Benefits of electroacupuncture and a swimming association when compared with isolated protocols in an osteoarthritis model

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1. Introduction

Joint cartilage degradation, bone remodeling, inflammation, and the loss of mobile joint functions characterize osteoarthritis (OA). The synovial membrane, the synovial fluid, ligaments, tendons, and joint capsules are also included in OA. Other associations with OA include chronic pain and that reflects on a poor quality of life, morbidity, mortality, and physical inability. The high cost of an OA treatment and its management were the main associated problems. However, patient education, exercise, physical therapy, acupuncture, and electroacupuncture are other options required. Promising drugs have failed in preclinical studies and in clinical trials, thus studies with non-pharmacological interventions are standing out. New therapeutic strategies, with different combinations of conduct must be discovered.

The mechanisms of action of the therapeutic effects of electroacupuncture (EA) and swimming (SW) are non-pharmacological interventions recommended for treating OA. The study evaluated the benefits of electroacupuncture (EA) and swimming (SW) association when compared with isolated protocols in an OA rodent model. Experimental.

Background and aim: Osteoarthritis (OA) is characterized by pain and inflammation. Electroacupuncture (EA) and swimming (SW) are non-pharmacological interventions recommended for treating OA. The study evaluated the benefits of electroacupuncture (EA) and swimming (SW) association when compared with isolated protocols in an OA rodent model.

Procedures: An ankle monoarthritis model was induced in rats by applying Complete Freund’s Adjuvant (CFA). After seven days of induced OA, the groups were submitted to EA (ST36 and the GB 30 Acupoint), SW, or the EA + SW protocol. The nociceptive behavior was measured by the Von Frey test, the Cold Stimulation test, and the Paw Flick Immersion test. Inflammatory activity was evaluated by measuring TNF levels, myeloperoxidase, NAGase, immunological parameters and the histology from the subcutaneous tissue.

Results: Compared to CFA group, EA decreased the nociceptive scores in the cold stimulation test (p < 0.05), and it also increased the latency time in thermal cold (p < 0.01) and heat hyperalgesia (p < 0.001). Also, EA reduced NAGase (p < 0.01). SW reduced the edema (p < 0.05) and did not increase the inflammatory infiltrates or congestion, neither in the histological measurements nor by analyzing the levels of TNF. The association of EA + SW decreased the neutrophils and the monocytes, MPO (p < 0.05), and the glutamate levels in the cerebrospinal fluid (CSF, p < 0.001).

Conclusion: There were statistical differences between combination therapy and monotherapy as seen by the inflammatory parameters, which could be associate to the delay of the chronicification osteoarthritis retardation. However, EA + SW did not show benefits when compared to isolated protocols in nociceptive behavior.

Keywords: osteoarthritis, Pain behavior, Electroacupuncture, Swimming, Non-pharmacological approaches.
electroacupuncture (EA) are unclear. There may be a delayed cartilage degeneration by treatment with EA, in part, due to the inflammatory factors. EA in ovariectomized rats inhibited the bone loss and also shielded the articular cartilage. EA has shown analgesic effects in peripheral pain by spinal and supraspinal mechanisms, desensitizing the peripheral nociceptors, and by reducing pro-inflammatory cytokines in the spinal cord. Moreover, EA showed antinociceptive effects, remarking that they were not dependent on the stimulation period. Systematic reviews of clinical trials have suggested the unproven efficacy of EA. The contrary, there are some clinical studies that show the benefits of EA in the early stages of the inflammatory process in OA, or at least during two weeks of pain modulation.

Exercise plays a role in modulating the pain pathways. There is a protective effect that is described in low impact sports, such as walking, swimming, or cycling, with a recognized action in the delayed progression of OA. Swimming has also been shown to decrease the inflammatory markers and pain in OA patients.

