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ORIGINAL ARTICLE

Effects of photobiomodulation therapy, pharmacological therapy, and physical exercise as single and/or combined treatment on the inflammatory response induced by experimental osteoarthritis

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Abstract Osteoarthritis (OA) triggers increased levels of inflammatory markers, including prostaglandin (PG) E_2 and proinflammatory cytokines. The elevation of cytokine levels is closely associated with increased articular tissue degeneration. Thus, the use of combination therapies may presumably be able to enhance the effects on the modulation of inflammatory markers. The present study aimed to evaluate and compare the effects of photobiomodulation therapy (PBMT), physical exercise, and topical nonsteroidal anti-inflammatory drug (NSAID) use on the inflammatory process after they were applied either alone or in different combinations. OA was induced by intra-articular papain injection in the knee of rats. After 21 days, the animals began treatment with a topical NSAID and/or with physical exercise and/or PBMT. Treatments were performed three times a week for eight consecutive weeks, totaling 24 therapy sessions. Analysis of real-time polymerase chain reaction (RT-PCR) gene expression; interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α) protein expression; and PGE_2 levels by enzyme-linked immunosorbent assay (ELISA) was conducted. Our results showed that PBMT alone and Exerc + PBMT significantly reduced IL-1β gene expression ($p < 0.05$) while no treatment changed both IL-6 and TNF-α gene expression. Treatment with NSAID alone, PBMT alone, Exerc + PBMT, and NSAID + PBMT reduced IL-1β protein expression ($p < 0.05$). All therapies significantly reduced IL-6 and TNF-α protein expression ($p < 0.05$) compared with the OA group. Similarly, all therapies, except Exerc, reduced the levels of PGE_2 ($p < 0.05$) compared with the OA group. The results from the present study indicate that treatment with PBMT is more effective in modulating the inflammatory process underlying OA when compared with the other therapies tested.

Keywords Photobiomodulation therapy · Nonsteroidal antiinflammatory drug · Osteoarthritis · Papain · Physical exercise · Rats

Introduction

Osteoarthritis (OA) is the most prevalent articular disease worldwide, affecting approximately 10 % of men and 18 % of women over 60 years of age [1]. It is a key trigger of pain and functional disability, and its management is responsible for incurring high socioeconomic costs in all countries worldwide [2].

OA is long considered a degenerative process of the cartilage that was difficult to treat because chondrocytes, the cartilage cells, have low levels of metabolism and cartilage has no vascularization or innervation, which hinders repair and inflammatory response to injury [3]. However, advances in the field of molecular biology have shown that inflammatory mediators, including cytokines and prostaglandins (PGs), may enhance chondrocyte production of matrix metalloproteinases (MMPs). Furthermore, studies have shown that the

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subchondral bone also plays a key role in pain and cartilage degeneration caused by OA [4–6]. Patients with OA reportedly have high levels of proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α) and interleukins (ILs) 1β, 6, and 8, and increased prostaglandin E_2 (PGE_2) levels [7].

Thus far, the use of a therapeutic approach with the goal of reducing proinflammatory cytokine levels by using anti-IL-1 and anti-TNF antibodies, for example, showed no significantly effective improvements in OA symptoms [8, 9]. Despite this, there was a noticeable increase in the level of inflammatory markers, which demonstrates the contribution of the inflammatory process to articular cartilage degeneration in OA [8, 9]. The wide variety of therapies available to treat OA usually aim to control symptoms, prevent disease progression, and minimize functional disability [10] without necessarily seeking to modulate the underlying inflammatory process.

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is one of the most commonly prescribed treatments for OA cases, and diclofenac has already been shown to reduce PGE_2 levels in experimental OA [11]. Furthermore, performing physical exercise is also widely recommended for treating OA and may affect the release of inflammatory markers, depending on the type of physical exercise that is practiced [12]. Lastly, previous studies have shown that photobiomodulation therapy (PBMT) effectively reduces the number of proinflammatory cells and the expression of proinflammatory cytokines and is able to modulate the underlying inflammatory process of experimental OA [13, 14].

