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RESEARCH ARTICLE

Optimization of Pulsed Electromagnetic Field Parameters Attenuates Mechanical Hyperalgesia and Selectively Modulates Inflammatory and Oxidative Markers in a CFA-Induced Inflammatory Pain Mouse Model

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ABSTRACT

Pain poses a global challenge involving sensory, cognitive, and emotional mechanisms, necessitating effective treatments. In this study, we evaluate the effects of pulsed electromagnetic field (PEMF), a noninvasive low-frequency signal with potential anti-inflammatory effects, in male Swiss mice after a complete Freund's adjuvant (CFA)-induced inflammation. The aim was to identify the optimal PEMF pulse frequency (5, 50, or 75 Hz) and treatment duration (10, 20, or 30 min) for analgesic effects, assessed by mechanical hyperalgesia, and to investigate peripheral (paw) and central (spinal cord, hippocampus, and prefrontal cortex) inflammatory and oxidative responses following CFA administration. Emotion-related behaviors were also assessed; however, CFA did not induce detectable anxiety- or depression-like alterations under the experimental conditions used. The best antihyperalgesic effect was at 75 Hz, particularly with 20 and 30 min of treatment, without affecting paw edema. A 20-min PEMF treatment at 75 Hz reduced TNF levels in the paw and increased SOD enzyme activity in the paw and spinal cord, indicating anti-inflammatory and antioxidant effects both peripherally and centrally. These results confirm PEMF's anti-hyperalgesic effect, identifying 75 Hz and 20 min as optimal parameters. Understanding PEMF mechanisms could lead to new therapeutic targets, improving pain management in patients with inflammatory conditions, and altering the underlying pathological process.

1 | Introduction

Pain is a complex experience that involves sensory, cognitive, and emotional mechanisms and has become a global problem (Gomes et al. 2020). Around 20% of the worldwide population presents clinical signs of chronic pain, which is often accompanied by anxiety, depression, irritability, and attention deficits—affecting their quality of life and productivity (Treede et al. 2019). Pain originates from harmful stimuli that compromise the integrity of biological tissues, resulting in nociceptive (often accompanied by inflammation), neuropathic, and nociplastic syndromes—the most common types of acute and chronic pain based on etiology and clinical presentation (Orr et al. 2017; Woolf 2010). Acute pain can occur within a

Summary

- Optimal pulsed electromagnetic field parameters identified: This study identifies a 75 Hz frequency and 20 min as the optimal parameters for pulsed electromagnetic field (PEMF) therapy to achieve significant antihyperalgesic effects in a chronic inflammatory pain model.
- Anti-inflammatory and antioxidant effects: PEMF treatment at 75 Hz for 20 min demonstrated anti-inflammatory and antioxidant effects by reducing TNF levels and increasing SOD enzyme activity in both peripheral (paw) and central (spinal cord) regions.
- Potential therapeutic target: Understanding the mechanisms of PEMF therapy opens new avenues for therapeutic targets, offering improved pain management and altering pathological processes in patients with inflammatory conditions.

few minutes up to 3 months after an injury, while chronic pain can persist for a more extended period (Raja et al. 2020), leading to neuroplasticity throughout all nociceptive pathways—from the periphery to the central nervous system, affecting areas such as the spinal cord, hippocampus, and prefrontal cortex. These modifications in the prefrontal cortex can influence cognitive functions (such as memory and expectation), emotional responses (including anxiety and depression), and behavioral responses to the environment (such as reinforcement and conditioning) (Guida et al. 2015; Hung et al. 2014).

Chronic inflammatory pain was reproduced in this research by administering complete Freund's adjuvant (CFA), an emulsified *Mycobacterium tuberculosis* suspended in paraffin oil) in mice, a widely recognized model for studying chronic inflammatory pain. CFA stimulates the release of inflammatory mediators such as cytokines, reactive oxygen species (ROS), and nitrogen, inducing neutrophil infiltration at the injury site and causing damage to peripheral tissue, a characteristic of this model (Martins et al. 2016). Inflammatory cytokines (such as interleukin-1 beta [IL-1b], IL-6, and tumor necrosis factor-alpha [TNF-a]) and ROS increase the sensitivity to painful stimuli by sensitizing peripheral afferent nociceptive fibers and activating the cascade of central sensitization events (W. Zhang et al. 2021), producing behavioral, emotional, and cognitive changes (Altarifi et al. 2019). Inflammatory cytokines and ROS modulate the complex pathways associated with acute and chronic pain, and their inhibition has been emerging as a potential therapeutic approach to pain management (Altarifi et al. 2019; Martins et al. 2016).

The therapeutic integration of PEMF into various medical conditions has increased in recent years due to the physiological effects that are activated in different systems (Mayer et al. 2024). Previous studies demonstrated that pulsed electromagnetic field (PEMF) therapy exhibits cellular effects, including modulation of pro-inflammatory cytokines and releasing anti-inflammatory cytokines (Vincenzi et al. 2013; Mert et al. 2017), increased adenosine receptor expression and promotion of neuroprotective action (Varani et al. 2012), improved blood circulation and protection against tissue damage after strokes (Grant et al. 1994), increased vascular perfusion and tissue oxygenation (Bragin et al. 2015), regeneration of peripheral nerve (Hei et al. 2016), prevention of cartilaginous destruction in osteoarthritis (OA) (Fini et al. 2005), and improved cognition and memory in models of Alzheimer's Disease (Dragicevic et al. 2011). In addition, PEMF has shown promising therapeutic potential across a wide spectrum of conditions, including musculoskeletal and bone-related disorders such as sarcopenia and osteoporosis (Venugobal et al. 2023; Lang et al. 2022; J. Zhou et al. 2022). Benefits have also been reported in neurodegenerative diseases such as Alzheimer's disease (Merighi et al. 2024) and multiple sclerosis-related pain (Hochsprung et al. 2021), neuropathic pain associated with diabetes (Graak et al. 2009), patellofemoral pain syndrome (Servodio Iammarrone et al. 2016), and oncology settings (Pantelis et al. 2024; Vadalà et al. 2016). Beyond these clinical applications, indirect vagus nerve stimulation by PEMF has also been shown to modulate cellular mechanisms with therapeutic potential (Sprunks et al. 2024).

PEMF therapy consists of applying a low-frequency signal, which can induce cell membrane changes and activate several intracellular pathways (Andrade et al. 2016). PEMF treatment is noninvasive, has rapid effects on the cell, and is easy to handle and apply (T. Wang et al. 2019). Although PEMF has been used in various pathological conditions, relevant questions about pulse frequency and application time directly influencing the final dose administered are still open. It varies according to each professional and does not obtain parameterization, thus making data collection and conducting clinical studies difficult.

An important gap in this field concerns the rationale for frequency selection. Different frequencies can trigger distinct cellular responses, including modulation of signaling pathways involved in inflammation and oxidative stress (Ross et al. 2019; Gaynor et al. 2018; Mansourian and Shanei 2021). Evidence shows that PEMF may regulate homeostatic mechanisms in pro- and anti-inflammatory responses, promote tissue regeneration, and provide analgesic effects depending on the chosen parameters (Ross et al. 2019; Ross and Harrison 2013; T. Wang et al. 2019; Chan et al. 2019). Clinical studies and meta-analyses in osteoarthritis further highlight that specific frequencies are associated with improved pain outcomes (Viganò et al. 2021; Tong et al. 2022; Wu et al. 2018). Despite these findings, there is no consensus on optimal frequencies for chronic inflammatory pain. Thus, investigating the effects of different PEMF frequencies represents a key objective of the present study.

Considering that scientific studies demonstrate the applicability of PEMF in cases of joint changes, fractures, and orthopedic and neurological diseases; its biological effects with anti-inflammatory, analgesic, tissue-healing potential, maintenance of joint integrity, and potential antioxidant effect; and its painless, low-cost, easy-to-apply therapeutic application, leading to a potential adjuvant treatment in human conditions involving chronic inflammatory pain. This study aimed to identify the best pulse frequency (5, 50, or 75 Hz) and the best treatment time (10, 20, or 30 min) with PEMF in the analgesic (mechanical hyperalgesia), anti-inflammatory (edema), and emotional aspects of chronic inflammatory pain (anxiety and depression-like behaviors). In addition, this study investigates the effect of PEMF in the modulation of peripheral (paw) and central (spinal cord, hippocampus, and prefrontal cortex)

inflammatory and oxidative responses in mice after CFA-induced inflammatory arthritis.

This study enables the analysis of some physical parameters of PEMF and, due to its originality, brings relevance to the scientific community by testing treatment with PEMF on inflammatory lesions induced by CFA in mice.

2 | Materials and Methods

2.1 | Animals

Male Swiss mice (25–35 g, $n = 8$ per group) were obtained from the animal facility of the Federal University of Santa Catarina (Florianopolis, Brazil). The animals were housed in the Experimental Neuroscience Laboratory (LaNEx) of the University of Southern Santa Catarina, acclimatized at 22°C ± 2°C, under a 12-h light/dark cycle, with access to food and water *ad libitum*. The sample size was carried out through statistical calculations based on the equation determining the number of animals in a group in a sample without replacement (Vierck and Yezierski 2015). Using the following formula: $n = (((z\ \text{alpha} + z\ \text{beta}) \times s)/\text{sigma})^2$. The alpha value was 0.05, and the *z*-alpha value was 1.96. The beta value was determined to be 0.10, and the *z*-beta value was 1.28. Based on previous experimental data, a minimum of 40% was established as the difference value between the group means (sigma), and the standard deviation (SD) value was established at 35% of the mean value (s). With the values of the formula applied, we have $n = (((1.96 + 1.28) \times 35)/40)^2 = 8$. In this way, each group was made up of 8 animals. The literature indicates that in this animal model of inflammation induction by CFA, groups can contain between 6 and 8 animals due to the minimal interindividual variations during the progression of the disease (Bolon et al. 2011). All animals were randomized. For this, the allocation sequence was generated by drawing, and a blinded subject carried out the random distribution of the animals between the experimental groups. All experimental protocols were approved by the Ethics Committee on Animal Use (CEUA-UNISUL, protocol n° 23.006.4.01.IV) following the ARRIVE guideline (Animals in Research: Reporting In Vivo Experiments) (Percie du Sert et al. 2020).

