1. Introduction

Acupuncture (Ac) and electroacupuncture (EA) treatments have yielded good results for alleviating neuropathic pain (NP) pain in humans and experimental animals [1,2], though the exact mechanisms of action are unknown. Owing to the practical difficulties in Ac and EA application to awake and freely moving animals, most studies have used restraint [3] or anesthesia [4]. However, anesthetization or immobilization during treatment may lead to physiological changes which could potentially affect treatment efficacy.

The evaluation of Ac and EA may be biased by restraint stress or habituation in conscious animals [5] or by the anesthetics used in sedated animals. The application of Ac or EA treatment to awake animals (insertion of a needle or manual or electric stimulation of animals) may be viewed as stressors. Restraint, shock, and fear are known to trigger stress-induced analgesia when animals are awake, as shown in models of Ac and EA analgesia [6]. Therefore, Ac and EA analgesia can be significantly reduced if concomitant stressors are not adequately controlled.

In animal studies of Ac or EA, isoflurane is the most commonly used anesthetic [4]. It is easily administered and produces the behavioral and physiological characteristics of general anesthesia without an adjunct [7]. Nevertheless, whether it can alter the analgesic response to Ac or EA treatment is unknown. Interestingly, anesthesia may influence biomarker expression. Mice exposed to isoflurane during postnatal brain development showed increased serum levels of calcium-binding protein β (S100β), a protein used as a neurodegenerative biomarker [8], though this has not been studied in adult rats.

Neurotrophins help in neuronal survival, growth, and differentiation and may also be affected by isoflurane. Nerve growth factor (NGF) is a pain-related neurotrophin that can exert pronociceptive or antinociceptive effects, depending on concentration and site of administration [9]. Chen et al. demonstrated the neuroprotective effect of EA-induced neurotrophins in an animal model of spinal cord injury [10], indicating that EA may reduce pain by neurotrophic modulation.

Based on these findings, we believe that isoflurane can potentiate the analgesic effect of Ac and EA treatments and modify neuromodulation parameters. To test this hypothesis, we evaluated the nociceptive response induced by isoflurane, the serum levels of S100β and NGF in the left sciatic nerve of Ac- or EA-treated NP rats, using the von Frey test. Concurrently, we assessed locomotor behavior to demonstrate the extent to which the animals were affected by anesthesia.

2. Materials and Methods

2.1. Animals

A total of 140 male Wistar rats (weight ≥250 g) aged 55—65 days were used in the experiment. Based on our previous studies, 140 animals were deemed to produce reliable scientific data [11, 12, 13]. Animals housed individually in polypropylene cages (49 × 34 × 16 cm) in a controlled environment (22 ± 2°C) under a standard light—dark cycle (lights-on/lights-off: 0700 h/1900 h), with free access to water and chow (Nuvital, Porto Alegre, Brazil). All experimental procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol no. 13-0298) and conformed to the Guide for the Care and Use of Laboratory Animals (8th ed., 2011). Animal maintenance followed the Brazilian Law 11794 (specifying procedures for the use of animals in scientific research). The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines [14].

2.2. Experimental design

The animals were acclimated to the study environment for 2 weeks before experimental initiation. It is important to emphasize that the application of Ac and EA in awake animals is complex and generates discomfort which may alter treatment-induced analgesia. Furthermore, anesthesia with isoflurane was used during treatments, and its use may have generated a bias in the study, considering the possible interference of the drug in behavioral and

neurochemical results. To avoid possible bias, the animals were randomized and divided into 14 groups: control (no manipulation), sham surgery (Ss; surgery without nerve constriction), sham surgery + Ac (surgery without nerve constriction + 8 Ac sessions), sham surgery + EA (surgery without nerve constriction + 8 EA sessions), NP (Np; surgery with nerve constriction), NP + Ac (NpAc; surgery with nerve constriction + 8 sessions of Ac freely movements), NP + EA (NpEA; surgery with nerve constriction + 8 sessions of EA freely movements), control + anesthesia (no manipulation + 8 days of isoflurane anesthesia), sham surgery + anesthesia (surgery without nerve constriction + 8 days of isoflurane anesthesia), sham surgery + Ac + anesthesia (surgery without nerve constriction + 8 Ac sessions with isoflurane anesthesia), sham surgery + EA + anesthesia (surgery without nerve constriction + 8 EA sessions with isoflurane anesthesia), NP + anesthesia (NpAn; surgery with nerve constriction + 8 days of isoflurane anesthesia), NP + Ac + anesthesia (surgery with nerve constriction + 8 Ac sessions with isoflurane anesthesia), and NP + EA + anesthesia (surgery with nerve constriction + 8 EA sessions with isoflurane anesthesia) (Fig. 1). Subsequently, the Np and Ss groups received appropriate interventions (chronic constriction injury [CCI] or sham surgery). Fourteen days after surgery, hyperalgesia was evaluated by the von Frey test to confirm NP establishment. Treatments were initiated soon after the establishment of NP; animals were treated for 8 days according to group-specific protocols (Ac, EA, or no treatment). The von Frey test was performed at baseline, 14 days postoperatively, and immediately, 24 h and 48 h after the last treatment session.