Using each of the abovementioned therapies as monotherapy to treat OA has already been examined [11–14]. However, combination therapies could presumably enhance the positive effects observed with each therapy alone (NSAID, physical exercise, and PBMT). However, no studies combining the three commonly prescribed therapies and assessing whether there are benefits from reducing symptoms and decreasing disease progression through combination therapies have been published thus far. Considering the above issues, the present study aimed to evaluate and compare the effects of PBMT, physical exercise, and topical NSAID use, applied alone and in different combinations of these therapies, on the inflammatory process, more specifically, on the release of proinflammatory mediators triggered in an experimental model of OA.

Method

Animals

Male Wistar rats were used, weighing approximately 200–250 g, which were derived from the central animal house of the university. The animals were housed under standard temperature (from 22 to 24 °C), relative humidity (from 40 to 60 %), and 12-h light-dark cycle conditions, with water and feed ad libitum. Experimental protocol was approved by the Ethics Committee for Animal Experimentation (number 077, page 130, book 02) of the Institute of Biomedical Sciences, University of São Paulo (ICB-USP).

Experimental groups

Fifty-four animals were randomly assigned to 9 groups of 6 animals per group as follows:

- Control group—the animals were not subjected to any procedure or treatment.
- OA group—the animals were subjected to OA induction, albeit without receiving any type of treatment.
- OA + physical exercise (Exerc) group—the animals were subjected to OA induction and treated with swimming.
- OA + NSAID (NSAID) group—the animals were subjected to OA induction and treated with NSAID.
- OA + PBMT (PBMT) group—the animals were subjected to OA induction and treated with PBMT.
- OA + physical exercise + NSAID (Exerc + NSAID) group—the animals were subjected to OA induction and treated with the swimming and NSAID combination.
- OA + physical exercise + PBMT (Exerc + PBMT) group—the animals were subjected to OA induction and treated with the swimming and PBMT combination.
- OA + NSAID + PBMT (NSAID + PBMT) group—the animals were subjected to OA induction and treated with the NSAID and PBMT combination.
- OA + physical exercise + NSAID + PBMT (Exerc + NSAID + PBMT) group—the animals were subjected to OA induction and treated with the swimming, NSAID, and PBMT combination.

All treatments began 21 days after the final papain injection and were administered once daily, 3 times a week (on alternate days) for 8 consecutive weeks, totaling 24 therapy sessions, according to the experimental groups. Seventy-two hours after the final treatment, the animals were sacrificed by cervical dislocation to collect biological material.

Procedures

OA induction in the knee Initially, the animals were anesthetized with a mixture of ketamine and xylazine (90 and 10 mg/kg, respectively; König, Avellaneda, Argentina) injected intraperitoneally. Subsequently, they were subjected to OA induction in the right knee according to Murat et al. [15]: 0.2 mL of 4 % papain solution, with 0.1 mL 0.03 M cysteine (activator), was injected intra-articularly with a microsyringe into the right knee of the animal. This procedure was repeated on the fourth and seventh days after the first injection.

Treatments

The animals were shaved in the knee area before every treatment. One week after the last injection of papain, all animals of the treated groups were subjected to treatment (with exercise, NSAIDs, and/or PBMT) in accordance with the aforementioned experimental groups:

Single therapies

- *NSAID application*: A dose of 1 g diclofenac sodium gel generic 10 mg/g (EMS®, Santo André, São Paulo, Brazil) was used on the knee joint previously subjected to OA induction.
- *Physical exercise protocol*: The physical exercise protocol used was adapted from the study by Lima et al. [16]. A 100 × 50 × 75-cm glass tank with water at a temperature of 31 °C was used for this purpose. The animals were subjected to a physical exercise protocol that included swimming. The first 2 weeks of treatment were intended for enabling the animals to adapt to physical exercise, and the other 6 weeks were intended for the training itself, totaling 8 weeks of treatment. The application of the physical exercise protocol occurred according to the aforementioned experimental groups. The adaptation and training program occurred as shown in Table 1.
- *PBMT application*: The PBMT parameters used were based on the study by Pallotta et al. [11]. A diode laser with an 830-nm wavelength (infrared) was used in continuous mode, with a 0.028-cm^2 spot size, 100 mW power output, continuous mode, 3.57 W/cm^2 power density, 214.2 J/cm^2 energy density, and energy of 6 J per point, 60 s per point, at 1 point on the joint subjected to OA induction. The spot was kept in direct contact with the skin while applying light pressure on the tissue in order to irradiate the animals.

Combination therapies

All combination therapies were applied similarly and with the same parameters used as when the therapies were applied alone.