2.2 | Experimental Design

This study was conducted in three sets of experiments (Figure 1), as described below:

2.2.1 | *Experiment 1*

The animals underwent intraplantar (i.pl.) injection of CFA or saline and were treated with a placebo or PEMF (5, 50, or 75 Hz) to parameterize the frequency, with a fixed treatment time of 30 min and a fixed intensity of 1.5 mT. The following experimental groups ($n = 8$) were used: (1) saline/placebo, (2) saline/PEMF, (3) CFA/placebo, (4) CFA/PEMF 5 Hz, (5) CFA/PEMF 50 Hz, and (6) CFA/PEMF 75 Hz. The animals were evaluated for 7 days with the von Frey test and measurement of paw edema. The group that had the best response to treatment was chosen to continue the experiments.

2.2.2 | *Experiment 2*

The animals underwent i.pl. injection of CFA or saline, and were treated with a placebo or PEMF (frequency defined in the

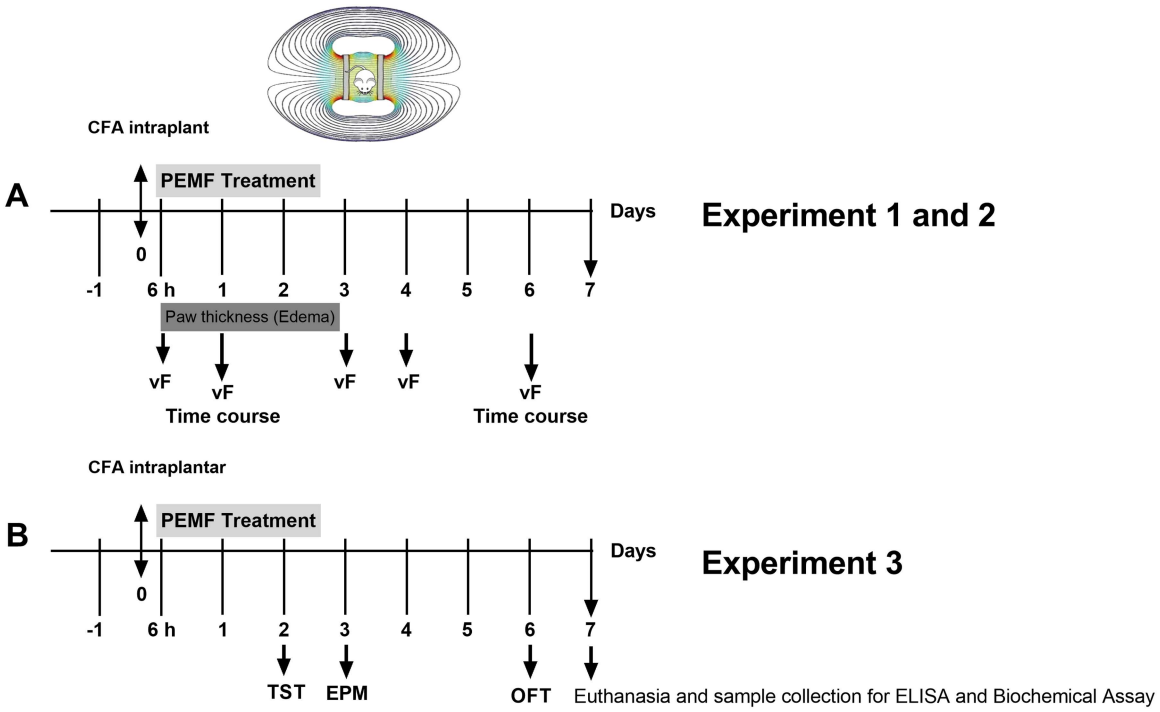

FIGURE 1 | Schematic representation of the experimental design. Timeline of experiments to evaluate the effects of PEMF on mechanical hyperalgesia and edema after CFA-induced inflammation (A). Timeline of behavioral tasks performed to evaluate the effects of PEMF on depression and anxiety-like behavior, and locomotor activity after CFA-induced inflammation (B). CFA, complete Freund's adjuvant; EPM, elevated plus maze; PEMF, pulse electromagnetic field; OFT, open field test; TST, tail suspension test; vF, von Frey test.

previous experiment) to parameterize the best treatment time. The following experimental groups ($n = 8$) were used: (1) saline/placebo, (2) saline/PEMF 75 Hz for 30 min, (3) CFA/placebo, (4) CFA/PEMF 75 Hz for 10 min, (5) CFA/PEMF 75 Hz for 20 min, and (6) CFA/PEMF 75 Hz for 30 min. The animals were evaluated for 7 days with the von Frey test and measurement of paw edema, and the group that had the best response to treatment was chosen to continue the experiments.

2.2.3 | *Experiment 3*

The animals were subjected to i.pl. injection of CFA or saline, and were treated with a placebo or PEMF (frequency and time defined in previous experiments). The following experimental groups ($n = 8$) were used: (1) saline/placebo, (2) saline/PEMF 75 Hz for 20 min, (3) CFA/placebo, and (4) CFA/PEMF 75 Hz for 20 min. The animals were evaluated with the tail suspension test (TST) on Day 2, the elevated plus maze test on Day 3, and the open field test on Day 6. On the 7th day, 1 h after the treatment with PEMF, the animals were euthanized, and samples were collected for biochemical evaluations.

2.3 | Experimental Protocols

2.3.1 | *PEMF*

The PEMF device consists of a pair of Helmholtz coils with an approximate wire volume of 41.82 cm^3, with a total of 800 spirals of 27 AWG enameled copper wire, connected to a 220 Volt electronic device, which generates a wave square (VH Equipamentos, Magnum, Brazil). The frequency emitted by the equipment can vary from 1 Hz to 100 Hz, with a duty cycle that ranges between 10% and 90%. The two Helmholtz coils were placed vertically on the ground, connected simultaneously to the electronic device, and in parallel. The alternating current passing through the coils produces an electromagnetic field ranging from 0 to 200 Gauss. The PEMF was placed in the treatment room with a controlled temperature of (22°C ± 2°C). PEMF treatment was carried out on the animal's entire body. The animals were placed individually in acrylic chambers without a bottom and covered with a lid (9 cm × 7 cm × 11 cm) on a table made of diamagnetic material (wood). The Helmholtz coils were placed on the side faces of the acrylic chamber box, providing a uniform magnetic field throughout the treatment.

The equipment emits the pulsed magnetic field at a fixed average intensity of 1.5 mT, with pulse frequency and exposure duration programmed according to the experimental protocol and a fixed duty cycle of 12.5%. The pulse frequency (Hz) and time (minutes) parameters were programmed into the equipment during Experiments 1 and 2, respectively, to determine the best protocol to be adopted in the other experimental sets. The devices were properly calibrated in a specialized laboratory (Return Projetos Ltda., São Paulo, Brazil). PEMF treatment was carried out every day until the 7th day after CFA application in all experiments.

The choice of frequencies (5, 50, and 75 Hz) was based on previous studies indicating that different frequency ranges can modulate distinct biological processes, such as inflammatory and oxidative responses, tissue regeneration, and analgesia. Low (5 Hz), intermediate (50 Hz), and higher (75 Hz) frequencies were therefore selected to allow a systematic evaluation of their relative efficacy in the CFA-induced inflammatory pain model (Ross et al. 2019; Gaynor et al. 2018; Mansourian and Shanei 2021; Chan et al. 2019; Ross and Harrison 2013; T. Wang et al. 2019; Viganò et al. 2021; Tong et al. 2022; Wu et al. 2018).

2.3.2 | *CFA-Induced Pain and Inflammation Model*

A single i.pl. injection (20 μL) was performed in the right hind paw with CFA (1 mg/mL heat-killed *M. tuberculosis*, F5881, Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline solution (70%) (Omura et al. 2022). Animals in the saline groups received an injection of 0.9% NaCl (20 μL, i.pl.) in the right hind paw.

2.3.3 | *Assessment of Mechanical Hyperalgesia*

Mechanical hyperalgesia assessments were carried out randomly between the animals in the groups. A 0.6 g von Frey monofilament (Stoelting, IL, USA) was used. The animals were placed individually in acrylic chambers without a bottom and covered with a lid (9 cm × 7 cm × 11 cm) on a platform (70 cm × 40 cm) containing a 6-mm wire mesh, which made it possible to apply the monofilament that was used for 5 s 10 times, perpendicular to the plantar surface of the animal's right paw. The curvature of the filament indicated that the pressure was sufficient, and the lifting of the paw corresponds to a positive nociceptive response. Paw withdrawal was counted and recorded as a response percentage (Omura et al. 2022). A baseline assessment was carried out (before induction), 6 h after CFA administration, after the first treatment, daily until the 7th day after CFA, and post-treatments (PEMF or placebo).

2.3.4 | *Measurement of Paw Edema*

Paw edema was measured using a universal digital micrometer (Salm et al. 2023). The thickness (μm) of the back of the plantar part of the right paw of each animal (injected with CFA or saline) was determined pre- and post-injection of CFA and after treatments with PEMF. The following evaluations were performed: baseline (before induction), 6 h after CFA administration, 0.5 h after the first treatment, and daily until the edema disappeared or until the 7th day after CFA, post-treatments (PEMF or placebo).

2.3.5 | *TST*

The TST evaluates the immobility time of mice during the test. The TST consists of a matte white acrylic box-type apparatus measuring W 20 cm × L 40 cm × H 60 cm, with one of the walls open for video recording. The center of the apparatus was illuminated with approximately 250 lux. The mice were suspended by their tails 60 cm from the ground (Ueno et al. 2022), maintaining 150 mm from any object, and acoustically and visually isolated them (Steru et al. 1985). Suspension occurs by capturing the tail with adhesive tape, placed approximately 1 cm from the tip of the tail (Maciel et al. 2013), and observing the animal's behavior recorded on video for 6 min. The number of episodes that presented immobility and the total duration of immobility were observed. Time was measured in seconds. It

was considered immobile when the animal remained for a time greater than or equal to 1 s without escaping movement (Ueno et al. 2022). The TST was performed on day two after CFA.