2.3. NP model: CCI of the sciatic nerve

The CCI of the sciatic nerve was performed (as per Bennett and Xie [15]) to induce NP. The animals were anesthetized with isoflurane (5% for induction, 2.5% for maintenance), the surgical site shaved, and the skin cleaned with 2% alcoholic iodine [15]. The left sciatic nerve was approached in the mid-thigh by removing part of the biceps femoris muscle. Three ligatures (4-0 Vicryl) were tied 1 mm apart, close to the sciatic trifurcation, and tightened until muscle contraction of the leg could be observed, ensuring epineural blood flow. The same investigator performed the ligatures in all animals. In the Ss

Groups	
Control (C)	No manipulation
Sham Surgery (Ss)	surgery without nerve constriction
Sham Surgery + Ac (SsAc)	surgery without nerve constriction+ 8 sessions of acupuncture
Sham Surgery + EA (SsEA)	surgery without nerve constriction+ 8 sessions of eletroacupuncture
Neuropathic Pain (Np)	surgery with nerve constriction
NeuropathicPain + Ac (NpAc)	surgery with nerve constriction+ 8 sessions of acupuncture freely movements
NeuropathicPain + EA (NpEA)	surgery with nerve constriction+ 8 sessions of eletroacupuncture freely movements
Control+Anesthesia (CAn)	No manipulation + 8 days of isoflurane
ShamSurgery+ Anesthesia (SsAn)	surgery without nerve constriction+ 8 days of isoflurane anesthesia
Sham Surgery + Ac + Anesthesia (SsAc An),	surgery without nerve constriction+8 sessions of acupuncture with isoflurane anesthesia
Sham Surgery + EA + Anesthesia (SsEAAn)	surgery without nerve constriction+8 sessions of eletroacupuncture with isoflurane anesthesia
Neuropathic Pain+ Anesthesia (NpAn)	surgery with nerve constriction+ 8 days of isoflurane anesthesia
Neuropathic Pain + Ac + Anesthesia (NpAc An)	surgery with nerve constriction+8 sessions of acupuncture with isoflurane anesthesia
Neuropathic Pain + EA + Anesthesia (NpEAAn)	surgery with nerve constriction+8 sessions of eletroacupuncture with isoflurane anesthesia

Figure 1 Groups. Description of the procedures performed in the animals in each study group.

groups, animals were anesthetized and the left sciatic nerve was exposed but not constricted. The control groups did not undergo any surgical procedure.

2.4. Acupuncture

Two stainless steel Ac needles with guide tubes (Suzhou Huanqiu Acupuncture Medical Appliance Co. Ltd., 218, China; length: 0.18 mm; diameter: 0.8 mm) were inserted bilaterally approximately 2—3 mm into the acupoint BL-24, which is located on the side depression of the lower edge of the L3 spinous process of the third lumbar vertebra (Fig. 2).

2.5. Electroacupuncture

In rats that were either freely moving or anesthetized with isoflurane (under oxygen flow; 2% for induction, 0.5% for maintenance), Ac needles were connected bilaterally to an electro-stimulation device (Model EL 608 NKL, Brusque, SC, Brazil), with an alternating frequency of 2 Hz and 100 Hz (2/10 Hz, 0.3 ms width), and treatment performed for 20 min.

