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RESEARCH ARTICLE

Participation of Potential Transient Receptors in the Antinociceptive Effect of Pharmacopuncture

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Background: Despite the widespread clinical use of acupuncture in painful situations, the use of this treatment should be further clarified. Nociception is mediated by the activation of nociceptors, such as transient receptor potentials (TRPs). The family of TRPs includes TRPV1, TRPM8, and TRPA1, which can be stimulated by substances such as capsaicin, menthol, and methyl salicylate, respectively.
Objectives: This study aimed to investigate the role of TRPs in antinociception via the administration of agonists of these receptors in the Zusanli acupoint (ST36) in models of inflammatory, acute, and neuropathic pain.
Methods: Male Wistar rats were used for this experiment. All rats received a subcutaneous injection of TRP agonists (capsaicin, menthol, or methyl salicylate) in ST36; saline was injected as control. Nociception was evaluated using the electronic mechanical threshold test and tail-flick test before the administration of complete Freund's adjunct or chronic constriction injury of the sciatic nerve and after the administration of TRP agonists.
Results: Nociception was found to be attenuated after treatment with TRP agonists. The administration of different doses (0.03, 0.3, and 3.0 μg/20 μL) of capsaicin, menthol, and methyl salicylate in the different pain models (neuropathic, inflammatory, and nociceptive) induced antinociception in most of the evaluated time points.
Conclusion: Based on the findings, we suggest that the activation of TRPV1, TRPM8, and TRPA1 receptors results in the antinociceptive effect of the stimulation of the ST36 acupoint. Thus, TRP receptors may present a new therapeutic opportunity for the control of inflammatory and neuropathic pain.

Keywords: Pain, Nociceptors, Acupuncture

INTRODUCTION

Acupuncture is a Chinese medicine treatment that consists of a complex therapeutic system that involves the insertion of needles into specific sites, or acupoints [1]. Acupuncture techniques for therapeutic purposes include manual acupuncture and electrical sand thermal stimulation [2]. Although little is known about its biological mechanism, acupuncture has become a common treatment method in painful conditions and a wide variety of chronic disorders [3].

The analgesic effects of acupuncture, through the stimulation of the acupoint, are caused by tissue damage around these points and the release of pro-inflammatory mediators such as histamine, bradykinin, PGE2, serotonin, and adenosine triphosphate (ATP), all of which simultaneously sensitize mechanoreceptors and nociceptors [4,5]. This activation converges to the conduction of mechanical and painful information from the myelinated type A-β afferent and unmyelinated type C fibers, respectively, to the dorsal horn of the spinal cord. Thus, the mechanical fiber with the highest conduction speed activates pain-related inhibitory interneurons, resulting in an antinociceptive effect [6].

Pharmacopuncture is a form of acupuncture therapy that combines acupuncture and herbal medicine and involves the point injection of the herbal extract into the acupoint related to the diseases. Pharmacopuncture has application in traditional Korean medicine [7]. Goldman et al. [8] reported that acupuncture at the Zusanli (Stomach 36, ST 36) acupuncture point in mice significantly reduced chronic pain and increased the extracellular concentration of ATP and

adenosine in the tissues surrounding the acupuncture points; furthermore, local application of an adenosine A1–receptor agonist replicated the analgesic effect of acupuncture. This technique obtained analgesic effects similar to those of manual acupuncture and electroacupuncture through the administration of 2-chloro-N (6)-cyclopentyladenosine, an agonist of the A1 receptor in the ST36 acupoint [8]. ST36 is one of the most frequently used points for acupuncture analgesia [1,2,5]. It is located on the anterior aspect of the leg in the tibialis anterior muscle, 3.0 cun inferior to the lateral depression underneath the knee cup and one fingerbreadth lateral to the tibial crest. Regarding neural processing, studies have shown an abundant expression of ion channels and response to chemical stimulation in the region of the ST36 acupoint, contributing to the analgesic effect [2].

Numerous ion channels within the human genome have fundamental characteristics for activating their function. The discovery of the class of channels called transient receptor potentials (TRPs) led to the understanding of the initiation and conduction of nociceptive information [9]. These channels are divided into subfamilies, such as the transient receptor potential vanilloid 1 (TRPV1), which is stimulated by painful stimuli and heat; transient receptor potential melastatin type 8 (TRPM8), which is an inferior thermal transmission integrator; and the transient receptor potential ankyrin 1 (TRPA1), which is stimulated by harmful cold. They fit in these subfamilies according to the molecules that enable their activation, such as capsaicin, menthol, and methyl salicylate, respectively [10-12].

