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ORIGINAL INVESTIGATION

Therapeutic ultrasound ameliorates hyperalgesia and edema on CFA-induced persistent inflammatory response in mice

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Abstract

Background: The present study investigated the effects of pulsed and continuous ultrasound (USP and USC) in edema and hyperalgesia after chronic inflammatory process induced by Complete Freund's Adjuvant-CFA and analyzing the relationship of the application frequency of ultrasound, in pro- and anti-inflammatory cytokine production.

Methods: Forty-five animals were divided into 9 groups; all animals from groups 2 to 9 were subjected to a persistent inflammation model induced by CFA in mice. We report the effects and the underlying action mechanisms of USP and USC in the animals which were irradiated two, three or five times a week on the left hind paw. The analyses performed in this study were: evaluation of hind paw edema through the plethysmometer, evaluation of thermal hyperalgesia through withdrawal test using a water container at 44.5°C (± 0.5°C), and the plantar region of the left paw which was removed for analysis of cytokines.

Results: Our results showed that USP and USC consistently reduced paw edema, and pulsed ultrasound showed a higher significant effect than the continuous mode. Moreover, groups with irradiation frequency of five times a week presented an inhibition of the edema, and groups with frequency of three or two times a week reduced mainly hyperalgesia, in comparison with the control group. The beneficial effects of the US then seem to be associated

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with upregulation of anti- and pro-inflammatory mediators, such as IL-10 and IL-6, respectively.
Conclusion: This study provided evidence that ultrasound constitutes an important non-pharmacological intervention for the management of inflammatory and pain states.

Introduction

The inflammatory process involves a complex cascade of biochemical and cellular events, which are started by innate immune response.[1] Clinically, five cardinal signs characterize inflammation: redness, increased heat, swelling, pain, and loss of function.[2] It consists in the exudate overflow to the interstitial medium and generates edema. Although edema formation is a common response after injury, it may be detrimental to the resolution of the clinical condition, since it can aggravate the inflammatory process, worsening the functionality and leading to pain-spasm-pain.[3] When the resulting pain evolves into the chronic state, it becomes a public health problem, inducing morbidity and temporary or permanent incapacity, which represents high costs to the health system.[4,5]

There are different stimuli to induce the inflammatory process in the mice's paw, such as the Complete Freund's Adjuvant (CFA), which acts as an irritative agent.[6,7] This inflammatory response induces edema, increase of tissue volume, and consequent hypersensitivity to thermal and mechanical stimuli,[6-8] which is due to changes on the production of peripheral inflammatory mediators, thereby facilitating prolonged depolarization of the neuronal membrane and exacerbating hyperalgesia or allodynia.[9]

Besides drug therapy, therapeutic ultrasound action in inflammatory treatment has been investigated by different studies, which include the use of US and other resources for inflammatory response control and consequent inhibition of edema and pain.[10] Some evidence indicates that US treatment increases synthesis and improves the aggregation and alignment of collagen fibers, besides inducing stimulation of proliferation tissue and, consequently, onset of the repair process.[11,12] Some authors suggest that US has a pro-inflammatory action, since it accelerates the inflammatory response in the process of lesion repair and promotes the release of histamine, which increases venous and lymphatic return, facilitating edema absorption.[13-15]

Previous studies have investigated electrophysical agents action on the acute and chronic inflammatory process.[10,13,15] Therapeutic ultrasound is widely used for clinical treatments. However, in general, few use US in preclinical research, and few authors associate therapeutic ultrasound with a model of persistent inflammation induced by the application of CFA. Thus, the present study investigated the effect of the pulsed or continuous beam regimen of therapeutic ultrasound and demonstrated a comparison of different application frequencies (two, tree or five days for week), during persistent inflammatory process induced by CFA in mice.

Methods

Ethical aspects

All protocols and procedures used in this study were approved by the Federal University of Santa Catarina, Ethics Committee of Animal Experimentation (CEUA/UFSC, protocol number PP00956). The ARRIVE checklist was used.[16]

Experimental procedure

Forty-five 1−2 months old male Swiss mice (30−40 g) were obtained from the Universidade Federal de Santa Catarina (UFSC), Campus Florianópolis, and kept in the Araranguá center (BIO-ARA/UFSC). Each experimental group comprised five animals kept in isolated cages with ventilation, temperature control (22°±2°C) and humidity control (60−80%), in a light-dark cycle of 12 hours, with free access to water and food. The behavioral experiments were performed at the Laboratory of Autoimmunity and Immunopharmacology (LAIF) between 7 a.m. and 11 a.m. The animals were kept in the laboratory for acclimatization for at least 30 minutes prior to the evaluation. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Experimental groups

Sample calculation was performed using the G*Power software, version 3.1.[17] Analyses included F tests, ANOVA with repeated measures, and intra and intergroup interaction, with effect size F = 0.25, significance level α = 0.05, and power (1-β) 0.95. All experiments were divided into two independent experiments, the first with 27 animals divided into 9 groups and after a week the second experiment with 18 animals divided into 9 groups, totalling 45 evaluated animals. The animals were randomly separated into the groups (n = 5 animals/group) G1: Naïve (Saline Injection); G2: Sham; G3: Placebo; G4: US Continuous − 5 × week; G5: US Continuous − 3 × week; G6: US Continuous − 2 × week; G7: US Pulsed − 5 × week; G8: US Pulsed − 3 × week; G9: US Pulsed − 2 × week.