- *Physical exercise combined with PBMT*: The physical exercise protocol was performed, immediately followed by PBMT application.
- *NSAID combined with PBMT*: NSAID was applied, immediately followed by PBMT application.
- *Physical exercise combined with NSAID*: The physical exercise protocol was performed, immediately followed by NSAID application.
- *Physical exercise combined with NSAID and PBMT*: The physical exercise protocol was performed, immediately followed first by NSAID application, and then by PBMT.

Collection of biological material

- *Articular tissue collection—cartilage*: The animals were anesthetized with a mixture of ketamine and xylazine (90 and 10 mg/kg, respectively; König, Avellaneda, Argentina) intraperitoneally. Then, the articular tissue was collected through an incision in the knee area, exposing the joint of the animal.
- *Blood plasma collection*: Immediately before sacrificing, 3 mL of blood samples was collected from the animals by cardiac puncture using a syringe containing heparin.

Table 1 Physical exercise protocol

Week	Day	Series	Active/recovery	Overload	Total time
Adaptation protocol					
1st week	1st day	5	1 min:1 min	3 % body weight	10 min
	2nd day	10	1 min:1 min	3 % body weight	20 min
	3rd day	8	1 min:1 min	5 % body weight	16 min
2nd week	4th day	10	1 min:1 min	7 % body weight	20 min
	5th day	10	1 min:1 min	9 % body weight	20 min
	6th day	10	1 min:1 min	10 % body weight	20 min
Training protocol (swimming)					
3rd week	–	11	1 min:1 min	10 % body weight	22 min
4th week	–	12	1 min:1 min	10 % body weight	24 min
5th week	–	13	1 min:1 min	10 % body weight	26 min
6th week	–	14	1 min:1 min	10 % body weight	28 min
7th week	–	15	1 min:1 min	10 % body weight	30 min
8th week	–	15	1 min:1 min	10 % body weight	30 min

After collection, the material was centrifuged and the supernatant was stored as plasma.

Analyses

Real-time polymerase chain reaction (RT-PCR) gene expression analysis IL-1β, IL-6, and TNF-α gene expression analysis was performed. The articular cartilage was removed, immediately frozen in liquid nitrogen, and stored at −80 °C until processing and analysis of the material. Total RNA was extracted using the reagent TRIzol® (Gibco BRL, USA), according to the manufacturer's instructions. After treatment with DNAse, cDNA synthesis was performed using the reverse transcriptase method and the enzyme SuperScript (Invitrogen) with 2 μg of total RNA and a mixture of random primers and oligo-dT. RT-PCR experiments were performed using the following program: one initial denaturation cycle of 10 min at 95 °C and 40 amplification cycles (30 s denaturation at 95 °C and 1 min annealing and extension at 60 °C). Primer sequences were the same used by Wang et al. [17] and are outlined in Table 2. Results were interpreted using the formula $2^{-\Delta\Delta Ct}$ (Ct is the number of cycles required to reach the threshold fluorescence above the background signal), which relates the expression of the gene of interest to the expression of the control gene hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Analysis of cytokine levels using the enzyme-linked immunosorbent assay (ELISA) method Samples were homogenized in 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM phenylmethylsulfonyl fluoride (PMSF; 1 g tissue: 5 mL buffer). Then, they were centrifuged at 12,000×*g* for 10 min to form a pellet. Subsequently, the supernatant resulting from sample homogenization was collected and analyzed. The concentrations of the cytokines TNF-α, IL-1β, and IL-6 of the articular cartilage samples were measured by ELISA according to the manufacturer's instructions (R&D Systems, USA). For this purpose, 96-well plates were sensitized with 100 μL/well monoclonal antibody against each cytokine: anti-IL-1β and anti-IL-6 antibodies were diluted in sodium carbonate buffer (0.1 M, pH 9.6), whereas the anti-TNF-α antibody was diluted in sodium phosphate buffer (0.2 M, pH 6.5). The plates were incubated at 4 °C for 18 h. For blocking, the plates were washed four times in phosphate-buffered saline solution with 0.05 % Tween 20 (PBST) and then filled up with 300 μL/well blocking solution (3 % gelatin in PBST, Sigma), which remained in the plates for 3 h at 37 °C. Then, they were subjected to a new cycle of four washes. Subsequently, 100 μL/well properly diluted samples or recombinant cytokine standards was added to the plate, which was stored for 18 h at a temperature of 4 °C. After washing, 100 μL/well of the respective biotinylated antibodies specific for each cytokine was added and the plate was left standing for 1 h at room temperature (22 °C). After washing the plates, 100 μL/well streptavidin-peroxidase was added and the plate was left standing for 1 h at room temperature (22 °C), followed by a new cycle of four washes. The reaction was developed by adding 100 μL/well 3,3′,5,5′-tetramethylbenzidine (TMB) solution and stopped by adding 50 μL/well sulfuric acid (H_2SO_4, 2N). Spectrophotometric readings were performed in a SpectraMax® Plus 384 Absorbance Plate Reader (Sunnyvale, CA, USA) with 450 nm wavelength and correction to 570 nm. Sample concentrations were calculated using the standard curves obtained with recombinant cytokines.