Therefore, in the present study, we aimed to evaluate the participation of TRPs via the administration of the agonists, capsaicin, menthol, and methyl salicylate, in the ST36 acupoint as a treatment method using inflammatory and neuropathic pain models.

MATERIALS AND METHODS

All experimental models had 10 groups of six rats each, respecting the randomness of the study regardless of the initial treatment allocation.

A total of 180 Wistar rats, weighing 250-300 g, were included in the study, for 6 weeks. The animals were provided by the Central Vivarium of the Federal University of Alfenas Minas Gerais (UNIFAL-MG) and kept in the vivarium of the Physical Therapy department. All experiments followed the ethical standards established for animal experimentation recommended by the International Association for the Study of Pain (IASP) and standards regulating the ethical aspects for the use of laboratory animals by the Ethics Committee on Animal Experimentation at UNIFAL-MG (Protocol number: 632/2015).

Inflammatory hyperalgesia was induced by intraplantar (i.pl.) administration of 100 μL of complete Freund's adjuvant (CFA) in the right paw of all rats, and the effect on the mechanical nociceptive threshold was assessed using an electronic analgesiometer (Insight Instruments, Ribeirão Preto, Brazil) 4 hours after administration. The control group of rats received an i.pl injection of 100 μL of sterile saline (0.9%).

The experimental model of neuropathic pain was induced by chronic constriction injury (CCI) of the sciatic nerve, as previously described [13,14]. Briefly, the animals were anesthetized with 2% isoflurane by inhalation. The common sciatic nerve in the right paw was carefully exposed, and then four ligatures with silk thread, separated by 2 mm, were made. The muscles and skin over the nerves were sutured, with a pattern of three ties. Three days after the procedure, the animals were tested and considered hyperalgesic when the response threshold to the application of mechanical stimuli in the mechanical threshold test corresponded to at least 50% of the baseline response threshold.

Capsaicin, menthol, or methyl salicylate (Sigma-Aldrich, St. Louis, MO) were dissolved in sterile saline (0.9%), at concentrations of 0.03, 0.3, and 3.0 μg/20 μL each, and stored at –20℃. The animals were divided into groups according to the agonist they received; rats in the control groups received an injection of sterile saline (0.9%). To our best knowledge, these agonists have yet to be described in the literature regarding their use on acupoints. To that effect, the doses of the agonists were established using a dose-effect test with the subcutaneous (s.c.) route as the form of application (0.03, 0.3, and 3.0 μg/20 μL).

Syringes (paired with 22 G, 0.70 × 30 mm needles) were used to inject the ST36 acupoint on the right side ipsilateral to the lesion with an application guide to obtain a depth of 5 mm [15,16]. All groups received an injection of the corresponding TRPV1, TRPM8, or TRPA1 agonist in different doses, and the control group received an injection of sterile saline (0.9%).

The tail-flick test (Insight Instruments, Ribeirão Preto, Brazil) was used to evaluate acute pain. The electronic mechanical threshold test was used to evaluate the mechanical nociceptive threshold of inflammatory pain by CFA and neuropathic pain induced by CCI. In the tail-flick test, the animal was gently immobilized, and the tail portion, at 2 cm from its end, was positioned on a nickel-chromium filament that was heated progressively (approximately 9℃/second) from room temperature (23℃ ± 1℃) until it reached a harmful temperature (53℃) in approximately 3 seconds. The heating was automatically interrupted at 6 seconds to avoid tissue damage. For the electronic mechanical threshold test, the rats were placed in acrylic cages (12 × 20 × 17 cm high) with a wire grid floor before beginning the tests in a quiet room.

Before paw stimulation, the animals were quiet, without exploratory defecation, and the mechanical threshold of each animal was tested at different times by three consecutive measurements in an interval of 5 minutes, applying a probe perpendicularly to the plantar surface of the right hind paw [13]. The force applied was sufficient to generate a positive or nociceptive response of removing the paw, followed by licking and/or "flinch." The average of the three measurements was considered the mechanical nociceptive threshold.