In group 1 (naive), saline was injected into the animals' left hind paws. The animals of groups 2 (Sham, was submitted only to CFA injection and not to Ultrasound) to 9 were induced to the chronic inflammatory process in the left hind paw through CFA injection as described below. Groups 3 to 9 were subjected to ultrasound application. The evaluation procedures were performed by blind evaluator.

Edema induction

According to Dutra et al.,[18] the following substance was used for induction of the persistent inflammatory process, 30 μL of CFA (1 mg.mL^{-1} *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monooleate from Sigma Chemical Co., St. Louis, MO, USA) which was injected into the animals' subcutaneous plantar surface in the left hindpaw.[14,15,19]

Therapeutic ultrasound

The therapeutic ultrasound device produced by the Ibramed® Brazilian Industry of medical equipment (Amparo, São Paulo/Brasil) was applied to the mice's left hind paw following the US continuous parameters (Frequency: 1 MHz; ERA: 1 cm^2; Duty cycle: 100%; Intensity: 0.4 W.cm^{-2}; BNR: 8:1; Time: 3 min) and US Pulsed (Frequency: 1 MHz; ERA: 1 cm^2; Duty cycle: 20%; Pulse frequency: 100 Hz; Intensity: 2 W.cm^{-2}; SATA: 0.4 W.cm^{-2}; BNR: 8:1; Time: 3 min).[20-23] Both pulsed and continuous US groups were submitted to the underwater technique in the plantar region of the left hind paw, with a distance of 0.5 to 1.0 cm between the paw and the US transducer. The placebo group received an application simulation with the equipment switched off. US application was initiated one hour after CFA administration and weekly, according to each group frequency, for up to 4 weeks.

Edema evaluation

Paw edema was evaluated by a plethysmometer (Model 7150, Ugo Basile, Varese, Italy). The samples were collected at 1, 2, 3, 4, 5, 6, 7, 8, 24 and 48 hours, and continued daily for up to 4 weeks after the edema induction. Data was expressed by the volume displaced in milliliters (mL),[24] and the analyses were performed by the difference of times evaluated with the first baseline assessment, so the smaller the delta, the smaller edema in the animals' paws.

Thermal hyperalgesia evaluation

Thermal hyperalgesia was performed through a withdrawal test using a water container at 44.5°C (±0.5°C). After immersing the animal's foot in the heated bath, the withdrawal time was measured in seconds, with the maximum set time of the animal's foot immersion of 30-second cut-off to avoid tissue injury. The samples were collected at the same time as the edema measurement, as described above.[24]

Cytokine evaluation

After 28 days of the CFA injection, animals were submitted to the last analysis and later submitted to euthanasia by anesthetic overdose. The levels of cytokines in the plantar tissue of CFA-injected mice were determined according to the following protocol: The subcutaneous paw tissue was removed from each mouse, and homogenized in a phosphate buffer solution containing 0.05% Tween 20, 0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 UI aprotinin A. The homogenate was centrifuged at 3000 × g for 10 min, and supernatants were stored at -20°C until further analysis. IL-10 and IL-6 levels were evaluated using ELISA kits (Organon-Teknika, Roseland, NJ, USA), according to the manufacturer's recommendations.[24]

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 software. Before each analysis, the normality in data distribution was verified by the Shapiro-Wilk test. After the normality test, a two-way Analysis of Variance (ANOVA) was performed to evaluate edema and thermal hyperalgesia, and a one-way ANOVA was performed to evaluate cytokines IL6 and IL10. The significance coefficient was $p < 0.05$, and the post-hoc Bonferroni test was performed after the ANOVAs. Pearson's test was used for data correlation.

Results

The chronic inflammatory response was induced in the animals' left hind paws from groups 2 to 9 after a CFA injection. The evaluation of the animals was through the measurement of edema and thermal hyperalgesia. The results are shown in figures, mainly the analyses performed on the 7th, 14th, 21st, and 28th days.

Continuous and Pulsed Therapeutic ultrasound in the edema

There was no difference among groups considering the baseline analysis. After inducting the inflammatory process, edema formation was observed in all groups. We emphasize that the sham and placebo groups kept the same edema behaviour ($p < 0.05$) compared to the naive group in all assessments. Experimental groups with continuous therapeutic ultrasound presented significant differences ($p < 0.05$). On the 14th day, the placebo group presented a significant increase in edema compared to groups 2 (Sham) and 5 (CFA+USC, three times a week). On the 21st day, group 4 (CFA+USC, five times a week) significantly decreased edema volume compared to the placebo group. Finally, on the 28th day, group 2 showed higher edemas than groups 4 and 6 (CFA +USC, two times a week) (Fig. 1).