Table 2 Primer sequences

Primer	Following primer
HPRT	Forward: AAGCTTGCTGGTGAAAAGGA Reverse: TGATTCAAATCCCTGAAGTGC
IL-1β	Forward: TGACCCATGTGAGCTGAAAG Reverse: GGGATTTTGTCGTTGCTTGT
IL-6	Forward: ACCACCCACAACAGACCAGT Reverse: CAGAATTGCCATTGCACAAC
TNF-α	Forward: CAGAGGGAAGAGTTCCCCAG Reverse: CCTTGGTCTGGTAGGAGACG

Assessment of PGE_2 levels present in blood plasma measured using the ELISA method Immediately before sacrificing, 3 mL of blood samples were collected from the animal by cardiac puncture using a heparinized syringe to collect plasma. After collection, the material was centrifuged and only the supernatant was analyzed. Quantification of PGE_2 levels was performed using the kit (R&D Systems, Minneapolis, MN, USA) containing anti-PGE_2 antibody adhered to the plate. Initially, 150 μL/well solution with PGE_2 antigen at different concentrations was added to draw the standard curve. Then, 100 μL sample was added to the microtiter plate wells with 50 μL primary antibody and 50 μL PGE_2 conjugate. The plate was incubated for 16 h under stirring. Following this, plate washing was performed with 200 μL/well wash buffer solution for four times. Then, the kit substrate solution (solution A + solution B, 1:1) was added to the plate. After the time indicated in the kit leaflet elapsed, 50 μL/well 2N H_2SO_4 was added. Spectrophotometric readings were performed in a SpectraMax® Plus 384 Absorbance Plate Reader (Sunnyvale, CA, USA) with 450 nm wavelength and correction to 570 nm.

Statistical analysis

Initially, data were tabulated and assessed for normality using the Shapiro-Wilk test. An analysis of variance (ANOVA) was used, followed by application of the Tukey's test for multiple comparisons, since a normal distribution was present. Results were expressed as mean and standard error of the mean (± SEM), and values of $p < 0.05$ were considered statistically significant.

Results

RT-PCR gene expression analysis

Figure 1 shows the levels of IL-1β, IL-6, and TNF-α gene expression in the articular cartilage of all experimental groups. We only observed changes in IL-1β gene expression, with increased expression after OA induction in our experimental model in OA groups and in groups treated with Exerc, NSAID, Exerc + NSAID, and Exerc + NSAID + PBMT. Furthermore, we observed that the PBMT and Exerc + PBMT were the only therapies effective in reducing IL-1β gene expression compared to the control group.

Protein expression analysis by ELISA

Figure 2 shows the IL-1β, IL-6, and TNF-α protein expression levels in the articular cartilage of all experimental groups. We observed increased protein expression after inducing OA in our experimental model. We found that the NSAID, PBMT, Exerc + PBMT, and NSAID + PBMT therapies effectively reduced IL-1β protein expression. Furthermore, all treatments effectively reduced IL-6 and TNF-α protein expression. However, PBMT and Exerc + PBMT were more efficient in reducing IL-1β, IL-6, and TNF-α protein expression than the other treatments.

Analysis of PGE_2 levels by ELISA

Figure 3 shows the blood plasma PGE_2 levels of all experimental groups. Our experimental model of OA induction noticeably caused increased PGE_2 levels, whereas all therapies, except Exerc, reduced its plasma levels. However, PBMT and Exerc + PBMT were more effective in reducing PGE_2 than the other therapies.