2.3.6 | *Elevated Plus Maze Test*

The use of an acrylic apparatus, which consists of two open arms (L 6 cm × L 35 cm without walls) and two closed arms (W 6 cm × L 35 cm flanked by 17 cm dark walls), which extend a shared central area (6 cm × 6 cm), 70 cm above the floor. The animals were transferred to the experiment room 30 min earlier for acclimatization. For each test, the animal was placed in the central area of the cross, with its head toward the closed arm (Komada et al. 2008), and was allowed to freely explore each apparatus space for 5 min while being recorded with a camera fixed above the maze for later evaluation using ANYmaze software (Stoelting, USA). The number of entries in each arm and the total time spent in each arm were calculated as a percentage (Komada et al. 2008). The elevated plus maze test was performed on day three after CFA.

2.3.7 | *Open Field Test*

The open field test evaluated the animals' locomotor activity and anxiety-like behavior. The apparatus comprises a square, matte light gray wooden box with dimensions W 40 cm × L 40 cm × H 40 cm (Kraeuter et al. 2019). The animals were previously acclimatized for 1 h in the testing room and then positioned individually in the center of the apparatus (Kraeuter et al. 2019) under the dim lighting of approximately 25 lux (Burek et al. 2021), allowing exploration of the environment for 5 min. The animal's exploratory behavior in the center and edges of the box was filmed and subsequently evaluated, analyzing the total distance covered (in meters) using the ANYmaze software (Stoelting, USA). The open field test was performed on the 6th day after CFA.

2.3.8 | *Colorimetric and Immunological Biochemical Assays*

The biochemical tests were carried out 7 days after the administration of CFA or Saline and around 1 h after the last treatment with PEMF or placebo. The animals were anesthetized (1%–2% isoflurane to 100% oxygen) for euthanasia by decapitation. Tissues were removed from the right hind paw (skin and muscle), spinal cord (lumbar segment), prefrontal cortex, and hippocampus (right and left). Immediately after dissection, the samples were frozen in liquid nitrogen and stored at −80°C until further analysis.

2.3.8.1 | *Enzyme-Linked Immunosorbent Assay (ELISA)*

Homogenization of skin and muscle paw, spinal cord, prefrontal cortex, and hippocampus samples was performed with the Ultra-Turrax Homogenizer (T-18, IKA Works, Wilmington, NC, USA) in phosphate-buffered saline containing Tween 20 (0.05%), PMSF (0.1 mM), EDTA (10 mM), aprotinin (2 ng/mL), and benzethonium chloride (0.1 mM) (Sigma-Aldrich, St. Louis, MO, USA). The samples were then centrifuged at 3000× g for 15 min (4°C), and the supernatant was collected and used to evaluate the levels of IL-6 and TNF using the sandwich ELISA method (Omura et al. 2022). Levels were analyzed using DuoSet ELISA kits (R&D Systems, Minneapolis, MN) for mice according to the manufacturer's recommendations. The total protein content was measured in the supernatant using the Bradford method (Bradford 1976). Absorbance, after the assay, was measured using a plate reader at 450 and 545 nm, and the values were interpolated into a standard curve (for TNF, inter-assay CV 5.67% and intra-assay CV 9.8%; for IL-6, inter-assay CV 7.6% and intra-assay CV 14.5%) and expressed as pg of cytokine/mg of protein.

2.3.8.2 | *Biochemical Assay*

Oxidative damage to proteins was assessed by the formation of carbonyl groups. Initially, the proteins were precipitated with 20% trichloroacetic acid and dissolved in dinitrophenylhydrazine. Absorbance was read at 370 nm, with results expressed in nmol/mg of protein (Levine et al. 1990).

Lipid damage was quantified by determining thiobarbituric acid reactive substance levels. The samples were precipitated with 10% trichloroacetic acid, then 0.67% thiobarbituric acid was added. Absorbance reading was taken at 535 nm using 1,1,3,3-tetramethoxypropane as an external standard. The results were expressed in malondialdehyde (MDA) equivalents (nmol/mg of protein) (Draper and Hadley 1990).

Neutrophil infiltrate was measured by determining myeloperoxidase (MPO) activity (Young et al. 1989). Samples were homogenized (50 mg/mL) in 0.5% hexadecyltrimethylammonium bromide and centrifuged at 15,000× g for 40 min. The suspension was then sonicated three times for 30 s. An aliquot of the supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 1 mM H_2O_2. Activity was measured by a spectrophotometer as the change in absorbance at 650 nm at 37°C. Data were expressed as mU per mg of protein.

The concentration of nitrite and nitrate was determined by the Griess reaction (Green et al. 1982). Samples were sonicated in phosphate buffer (pH 7.0), and the N/N concentration was assessed spectrophotometrically in the resulting suspension using Griess reagents (1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride in double-distilled H_2O) and vanadium (III) chloride. The standard curve was run simultaneously with each set of samples, and the optical density at 550 nm was measured using a plate spectrophotometer. Data were expressed as nmol/mg of protein.

Catalase (CAT) activperoxide dismutity was determined by decreasing absorbance at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and the supernatants containing 0.1–0.3 mg protein per mL. Specific activity was expressed as mU per milligram of protein (Aebi 1984). The results were expressed in U/mg of protein.

Superoxide dismutase (SOD) activity was determined by inhibition of adrenaline autoxidation. The samples were homogenized in glycine buffer (pH 10.2) and centrifuged at 3000 rpm for 10 min at room temperature. The blank reagent was prepared with catalase in purified water and glycine buffer at 32°C, followed by the addition of adrenaline. The blank reagent reading was taken every 10 s for 180 s for calibration. The samples were prepared with catalase, sample, and glycine buffer at 32°C and read every 10 s for 180 s in a spectrophotometer at 480 nm. SOD activity was expressed in U of SOD/mg of protein. (Bannister and Calabrese 1987).

Total protein content was measured in the supernatant using the Bradford method (Bradford 1976), and all analyses were normalized by tissue protein concentration.

2.3.9 | *Statistical Analysis*

The results were analyzed using GraphPad Prism version 8.0 (La Jolla, CA, USA). Normality of the data was first assessed using the Shapiro–Wilk test. As the data followed a parametric distribution, results were expressed as mean ± SD. For Experiments 1 and 2, data were analyzed using two-way repeated measures ANOVA, with time as the repeated measure and the between-subject factors being induction of the CFA model (saline vs. CFA) and treatment (placebo vs. PEMF at different frequencies or durations). Interactions between these factors were also evaluated. For behavioral tests in Experiment 3 (TST, elevated plus maze, and open field test), as well for colorimetric and immunological biochemical assays, data were analyzed using two-way ANOVA. When significant main effects or interactions were detected, Tukey's multiple comparisons post hoc test was applied. A p value < 0.05 was considered statistically significant in all analyses. All analyses were performed with biological replicates ($n = 8$ animals per group), and the study was conducted in three independent experimental sets to ensure data stability and reproducibility.

3 | Results

3.1 | Effects of Treatment With 30 min of Different PEMF Frequencies on Mechanical Hyperalgesia and Paw Edema After CFA-Induced Inflammation

The first set of experiments (Figure 2) evaluated the effect of different frequencies of PEMF 5, 50, and 75 Hz, with a treatment time of 30 min. The administration of CFA into the paw resulted in accentuated hyperalgesia, as observed in the CFA/Placebo group through increased response to mechanical