Based on these scenarios, this study aimed at identifying the effects of EA, aquatic exercise, and their association, by using a model of monoarticular ankle osteoarthritis. The research evaluated the antinociceptive effects through behavioral, histological, hematological, and immunological parameter tests. Myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAGase) activities, besides the levels of TNF in the damaged tissue also were analyzed as potential biomarkers of OA severity and progression. Moreover, glutamate measurements in the CSF were assessed to correlate with nocxious stimulation. Accordingly, this study has shown that non-pharmacological therapies when used together, contributed to the anti-inflammatory effects in OA. Thus, it was investigated the effectiveness of swimming and electroacupuncture association on monoarthritis ankle CFA-induced. Currently, no similar investigation was reported in order to compare the association of the two treatments.

2. Materials and methods

2.1. Animals

Thirty-six male Wistar rats weighing between 300 ± 50 g at 10 weeks of age were maintained with food and water ad libitum, in a controlled temperature (25 °C), with a 12 h light and dark cycle. The study was approved by the Animal Use Ethics Committee of the Lutheran University of Brazil (protocol 2017/252) and the study was conducted at the same university. Besides, the study was carried out according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments).

2.2. Monoarthritis model

An ankle osteoarthritis model is well established in triggering signs and symptoms of OA. Briefly, 100 μL of CFA, inactivated M. butyricum (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA), or 100 μL of saline (control group), was injected into the intra-articular space of the left ankle. Seven days after the CFA application (sub-acute phase), the animals were randomly distributed as naïve (n = 6), saline (control) (n = 6), CFA (n = 6), EA (n = 6), SW (n = 6), and EA + SW (n = 6). The treatment protocols were in place until the chronic phase (20 days) (Fig. 1).

2.3. EA protocol

The stimulation of EA was performed at acupoints ST36 and GB30 on the left ankle. The frequency was alternated among the days between 10 Hz–250 μsec and 100 Hz–40 μsec, with intensities of 1.2 and 3 mA (these were increased every 10 min of treatment) starting with the lowest frequency. The sessions were held 5 times a week, lasting for 30 min (Fig. 1). The equipment used was the QUARK Tensiv 993 Dual® model (Quark Medical, São Paulo, Brazil) with 0.25 × 0.15 mm stainless steel needles (DONG-BANG®, South Korea, Seoul).

2.4. Swimming protocol

The protocol consisted of 10 min of swimming, three times a week. The EA + SW group received alternated EA sessions, three times swimming and twice a week EA (the frequency was alterned among the days between 10 Hz–250 μsec and 100 Hz–40 μsec - starting with the lowest frequency, with intensities of 1.2 and 3 mA - these were increased every 10 min of treatment).

2.5. Behavioral tests and assessment

Behavioral tests were performed before the CFA application (day 0), after 7 days of the induced OA (before the protocol initiation), and in the 20th day.

2.5.1. Edema assessment

Before the CFA injection, animals were held while right hind paw thickness was measured using a paquimeter.

2.5.2. Von Frey test

The assessments of the mechanical paw withdrawal threshold were performed by using the top-down paradigm. Rats were placed individually and habituated for 30 min into clear front Plexiglass boxes (9 × 7 × 11 cm) on an elevated mesh platform to allow access to the ventral surface of the hind paws. The tip of the pressure transducer of the analgesimeter was applied linearly through the holes in the mesh on the plantar surface of the hind paw at increasing pressure. Paw withdrawal caused by the stimulation was registered as a response and the corresponding force applied was recorded in grams to determine the mechanical sensitivity threshold. The average of five trials per paw was used to measure the mechanical algesia. Data were collected on both hind paws.

2.5.3. Cold stimulation

Stimulation to the left hind paw of the animal was performed according to the procedures as proposed by Choi et al. The number of beats and/or the act of licking the hind paw due to the stimulus-evoked by the evaporated acetone was collected within the first 2 min after the application. The nociceptive response rate to cold stimulation was calculated at each moment, with an average of two consecutive assessments, over a 5-min interval.

2.5.4. Paw-Flick Immersion Test

The immersed paw withdrawal test was measured according to the methodology as described by Yashpal et al. The measurement was determined by the averaging of two isolated measurements, before the protocols (baseline latency), and at different times after the treatment.