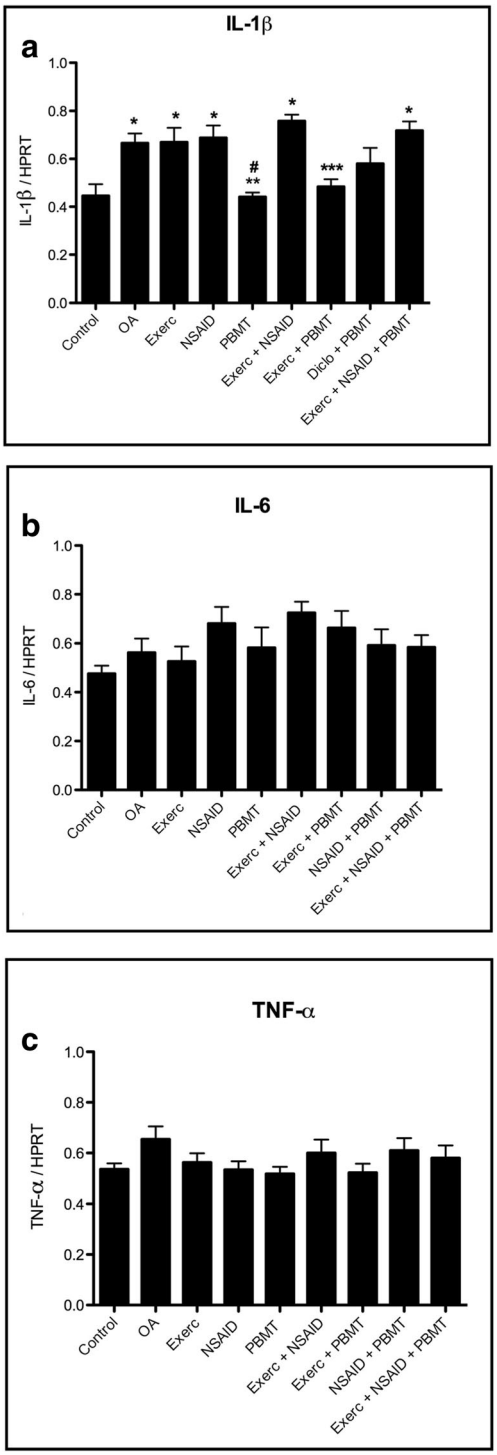
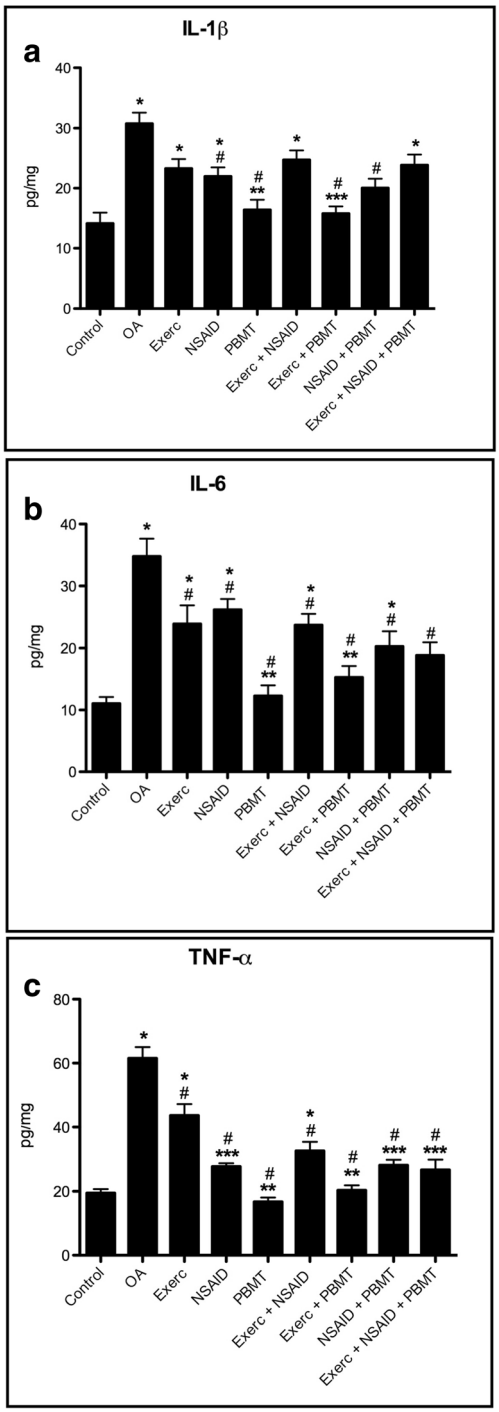