A significant improvement was found ($p < 0.05$) on the 7th day, for group 7 (CFA+USP, five times a week), compared to group 9 (CFA+USP, two times a week) (Fig. 2). On the 14th day, groups 2 and 7 showed a significant improvement compared to the placebo group. Finally, on the 28th day, group 2 presented significant differences from groups 8 (CFA+USP, three times a week) and 9.

Continuous and pulsed therapeutic ultrasound inhibited hyperalgesia

As previously described, CFA injection in mice's paws induced persistent pain and hyperalgesia.[18] Next, we evaluated whether US reduces hyperalgesia through a withdrawal test. Significant differences ($p < 0.05$) were found on the 7th day for group 4, which achieved a significant increase in withdrawal time when compared to the placebo group, and for group 6, which inhibited hyperalgesia when compared to

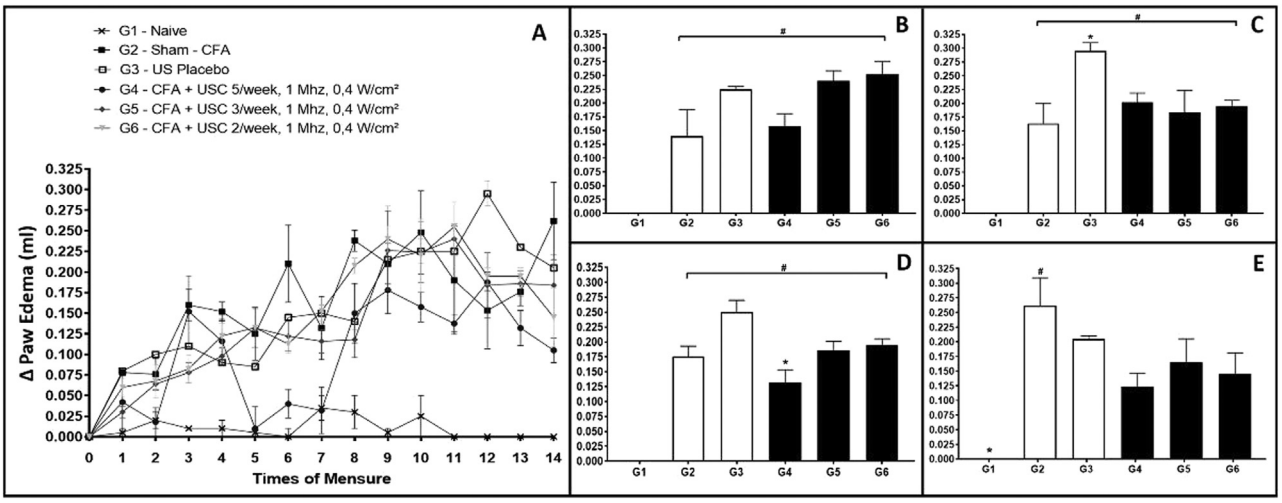

Figure 1 Data referring to the delta of the hindpaw edema volume of the animals submitted to treatment with continuous Terapeutic Ultrasound and naive, Sham and placebo groups at different evaluation times. (USC, Continuous Ultrasound; 5 ×, Five times a week; 3 ×, Three times a week and 2 ×, Twice a week). A, The evaluation times were (t) 9, Related to 24 h after induction; (t) 10, Referring to 48 h after induction; (t) 11, Equivalent to the 7th day; (t) 12, Equivalent to the 14th day; (t) 13, Equivalent to the 21st day; and (t) 14, Equivalent to the 28th day. B, Referring to the 7th day (# statistical difference Naïve with all groups [$p < 0.05$]). C, 14th day (# statistical difference Naïve with all groups [$p < 0.05$] and * statistical difference between placebo with sham groups [$p < 0.05$]). D, 21st day (# statistical difference Naïve with all groups [$p < 0.05$]). E, 28th day (# statistical difference Sham with G1, G4 and G6 groups [$p < 0.05$] and * statistical difference between naive with all groups [$p < 0.05$]).

placebo, 2 and 5 groups (Fig. 3). Also, group 2 showed different values concerning groups 1 (naive) and 9, whereas the placebo group presented an increase in hyperalgesia compared to all but the Sham group.

On the 14th day, group 9 showed significant ($p < 0.05$) improvement in hyperalgesia compared to groups Sham and seven (Fig. 4). Moreover, group 8 showed improvement of hyperalgesia compared to the Sham group. On the 21st day, the naive group showed significant differences to placebo, Sham and group 9. Finally, there was a significant decrease in hyperalgesia in group 7 compared to Sham and placebo groups.