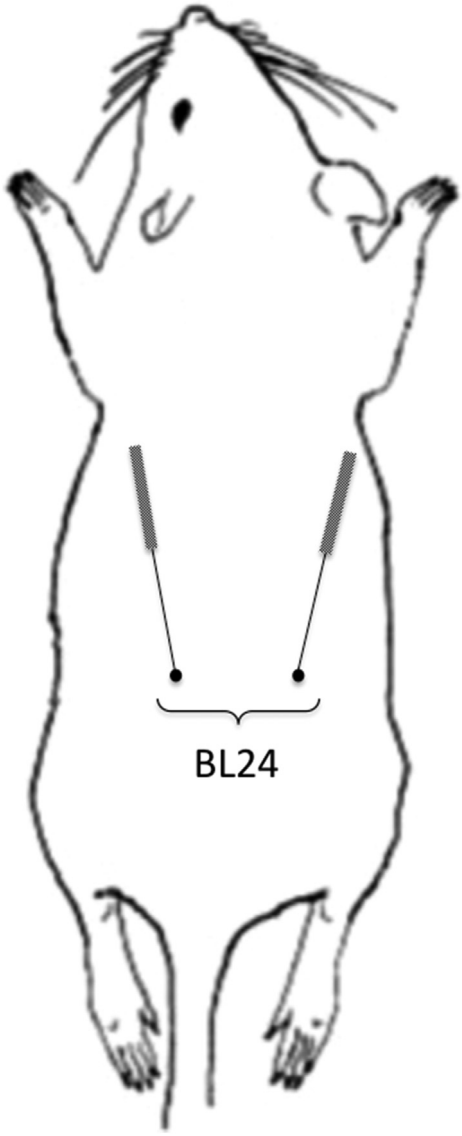

Figure 2 **Acupoint.** Description of the acupoint BL-24 in the rat.

2.6. Von Frey test

Twenty-four hours before the test, the rats were acclimated to the apparatus for 5 min. On the test day, rats were placed on the analgesiometer, and the sensor containment box was positioned next to the paw, guided by the reflection in the mirror. The test was based on the maximum pressure (grams) required for the animal to show sensitivity to a paw touch [16]. Three measurements were performed, and the mean value was considered the pain threshold. Measurements were made at baseline, 14 days postoperatively, and immediately, 24 h, and 48 h after the last treatment session. The same trained investigator, who was blinded to the treatment groups, performed all tests. All tests were performed in awake and freely moving animals, without any sedation.

2.7. Open field test

Behavioral assessment excluded acute phase effects and was performed 24 h after the sixth session and before the seventh session to determine the chronic effect of six sessions of Ac and EA with and without anesthesia. A 60 $\times$ 40 $\times$ 50 cm cage with the floor divided by lines into 12 squares of 13 $\times$ 13 cm each was used. The test started immediately after the animals were placed in the back left corner and allowed to explore the surroundings for 5 min [17,18]. The number of line crossings (all paws crossing the boundary into an adjacent marked-out area) was taken as a measure of locomotor activity [19].

2.8. S100β measurement in blood serum

Animals were killed by decapitation 24 hours after the last treatment. Blood serum was collected and frozen at −80°C until the time of testing. Serum S100β levels were measured by a competitive enzyme-linked immunosorbent assay kit (MyBiosource, California, USA), according to manufacturer's instructions.

2.9. NGF measurement in the left sciatic nerve

The nerve was removed and frozen at −80°C until the time of testing. NGF levels were determined by a sandwich enzyme-linked immunosorbent assay, using monoclonal antibodies specific for each measurement (R&D Systems, Minneapolis, United States). Total protein was measured by Bradford's method using bovine serum albumin as a standard.

2.10. Statistical analysis

Data were expressed as mean ± standard error of the mean. Nociception data were analyzed by generalized estimating equations followed by a Bonferroni test. The biochemical and open-field data of all groups were compared using a three-way analysis of variance (ANOVA)

followed by Student—Newman—Keuls (SNK) test or Fisher's least significant difference (LSD) test. A $p \leq 0.05$ indicated significance. SPSS, version 20.0, for Windows was used for all statistical analyses.

3. Results

3.1. Von Frey test showed that isoflurane potentiates analgesia induced by Ac and EA

Generalized estimating equations revealed a significant interaction between time and treatments ($\chi^2 = 1419.33$; 52; $p < 0.001$) (Fig. 3). At baseline, all groups had a pain threshold similar to that of the control group ($p \geq 0.05$). Fourteen days postoperatively, the pain threshold in pain groups was different from that in the control and sham groups, confirming the establishment of NP ($p < 0.001$). Immediately after the last treatment session (21 days after surgery), both treatments (Ac and EA) enhanced the mechanical pain threshold of animals exposed to the NP model (NpAc and NpEA groups), but these results did not differ from those of animals exposed to the NP model without treatment (Np and NpAn groups) ($p > 0.05$). Conversely, when all groups received isoflurane anesthesia, the increase in pain threshold was significantly different between the Np and NpAn groups ($p < 0.001$). This outcome was maintained 24 h and 48 h after the last treatment session (22 and 23 days after surgery).