Fig. 1 IL-1β, IL-6, and TNF-α gene expression levels in the articular cartilage of all experimental groups. In **a**, $*p < 0.05$ indicates a significant difference from the control group. $\#p < 0.05$ indicates a significant difference from the OA group. $**p < 0.05$ indicates a significant difference from the Exerc, NSAID, Exerc + NSAID, and Exerc + NSAID + PBMT groups. $***p < 0.05$ indicates a significant difference from the Exerc + NSAID and Exerc + NSAID + PBMT groups. Panels **b**, **c** show no significant changes. Data are expressed as mean ± SEM



◀ **Fig. 2** IL-1β, IL-6, and TNF-α protein expression levels in the articular cartilage of all experimental groups. In **a**, $*p < 0.05$ indicates a significant difference from the control group. $\#p < 0.05$ indicates a significant difference from the OA group. $**p < 0.05$ indicates a significant difference from the Exerc + NSAID group. $***p < 0.05$ indicates a significant difference from the Exerc + NSAID and Exerc + NSAID + PBMT groups. In **b**, $*p < 0.05$ indicates a significant difference from the control group. $\#p < 0.05$ indicates a significant difference from the OA group. $**p < 0.05$ indicates a significant difference from the Exerc, NSAID, and Exerc + NSAID groups. In **c**, $*p < 0.05$ indicates a significant difference from the control group. $\#p < 0.05$ indicates a significant difference from the OA group. $**p < 0.05$ indicates a significant difference from the Exerc and Exerc + NSAID groups. $***p < 0.05$ indicates a significant difference from the NSAID, NSAID + PTBM, and Exerc + NSAID + PTBM groups. Data are expressed as mean ± SEM

First, it is noteworthy that the results showed that our experimental model of OA induction by papain injection was both effective in triggering an inflammatory response as evidenced by the release of classical inflammatory markers and is consistent with the earlier findings of Alves et al. [13] and Dos Santos et al. [14].

Our results indicated that PBMT, applied at 6 J per point (100 mW, 60 s), effectively modulated the underlying inflammatory process in induced OA, reducing both the levels and expression of key inflammatory markers. Furthermore, PBMT possibly contributed to slowing disease progression because proinflammatory cytokines play a key role in this process.

The studies by Milares et al. [18] and Assis et al. [19] analyzed the effects of PBMT (alone), physical exercise (alone), and the combination of both therapies in OA induced in rats. However, Milares et al. [18] used an aquatic exercise program, whereas Assis et al. [19] used a treadmill running

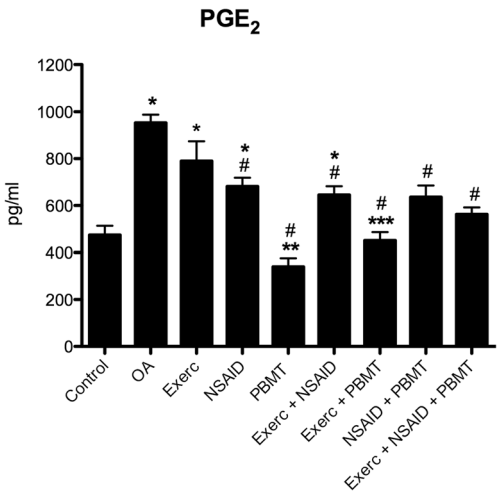


Figure 3 Blood plasma PGE_2 levels of all experimental groups. $*p < 0.05$ indicates a significant difference from the control group. $\#p < 0.05$ indicates a significant difference from the OA group. $**p < 0.05$ indicates a significant difference from the Exerc, NSAID, Exerc + NSAID, NSAID + PTBM, and Exerc + NSAID + PTBM groups. $***p < 0.05$ indicates a significant difference from the Exerc, NSAID, Exerc + NSAID, and NSAID + PTBM groups