2.6. Biochemical analysis

For the biochemical collections, on the 20th day of the protocol applications, the rats were euthanized by isoflurane inhalation (1ml/1 ml, BioChimico, Itatiaia, Rio de Janeiro, Brazil), which was associated with pure oxygen.
2.6.1. Hematological and immunological parameters

Blood collection was performed through the aortic vein after the euthanasia procedure of the animals. The blood was transferred to EDTA and gel separator biochemistry tubes and then processed for blood smears. These were stained with a panoptic kit and then read under an optical microscope on 40× and 100× magnification for the cell counting.

2.6.2. Measurement of glutamate in the CSF

The CSF was obtained by a puncture in the cisterna magna. The measurement of glutamate release was performed by using a continuous fluorimetric assay as described. In brief, fluorescence emission was recorded by using excitation wavelength of 360 nm and emission of 450 nm using a fluorimeter (Synergy TM2, BioTek®). Glutamate release was measured indirectly by following the increase in the fluorescence due to the production of NADPH in the presence of glutamate dehydrogenase type II and NADP⁺.

2.7. Inflammatory modulation

Myeloperoxidase. The subcutaneous tissue of the left ankle was injected into an Eppendorf® tube containing 0.75 mL of 80 mM sodium phosphate buffer (pH 5.4) and 0.5% hexadecyltrimethylammonium (HTAB). The enzyme assay was loaded as described by Bradley et al. The reaction product was determined colorimetrically by using a microplate reader (652 nm absorption), with a molar absorption coefficient of 3.9 × 10⁴ per 3.3 × 5.5'-tetramethyl benzene salt. The values were expressed as optical densities, corrected by a gram of homogenized tissue (OD/g tissue).

2.7.1. N-acetyl-β-D-glucosaminidase (NAGase)

The samples were homogenized in Eppendorf® tubes, with volumes of sodium acetate buffer containing 0.5% hexadecyltrimethylammonium, for 15 s at 4°C. They were centrifuged at 16,000 rpm for 20 min at 4°C and the supernatant was then separated. Soon after, 25 µL of each sample was transferred to each microplate well. 25 µL NAG (2.24 mM) and 100 µL Citrate Buffer (50 mM) were next added, incubated for 60 min at 37°C, and then placed on ice. The reaction was stopped by adding 100 µL of glycine buffer (0.2 mM, pH 10.4). The enzyme activity was determined colorimetrically at 405 nm.

2.7.2. Immunohistochemical analysis of TNF

The samples of the subcutaneous tissue that were taken from the left ankle were fixed in 10% formaldehyde and embedded in paraffin. After that, the samples were xylene deparaffinized and rehydrated in a graded ethanol series. Antigen recovery was performed in a 100°C citrate buffer and the endogenous peroxidase activities were blocked by an incubation of the slides in absolute methanol containing 3% hydrogen peroxide at room temperature. The sections were sequentially preincubated with 10% rabbit serum at room temperature, in order to block possible unwanted secondary antibody reactions. The slides were then incubated with the TNF monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, followed by an incubation with secondary antibodies for 1 h at room temperature. They were treated by the EnVision™ reagent kit and then three washes with PBS were performed. The nuclei were counterstained with hematoxylin. The primary antibodies were diluted in PBS, which contained bovine albumin as a negative control.
2.7.3. Subcutaneous tissue histology

The left ankle subcutaneous tissue was fixed in 10% formalin for 3 days and decalcified in 40% ethylenediaminetetraacetic acid (EDTA) for approximately thirty days. After the conventional ethanol gradient dehydration, the samples were sectioned at 7 μm in paraffin and stained with hematoxylin and eosin (HE). The histopathological parameters evaluated were the inflammatory infiltrate density and the vascular congestion through the scores, 0 = absent, 1 = discreet, 2 = moderate, 3 = abundant.

2.8. Statistical analysis

Data was expressed as mean ± standard error (S.E.M). The data from the in vivo analyses was compared using Two-Way Analysis of Variance (ANOVA) and the Bonferroni post-hoc test. The biochemical analysis data was compared using One-Way ANOVA, followed by Dunnett’s Multiple Comparison Test. The results were considered significant when p < 0.05.