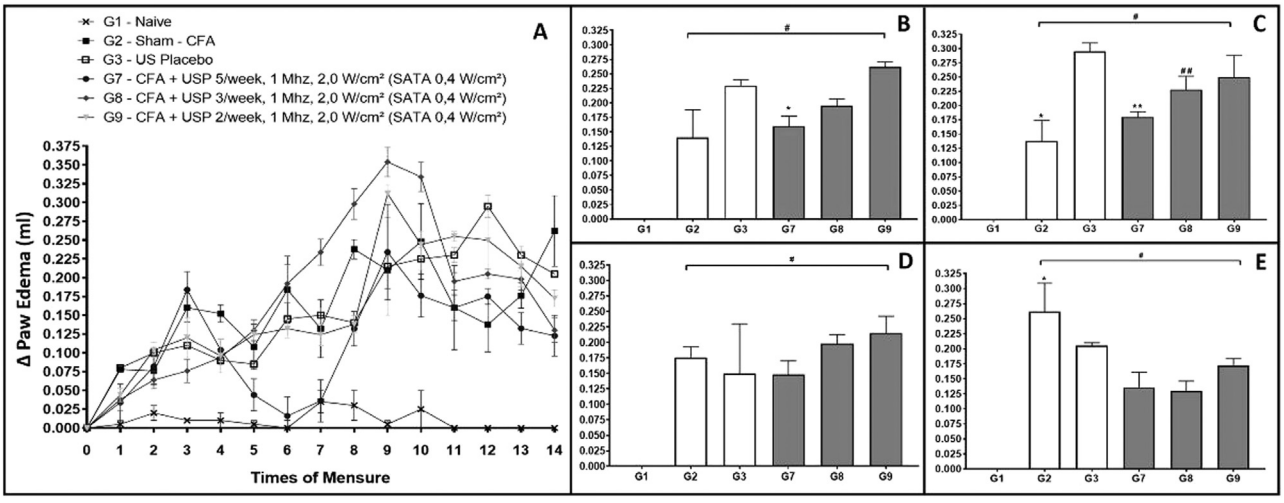

Figure 2 Data related to the delta of the hindpaw edema volume of the animals submitted to treatment with US pulsed and naive, Sham and placebo groups at the different evaluation times. (USP, Pulsed Ultrasound; 5 ×, Five times a week; 3 ×, Three times a week and 2 ×, Twice a week). A, The evaluation times were (t) 9, Related to 24 h after induction; (t) 10, Referring to 48 h after induction; (t) 11, equivalent to the 7th day; (t) 12, equivalent to the 14th day; (t) 13, equivalent to the 21st day; and (t) 14, equivalent to the 28th day. B, Referring to the 7th day (# statistical difference Naïve with all groups [$p < 0.05$] and *statistical difference G7 with G9 groups [$p < 0.05$]). C, 14th day (# statistical difference Naïve with all groups [$p < 0.05$], * statistical difference between sham with placebo and G9 groups [$p < 0.05$], ** statistical difference G7 with placebo groups [$p < 0.05$] and ## statistical difference G8 with placebo groups [$p < 0.05$]). D, 21st day (# statistical difference Naïve with all groups [$p < 0.05$]). E, 28th day (# statistical difference between naive with all groups [$p < 0.05$] and * statistical difference Sham with G7, G8 and G9 groups [$p < 0.05$]).