1. Statistical analysis

The behavioral results are expressed as mean ± standard error of the mean of six animals per group. Two-way (treatment and time factors) or one-way (treatment or time factor) analysis of variance (ANOVA) test, followed by the multiple comparisons Bonferroni post hoc test, were applied using the Statistical Package for the Social Sciences (SPSS) software (IBM Corp, Chicago, IL, USA). Differences were considered statistically significant if $p < 0.05$.

RESULTS

The first experiment is shown in Fig. 1, in which each animal received an i.pl injection of CFA and administration of the TRPV1, TRPM8, and TRPA1 agonists, capsaicin, menthol, or methyl salicylate, respectively, in the ST36 acupoint. Nociceptive evaluation was performed using an electronic mechanical threshold test before induction with CFA (B1); 4 hours after CFA (B2) administration; and 0, 1, 3, 6, and 24 hours after the injection of capsaicin, menthol, and

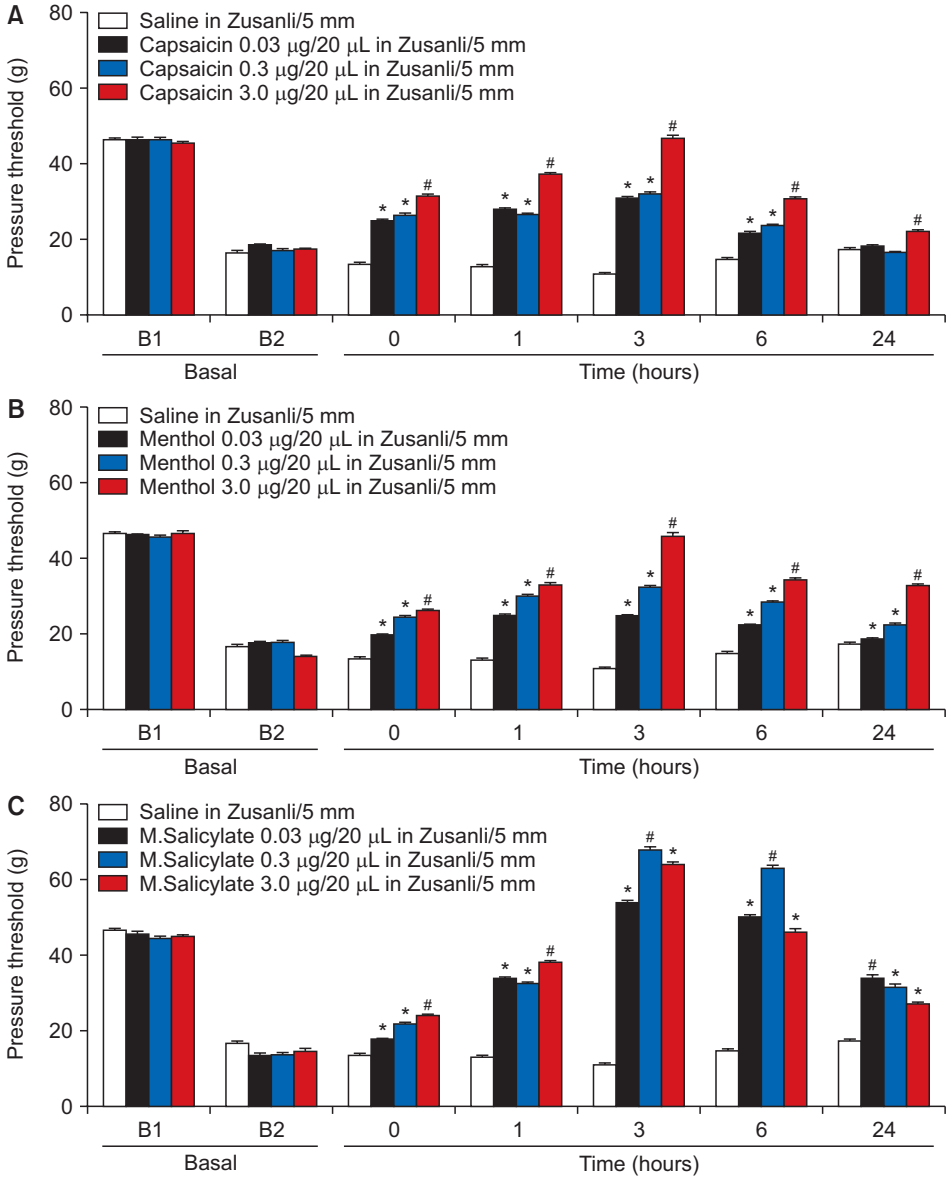

Fig. 1. Nociceptive threshold evaluated using the electronic mechanical threshold test after CFA-induced experimental pain model. (A) Effect of capsaicin on TRPV1 at 0.03, 0.3, and 3.0 μg/20 μL concentrations. (B) Effect of menthol on TRPM8 at 0.03, 0.3, and 3.0 μg/20 μL concentrations. (C) Effect of methyl salicylate on TRPA1 at 0.03, 0.3, and 3.0 μg/20 μL concentrations. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni post hoc test. *$p <$ 0.05 compared to saline; # represents $p < 0.05$ compared to the other treatments (#).