3. Results

3.1. Behavioral tests

3.1.1. The effects of EA, SW, and associated treatments in the paw edema

CFA-induced inflammation in the footpad can manifest as edema (Models of Inflammation: Zymosan, Carrageenan or Complete Freund’s Adjuvant-Induced Edema and Hypersensitivity in the Rat[39]). Thus, the study observed an increase in the paw edema after the application of CFA in all of the intervention groups (Fig. 2a). After 10 sessions of treatment, statistical differences were prominent among the SW (9.45 ± 0.19 mm) and CFA (11.86 ± 0.52 mm; p < 0.05) or EA + SW (11.83 ± 0.68 mm; p < 0.01) and EA (10.65 ± 0.76 mm; p < 0.01) groups. Data from the control naïve group was represented in Fig. 1S.

3.1.2. Effects on mechanical allodynia - Von Frey test

In order to measure the mechanical algesia the Von Frey test was performed. After 7 days of the OA-induced models, the groups CFA (10.67 ± 1.46 g), EA (13.33 ± 2.64 g), SW (20.43 ± 2.93 g), and EA + SW (18.02 ± 3.84 g) had a decrease in the threshold mechanical nociception when compared to the saline group (46.71 ± 2.16 g) (Fig. 2b). After 20 days, the EA + SW group (22.14 ± 5.69 g, p < 0.01) had the best result when compared to the EA (15.69 ± 2.02 g, p < 0.01) and SW (14.59 ± 2.76 g) when compared to other treatments or saline group (47.28 ± 7.08 g; p < 0.01).

3.1.3. Effects on thermal hyperalgesia - cold stimulation

To assess if CFA-induced nociception is reversed by non-pharmacological interventions, all tested groups showed a decreased cold latency score after the CFA application (7 days) when compared to saline group (1.00 ± 0.41 s), EA (2 ± 0.25 s), CFA (2.25 ± 0.25 s), EA + SW (2.0 ± 0.26 s), and SW (2.0 ± 0.26 s) (Fig. 2c). In the 20th day, EA achieved the best score compared with CFA group (1.75 ± 0.25 s, p < 0.01) and the EA + SW group showed the worst score (p < 0.05).

3.1.4. Effects on thermal hyperalgesia - Paw-Flick Immersion Test

To examine the effects of noxious thermal stimulation of one

Fig. 2. Effects of EA, SW, and the associated treatments in the paw edema (a); mechanical allodynia (b); cold allodynia (c) and heat hyperalgesia (d). The measurements were done after 10 intervention sessions of EA, SW, and the associated treatments. (***, **, *) indicate a significant difference compared to the saline group (p < 0.001, p < 0.01, p < 0.05). (++, +, +) indicates a significant difference compared to the CFA group (p < 0.001, p < 0.01, p < 0.05). (oo) indicates a significant difference compared to the EA group (p < 0.001). ($$) indicates a significant difference compared to the EA + SW group (p < 0.01). Each column represents the mean and SEM values that were obtained from 6 animals. Statistical analyses were performed using Two-Way ANOVA, followed by Bonferroni’s post-hoc test.
3.2. Biochemical analysis

3.2.1. Hematological and immunological parameters

Investigating alterations on hematological and immunological parameters in our study, the analysis showed that SW group presented low values of monocytes (2.5 ± 1.5%). However, the EA group decreased the neutrophil values (6.0 ± 1.0%). The EA + SW group showed higher values of leukocytes (8.7 ± 0.3%). Among all of the treatments, the EA group decreased the number of platelets (1732 ± 56.15/1000), and the saline group (1069 ± 92.5/1000) presented lower values (Table 1S).

3.2.2. Measurements of glutamate in the cerebrospinal spinal fluid

The experiment measured the levels of the glutamate in the CSF, which has been described to increase in response to inflammatory substances given peripherally.²⁹ The researchers demonstrated that which has been described to increase in response to inflammatory substances given peripherally.²⁹ The EA group decreased the number of platelets (1732 ± 56.15/1000), and the saline group (1069 ± 92.5/1000) presented lower values (Table 1S).