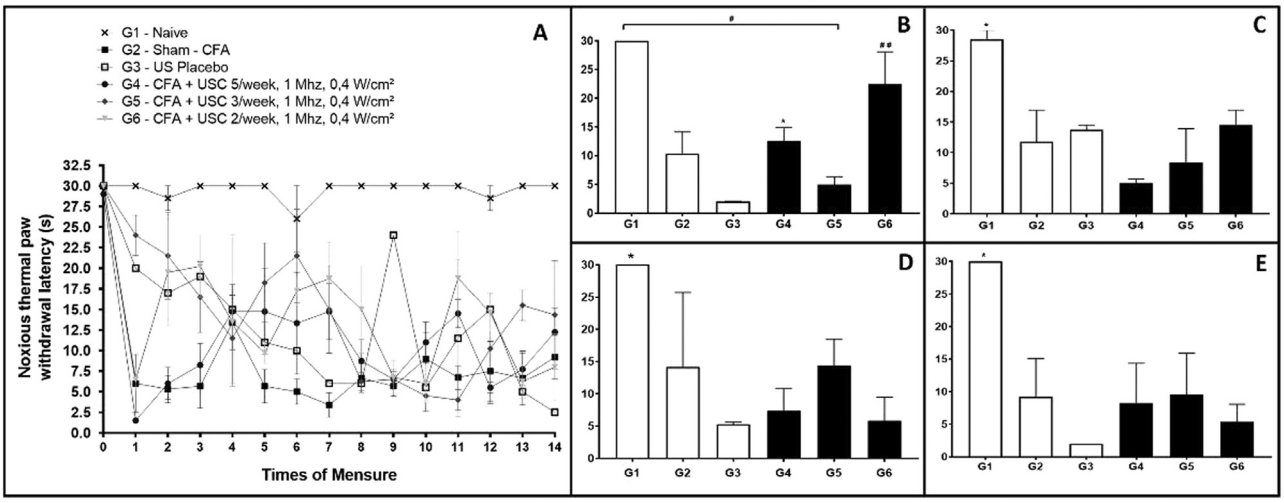

Figure 3 Data referring to the withdrawal time of the animals hindpaw of the groups submitted to the treatment with continuous US and in naïve, Sham and placebo groups at the different evaluation times. USC, Continuous Ultrasound; 5 ×, Five times a week; 3 ×, Three times a week an; 2 ×, Twice a week). A, The evaluation times were (t) 9, related to 24 h after induction; (t) 10, referring to 48 h after induction; (t) 11, equivalent to the 7th day; (t) 12, equivalent to the 14th day; (t) 13, equivalent to the 21st day; and (t) 14, equivalent to the 28th day. B, Referring to the 7th day (# statistical difference Naïve with all groups [$p < 0.05$] exept G6 group, * statistical difference G4 with G3 groups [$p < 0.05$] and ** statistical difference G6 with G3 and G5 groups [$p < 0.05$]). C, 14th day (* statistical difference Naïve with all groups [$p < 0.05$]). D, 21st day (* statistical difference Naïve with all groups [$p < 0.05$]). E, 28th day (* statistical difference between naive with all groups [$p < 0.05$]).

There was no difference between the groups submitted to the pulsed and continuous therapeutic ultrasound during the analyses among the groups submitted to the two types of US. In the thermal hyperalgesia analysis, the groups submitted to pulsed therapeutic ultrasound showed better results. However, there was a statistical difference ($p < 0.05$) when comparing group 9 (USP 3 × per week) with group 5 (USC 2 × per week). The analysis of thermal hyperalgesia has a cut-off point of 33 seconds, for the animals that stayed the 33 seconds it was considered 100%, thus

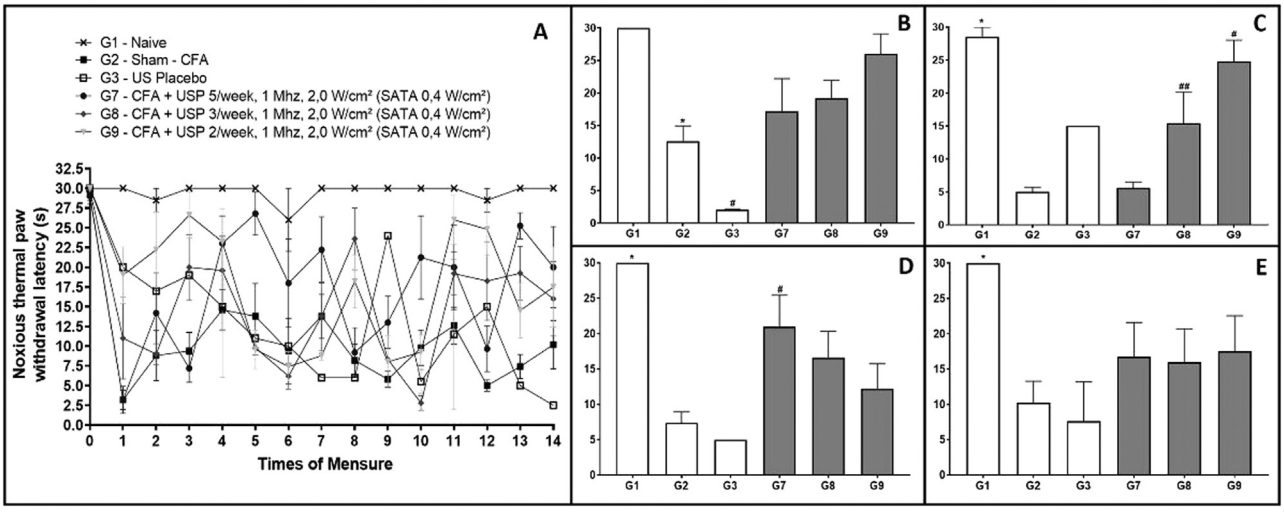

Figure 4 Data regarding the withdrawal time of the animals hindpaw of the groups submitted to the treatment with US pulsed and in naïve, Sham and placebo groups at the different evaluation times. USP, Pulsed Ultrasound; 5 ×, Five times a week; 3 ×; Three times a week an; 2 ×, Twice a week). A, The evaluation times were (t) 9, related to 24 h after induction; (t) 10, referring to 48 h after induction; (t) 11, equivalent to the 7th day; (t) 12, equivalent to the 14th day; (t) 13, equivalent to the 21st day; and (t) 14, equivalent to the 28th day. B, Referring to the 7th day (# statistical difference placebo group with all groups [$p < 0.05$] and * statistical difference sham group with G1, G7, G8 and G9 groups [$p < 0.05$]). C, 14th day (* statistical difference Naïve with G2, G3, G7 and G8 groups [$p < 0.05$], # statistical difference G9 with G2, G3, G7 and G8 groups [$p < 0.05$] and ## statistical difference between G8 with G2 and G7 groups [$p < 0.05$]). D, 21st day (* statistical difference Naïve with all groups [$p < 0.05$] axept G7 and # statistical difference between G7 with G2 and G3 groups [$p < 0.05$]). E, 28th day (* statistical difference Naive with G2 and G3 groups [$p < 0.05$]).