3.2. Isoflurane decreased locomotor activity

No interactions among pain, treatments, and anesthesia (open field test data) were observed (three-way ANOVA/SNK, $p > 0.05$). However, there was an interaction between anesthesia and treatments (three-way ANOVA/SNK, $F_{(2.125)} = 3.03$; $p = 0.052$). Significant effects of anesthesia were observed (ANOVA/SNK, $F_{(1.125)} = 13.92$; $p < 0.01$). Ac and EA induced an increase in locomotor activity, but anesthesia was able to reverse this effect (Fig. 4).

3.3. Isoflurane altered serum S100β

The analyses showed interactions among pain, treatments, and anesthesia (three-way ANOVA/SNK, $F_{(2.68)} = 4.21$; $p < 0.05$), between pain and treatments (three-way ANOVA/SNK, $F_{(2.68)} = 6.57$; $p < 0.05$) and pain and anesthesia (three-way ANOVA/SNK, $F_{(2.68)} = 8.05$; $p < 0.05$). Results indicate that animals exposed to pain and treatment showed increased S100β, while animals exposed to pain and anesthesia showed decreased S100β. Significant effects of anesthesia (three-way ANOVA/SNK, $F_{(1.68)} = 34.96$; $p < 0.01$) and treatments (three-way ANOVA/SNK, $F_{(2.68)} = 6.158$; $p < 0.01$) were also observed, with decreased serum S100β seen in anesthetized animals compared to those in nonanesthetized animals. EA treatment induced an increase in serum S100β, while Ac treatment did not affect the S100β (Fig. 5).

3.4. Isoflurane altered NGF levels in the affected nerve

The analyses showed interactions among pain, treatments, and anesthesia (three-way ANOVA/LSD, $F_{(2.116)} = 9.47$; $p < 0.05$). Significant independent effects of anesthesia (three-way ANOVA/LSD, $F_{(1.116)} = 30.33$; $p < 0.05$), pain (three-way ANOVA/LSD, $F_{(1.126)} = 101.15$; $p < 0.05$), and treatments (three-way ANOVA/LSD,

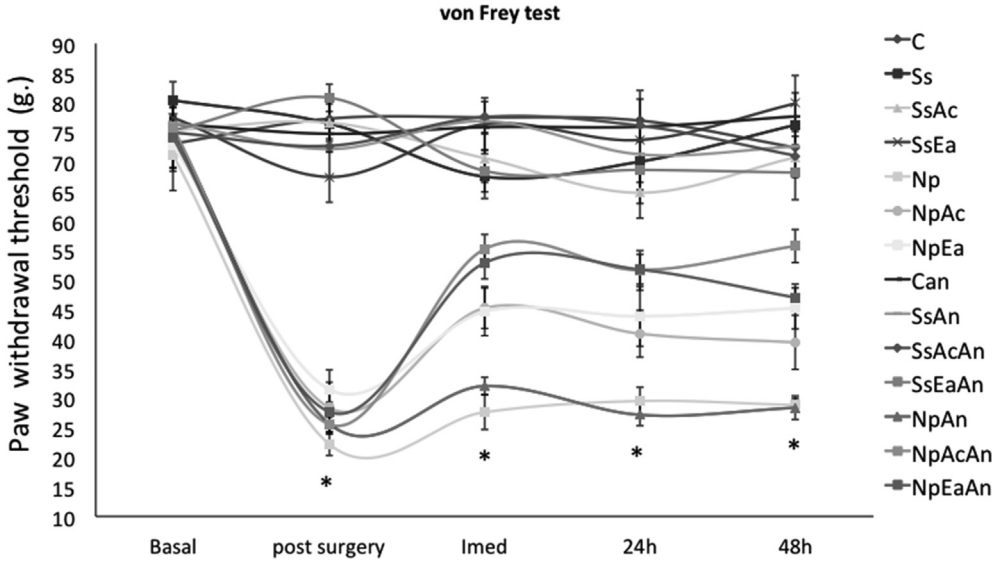

Figure 3 Von Frey test. Data are expressed as the mean ± standard error of the mean (SEM) of pain threshold in grams. Generalized estimating equation (GEE) statistics showing interaction between time and treatments ($\chi^2 = 1419.33$; 52; N = 8-10 animals per group). *Significantly different from control and sham groups. # Significantly different from Np and NpAn groups. Ac = acupuncture; EA = electroacupuncture; C = Control, Ss = sham surgery; SsAc = sham surgery + Ac; SsEA = sham surgery + EA; Np = neuropathic pain, NpAc = NP + Ac; NpEA = NP + EA, CAn = control + anesthesia; SsAn = sham surgery + anesthesia; SsAcAn = sham surgery + Ac + anesthesia; SsEAAn = sham surgery + EA + anesthesia, NpAn = NP + anesthesia; NpAcAn = NP + Ac + anesthesia; NpEAAn = NP + EA + anesthesia.