Discussion

Thus far, the present study is the first one, to our knowledge, to assess the effects of combining PBMT, topical NSAID use, and physical exercise on the inflammatory response underlying an experimental model of OA in rats. In addition, we evaluated the effects of these therapies when used as monotherapy in our study.

protocol. Both studies showed that all therapies effectively reduced some degenerative aspects and inflammatory mediators, including IL-1β, for example. Unlike the aforementioned studies [18, 19], we determined that not all therapies used effectively modulated the inflammatory response underlying experimental OA in our study, since PBMT had an advantage over the other therapies. Such a distinction most likely resulted from the different parameters used because the effects of PBMT are dose-dependent; that is, they depend on the dose of PBMT delivered to the tissue [20], thereby confirming that the parameters used in the present study were adequate.

Similar to our study, Alves et al. [13] and Dos Santos et al. [14] also observed that PBMT applied alone reduced the levels of proinflammatory cytokines in OA induced in rats. Likewise, Wang et al. [21] determined that PBMT (alone) was able to control synovial inflammation and has a protective role against cartilage degradation in OA induced in rabbits. Furthermore, Castano et al. [22] showed that PBMT effectively reduced the levels of inflammatory markers, including PGE_2, and the therapy effects vary depending to the parameters used. Lastly, our outcomes corroborate to Oshima et al.'s [23] study that investigated effectiveness of PBMT in rabbits' knee OA. The authors observed modulation of inflammatory process using PBMT, through decreased TNF-α.

Furthermore, we concluded that physical exercise alone was ineffective in modulating the underlying inflammatory process in experimental OA in rats, as previously shown by Muniz Renno et al. [24], Milares et al. [18], and Assis et al. [19]. However, the physical exercise protocol used in the present study was different from all aforementioned studies, and this factor may have negatively contributed to our results. Furthermore, the effects of physical exercise on proinflammatory cytokine expression in OA remain unclear and require further studies in this area.

Similarly, we observed that the use of topical NSAID alone was unable to modulate the underlying inflammatory response in induced OA, even though the benefits from using these drugs in OA cases are widely reported [25]. The study by Pallotta et al. [11] showed that intramuscular NSAID use was able to reduce the levels of PGE_2, which was corroborated by our study. Conversely, Pallotta et al. [11] reported that treatment with intramuscular diclofenac decreased the levels of IL-1β and IL-6, unlike the findings from the present study. However, it is noteworthy that the route of administration in both studies was different, thus possibly explaining the disparate results.

Finally, our results corroborate the findings of Milares et al. [18] and Assis et al. [19] regarding combination therapies, which show that combination therapies were unable to promote additional effects supporting its use (combination therapies). In the present study, we also determined that no extra benefits justifying the use of the combination therapies tested have been found. Furthermore, it is noteworthy that combining PBMT with physical exercise showed favorable results, most likely from the effects of PBMT (alone) and not from combining the therapies because the data were very similar to those observed when applying PBMT (alone).

It is difficult to figure out why combined treatments were less effective than PBMT as single therapy. It is possible that the order of treatments led to a negative interaction between therapies. In this sense, further studies investigating the same combination of treatments in different order are needed. For instance, maybe if PBMT was applied before exercises, a positive interaction could be observed [26–30].

We believe that our results are relevant because they show that PBMT (alone) may be used as an alternative therapy to pharmacological treatment in OA since PBMT effectively modulates the inflammatory process and has demonstrated no contraindications or adverse effects to date. Moreover, the practice of physical exercise (alone) used for treating OA must be further examined to enable establishing an effective protocol for modulating the underlying inflammatory process in this disease.

Lastly, based on the PBMT parameters, the type of physical exercise, and the dose and route of administration of the NSAID used in the present study, combination therapies should not be considered as first-line treatment for modulating the underlying inflammatory process in OA. Supporting evidence for this contention includes that they fail to enhance the beneficial effects observed using each therapy alone, lengthen treatment sessions, and increase treatment costs.

Conclusion

Our results indicate that PBMT used alone is the best alternative among the therapies tested in the present study because it reduces the expression of proinflammatory cytokines (both gene and protein) and PGE_2 levels. However, further studies are required for a more detailed analysis of combination therapies.

Compliance with ethical standards Experimental protocol was approved by the Ethics Committee for Animal Experimentation (number 077, page 130, book 02) of the Institute of Biomedical Sciences, University of São Paulo (ICB-USP).

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