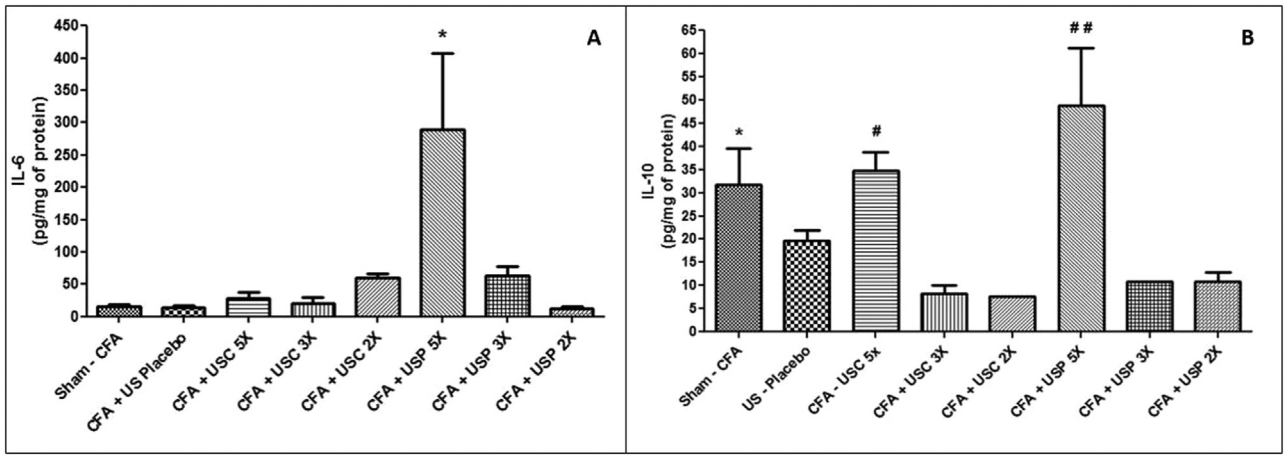

Figure 5 Data referring to 28 days after start of the experimentation. A, IL-6 levels in the tissue sample of the respective groups submitted to intra-plantar injection of CFA: Sham, US placebo, US continuous 5, 3, and 2 times a week and the US pulsed 5, 3, and 2 times a week ($p < 0.001$). B, IL-10 levels in the tissue sample of the respective groups submitted to intra-plantar injection of CFA: Sham, US placebo, US continuous 5, 3 and 2 times a week, and US pulsed 5, 3 and 2 times a week ($p < 0.001$).

facilitating the comparison between the groups. In the last two assessments of thermal hyperalgesia, the placebo group withdrew the paw earlier (shorter time) when compared to the other groups. There was a statistical difference ($p < 0.05$) when compared to naive and 7 (USP 5 × per week) groups.

Continuous and pulsed therapeutic ultrasound effects on the release of cytokines in the inflammatory process induced by CFA

Including cytokine secretion, both IL-6 and IL-10 can be involved in inflammation.[19] We evaluated whether the US attenuated the production of pro-inflammatory cytokines and increased the production of anti-inflammatory cytokines. The Sham group increased IL-10 levels compared to groups 5, 6 and 8 ($p < 0.05$). Group 4 showed a difference to groups 5, 6, 8 and 9 ($p < 0.05$). Finally, group 7 showed a significant increase of IL-10 (Fig. 5A) and significant differences regarding the number of IL-6 levels (Fig. 5B) when compared to all other groups ($p < 0.001$).

Correlation of the continuous and pulsed therapeutic ultrasound effects on the release of cytokines, Edema and Thermal hyperalgesia in the inflammatory process induced by CFA

Table 1 demonstrates all correlations between the variables Thermal hyperalgesia and Edema compared to cytokines IL-6 and IL-10, the analysis was performed by Pearson's test, and the data referring to the 28th day of the experiment was used for evaluation.

Discussion

This study aimed to investigate the effects of pulsed and continuous US in edema and hyperalgesia after the chronic inflammatory process induced by CFA, besides analyzing the relationship of the US application frequency and pro and anti-inflammatory cytokine production. There is a paucity of research evaluating the influence of the US on pain and chronic edema in animals. In order to discuss the present data, we used articles that evaluated acute pain and edema.

Table 1 Demonstrates the correlation between the variables Thermal hyperalgesia and Edema compared to the cytokines IL-6 and IL-10. The analysis was performed by Pearson's test.