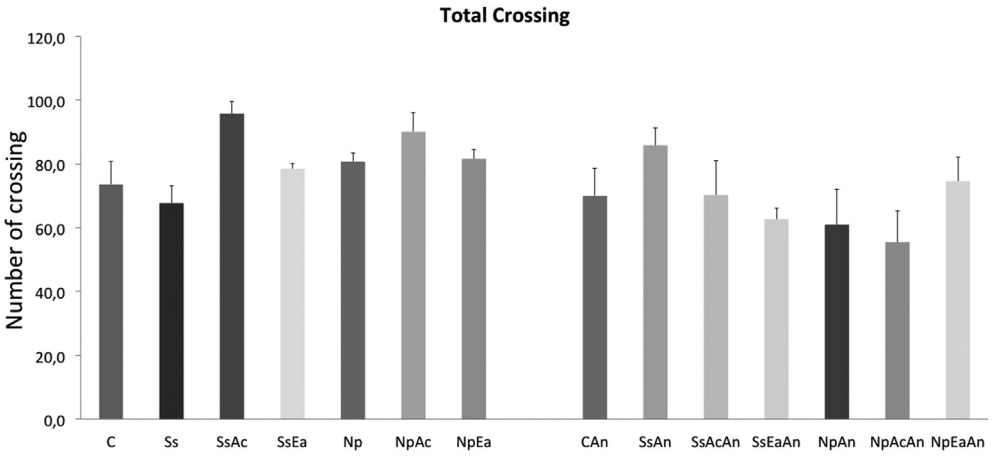

Figure 4 Open field test. Data are expressed as the mean ± standard error of the mean (SEM) of total number of crossings. There was an interaction between anesthesia and treatments (three-way ANOVA/SNK, $F_{(2.165)} = 3.03$; $p = 0.05$), and a significant effect of anesthesia on treatments (ANOVA/SNK, $F_{(1.125)} = 13.92$; $p < 0.01$) (N = 8-10 animals per group).
Ac = acupuncture; EA = electroacupuncture; C = Control, Ss = sham surgery; SsAc = sham surgery + Ac; SsEA = sham surgery + EA; Np = neuropathic pain, NpAc = NP + Ac; NpEA = NP + EA, CAn = control + anesthesia; SsAn = sham surgery + anesthesia; SsAcAn = sham surgery + Ac + anesthesia; SsEAAn = sham surgery + EA + anesthesia, NpAn = NP + anesthesia; NpAcAn = NP + Ac + anesthesia; NpEAAn = NP + EA + anesthesia; ANOVA/SNK = analysis of variance/Student–Newman–Keuls test.

$F_{(2.116)} = 6.54$; $p < 0.05$) were observed. Animals receiving isoflurane had decreased NGF levels, similar to those found in animals in the pain model. Conversely, animals that received EA treatment had increased NGF levels in the affected nerve (three-way ANOVA/LSD, $p < 0.05$) (Fig. 6).

4. Discussion

We demonstrated that repeated exposure to isoflurane enhances the analgesic efficacy of Ac and EA, though isoflurane alone did not have an analgesic effect. Use of needles in conscious rats may, however, complicate the interpretation of the results, since the needle may occasionally be erroneously inserted outside the acupoint in animals agitated during Ac. Additionally, needle insertion and electric stimulation are painful or uncomfortable stimuli [20], which could interfere with the analgesic response to Ac or EA. It is well established that Ac has segmental, extrasegmental, and central effects [21]. Therefore, a single Ac application may be an acute stressor leading to an analgesic response, though when applied