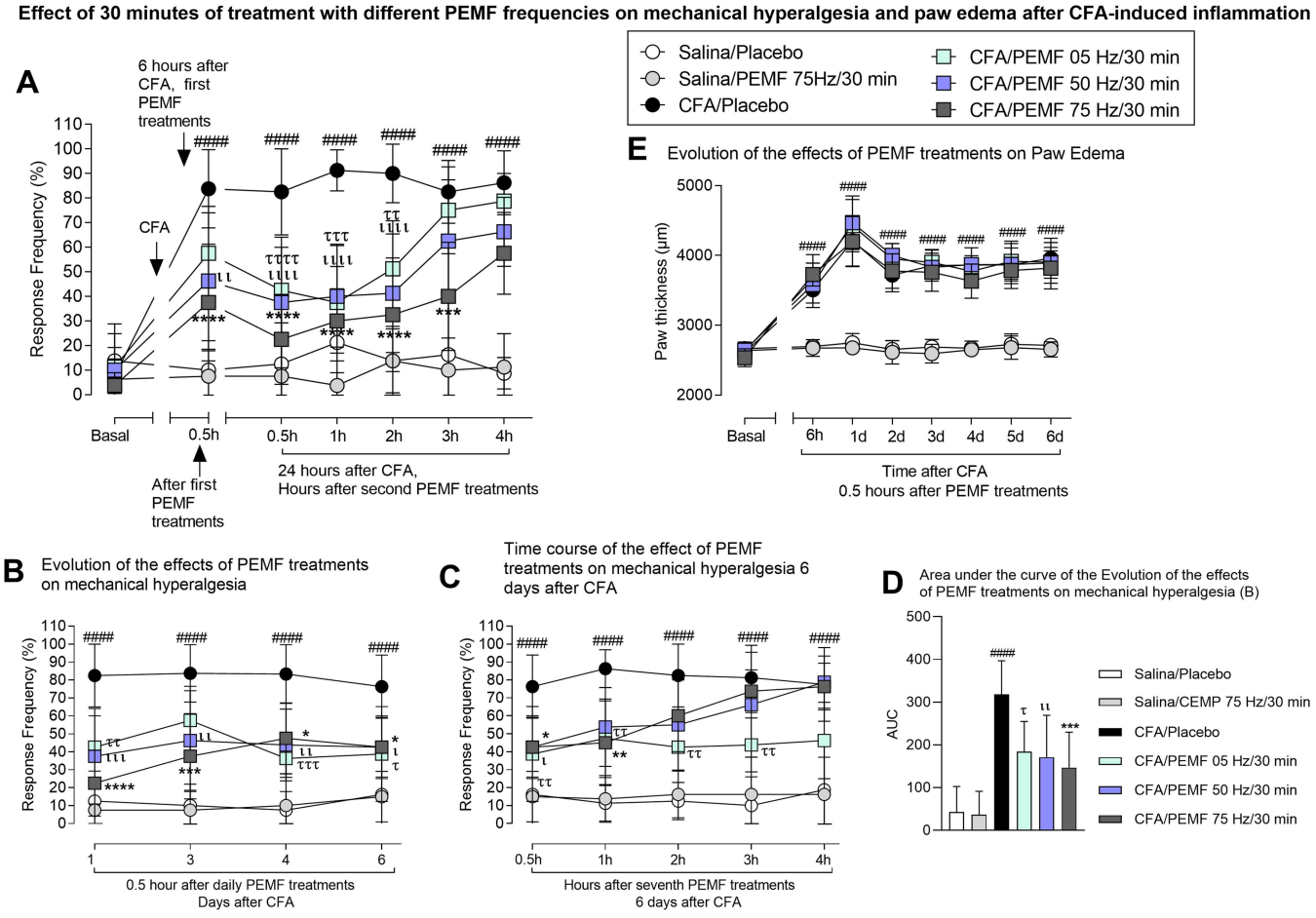

FIGURE 2 | Effects of treatment with 30 min of PEMF 5, 50, and 75 Hz on mechanical hyperalgesia and paw edema after CFA-induced inflammation in mice. Response frequency to mechanical stimulus before the procedure (A left), 6 h (A), and 24 h (A) after CFA administration, and after the first and the second treatment with PEMF (A right). Effects of treatment with 30 min of PEMF 5, 50, and 75 Hz on mechanical hyperalgesia on days 1, 3, 4, and 6 (B) after CFA administration. Time course of the impact of treatment with 30 min of PEMF 5, 50, and 75 Hz on mechanical hyperalgesia 6 days after CFA administration (C). The area under the curve of the effects of treatment with 30 min of PEMF 5, 50, and 75 Hz on mechanical hyperalgesia (D). Effects of treatment with 30 min of PEMF 5, 50, and 75 Hz on paw edema up to six days after CFA administration (E). The values represent the mean ± SD ($n = 8$ animals/group). Two-way ANOVA with repeated measures followed by Tukey's post hoc test. $^{\#\#\#\#}p < 0.0001$, when compared to the Saline/Placebo group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, $_{\upsilon}p < 0.05$, $_{\upsilon}p < 0.01$, $_{\upsilon\upsilon}p < 0.001$, and $_{\upsilon\upsilon}p < 0.0001$, $^{\square}p < 0.05$, $^{\square\square}p < 0.01$, $^{\square\square\square}p < 0.001$, and $^{\square\square\square\square}p < 0.0001$ compared to the CFA/Placebo group. CFA, complete Freund's adjuvant; PEMF, pulse electromagnetic field.

stimulus in the von Frey test. The heightened response frequency was observed from 6 h (Figure 2A) up to 6 days (Figure 2B,C) after CFA administration in the CFA/Placebo group compared to the control group (Saline/Placebo; $p < 0.0001$, Figure 2).

Our research underscored the immediate antihyperalgesic effect of a 30-min PEMF 75 Hz treatment, which was evident half an hour after the first application. Following this initial treatment, PEMF at both 50 and 75 Hz significantly reduced mechanical hyperalgesia induced by CFA administration compared to the CFA/Placebo group ($p < 0.05$, Figure 2A). After the second application, the treatment with 30 min of PEMF 5 Hz and 50 Hz significantly reduced CFA-induced mechanical hyperalgesia from 0.5 h up to 2 h compared to the CFA/Placebo group ($p < 0.05$, Figure 2A); the effect of treatment with 30 min of PEMF 75 Hz lasted from 0.5 h up to 3 h ($p < 0.05$, Figure 2A). The impact of different PEMF frequencies in reducing mechanical hyperalgesia remained consistent up to 6 days after CFA administration, as observed in the evolution of the effects of treatment on Days 1, 3, 4, and 6 (Figure 2B) and in the area under the curve on Day 6 (Figure 2D).

The treatment with 30 min of PEMF 5 Hz reduced mechanical hyperalgesia half an hour after treatment, as evaluated on Days 1, 4, and 6. Similarly, 30 min of PEMF 50 and 75 Hz were effective in reducing mechanical hyperalgesia on Days 1, 3, 4, and 6 compared to the CFA/Placebo group ($p < 0.05$, Figure 2B). A time course of mechanical hyperalgesia performed 6 days after CFA administration further confirmed the effectiveness of 30 min of PEMF in reducing the mechanical hyperalgesia. Treatment of PEMF at a frequency of 5 Hz was effective from 0.5 h up to 3 h after treatment, while treatment with PEMF 50 Hz was effective only during the first half an hour. Treatment with PEMF 75 Hz was effective up to 1 h after treatment compared to the CFA/Placebo group ($p < 0.05$, Figure 2C).

Our findings revealed persistent paw edema induced by i.pl. administration of CFA, as evidenced by an increase in the paw thickness of Swiss mice from 6 h up to 6 days after administration in the CFA/placebo group compared to the Saline/Placebo ($p < 0.0001$, Figure 2E). PEMF treatment did not affect the paw edema induced by CFA administration in mice, regardless of the frequency applied (Figure 2E). Treatment with 30 min of PEMF 75 Hz in the Saline group did not affect the response to the mechanical stimulus (Figure 2A–D) or paw edema (Figure 2E). After this set of experiments, the frequency of 75 Hz was chosen for the following experiments.

3.2 | Effect of 10, 20, or 30 min of Treatment With PEMF 75 Hz on Mechanical Hyperalgesia and Paw Edema After CFA-Induced Inflammation

Consistent with the first experiment set, i.pl. administration of CFA induced intense hyperalgesia—increasing the response to the mechanical stimuli—and edema—increasing the thickness of the paw—in the CFA/Placebo group compared to the Saline/Placebo group ($p < 0.0001$, Figure 3A,E). Figure 3A shows the mechanical hyperalgesia 6 h after CFA injection. Half an hour after the first treatment, both 20 min and 30 min of PEMF 75 Hz reduced the mechanical hyperalgesia induced by CFA compared to the CFA/Placebo group ($p < 0.0001$, Figure 3A). The time course of mechanical hyperalgesia evaluated at 0.5, 1, 2, 3, 4, and 5 h after the second treatment indicates that 20 min of PEMF 75 Hz reduced the mechanical hyperalgesia for up to 4 h compared to the CFA/Placebo group ($p < 0.05$, Figure 3A). The treatment with 30 min of PEMF 75 Hz was effective up to 3 h after the treatment compared to the CFA/Placebo group ($p < 0.05$, Figure 3A). The application of 30 min of PEMF 75 Hz in the Saline group did not affect the response to the mechanical stimulus ($p > 0.05$, Figure 3A).

An increase in response to the mechanical stimulus was observed from 24 h up to 6 days after CFA administration. Treatment with 20 min and 30 min of PEMF 75 Hz reduced the mechanical hyperalgesia on Days 1, 3, 4, and 6 compared to the CFA/Placebo group ($p < 0.05$, Figure 3B). A time course of the effects of PEMF 75 Hz performed 6 days after CFA administration indicated that 20 min of treatment reduced mechanical hyperalgesia for up to 1 h compared to the CFA/Placebo group ($p < 0.05$, Figure 3C). In contrast, the effects of 30 min of PEMF 75 Hz persisted up to 2 h after treatment ($p < 0.05$, Figure 3C), while 10 min did not have an effect. The AUC analysis indicated that treatment with 20 min and 30 min of PEMF 75 Hz reduced the mechanical hyperalgesia induced by CFA administration, whereas 10 min had no effect (Figure 3D).

Regarding the effects of PEMF on edema induced by CFA administration, the treatment with PEMF 75 Hz did not affect the paw edema, regardless of the time applied (Figure 3E). After this set of experiments, the treatment with 20 min of PEMF 75 Hz was chosen for the following experiments.

3.3 | Effect of Treatment With 20 min of PEMF 75 Hz on Anxiety- and Depression-Like Behaviors After CFA-Induced Inflammation

Depression- and anxiety-like behaviors were evaluated 2 and 3 days after CFA-induced inflammation, respectively, using the TST and elevated plus maze. Our results demonstrated that i.pl. administration of CFA in Swiss mice did not induce anxiety- and depression-like behaviors compared to the Saline/Placebo group. Specifically, there was no significant difference in the time spent in the open arms of the elevated plus maze ($p > 0.05$, Figure 4A) or the immobility time in the TST ($p > 0.05$, Figure 4B). In addition, treatment with 20 min of PEMF 75 Hz in animals treated with either CFA or Saline did not affect the behavioral parameters evaluated.

3.4 | Effect of Treatment With 20 min of PEMF 75 Hz on Locomotor and Anxiety-Related Behavior After CFA-Induced Inflammation

Our results demonstrated that CFA administration in Swiss mice did not alter locomotor activity compared to the Saline/Placebo group, as observed in the route, occupation, and distance traveled in the open field test ($p > 0.05$, Figure 5A–C). CFA administration also did not affect the time spent or the number of entries in the center of the apparatus—usually evaluated as indicative of anxiety-like behavior in rodents (Figure 5D,E). In addition, treatment with 20 min of PEMF