Groups	Hyperalgesia × IL-6	Hyperalgesia × IL-10	Oedema × IL-6	Oedema × IL-10
G1: Naive	−0.20	0.07	−0.21	0.72
G2: Sham	−0.81	−0.99	−0.66	−0.01
G3: Placebo	−0.67	−0.86	−0.42	−0.50
G4: USC – 5 × week	−0.34	−0.45	0.54	0.86
G5: USC – 3 × week	−0.37	0.82	−0.81	0.53
G6: USC – 2 × week	−0.81	0.83	0.68	−0.79
G7: USP – 5 × week	−0.73	0.71	0.30	−0.65
G8: USP – 3 × week	−0.26	0.58	−0.01	0.32
G9: USP – 2 × week	0.40	−0.61	−0.92	0.94

In the study conducted by Bertolini et al.,[20] the authors used the same parameters used in this study, considering the intensity of 0.4 W.cm^{-2} and a five-day treatment frequency. The authors observed that US treatment decreases the pain and acute edema in animals with calcaneus tendon injuries. The early effect of the pulsed ultrasound was observed, corroborating our findings that the USP minimized the animals' hyperalgesia and chronic edema. Bertolini et al.[10] used a 0.5 W.cm^{-2} intensity in the continuous and pulsed regime and observed that the US inhibited the progression of acute edema in mice's paw but did not diminish the already installed edema. In the present study, the groups submitted to a frequency of two and three times a week of continuous or pulsed US application inhibited chronic edema in animals but did not decrease edema already installed in the mice's paws. However, we found significant differences between groups 1, 2, and 3. We found that the groups treated five times a week were effective in minimizing edema.

This study observed the influence of the therapeutic ultrasound week application frequency and other parameters, such as the pulse regime.[19] The groups did not present a uniform behavior compared to the frequency and the US pulse regime. These results can be explained considering that the response dose of therapeutic US can be influenced by the time of application.[25] Spped in 2001 described the thermal and non-thermal activity of the therapeutic ultrasound and observed the pulse regime employed. The author described the US thermal effects in continuous pulses, such as tissue extensibility improvement, flow blood increase, and pain modulation. In non-thermal effects in a pulsed regime, the author described the action mechanism across the cavitation and the acoustic microstreaming, stimulating protein synthesis and increasing blood flow and tissue regeneration.

Continuous and Pulsed Therapeutic ultrasound in edema and thermal hyperalgesia

Chung et al.[26] evaluated the anti-inflammatory effects of low-intensity USC on CFA-induced arthritis. The US application occurred for 10 minutes in 5 days. They observed a significant decrease of edema in the group treated with US, compared to the control group, to which only the CFA application was performed. Maiti and Kumar[27] used a tendon injury model and demonstrated that USP, at an intensity of 1 W.cm^{-2}, influenced the inflammatory edema resolution and hyperalgesia. These studies corroborate our findings since we obtained an improvement of hyperalgesia and edema in the evaluated animals.

In the present study, pulsed ultrasound obtained better results for thermal hyperalgesia, corroborating the research by Coradini et al.[28] who compared photobiomodulation and ultrasound in formalin-induced acute knee pain in Wistar rats, both studies used the same ultrasound parameters. The authors concluded that both therapeutic modalities showed antinociceptive effects, although ultrasound was superior to photobiomodulation by laser. In the present study of the continuous and pulsed US obtained an improvement in hyperalgesia.

Tascioglu et al.[29] evaluated continuous (1 MHz, 2 W.cm^{-2}, 5 minutes) and pulsed (2 W.cm^{-2}, 20% cycle) 10-session US patients with knee osteoarthritis. They observed that only the pulsed group achieved significant improvements in pain and the WOMAC index. Their results corroborate our findings, considering that pulsed US significantly improved hyperalgesia, especially in groups 8 (CFA+USP, three times a week) and 9 (CFA+USP, twice a week).

Most of the studies cited above demonstrated anti-inflammatory results, but US also has pro-inflammatory effects. Bertolini et al.[20] reported that in periods prior to 24 hours, therapeutic US promoted edema increases, which probably occurred due to pro-inflammatory action. We also observed an increase prior to the decrease of edema and consequent resolution of hyperalgesia. When comparing the placebo group with the other groups, we can see that it was better compared to the Sham group in edema and thermal hyperalgesia analyses.

Maximo et al.[30,25] observed that continuous US (intensity of 0.2 W.cm^{-2}) did not improve hyperalgesia and chronic edema, but when associated with drugs, such as indomethacin, a decrease in the edema of rats with arthritis was noticed. However, we found that the therapeutic US in isolation was effective in resolving hyperalgesia and edema.

Continuous and pulsed therapeutic ultrasound effects on the release of cytokines in the inflammatory process induced by CFA

IL-6 and anti-inflammatory cytokines (IL-10), present in the animals' left hind paw tissue, was evaluated by the ELISA method and presented a significant increase in group 7 concentration (CFA+USP, five times a week). In the USP five times a week group, we observed that the increase in the pro-inflammatory cytokines IL-6 may have caused a proportional increase in anti-inflammatory cytokine IL-10. This same pattern was described in the study by Van Miert[31] which presented the hypothesis that a proportional elevation of interleukins 6 and 0 may occur if both have a counter-regulatory effect, and an improvement in pain and edema in the USP five times a week group was observed as consequence. Also, Wei et al.[32] observed an increase of IL-6 at the fourth week after the neuropathic pain induction. These studies may explain the present study outcomes, where the therapeutic US can stimulate inflammation while inhibiting it at any given time.