3.2.3. Qualitative TNF analysis in the subcutaneous tissue

The analyses of the qualitative immunohistochemical expression of the proinflammatory cytokine TNF in the subcutaneous tissue around the left foot at the end of the interventions showed that the saline (A) group did not display any staining. On the other hand, the CFA (B), EA (C) and EA + SW (E) groups showed in some regions near the periphery/surface, with staining for TNF. The SW group (D) did not present TNF staining (Fig. 4).

3.3. Anti-inflammatory effects

3.3.1. Neutrophilic infiltration – MPO

The group CFA showed an increase in the 20th day (3.22 ± 0.22 DO/g tissue). Nevertheless, the EA + SW (1.73 ± 0.21 DO/g tissue) group showed a decrease in the inflammatory marker when compared to the saline group (1.65 ± 0.69 DO/g tissue) level (Fig. 3b). It was noteworthy that the highest value in both treatments protocols was the isolated SW (3.10 ± 0.37 DO/g tissue), showing little efficacy in the inflammatory condition that was used in this study.

3.3.2. Macrophage infiltration – NAGase

The NAGase measurement showed that the EA group (1.28 ± 0.16) presented the lowest DO/gram tissue values, with a smaller amount of the inflammatory infiltrates by the macrophages (Fig. 3c).

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3.3.4. Subcutaneous tissue histology

The saline group did not present inflammatory infiltrates and/or vascular congestion (score 0). In contrast, CFA group showed score 2 for infiltration and 3 for congestion. EA + SW showed the worse score in infiltrate (3) and congestion (3). However, EA isolated improve these parameters (infiltrate 1 and congestion 1). But, the SW group reversed the inflammatory process at the end of the treatment (score 0 for both parameters) (Fig. 5).

![Fig. 3. Glutamate levels in CSF (a); Myeloperoxidase (b); NAGase values (c) after 10 intervention sessions of electroacupuncture (EA), swimming (SW) or the association. (***, **, *) indicates a significant difference compared to the saline group (p < 0.001, p < 0.01, p < 0.05). (oo, o) indicates a significant difference compared to the EA group (p < 0.001, p < 0.05). ($$, $) indicates a significant difference compared to the EA + SW group (p < 0.001, p < 0.05). (&&, &&) indicates a significant difference compared to the SW group (p < 0.001). Each column represents the mean ± standard error of 6 animals. One-Way ANOVA was used, followed by Dunnett’s Multiple Comparison Test.](image-url)
4. Discussion

According to Xiang et al., 100 Hz of EA stimulation in both short-term (3 days) and long-term (14 days) effectively produced analgesic effects on the CFA-induced inflammatory model, which were not dependent on the period of stimulation. Hydrotherapy is commonly used during the rehabilitation process, although there is no standard physical exercise protocol for OA treatment. 40 Thus, the present study decided to use low intensity EA even when associated therapy was applied. Notably, Mioto et al. 40 didn’t find any difference in the improvement of bone features of rats that received Zymosan into the knee and were submitted to different intensity of aquatic exercise for 5 weeks. On the other hand, a mild intensity swimming protocol in rats, three times per week, from 5 to 15 min for 4 weeks, revealed no aggravation of arthritis, besides no decrease on the response to paw pressure were observed in those animals. 41

OA is related to chronic pain, leading to disabilities of movement. 41, 42 The inflammatory process involved vascular permeability, contributing to the migration of the phagocytic cells, such as the neutrophils (acute phase) and the monocytes (chronic phase). 43 Biomarkers of OA could help to diagnose and to assess disease severity, predicting its future progression. 44 Moreover, the inflammatory process is enhanced by the inflammatory cytokine TNF. 42 Related to this, the study’s data showed that the SW protocol reduced edema and also the infiltrates in the histological analysis, besides reducing TNF in the injured subcutaneous tissue. The immunological activities in the SW group were verified by the peripheral blood biochemical parameters, showing the lowest monocyte levels when compared to all tested groups. The increased NAGase and MPO levels in the subcutaneous tissue of the rats in this group showed that the macrophage activity was increased. Thus, this may suggest that an aquatic exercise could slow the chronicity of OA.