methyl salicylate. In all control groups, the pain lasted since CFA injection up to 24 hours.

The results of capsaicin administration are shown in Fig. 1A. Inflammatory pain was significantly decreased in all groups at different doses up to 6 hours after its administration; only the group with the highest dose attenuated nociception at 24 hours, being more effective than the other doses. A significant influence of time ($F_{6,120} = 3.97$, $p < 0.001$), treatment ($F_{3,20} = 1.74$, $p < 0.001$), and interaction between treatment × time ($F_{18,120} = 313.80$, $p < 0.001$) was noted.

Fig. 1B displays the antinociceptive results of the TRPM8 agonist, menthol. Inflammatory pain was reduced in all groups at different doses up to 24 hours, compared to the control group; similarly, a higher analgesic effect was noted in the group that received 3.0 μg/20 μL of menthol than in the other groups. A significant influence of time ($F_{6,120} = 4.01$, $p < 0.001$), treatment ($F_{3,20} = 1.58$, $p < 0.001$), and interaction between treatment × time ($F_{18,120} = 348.30$, $p < 0.001$) was noted.

The antinociceptive effect of administering methyl salicylate in the inflammatory pain model is demonstrated in Fig. 1C. Injection of methyl salicylate in ST36 induced antinociception up to 24 hours at all doses. A significant influence of time ($F_{6,120} = 4.49$, $p < 0.001$), treatment ($F_{3,20} = 2.44$, $p < 0.001$), and interaction between treatment × time ($F_{18,120} = 725.49$, $p < 0.001$) was noted.