According to the study by Tanaka et al.,[33] the excessive production of IL-6 cytokine may be linked to autoimmune disease or other pathological mechanisms, but the authors report that the excessive and persistent production of IL-6 is still not clear. Thus, the present study presents a model of a chronic inflammatory process, and in group 7 we observed an increase in IL-6, and since the animals did not present significant edema or pain in the last evaluation of the experiment, we deduced that the experimental model may have influenced the excessive production of IL-6, however it was observed only in group 7.[34]

Lin et al.[35] also reported that IL-6 is one of the earliest and important mediators of protein synthesis induction and control and is released during the acute phase of injuries and infections. After injury, plasma IL-6 concentration has its peak between 4 and 6 hours and may persist for ten days. However, it also exerts anti-inflammatory properties during injury by releasing soluble TNF and IL-10. Therefore, an important finding in our report also corroborates another

study describing the pleiotropic effects of IL-6 and IL-10 in detail and cytokines cross-regulation.[34]

Correlation of the continuous and pulsed therapeutic ultrasound effects on the release of cytokines, Edema and Thermal hyperalgesia in the inflammatory process induced by CFA

When performing the correlation of the data of the present study, different results can be observed when compared to pain, edema and the cytokines IL-10 and IL-6. In group 7, for example, we observed a moderate inverse correlation of IL-10 with edema (-0.65) and a weak correlation of IL-6 with edema (0.30). Regarding pain, we observed a moderate correlation of IL-10 with pain (0.71) and a moderate inverse correlation of IL-6 with pain (-0.73). Table 1 demonstrates all the correlations among the various edema and pain variables compared to cytokines IL-6 and IL-10.

The present study used an experimental model of CFA-induced chronic pain/inflammation. The study by Hung et al.[36] describes the use of therapeutic US and treadmill exercise in a model of neuropathic pain in animals treated for 28 days with IL-10 which obtained a behavior similar to the present study with self values. The authors report that this behavior may be due to a possible lasting symptom of neuropathic pain. According to Ruohonen et al.,[37] neuropathic (chronic) pain may present a cyclic pattern of pro and anti-inflammatory cytokine release. Considering the cyclic pattern of pro- and anti-inflammatory cytokines,[37] the data observed in group 7 (pulsed US 5 × per week) with a tissue sample collected on the 28th day showed expressive values of IL-6 and IL-10, therefore, we can deduce that IL-10 values may be compensatory when increasing IL-6. Oliveira et al.[38] showed that IL-10 has an inhibitory function in relation to proinflammatory cytokines. In this sense, this compensatory increase shows the moderate and strong correlation of IL-10 with hyperalgesia.

In general, when observing the data on the 28th day, the groups submitted to continuous US 2 and 5 times a week obtained better results with less edema and better results in thermal hyperalgesia, however, there was no statistical difference between the groups. Regarding the groups submitted to pulsed US, there was little difference in the data presented with the group of 3 times a week with slightly better results than the other groups, however, there was no statistical difference between them.

The present research sought to approach this electrophysical agent by observing the clinical routine to the frequency of weekly applications and the employed pulse regime. In this context, we present some limitations such as 1) The lack of histological evaluation with cell type analysis and counting, which could enrich the discussion about the pro and anti-inflammatory substances, to understand their increases in group number seven; 2) Cytokines analysis at different times; 3) Pro-inflammatory and anti-inflammatory analysis of different cytokines. In future studies that use an experimental model of chronic inflammation with CFA, we suggest enhancing the time of intervention and the performance of histological analysis and evaluation of inflammatory cells.

Conclusion

Considering our sample, we concluded that continuous and pulsed US influenced the reduction of edema, hyperalgesia and the release of IL-6 and IL-10 induced by CFA. Although two emission regimes used for the resource application showed positive effects, USP had more significant effects on these two analyzed variables. We observed that the groups' behaviour was not uniform considering the application analysis frequency since the groups irradiated five times a week were more effective considering the animals' edema reduction. When assessing hyperalgesia, the frequencies of three and two times a week were more effective in its inhibition. As for cytokine analyses, a significant increase of IL-10 was found in the Sham, 4 and 7 groups, whereas in IL-6, it was only found in group 7.

The present study indicates that US application decreases clinical signs of inflammation, such as edema and hyperalgesia. Therefore, the US may be a promising non-pharmacological therapy for inflammatory treatment. Also, we indicate the best resource application frequency, which will aid in treatment protocols.

Ethical approval

This study was approved by the Ethics Committee of Animal Experimentation of the Universidade Federal de Santa Catarina (CEUA/UFSC, protocol number PP00956).

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Conflicts of interest

The authors declare no conflicts of interest.

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