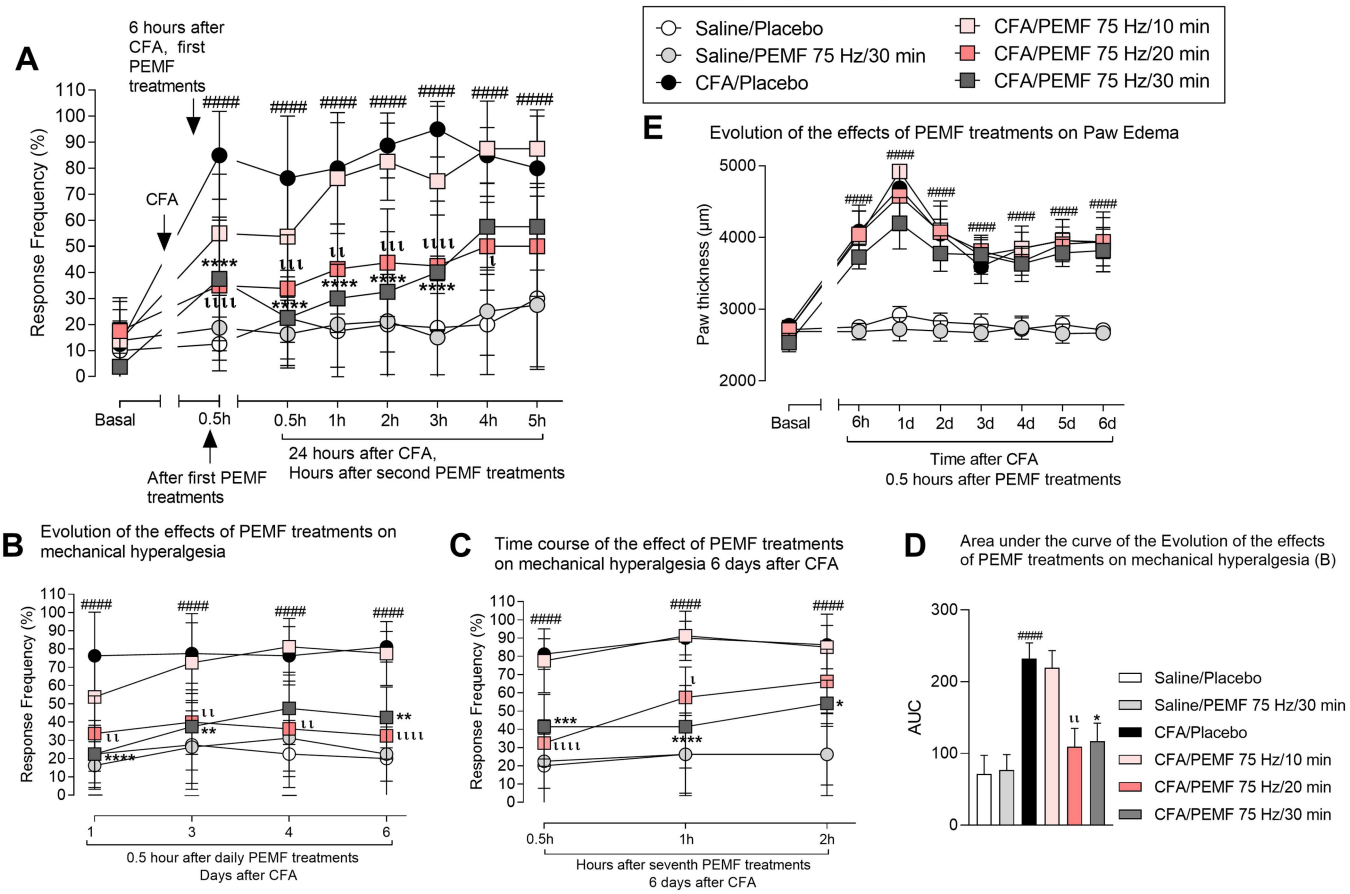

FIGURE 3 | Effects of treatment with 10, 20, or 30 min of PEMF 75 Hz on mechanical hyperalgesia and paw edema after CFA-induced inflammation in mice. Response frequency to mechanical stimulus before the procedure (A left), 6 h (A), and 24 h (A) after CFA administration and after the first and the second treatment with PEMF (A right). Effects of treatment with 10, 20, or 30 min of PEMF 75 Hz on mechanical hyperalgesia on Days 1, 3, 4, and 6 (B) after CFA administration. Time course of the effects of treatment with 10, 20, or 30 min of PEMF 75 Hz on mechanical hyperalgesia 6 days after CFA administration (C). The area under the curve of the effects of treatment with 10, 20, or 30 min of PEMF 75 Hz on mechanical hyperalgesia (D). Effects of treatment with 10, 20, or 30 min of PEMF 75 Hz on paw edema up to 6 days after CFA administration (E). The values represent the mean ± SD ($n = 8$ animals/group). Two-way ANOVA with repeated measures followed by Tukey's post hoc test. $^{\#\#\#\#}p < 0.0001$ compared to the Saline/Placebo group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, $^{\text{l}}p < 0.05$, $^{\text{ll}}p < 0.01$, $^{\text{lll}}p < 0.001$, and $^{\text{llll}}< 0.0001$ compared to the CFA/Placebo group. CFA, complete freund's adjuvant; PEMF, pulse electromagnetic field.

75 Hz did not affect the parameters assessed in the open field test in both the Saline and CFA groups ($p > 0.05$, Figure 5).

3.5 | Effect of Treatment With 20 min of PEMF 75 Hz on Pro-Inflammatory Cytokines After CFA-Induced Inflammation

CFA administration in Swiss mice increased the levels of pro-inflammatory cytokines TNF and IL-6 in the paw of animals in the CFA/Placebo group compared to the control group (Saline/Placebo; $p < 0.05$, Figure 6D,H). CFA administration did not affect the levels of TNF and IL-6 in the hippocampus (Figure 6A,E), prefrontal cortex (Figure 6B,F), and spinal cord (Figure 6C,G). Treatment with 20 min of PEMF 75 Hz demonstrated an anti-inflammatory effect by significantly reducing TNF levels in the paws of animals ($p < 0.01$, Figure 6D), although it did not alter IL-6 levels ($p > 0.05$, Figure 6H). Treatment with 20 min of PEMF 75 Hz did not have any effect per se.

3.6 | Effect of Treatment With 20 min of PEMF 75 Hz on Oxidative Stress Parameters After CFA-Induced Inflammation

Our findings indicated that CFA administration in Swiss mice modulates oxidative stress parameters in animals, as evidenced by a reduction in nitrite/nitrate levels in the paw (Figure 7P) and an increase in SOD activity in the hippocampus (Figure 7W). Treatment with 20 min of PEMF 75 Hz did not alter nitrite/nitrate levels in the paw compared to the CFA/Placebo group ($p > 0.05$, Figure 7P). However, it reduced SOD activity in the hippocampus compared to the CFA/Placebo group ($p < 0.05$, Figure 7W). Treatment with 20 min of PEMF 75 Hz per se increased SOD activity in the spinal cord ($p < 0.01$, Figure 7Y) and the paw of mice ($p < 0.01$, Figure 7Z). Also, according to our findings, CFA administration did not affect protein carbonyls, TBARS, MPO levels, and CAT activity in the hippocampus, prefrontal cortex, spinal cord, and paw of Swiss mice evaluated 7 days after administration (Figure 7).

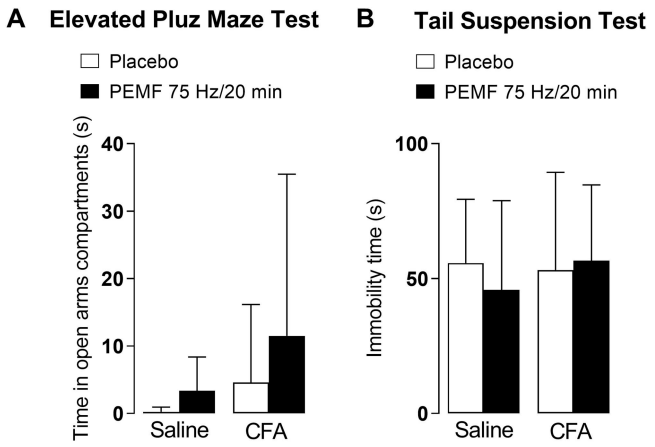

FIGURE 4 | Effects of treatment with 20 min of PEMF 75 Hz on anxiety- and depression-like behavior evaluated after CFA-induced inflammation in mice. Effects of treatment with 20 min of PEMF 75 Hz on time spent in the open arms of the plus maze apparatus (A) and the immobility time assessed by the tail suspension test (B). The values represent the mean ± SD ($n = 8$ animals/group). Two-way ANOVA. CFA, complete Freund's adjuvant; PEMF, pulse electromagnetic field.

4 | Discussion

The present study demonstrated for the first time the effect of PEMF treatment on pain behaviors in male Swiss mice with paw inflammation induced by CFA, with significant variations observed depending on the pulse frequency and time applied. This study also tested the effect of PEMF treatment on comorbidities associated with chronic inflammatory pain, such as anxiety and depression. Finally, inflammatory and oxidative stress parameters and antioxidant enzyme activity were measured.

The CFA-induced chronic inflammatory pain model is widely used and is a recognized method for studying experimental chronic pain, enabling analysis of the inflammatory and oxidative stress parameters involved in cellular alterations during the inflammatory process (Malik et al. 2023). After administration of CFA to the paw, persistent hyperalgesia and edema occur due to the development of the inflammatory process, cell activation, and phagocytosis. This process begins with the recognition of CFA components, such as lipoproteins, glycolipids, and peptidoglycans, by antigen-presenting cells through pattern recognition receptors (PRRs), such as Toll-like receptors 1, 2, 4, and 6 (Zhao et al. 2015), nucleotide-binding