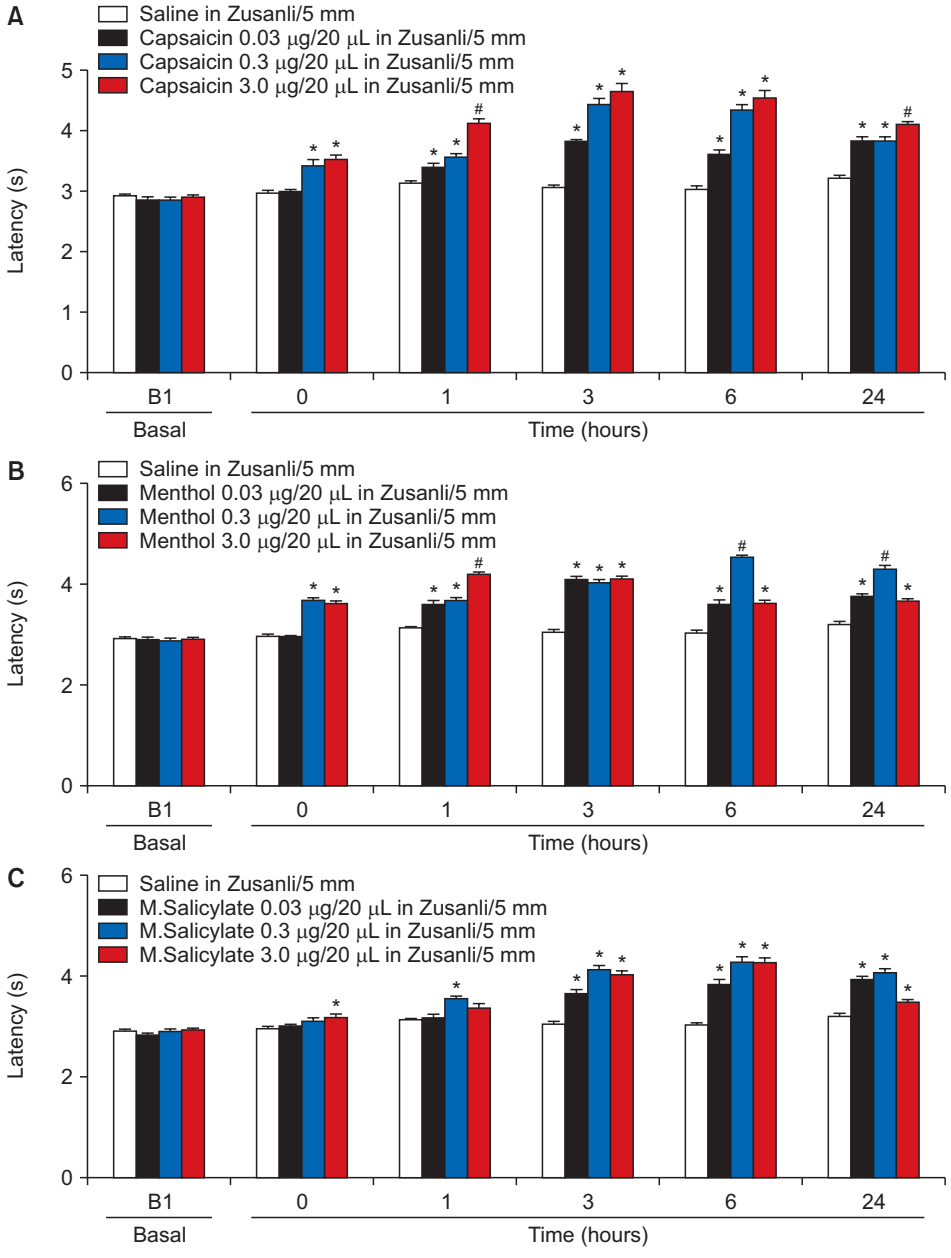

Fig. 2. Withdrawal latency using the tail-flick test (TF). (A) Effect of capsaicin on TRPV1 at 0.03, 0.3, and 3.0 μg/20 μL concentrations. (B) Effect of menthol on TRPM8 at 0.03, 0.3, and 3.0 μg/20 μL concentrations. (C) Effect of methyl salicylate on TRPA1 at 0.03, 0.3, and 3.0 μg/20 μL concentrations. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni post hoc test. *$p < 0.05$ compared to saline; # represents $p < 0.05$ compared to the other treatments (#).

Results of the second experiment, which was conducted to assess the withdrawal latency by the tail-flick test, are shown in Fig. 2. The rats received an s.c. injection of capsaicin, menthol, or methyl salicylate in the ST36 acupoint at three different concentrations: 0.03, 0.3, and 3 μg/20 μL, or 20 μL of sterile saline as control. Nociceptive evaluation was performed before the administration of agonists (B1) and after 0, 1, 3, 6, and 24 hours of the administration. In the control groups, nociception was maintained until 24 hours.

Fig. 2A demonstrates the analgesic effect of capsaicin assessed using the tail-flick test. A difference in withdrawal latency was noted up to 24 hours at all doses administered, except at time 0 (hours) with the lowest dose. Further, a significant influence of time ($F_{5,100}$ = 211.436, p < 0.001), treatment ($F_{3,20}$ = 225.635, p < 0.001), and interaction between treatment × time ($F_{15,100}$ = 25.774. p < 0.001) was noted.

A similar effect was obtained after the s.c. administration of menthol in ST36 acupoint (Fig. 2B) at all doses administered, except at time 0 (hours) with the lowest dose. A significant influence of time ($F_{5,100}$ = 307.629, p < 0.001), treatment ($F_{3,20}$ = 448.886, p < 0.001), and interaction between treatment × time ($F_{15,100}$ = 60.368, p < 0.001) was noted.