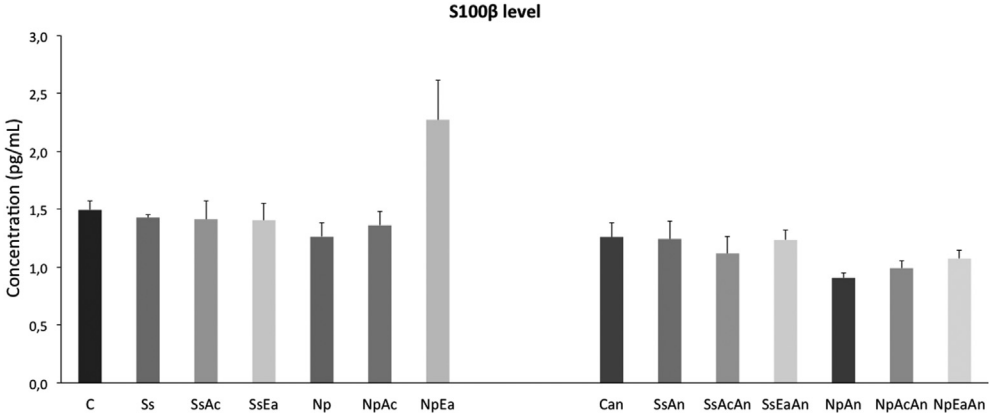

Figure 5 S100β levels in blood serum. Data are expressed as the mean ± standard error of the mean (SEM) of serum S100β levels in pg/mL. There was an interaction among pain, treatments, and anesthesia (three-way ANOVA/SNK, $F_{(2.68)} = 4.21$; $p < 0.05$), between pain and treatments (three-way ANOVA/SNK, $F_{(2.68)} = 6.57$; $p < 0.05$), and pain and anesthesia (three-way ANOVA/SNK, $F_{(2.68)} = 8.05$; $p < 0.05$) (N = 5-7 animals per group).
Ac = acupuncture; EA = electroacupuncture; C = Control, Ss = sham surgery; SsAc = sham surgery + Ac; SsEA = sham surgery + EA; Np = neuropathic pain, NpAc = NP + Ac; NpEA = NP + EA, CAn = control + anesthesia; SsAn = sham surgery + anesthesia; SsAcAn = sham surgery + Ac + anesthesia; SsEAAn = sham surgery + EA + anesthesia, NpAn = NP + anesthesia; NpAcAn = NP + Ac + anesthesia; NpEAAn = NP + EA + anesthesia; ANOVA/SNK = analysis of variance/Student–Newman–Keuls test.

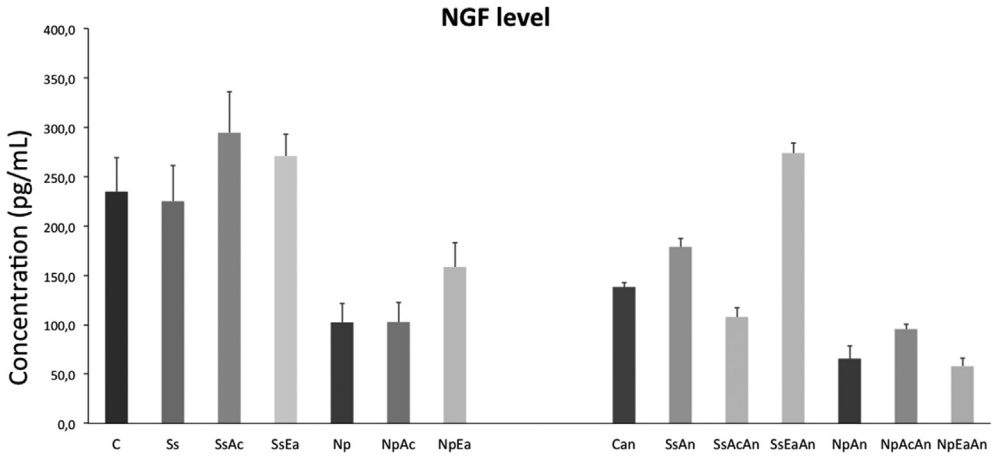

Figure 6 Nerve growth factor (NGF) levels in the left sciatic nerve. Data are expressed as the mean ± standard error of the mean (SEM) of left sciatic nerve growth factor (NGF) levels in pg/mL. Interactions among pain, treatments, and anesthesia (three-way ANOVA/LSD, $F_{(2.116)} = 9.47$; $p < 0.05$) were apparent. There were significant independent effects of anesthesia (three-way ANOVA/LSD, $F_{(1.116)} = 30.33$; $p < 0.05$), pain (three-way ANOVA/LSD, $F_{(1.126)} = 101.15$; $p < 0.05$), and treatments (three-way ANOVA/LSD, $F_{(2.116)} = 6.54$; $p < 0.05$) (N = 5-7 animals per group).
Ac = acupuncture; EA = electroacupuncture; C = Control, Ss = sham surgery; SsAc = sham surgery + Ac; SsEA = sham surgery + EA; Np = neuropathic pain, NpAc = NP + Ac; NpEA = NP + EA, CAn = control + anesthesia; SsAn = sham surgery + anesthesia; SsAcAn = sham surgery + Ac + anesthesia; SsEAAn = sham surgery + EA + anesthesia, NpAn = NP + anesthesia; NpAcAn = NP + Ac + anesthesia; NpEAAn = NP + EA + anesthesia; ANOVA/LSD = analysis of variance/Fisher's least significant difference test.

several times, Ac stimuli may act as chronic stressors [22], potentially altering the analgesic effect of Ac or EA.