Platelets are inflammatory cells that contain and release metalloproteinases, which degrade cartilage and subchondral bone. 43 They may also contribute to joint degeneration in OA, favoring the accumulation of metalloproteinases in the synovial fluid. 43 According to Büyükavcı et al., 45 those patients with more severe OA have an increased platelet blood distribution, suggesting a possible new marker for the progression of this disease. The present results demonstrated that all of the OA-induced groups showed increased platelet levels in the peripheral blood. Here, the findings demonstrate that EA reduced these cells. It is known that EA, with frequencies of 2–10 Hz, is more effective in inflammatory models. 15 Thus, in this experiment, the EA group was interspersed with the applications of high (100 Hz, 2x/week) and low frequencies (10 Hz, 3x/week) and showed better results.

Related to the nociceptive behavior, acupuncture stimulation of GB30 was associated to an increase in the heat thermal threshold in the hind paw of the rats with CFA-induced monoarthritis. 46 The reversal of mechanical allodynia and thermal hyperalgesia has been proven significantly through 15 and 100 Hz stimulation, 47, 48 mainly in the relief of inflammatory pain that was CFA-induced. 49 Wang et al. 50 started treatments with EA, the day after the intra-plantar CFA application, and they managed to improve the mechanical and thermal nociception threshold. As can be observed in the current study’s results, the EA group attenuated the threshold for heat tolerance, even starting on the seventh day.

The analgesic effects of ST36 acupuncture EA stimulation may have been mediated by the N-methyl-D-aspartate acid receptors (NMDAR) on the spinal cord. 51 Thus, the elevated levels of glutamate in the CSF are likely to be correlated with high levels of the inflammatory mediators. This correlation is also related to increased edema and sensitization to thermal hyperalgesia in experimental arthritis models. 52, 53 In this study, the glutamate levels in the CSF were decreased by the association of EA + SW, and this fact is in line with the improvement in the nociceptive behavior and inflammatory parameters evaluated like macrophages and neutrophils in the injured tissue. Likewise, Eftekharasadat et al. 53, 54 found that visual analog scale and pressure pain threshold were significantly improved in both groups with myofascial pain syndrome treated either with the combination of exercise and acupuncture or acupuncture alone throughout the 10 evaluated sessions.

There were slight weight losses in most of the groups throughout the study, but also with weight recoveries during the study time (unpublished data). In addition, the SW group was able to reduce paw edema when compared to the other groups. It is noteworthy that after the application of CFA in the intra-articular space of the ankle of the animals, all of the intervention groups presented severe edema and excessive motor losses. These characteristics refer to the acute inflammatory phase, requiring more days to commence the interventions in a supposed subacute and later chronic phase. This would probably influence better on the therapeutic responses of the study, disagreeing with the deadlines in the literature.

Therefore, the present findings reinforce the immunological
factor involved in the EA and SW treatments. Besides, it is the first time that a study investigated the effects of the association in the treatment of chronic pain OA-induced. Furthermore, the pain decreasing is correlated with the glutamate levels of the CSF and inflammatory biomarkers. In summary, the current work brings new perspectives to the association of non-pharmacological approaches in the management of pain triggered by OA-related diseases and its disabilities. The association therapy when compared to the monotherapy showed improvement on the inflammatory parameters tested, which could be associated with a decrease of the chronification of the osteoarthritis. However, EA + SW did not show significant beneficial effects as compared to

Fig. 5. Histological section of the subcutaneous left ankle tissue of the animals after 10 sessions of interventions: Saline (A1,2,3), CFA (B1,2,3), EA (C1,2,3), SW (D1,2,3), and EA + SW (E1,2,3). The black arrows indicate the infiltrate, and the empty arrows indicate congestion. 1 – 4x, 2 – 10x, and 3 – 40x magnification by the HE coloration.
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