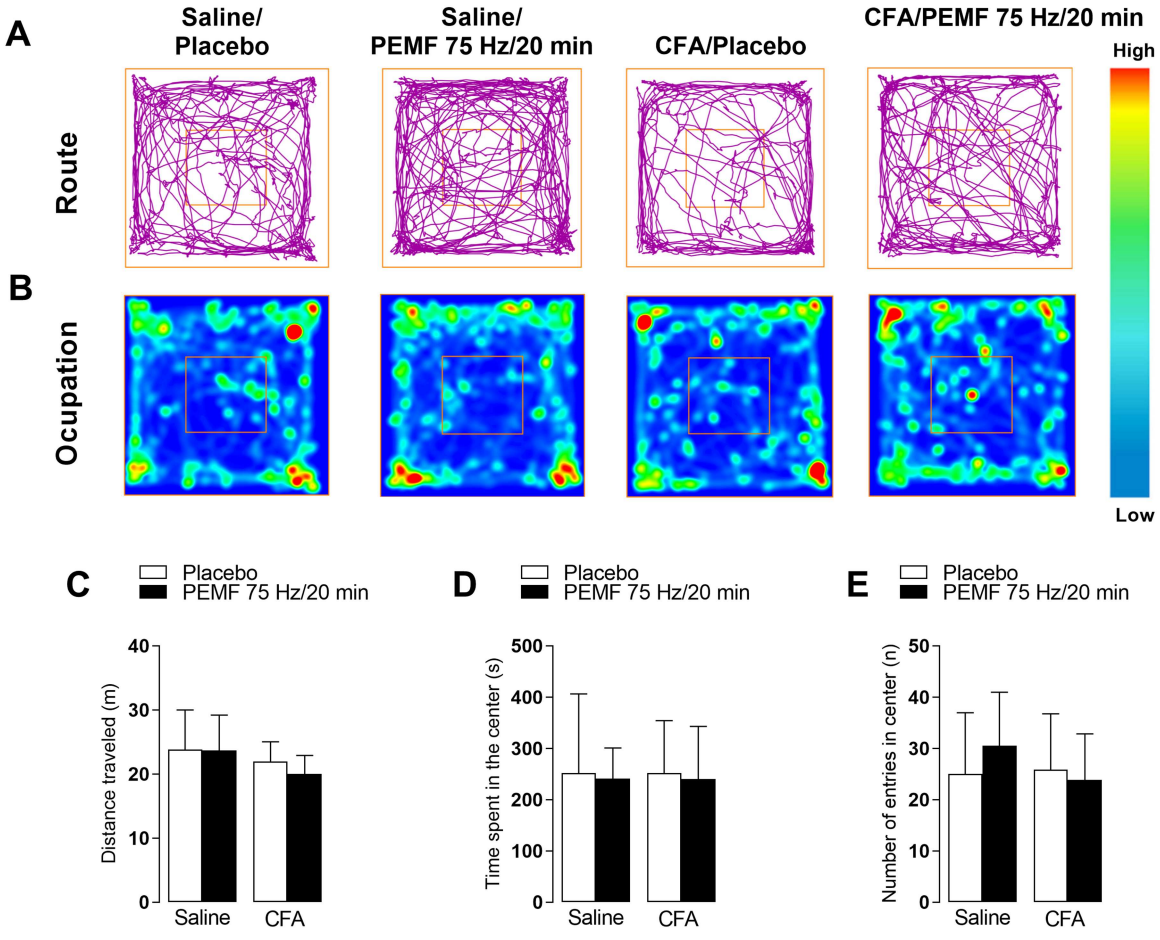

FIGURE 5 | Effects of treatment with 20 min of PEMF 75 Hz on locomotor activity and anxiety-like behavior after CFA-induced inflammation in mice. Representative track plots of the route (A) and the occupation (B) performed by mice in the open field test. Effects of treatment with 20 min of PEMF 75 Hz on the distance traveled (C), time spent in the center (D), and the number of entries in the center (E) of the open field. The values represent the mean ± SD ($n = 8$ animals/group). Two-way ANOVA. CFA, complete Freund's adjuvant; PEMF, pulse electromagnetic field.

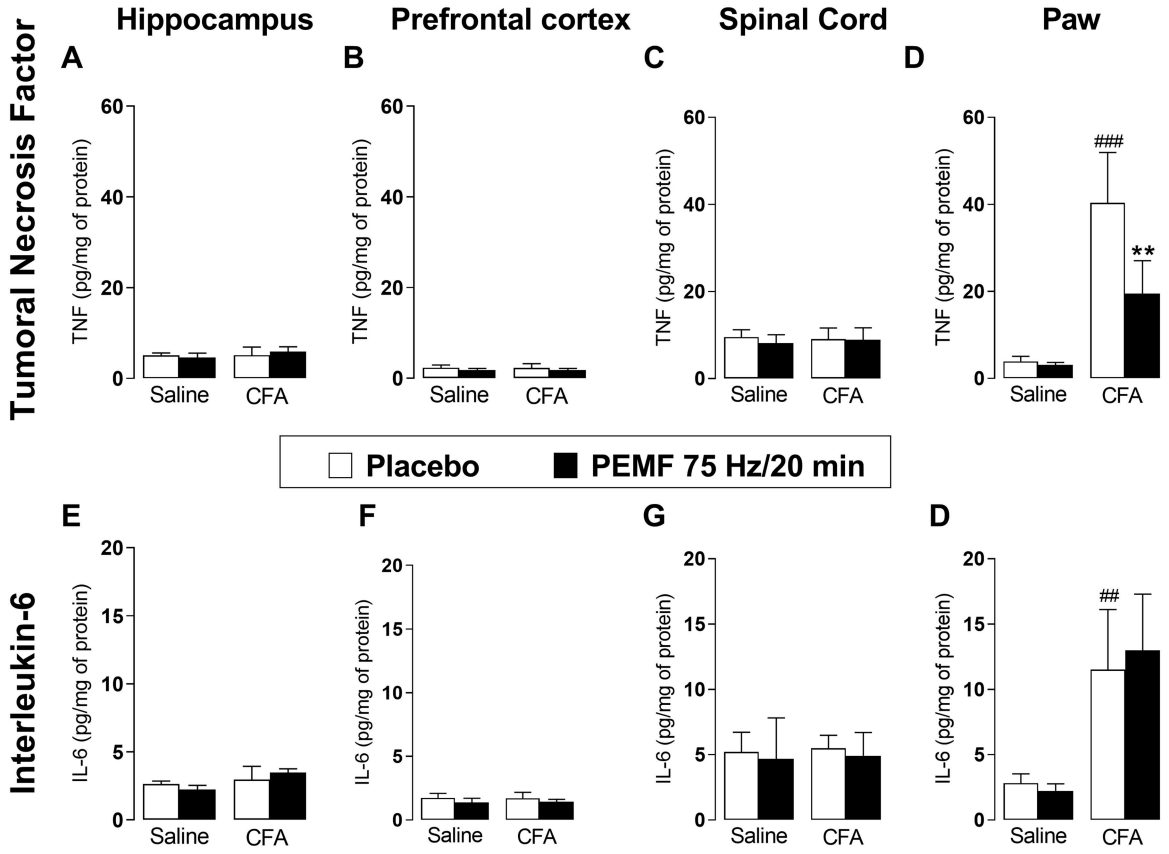

FIGURE 6 | Effects of treatment with 20 min of PEMF 75 Hz on pro-inflammatory cytokines TNF (A–D) and Interleukin-6 (E–H) after CFA-induced inflammation in mice. Effects of treatment with 20 min of PEMF 75 Hz on TNF levels in the hippocampus (A), prefrontal cortex (B), spinal cord (C), and paw (D). Effects of treatment with 20 min of PEMF 75 Hz on Interleukin-6 levels in the hippocampus (E), prefrontal cortex (F), spinal cord (G), and paw (H). The values represent the mean ± SD ($n = 8$ animals/group). Two-way ANOVA followed by Tukey's post-hoc test. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to the Saline/Placebo group. $^{**}p < 0.01$ compared to the CFA/Placebo group. CFA, complete Freund's adjuvant; IL-6, interleukin-6; TNF, tumor necrosis factor; PEMF, pulse electromagnetic field.

oligomerization domain-containing protein 2, and beta-glucan receptor dectin-1. PRRs activate intracellular mechanisms, for example, nuclear factor-kB and inflammasome transcription pathways, which control the inflammatory response, producing and releasing cytokines, such as TNF, and interleukins, such as IL-1β and IL-6. Inflammatory pain is mainly caused by the sensitization of primary nociceptive neurons due to the direct action of these inflammatory mediators (Ferwerda et al. 2005; Kleinnijenhuis et al. 2009; Means et al. 2001; Tapping and Tobias 2003; Underhill et al. 1999; Yadav and Schorey 2006).

Initially, frequencies of 5, 50, and 75 Hz were tested under mechanical hyperalgesia and edema, with a fixed exposure time of 30 min. The best antihyperalgesic effect was observed with a frequency of 75 Hz, with no effect on the generation of paw edema. Mechanical hyperalgesia and edema were subsequently assessed according to variations in exposure time: 10, 20, and 30 min. The most significant and long-lasting antihyperalgesic effect was observed within 20 and 30 min of treatment, and, consistent with Experiment 1, there was no improvement in paw edema. These experiments determined the most effective frequency and time parameters for using PEMF to facilitate an antihyperalgesic effect: 75 Hz for 20 min. The third experiment was conducted using these parameters; however, under the experimental conditions employed, the CFA protocol did not produce detectable anxiety- or depression-like alterations in Swiss mice within the evaluated time window (7-day follow-up), as assessed by the elevated plus maze, open field, and TSTs. As a result, the evaluation of potential PEMF effects on emotion-related behaviors was precluded, representing a limitation of the model and experimental timeline rather than a lack of treatment efficacy. Furthermore, a 20-min treatment with PEMF at 75 Hz reduced the concentration of TNF in the paw, as well as demonstrated greater SOD enzyme activity in the paw and spinal cord, suggesting an inflammation-modulating effect in the paw and an antioxidant effect both peripherally and in the central nervous system.

In the CFA model, mechanical hypersensitivity peaks in the first 6–24 h after induction and remains after 14 days (Ghasemlou et al. 2015). In the present study, we observed a significant increase in mechanical hypersensitivity in the groups induced with CFA, compared to the saline groups, corroborating the literature. The antihyperalgesic effect of PEMF found in this study is consistent with previous studies on the use of this therapeutic modality in the treatment of pain and inflammation in other experimental models and clinical trials (Chan et al. 2019; Shafford et al. 2002; Zidan et al. 2018). The first experiment of this study showed that 30 min of exposure to PEMF at frequencies of 5, 50, and 75 Hz demonstrated an antihyperalgesic effect that varied during the evaluated period. However, when analyzing the area under the curve during