Acute stress reduces pain sensitivity (stress-induced analgesia), which is probably mediated by brainstem modulation. In contrast, chronic stress increases pain sensitivity, inducing hyperalgesia [12,23] and allodynia [12,23]. This relationship between stress and pain sensitivity probably occurs because of activation of the hypothalamic—pituitary—adrenal axis, leading to glucocorticoid release, which can alter the serotonergic and dopaminergic systems involved in the nociceptive response [24]. Previous data from our group suggest the involvement of the opioid system in the hyperalgesic response induced by repeated restraint stress [12]. It is known that the Ac and EA analgesia pathways are also related to the opioid system [25]. Therefore, chronic stress caused by needle insertion and electric current may lead to decreased analgesic efficacy of Ac and EA in awake rats, since they are most likely to have reverse actions in the opioid system.

The effects of inhaled anesthetics have been previously evaluated in Ac studies with rats [20,26]. Wen et al. [20] evaluated the role of reducing EA-induced stress and found that 0.5% halothane, at a sub-anesthetic minimum alveolar concentration (sub-MAC dose), reduces the influence of stress during EA, allowing strong needle stimulation and rapid recovery of the animal. Kung et al. [26] assessed the use of isoflurane and showed that 0.75% isoflurane (sub-MAC dose) is also an effective anesthetic dose for EA studies. However, an important issue regarding the use of anesthetics in animal models is motor impairment resulting from anesthetic effects. Antognini et al. [27] showed that the MAC of isoflurane (0.6—0.9%) is able to preserve the integrity of the motor system in rats receiving this anesthetic. Silva et al. 2010 [28], in a study evaluating the effects of EA on the role of the anterior pretectal nucleus, used isoflurane at the same concentrations used in the present study for induction and maintenance of anesthesia (2% and 0.5% respectively) and, likewise, found no motor impairment in the animals.

Inhaled anesthetics hyperpolarize neuronal membranes and inhibit C-fiber latency, reducing neuronal excitability. However, isoflurane at sub-MAC doses does not affect wind-up phenomenon and thus is a candidate anesthetic for evaluating antinociceptive effects of Ac and EA [20,29]. Moreover, isoflurane alone at sub-MAC doses does not influence motor or nociceptive responses. It is important to highlight that the present study behavioral tests were performed in awake animals that had completely recovered from anesthesia.

In the open field test, all animals were able to move, indicating that they were not sedated. However, there was a significant effect of anesthesia on treatment, i.e., Ac induced an increase in locomotor activity, and anesthesia was able to reverse this effect. We observed that the anesthetic effect on locomotion depended on the state of the animal. Isoflurane led to decreased locomotion in animals with increased locomotion, while in sham animals, isoflurane had no significant effect on locomotion. It is possible that since isoflurane was administered for six consecutive days before the test; this effect was due to repeated exposure to the drug. Literature evidence suggests that isoflurane decreases locomotion and neuronal excitability by inhibiting glutamate release [30]. It is known that N-methyl-D-aspartate receptor, a glutamate receptor, is related to locomotor activity [31] and nociception response [32]. We may also speculate that isoflurane exerts a protective effect in animals submitted to the NP model. Ac-induced increase in locomotion in the Ss and Np groups is another interesting finding of this study. Isoflurane administration reversed this effect, indicating

an interaction between isoflurane and Ac. Manual Ac has been shown to increase dopamine release, improving locomotor function in mice [33]. Dopamine plays an important role in peripheral and central neurons, including those in the substantia nigra, hypothalamus, midbrain, and the ventral tegmental area [34]. In the present study, rats in the NpEaAn group showed greater locomotor activity than those in the NpAn and NP + Ac + anesthesia groups. This improvement may be due to stimulation that reached the motor threshold, maintaining skeletal muscle integrity in the NP model and improving locomotion. Another hypothesis is related to the increase of monoamines in the brain, which has been shown to block anesthetic action and increase locomotor activity in rabbits [35]. However, further studies are necessary to clarify Ac-induced locomotion.