In contrast, the TRPA1 agonist, methyl salicylate, only demonstrated antinociception with all doses from 3 to 24 hours after its administration (Fig. 2C). However, a significant influence of time ($F_{5,100}$ = 185.989, p < 0.001), treatment ($F_{3,20}$ = 101.243, p < 0.001), and interaction between treatment × time ($F_{15,100}$ = 22.279, p < 0.001) was noted.

Lastly, the results of the third experiment are shown in Fig. 3. The nociceptive threshold was assessed using the electronic

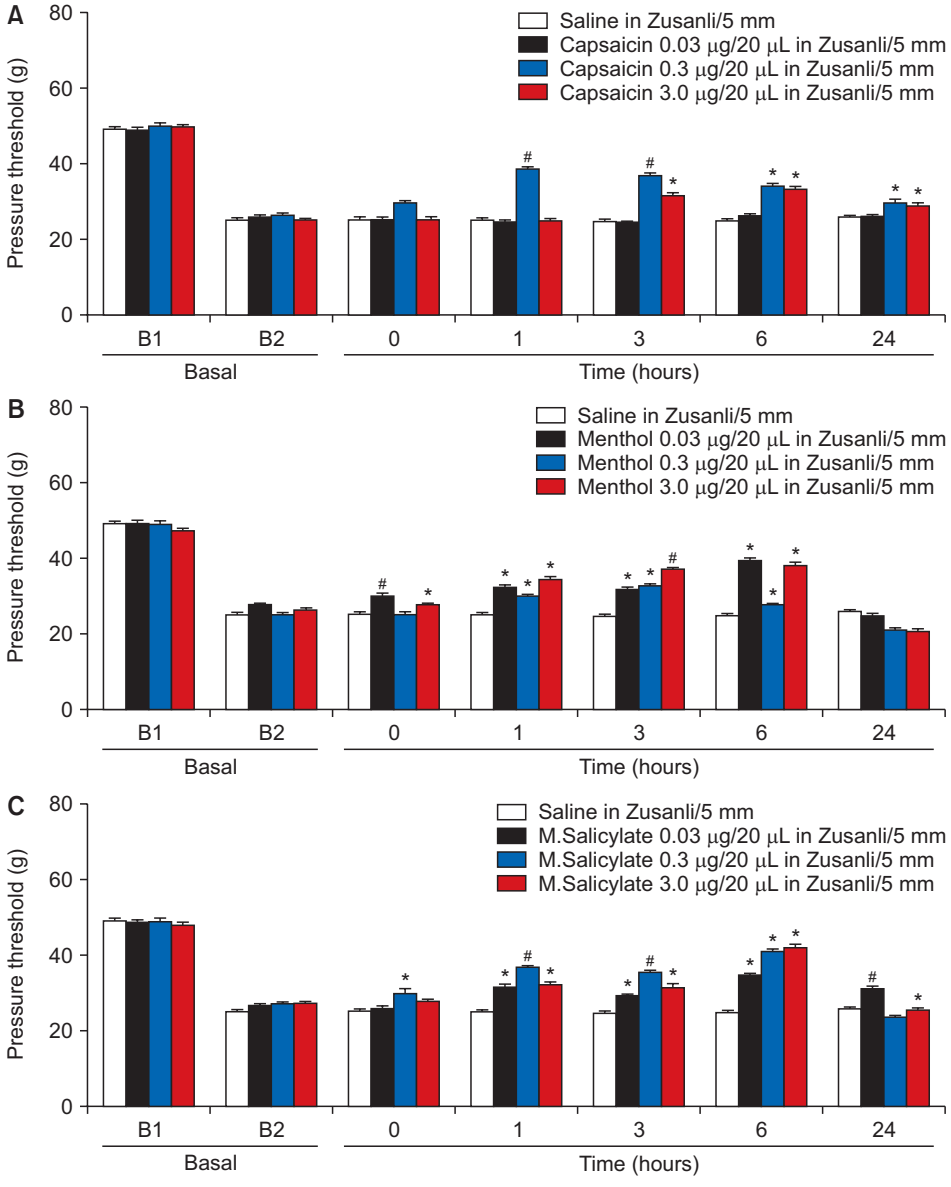

Fig. 3. Nociceptive threshold evaluated using the electronic mechanical threshold test following CCI experimental model. (A) Effect of capsaicin on TRPV1 at 0.03, 0.3, and 3.0 μg/20 μL concentrations. (B) Effect of menthol on TRPM8 at 0.03, 0.3, and 3.0 μg/20 μL concentrations. (C) Effect of methyl salicylate on TRPA1 at 0.03, 0.3, and 3.0 μg/20 μL concentrations. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni post hoc test. *p < 0.05 compared to saline; # represents p < 0.05 compared to the other treatments (#).