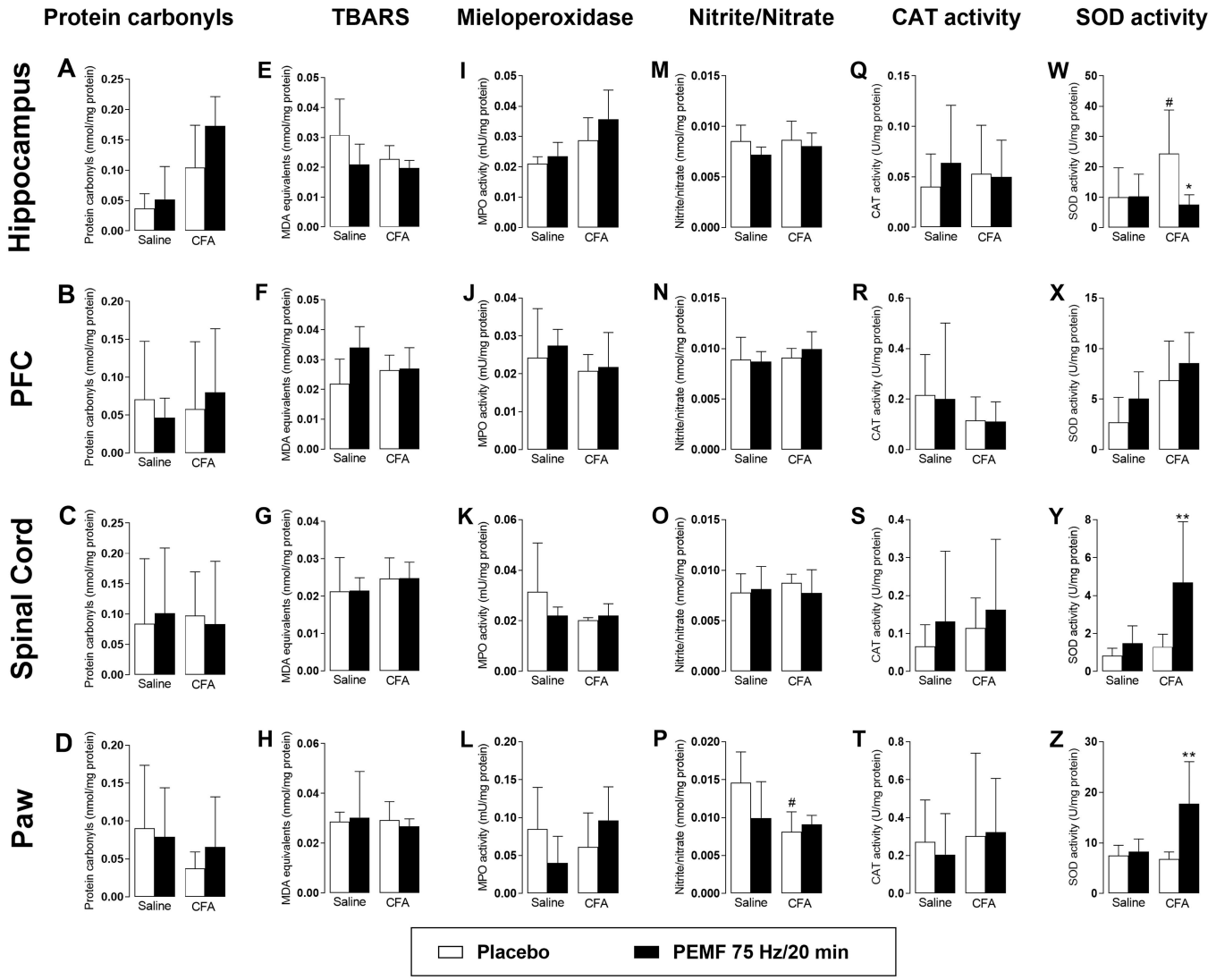

FIGURE 7 | Effects of treatment with 20 min of PEMF 75 Hz on oxidative parameters in the hippocampus, prefrontal cortex, spinal cord, and paw after CFA-induced inflammation in mice. Effects of treatment with 20 min of PEMF 75 Hz on protein carbonyls levels (A–D), TBARS (E–H), MPO (I–L), nitrite/nitrate (M–P), CAT activity (Q–T), and SOD activity (W–Z) in the hippocampus, prefrontal cortex, spinal cord, and paw. The values represent the mean ± SD ($n = 8$ animals/group). Two-way ANOVA followed by Tukey's post hoc test. $^{\#}p < 0.05$ compared to the Saline/Placebo group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the CFA/Placebo group. CAT, catalase; CFA, complete Freund's adjuvant; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive species; PEMF, pulse electromagnetic field; PFC, prefrontal cortex.

treatment with PEMF, the 75 Hz frequency was shown to be superior. Regarding exposure time, as the effect was similar with 20 or 30 min of exposure to PEMF at 75 Hz, it is more effective to use it for 20 min. On the other hand, PEMF at 75 Hz for 10 min did not show an antihyperalgesic effect.

Although the analgesic effect of PEMF has not been previously tested in this model, previous studies with an inflammation model using an intraplantar injection of carrageenan demonstrated an antihyperalgesic effect with different frequencies (Mert and Yaman 2020). However, the PEMF device by Mert and Yaman (2020) differs from the one used in the current work, as it simultaneously offers trains of pulses of different frequencies. The group that showed an antihyperalgesic effect was treated with pulse trains of 1, 3, 5, and 7 Hz, while the group that received PEMF with pulse trains of 7, 9, 12, and 14 Hz curiously demonstrated the opposite effect. Understanding that frequency variation can interfere with cellular response, it is crucial to study frequencies individually, as in the current study.

The choice of parameters, such as pulse frequency, magnetic field intensity, and exposure time, can influence cellular activation mechanisms and final effects. Studies are found in the literature with great variation in the choice of parameters, with positive effects on the modulation of inflammatory mediators, such as TNF and prostaglandins, in addition to reductions in edema with the use of PEMF at frequencies 5, 50, and 75 Hz (Pagani et al. 2017). Although different frequencies have also provided positive results regarding analgesia in other animal studies (Alvarez et al. 2019; Shafford et al. 2002; Zidan et al. 2018), our experiment took into account the evaluation of behavioral effects secondary to pain, such as anxiety and depression, and previous studies have demonstrated positive results on these variables using the frequencies chosen in this work (Faraji et al. 2021; Trzeciak et al. 1993). Another variable

that greatly diverges in the literature is the time of exposure to PEMF, which varies from 5 min (Rasouli et al. 2012) to 8 h of daily exposure (Eraslan et al. 2007). However, considering clinical applicability, it was decided to choose times closer to the reality of a therapeutic approach (Alvarez et al. 2019; Shafford et al. 2002; Zidan et al. 2018).

In addition to these parameters, the type of device used to generate the electromagnetic field also appears to influence outcomes. The use of PEMF in different configurations can alter the quality of the cellular response and contribute positively or negatively to the desired effect. While frequency, intensity, time, and waveform are important, the field generated by Helmholtz coil pairs compared to single-loop systems also shows differences in analgesic and anti-inflammatory effects (Gaynor et al. 2018). Helmholtz coils are more commonly applied for deeper tissues, such as bone fracture repair, whereas single-loop devices have been associated with effects on soft tissues, promoting pain relief and edema reduction (Gaynor et al. 2018). For example, PEMF single-loop systems delivering a 2-msec sine wave at 27.12 MHz in 2 bursts/s pulse trains have demonstrated significant benefits in reducing postoperative soft tissue pain and edema (L. Zhang et al. 2020; Shafford et al. 2002), as well as in spine surgery recovery (Zidan et al. 2018; Alvarez et al. 2019). Considering the inflammatory pain model of the present study and the results obtained, there remains a gap for future research directly comparing different PEMF devices with respect to their effects on analgesia and edema.

In the CFA inflammation model, significant neutrophil migration occurs in the first 3 h after administration, reaching a peak at 24 h, while pro-inflammatory monocytes predominate between the 3rd and 7th day after intraplantar injection (Ghasemlou et al. 2015). Neutrophils, in addition to releasing granules containing enzymes and proteins to combat local infection, also produce ROS, such as superoxide and hydrogen peroxide; chemotactic factors; lipid mediators, such as leukotriene and prostaglandin; and pro-inflammatory cytokines, such as TNF, IL-1, and IL-6, responsible for amplifying the inflammatory response and activating other immune cells (Yin and Heit 2018). TNF is a cytokine that stimulates acute phase reactions (Marques et al. 2009) and is very relevant in the regulation of apoptosis, inflammation, and immunity, in addition to being a potent propagator of the inflammatory response, both local and systemic (Ross and Harrison 2013). One of its main functions is to recruit specific T lymphocytes and monocytes, as well as promote their maturation into macrophages (Yasui 2014). This massive inflammatory release sensitizes peripheral nociceptors, triggering neuronal hyperexcitability and amplifying the painful response (Omorogbe et al. 2018). The antihyperalgesic effect found in our study can be supported by the biochemical findings that demonstrated a significant reduction in the concentration of TNF in the paw of the group treated with PEMF at 75 Hz for 20 min. Although the concentration of IL-6 was not changed with the use of PEMF in our study, the reduction in the concentration of TNF and IL-6 has been reported in studies on OA (Ciombor et al. 2003; Yang et al. 2021), musculoskeletal alterations (Hu et al. 2020), and in acute inflammation of the intervertebral disc (Chan et al. 2019). Since there are no studies using PEMF in the CFA model, the results obtained from using PEMF in inflammatory modulation come from other experimental models. Yang et al. (2021) demonstrated that IL-6 and TNF are critical factors in the functional and structural effects of PEMF on OA progression. The use of PEMF at 75 Hz for 60 min over 8 weeks demonstrated a persistent antihyperalgesic effect, resulting in lower expression of TNF and IL-6 for 4 weeks (Yang et al. 2021). Chan et al. (2019) also identified significant inhibition in the expressions of IL-6, IL-1β, and TNF after acute inflammation of intervertebral discs in animals exposed to PEMF at 15 Hz for 4 h daily for 7 days, suggesting an anti-inflammatory effect and therapeutic potential in inflammation associated with disc degeneration (Chan et al. 2019).

Building on these biochemical effects, mechanistic evidence indicates that PEMF can modulate rapid signal transduction by regulating intracellular Ca^{2+} influx and Ca^{2+}/calmodulin coupling, leading to nitric oxide production and activation of the Cyclic Guanosine Monophosphate/Protein Kinase G pathway, with crosstalk to Extracellular Signal-Regulated Kinase/cAMP Response Element-Binding Protein, which contributes to early antinociceptive and anti-inflammatory responses (Pilla 2012; Petecchia et al. 2015; Park et al. 2022). In parallel, activation of adenosine A_2A and A_3 receptors elevates cAMP and suppresses the release of pro-inflammatory cytokines such as TNF and IL-6 (Varani et al. 2012; Vincenzi et al. 2013; Varani et al. 2017). At the transcriptional level, PEMF has been associated with inhibition of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity, decreasing expression of inflammatory mediators, while concomitant activation of the Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2) axis upregulates antioxidant enzymes including SOD, CAT, and GPx, offering a plausible link between the observed TNF reduction and SOD increases and the antihyperalgesic outcomes reported here (Ross and Harrison 2013; Ross et al. 2019; Ehnert et al. 2017; Pi et al. 2019; Zhai et al. 2023; S. Zhou et al. 2025).