Another important finding was the association between serum S100β and pain, treatments, and anesthesia. It is known that chronic pain, such as fibromyalgia, correlates with higher serum S100β and BDNF levels [36], but our results were not in agreement. Some studies suggest that rats exposed to isoflurane during postnatal brain development have increased serum S100β [8], which could be related to brain damage. Nevertheless, according to the present results, isoflurane prevented an increase in S100β in adult male rats with NP treated with EA. Although Ac and EA are known to reduce S100β after neural injuries [37], our results showed the opposite effect. Since the animals underwent treatment while awake, the treatment may have mimicked a chronic stress condition, leading to increased S100β, which would be consistent with literature data [38]. Considering that the NpEA group had the highest serum S100β, our data suggest that animals submitted to NP and EA without isoflurane show increased stress levels due to NP induction and electric stimulation while awake. The NpAc and Np groups showed different results, indicating that electric stimulation has effects distinct from those of manual stimulation. The NP + EA + anesthesia group had S100β levels equal to those of the control group, indicating a protective effect of isoflurane anesthesia possibly by maintaining serum S100β similar to that of controls. Similarly, Garcia-Sanchez et al. (1993) [39] showed that isoflurane anesthesia increases plasma beta-endorphin after surgery, supporting the present study in that it suggests a protective effect of isoflurane against stress.

Isoflurane administration, NP, and Ac and EA treatments also altered NGF levels in the left sciatic nerve. When the control and control + anesthesia groups were compared, isoflurane clearly decreased NGF levels in the left sciatic nerve, as did NP, suggesting that isoflurane modifies the effect of Ac and EA in the left sciatic nerve (injured nerve). Although the groups receiving Ac or EA showed an increase in NGF levels, *post hoc* tests showed that this effect was linked to EA treatment, but not to Ac treatment. Despite extensive literature on the topic, there is still no consensus on the role of NGF in NP. It has been suggested that NGF, a neurotrophin involved in the growth, maintenance, and apoptosis of neurons [40], is decreased in sensory neurons of the dorsal root ganglion and of the spinal dorsal horn in diabetic NP models [41]. In contrast, Watson et al. (2008) [42] found increased NGF expression in an NP model [43]. This finding suggests that, after nerve injury, many substances, including NGF, are released mainly by the astrocytes of the central nervous system. In the dorsal horn of the spinal cord, increased NGF levels are associated with central sensitization [44], leading to NP. Similarly, increased NGF levels in the CNS [45] have been associated with an increase in pain. However, some studies suggest that the increase in NGF levels in the peripheral nervous system is associated with NP [46]. In contrast, another study suggests that neural mobilization increases the level of NGF in injured nerves, promoting nerve regeneration and reducing painful symptoms [47].

In the present study, treatment with EA induced an increase in NGF levels although a previous study showed that Ac and EA in NP decreased NGF levels, which was associated with decreased hyperalgesia [48]. Nevertheless, studies have shown a 52% increase in NGF levels after CCI and neural mobilization in rats, indicating that NGF contributes to regeneration of the sciatic nerve after CCI [47]. Considering this later study, the data from the present study suggest that the decrease in NGF levels in the injured nerve after NP may be due to reduced nerve regeneration involving pain. Although isoflurane also reduces pain, EA may have promoted an increase in the levels of this neurotrophin.

In conclusion, our data show that isoflurane administered during Ac or EA treatment in an animal model of NP decreased allodynia as determined by the von Frey test. Similarly, isoflurane prevented the increase in serum S100β in rats with NP treated with EA while awake and, most likely, reduced the harmful effects of chronic stress exposure. Inhaled anesthesia decreased NGF levels in the left sciatic nerve, while EA increased the levels of this neurotrophin. These results suggest that these effects are related to nerve regeneration rather than to increased analgesia.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgments

This research was supported by the following Brazilian funding agencies: National Council for Scientific and Technological Development—CNPq (Dr. ILS Torres; Dr. W Caumo; Dr. VL Scarabelot); Brazilian Federal Agency for Support and Evaluation of Graduate Education—CAPES (LNS Adachi; Dr. LF Medeiros); CAPES/PNPD Edital PPGCM 07/2016 (Dr. C de Oliveira); Graduate Research Group of Hospital de Clínicas de Porto Alegre—GPPG (Dr. ILS Torres—Grant 130298). The research was also supported by Research and Event Incentive Fund (FIPE-GPPG).

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