mechanical threshold test in the groups with neuropathic pain. The animals received an s.c. injection of three different doses—0.03, 0.3, and 3.0 μg/μL—of capsaicin, menthol, or methyl salicylate in the ST36 acupoint, while control animals received an injection of 20 μL of sterile saline (SAL). Nociceptive evaluation was performed before the induction of neuropathic pain (B1); 72 hours after the execution of the technique (B2); and 0, 1, 3, 6, and 24 hours following agonist administration.

In the control group, the pain lasted from the B2 up to 24 hours of evaluation. Fig. 3A demonstrates that the antinociceptive effect of capsaicin occurred only with the highest and intermediate dose at 3, 6, and 24 hours, with a predominance with the intermediate dose. A significant influence of time ($F_{6,120} = 1398.450$, $p < 0.001$), treatment ($F_{3,20} = 131.791$, $p < 0.001$), and interaction between treatment × time ($F_{18,120} = 42.739$, $p < 0.001$) was noted.

As shown in Fig. 3B, menthol obtained attenuated nociception at 1, 3, and 6 hours after its administration, with the different doses. A significant influence of time ($F_{6,120} = 1399.587$, $p < 0.001$), treatment ($F_{3,20} = 91.073$, $p < 0.001$), and interaction between treatment × time ($F_{18,120} = 58.749$, $p < 0.001$) was noted.

Finally, the TRPA1 agonist, methyl salicylate, induced antinociception mainly at 1, 3, and 6 hours after its injection in the ST36 acupoint, with three different doses; however, at 24 hours, only the lowest and highest dose reduced nociception (Fig. 3C). A significant influence of time ($F_{6,120} = 953.547$, $p <$ 0,001), treatment ($F_{3,20} = 116.209$, $p < 0.001$), and interaction between treatment × time ($F_{18,120} = 45.654$, $p < 0.001$) was noted.

DISCUSSION

The results obtained in this study demonstrated the characterization of transduction pathways resulting from the activation of TRPV1, TRPM8, and TRPA1 receptors in pain, along with antinociception induced by the administration of their agonists capsaicin, menthol, and methyl salicylate, respectively, in the ST36 acupoint.

This idea is supported by experimental data that confirm this nociceptive transduction in different types of pain [17]. Furthermore, the release of a variety of inflammatory mediators results in an increase in the expression of TRPV1, TRPM8, and TRPA1 [10]. In other studies, for example, this sensitization occurred after tissue injury [18] and by the administration of CFA in rats [19,20]. Furthermore, studies that targeted desensitization and analgesia have also administered high concentrations of the agonists of these receptors [17,21-23]. Therefore, the utilization of these agonists to control hyperalgesia, induced by increased expression of TRPs, seems a relevant approach for reducing painful conditions [17]; however, no relationship with pharmacopuncture has been found in ST36.

In this context, in an experiment on rats, previous studies have reported a high concentration of sensory receptors and their afferent fibers in an area located in muscle tissue [24,25]. Among these points was the ST36 acupoint, which can be used in events of decrease or absence of immune responses, attenuation of the inflammatory response, and other pathological manifestations [2,7]. Thus, one of the main findings of the present study was that the administration of the agonists of TRPs receptors in the ST36 acupoint induced antinociception in the models of acute, inflammatory, and neuropathic pain.

In this study, the administration of capsaicin, menthol, and methyl salicylate in the inflammatory pain model resulted in analgesia, which was mediated by the TRPV1, TRPM8, and TRPA1 receptors, in that order. In previous studies, capsaicin demonstrated analgesic effects in inflamed tissues that reflected a greater response to TRPV1 [26,27]. Another study in rats that used two types of experimental models for inflammatory pain demonstrated the antinociceptive effect of capsaicin through a single administration [28].