During an inflammatory injury, as in the intraplantar CFA model, an imbalance occurs between oxidant and antioxidant parameters (Malik et al. 2023), leading to exacerbated production of free radicals that promote the oxidation of biomolecules, compromising the biological functions of these structures (Barbosa et al. 2010). The metabolites arising from this oxidative process can be identified and quantified as markers of lipid oxidation, such as TBARS, proteins, carbonyl groups, and deoxyribonucleic acid (Barbosa et al. 2010). ROS released by neutrophils are responsible for initiating the oxidation process of proteins and enzymes, leading to loss of function and consequent formation of carbonyl groups (Vignola et al. 2012). In addition to ROS, neutrophil granules, when activated, secrete the enzyme MPO, which contributes to the oxidation of lipoproteins (Roman et al. 2008). On the other hand, nitric oxide (NO) released by oxidative stress promotes the production of antioxidant enzymes, such as SOD, which is an enzyme commonly expressed by neurons and glial cells in the CNS, with the ability to neutralize superoxide radicals, and represents the front line in antioxidant enzymatic defense (Chidambaram et al. 2024). In addition to the production of SOD, NO can generate metabolites, such as nitrite and nitrate, that act as ROS neutralizers, thus contributing to the cell's balance (Dusse et al. 2003). As well as these antioxidants, the activity recruited by enzymes such as catalase (CAT) and glutathione peroxidase (GPx) also removes excess ROS, preventing the cell from irreversible damage (Vignola et al. 2012). However, excessive

release of NO can contribute negatively, increasing imbalance and enhancing oxidative action through its connection with superoxide anions (Dusse et al. 2003) and altering the excitability of nociceptive neurons, stimulating the persistence of allodynia and hyperalgesia in chronic pain conditions (Yonehara et al. 1997). During the inflammatory process triggered by CFA injection, NO levels in the spinal cord increase substantially, contributing to the persistence of pain in this experimental model (W. Zhang et al. 2021).

Although the induction of ROS appears to be related to the intensity and frequency of PEMF (Ehnert et al. 2017), exposure to low-intensity fields (< 100 Hz) (Hu et al. 2020) demonstrated a 26.5% reduction in ROS levels in cultured human osteoblasts, after 7 days of daily exposure to PEMF at 16 Hz, in addition to increasing the expression of the SOD enzyme by 2.4 times and the expression of CAT by 8.1 times in groups of cells exposed to PEMF (Ehnert et al. 2017). The study by Vignola et al. (2012), carried out in a model of myopathy induced by subplantar injection of carrageenan, resulted in an antioxidant effect attributed to the group treated with PEMF at 50 Hz for 30 min, with levels of NO, carbonyl groups, and SOD that were similar to the control group (Vignola et al. 2012).

The current study evaluated peripheral structures (paw) and structures of the central nervous system (spinal cord, prefrontal cortex, and hippocampus) involved with the pain pathway and modulation and demonstrated a significant increase in SOD activity in the paw and spinal cord in the groups treated with PEMF at 75 Hz for 20 min, which suggests a positive control in the balance of oxidative stress, contributing to the anti-hyperalgesic and anti-inflammatory process. However, we observed that SOD activity in the hippocampus was greater in the CFA/Placebo group compared to saline, whereas PEMF-treated animals presented reduced enzymatic activity.

This apparent divergence raises the question of why PEMF attenuated hippocampal SOD despite enhancing activity in the paw and spinal cord. A plausible explanation lies in the distinct cellular and temporal dynamics of each structure. In the periphery and spinal cord, CFA induces robust immune infiltration and mediator release, with monocytes and macrophages predominating at later stages of inflammation, while significant neutrophil migration is no longer observed after day 7 (Ghasemlou et al. 2015; Omorogbe et al. 2018). The increased SOD observed after PEMF in the paw and spinal cord may therefore reflect direct antioxidant modulation in infiltrating immune cells. In contrast, the hippocampus is less exposed to peripheral immune infiltration and exhibits region- and time-dependent redox regulation (Sullivan et al. 2004; Naseri Kouzehgarani et al. 2020). The elevated hippocampal SOD in CFA/Placebo animals is consistent with an endogenous compensatory response to neuroinflammatory stress (Kölliker-Frers et al. 2021). Importantly, interventions that reduce upstream oxidative/inflammatory burden can prevent or normalize such elevations, indicating that the absence of further SOD upregulation after PEMF does not imply a lack of antioxidant effect but rather a reduced demand for enzymatic compensation (W. Zhang et al. 2021).

This interpretation is consistent with evidence that spinal microglia are robustly activated in CFA-induced pain, producing high levels of ROS and cytokines (Zhao et al. 2015), whereas hippocampal antioxidant responses vary dynamically depending on the stage of injury and regulatory context (Chidambaram et al. 2024). In this sense, the reduction of hippocampal SOD activity after PEMF exposure may indicate that the therapy attenuated upstream oxidative and inflammatory signaling, diminishing the demand for compensatory antioxidant responses. This effect is particularly relevant considering that, in the later phase of CFA inflammation (Days 3–7), macrophages become the predominant immune population at the site of injury, sustaining ROS, NO, and cytokine release that can drive peripheral sensitization and downstream microglial activation in the spinal cord and hippocampus (Ghasemlou et al. 2015).

Once activated, macrophages are responsible for releasing ROS, NO, IL-6, TNF, and other inflammatory mediators, which are then responsible for recruiting more cells. This massive inflammatory response triggers neuronal sensitization from the peripheral inflammatory lesion, activating microglia in the spinal cord (W. Zhang et al. 2021). Activated microglia initiate the production of more cytokines, such as IL-6 and TNF, increasing neuroimmune interaction during the neuroinflammation process (Kölliker-Frers et al. 2021). In the hippocampus, resident microglia participate in neurogenesis in their natural role. However, under persistent inflammatory conditions, they assist in the propagation of the neuroinflammatory process through the release of TNF and IL-6, impacting cognitive and memory function. This pro-inflammatory state has been associated with mental disorders, such as anxiety and depression (Kölliker-Frers et al. 2021). Alterations in behaviors related to anxiety, stress, depression, and reward in rats and mice are present in the CFA-induced pain model, being an important experimental model to study comorbidities related to chronic pain (Burek et al. 2022). The shorter exploration time in the open arms of the EPM, less exploration in the center of the open field, and a significant increase in immobility in the TST are behavioral alterations observed in this model. However, specific species and strains may more effectively express these anxiety and depression behaviors (Burek et al. 2022). The animals most used to replicate these behaviors are Wistar Han and Sprague–Dawley rats, and the C57BL/6 mouse (Burek et al. 2022). Few studies use Swiss mice (Maciel et al. 2013; Omorogbe et al. 2018) as a species favorable to the reproduction of these behavioral patterns, and in our study it was not possible to reproduce anxiety and depression behaviors with EPM, OFT, and TST in the intraplantar CFA model. We hypothesize that it was not possible to replicate the behavioral alterations due to the shorter experiment time. Previous experiments that evaluated anxiety and depression behaviors in Swiss mice after CFA injection carried out OFT and TST analyses 14 days after inflammatory induction (Maciel et al. 2013; Omorogbe et al. 2018). Although Maciel et al. conducted the TST assessment in Weeks 1, 2, 3, and 4 after CFA injection, and obtained a significant difference in the increase in immobility time in the first week, an even greater immobility time was observed in Weeks 2 and 3, returning to normal in the 4th week after inflammatory induction (Maciel et al. 2013).

Some limitations can be highlighted in our study, such as the early assessment, in the first week after CFA administration, of anxiety- and depression-like behaviors. Under the experimental conditions employed, the CFA model did not induce detectable emotional alterations in Swiss mice, which precluded the evaluation of potential PEMF effects on these outcomes; the relatively short follow-up period of 7 days may have contributed to

the absence of behavioral changes, as emotional alterations in CFA-induced inflammatory pain models may emerge at later time points. The use of the Swiss lineage may also have influenced these outcomes. The apparatus used for PEMF treatment allowed animal mobility, resulting in whole-body PEMF exposure, which could have introduced variability in tissue-specific field distribution. Although full containment would ensure more uniform exposure, it was avoided to reduce stress, which could itself affect behavior. Future studies comparing localized PEMF application or controlled confinement may help clarify these effects. In addition, a single magnetic field intensity was used, precluding dose–response analyses.

Another limitation is that only male Swiss mice were used. Although this reduced variability related to hormonal cycles, it limits generalizability. Sex differences are increasingly recognized as relevant: Knight et al. (2021) showed lower anxiety-like behavior in females than males in the elevated plus maze and open field tests, and Burek et al. (2022) identified sex as a source of heterogeneity in CFA-induced behavioral outcomes. Clinical evidence also supports sex-specific responses, as women exhibited greater muscle strength gains while men experienced more pronounced pain relief when PEMF was combined with exercise in knee osteoarthritis patients (Q. Wang et al. 2024). These findings emphasize that the absence of females is a limitation and that future studies should include both sexes, longer follow-up periods, and multiple field intensities to improve translational relevance.

In conclusion, the present study demonstrates the anti-hyperalgesic efficacy of PEMF in a CFA-induced inflammatory pain model, identifying a frequency of 75 Hz and a treatment duration of 20 min as the most effective parameters for reducing mechanical hyperalgesia. These behavioral effects were accompanied by selective biological changes, including a reduction in TNF levels in the inflamed paw and modulation of SOD activity in specific tissues, notably the paw and spinal cord. Importantly, PEMF did not produce widespread anti-inflammatory or antioxidant effects across all evaluated markers or brain regions. In addition, no anxiety- or depression-like behaviors were detected following CFA administration under the experimental conditions employed, precluding the assessment of PEMF effects on these behavioral outcomes. Together, these findings indicate that PEMF exerts targeted peripheral and spinal modulatory effects associated with pain relief, rather than broad systemic or central actions. From a translational perspective, these results support the potential of PEMF as a non-invasive and low-cost adjunctive strategy for inflammatory pain, warranting further investigation. Future studies should expand preclinical evaluation to include both sexes, different species, additional exposure intensities, and longer follow-up periods, as well as further explore behavioral comorbidities under optimized conditions. In parallel, translational and clinical studies will be necessary to refine PEMF treatment parameters and to determine the therapeutic scope and safety profile of this intervention across patient populations.

Author Contributions

Study conception and design: Franciane Bobinski and Jennifer Hummel. Acquisition of data: Heloiza dos Santos Baldança, Rafaela Hardt da Silva, Khiany Mathias, Anita dal Bó Tiscoski, Naíla Maciel Andrade, and Helena Mafra Martins. Analysis and interpretation of data: Franciane Bobinski, Josiel Mileno Mack and Jennifer Hummel. Drafting of manuscript: Jennifer Hummel and Franciane Bobinski. Critical revision: Franciane Bobinski, Jennifer Hummel, Josiel Mileno Mack, Daniel Fernandes Martins and Fabricia Petronilho.

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Ethics Statement

All experimental protocols were approved by the Ethics Committee on Animal Use (CEUA-UNISUL, protocol n° 23.006.4.01.IV).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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