A previous study described menthol as a potent anti-inflammatory agent, observing the inhibition of pro-inflammatory cytokine expression via the use of L-menthone [29]. Another study demonstrated its role in a model of inflammatory pain induced by CFA. Pre-treatment with a TRPM8 antagonist, AMG2850, followed by menthol administration, resulted in the inactivation of these receptors in the nerve terminals, thus causing inflammatory pain to persist [30].

TRPA1 is a thermoreceptor activated at low temperatures, around 17℃, and by several natural substances. In this study, we observed an important role of methyl salicylate in the analgesic effect at the ST36 acupoint, in different painful conditions. In a study in which methyl salicylate was administered in ST36, western blotting results revealed a high expression of TRPA1 receptors in this acupoint associated with ST37, suggesting the transduction pathway by TRPA1 [31]. To the best of our knowledge, studies regarding the action of capsaicin and menthol in the ST36 acupoint are scarce, highlighting the necessity of further exploration in this matter.

The TF test, which has been widely used to assess the antinociceptive effect of various drugs, was employed in this study. This test involves both spinal and supraspinal structures, depending on the intensity of the stimulus [32]. Another study administered capsaicin and RTX (a capsaicin analog), intrathecally, and reported the participation of TRPV1 and the analgesic effect. Our findings agree with the reduction of nociception by the activation of TRPV1 [33].

In our study, menthol, the TRPM8 agonist, demonstrated an antinociceptive effect in the pain models employed, reinforcing the data of a study in which it was administered intracerebroventricularly and the nociception was assessed through the hot plate test [34]. This possibly occurred due to the inhibition of calcium currents. In addition, plantar administration of menthol resulted in increased paw withdrawal latency, supporting the notion that TRPM8 would be a peripheral target for pain modulation [35], in addition to nociceptive suppression following a harmful thermal stimulus [36]. Methyl salicylate also showed significant results in our study; however, data regarding the use of this compound in a similar way are lacking in the literature.

To investigate the antinociceptive effect of TRP agonists, we used the CCI model. The antinociceptive results corroborate with those reported in a study that performed intrathecal administration of capsaicin and resulted in antinociception through thermal and mechanical evaluation [37].

In addition, our study demonstrated a reduction in post-surgical hyperalgesia following menthol application in ST36, agreeing with another study that applied menthol topically to the hind legs of animals with spinal nerve ligation, associated with a decrease in the expression of TRPM8 [38]. In our study, methyl salicylate induced an antinociceptive effect when injected s.c. in the ST36 in the CCI model; however, to our best knowledge, no study has explored this hypothesis thus far, warranting the need for further studies on this topic.

One limitation inherent to this study concerns ipsilateral pharmacopuncture. Although many theories exist regarding the mechanisms underlying the analgesic effect of electrical or manual acupuncture, including the gate control and endogenous opiates theories, the mechanism underlying the analgesic effect of contralateral manipulation remains unclear [15,16]. We suggest that these effects presented are associated with the acupuncture administered ipsilateral to the lesion; however, additional experimentation and/or binding studies are required to compare and define the exact type of manipulation that is better suited.

According to the present results, we suggest that TRPV1, TRPM8, and TRPA1 actively participate in the control of acute, inflammatory, and neuropathic nociception, as demonstrated by the administration of their agonists capsaicin, menthol, and methyl salicylate in ST36 acupoint, inducing analgesia. Thus, minimizing the oral administration of these drugs can prevent their deleterious effects on the gastrointestinal system.

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AUTHORS' CONTRIBUTIONS

All authors have full access to all the data in the study and are responsible for the integrity of the data and accuracy of the data analysis. Conceptualization: I.P.R.A., M.L.S.; Data curation: I.P.R.A., M.L.S.; Formal analysis: I.P.R.A., M.L.S.; Funding acquisition: I.P.R.A., J.R.T.S., M.L.S.; Investigation: I.P.R.A., J.R.P., J.R.T.S., M.L.S.; Methodology: I.P.R.A., M.L.S.; Project administration: J.R.T.S., M.L.S.; Resources: J.R.T.S., M.L.S.; Supervision: J.R.T.S., M.L.S.; Validation: I.P.R.A., J.R.P.; Visualization: I.P.R.A., J.R.P.; Writing - original draft: I.P.R.A., J.R.P.; Writing - review & editing: I.P.R.A., J.R.P., J.R.T.S., M.L.S.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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