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Photobiomodulation (λ=808nm) and Platelet-Rich Plasma (PRP) for the Treatment of Acute Rheumatoid Arthritis in Wistar Rats

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Abstract

Introduction: Rheumatoid arthritis (RA) causes inflammation, pain, edema, and articular degradation and its treatment can be based on anti-inflammatory drugs, photobiomodulation (PBM) and/or platelet-rich plasma (PRP) that can decrease cell flow and promote local healing. In the present study, we evaluate the effects of PBM and PRP on acute arthritis in Wistar rats through inflammatory and oxidative stress parameters.

Methods: Thirty female Wistar rats were assigned to five groups (n=6, each group): Control, Sham, PRP, Laser, and PRP+Laser. For arthritis induction, all animals of groups Sham, PRP, Laser and PRP+Laser received an intraarticular injection of Zymosan® (200µg) in the right knee. Twenty-four hours post-arthritis induction, PRP was prepared and injected (8×10^5 of platelets) in animals of PRP and PRP+Laser groups. PBM was performed in Laser and PRP+Laser groups by single-dose therapy with the GaAlAs laser (λ=808 nm, P=25 mW, fluence=30 J/cm², beam area=0.02 mm², t=33 seconds, E=0.825 J, punctual application). After seven days of induction, serum samples were collected and thiobarbituric acid reactive substances (TBARS), nitric oxide (NO) and catalase activity were analysed. Morphological parameters were measured for inflammation areas, cartilage thickness, and C3 protein expression in knee samples. Statistical analysis was performed with an ANOVA test and Tukey's post-hoc test with a significance level of 5% ($P<0.05$).

Results: NO was lower in the treated groups compared to the Sham group, and TBARS did not show any differences, while catalase showed greater activity between PRP+Laser versus PRP ($P<0.05$). Inflammatory areas and cartilage thickness were lower in the treated groups compared to Sham ($P<0.05$), while no differences in C3 protein expression was observed.

Conclusion: PBM associated with PRP is better for anti-inflammatory and joint preservation by morphological aspects and NO levels that concern a potential clinical application.

Keywords: Arthritis, Joint diseases, Platelet-rich plasma, Laser therapy

Introduction

Rheumatoid arthritis (RA) is an autoimmune pathology of unknown etiology, and it is considered a chronic inflammatory process, but with periods of acute exacerbation. In addition, it affects three times as many women as men, and its prevalence increases with age.[1] It is characterized by symmetrical peripheral polyarthritis, which causes deformity and destruction of small and large joints. Articular changes include pain, swelling, and limited movement of the affected joints by several pro-inflammatory markers.[2] The diagnosis of arthritis consists of the association of several clinical symptoms and signs, laboratory findings, and radiological exams, which are based on the classification criteria of the American College of Rheumatology (ACR).[3]

RA is basically treated with pharmacological drugs including nonsteroidal anti-inflammatory drugs, steroids and disease-modifying antirheumatic drugs associated with physiotherapy and electrotherapy, while in severe cases, a surgical procedure may be recommended.[3] Among the electrotherapeutic resources, photobiomodulation (PBM) by a low-level laser is highlighted due to its anti-inflammatory, analgesic and healing effects, being a non-invasive alternative and without the side effects of the

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drugs.[4]

Due to the low level of the laser, indirect pathways are active in mitochondria by near-infrared light, leading to physiological effects such as an increase in vascular vasodilation without thermal injury, improvements in local cell tropism, changes in gene expression, pro-inflammatory mediators, and acceleration in healing.[5]

Angiogenesis is crucial in the healing process and platelets are important to promote hemostasis; these compounds are released at the beginning of the injury process and responsible for secreting growth factors such as vascular growth factor (VGF) and fibroblast growth factor (FGF), which provide an increase in collagen synthesis, thus contributing to the tissue regeneration process.[6] Platelet-rich plasma (PRP) contains a high concentration of growth factors, such as platelet-derived growth factor (PDGF), transforming growth factors (TGF), vascular endothelial growth factor (VEGF), and Epidermal growth factor (EGF).[6,7] However, the role of PRP in the repair of articular cartilage is still poorly understood and requires further investigation.[6,8]

Considering the lack of studies on the role of PBM and PRP in acute arthritis, the aim of this study is to observe the effects of PBM with laser therapy (λ = 808 nm) associated with PRP on improving the articular tissues reorganization by modulation of the inflammation process and oxidative stress.

Materials and Methods

Animals, Experimental Groups, and Arthritis Induction

Thirty female Wistar rats (60 days old, 180±20 g) were provided with regular standard rat food and water ad libitum throughout the experiment in a room with a 12-hour light–dark cycle. Animals were randomly assigned into five groups: Control, without arthritis induction (n = 6); Sham, Zymosan-induced arthritis (n = 6); PRP, Zymosan-induced arthritis and treated with PRP (n = 6); Laser, Zymosan-induced arthritis and treated with laser (n = 6); PRP+Laser, Zymosan-induced arthritis and treated with laser and PRP (n = 6).

At time zero, all animals of groups Sham, PRP, Laser, and PRP+Laser were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) solution, and arthritis induction was performed in the right knee of the posterior limb with an intraarticular injection of Zymosan® (200 µg/50 µL saline solution).

PRP Preparation

On day one, after anesthesia with ketamine (80 mg/kg) and xylazine (10 mg/kg), whole blood was collected from 4 healthy male animals (volume approximately 20mL) by a cardiac puncture to obtain the PRP. The blood was centrifuged for the first time at 760 rpm for 20 minutes. Then, only the plasma with the buffy coat was centrifuged at 1.200 rpm for 10 minutes. Finally, a portion of the plasma (50%, corresponding to platelet-poor plasma) was discarded to obtain the PRP. The final volume of PRP was approximately 10% of the total blood volume, and thus the volume of PRP was approximately 2 mL.[9]

PRP and Photobiomodulation Treatment

The animals of groups PRP and PRP+Laser, previously anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), received 50 µL of PRP (8×10^5 of platelets) in the intraarticular region (joint infiltration type application) one day after the induction of arthritis by Zymosan.[10]

PBM by low-level laser therapy was performed with a gallium and aluminum arsenide device (GaAlAs - DMC Equipamentos, São Carlos, Brazil), Magnus Plus model, λ = 808 nm, nominal power of 25 mW, fluency of 20 J/cm^2, beam area of 0.02 mm^2, time of 33 seconds, total energy of 0.825 J with punctual application in the right patellar region of groups Laser and PRP+Laser animals by single-dose therapy. Laser therapy was performed 24 hours after induction. In the animals of the control and Sham groups, the laser was applied in an off position, simulating the time and stress that can be caused.

Euthanasia and Sample Collection

Samples were collected from all groups after seven days of induction. The animals were euthanized by anesthetic overdose with ketamine solution (240 mg/kg) and xylazine (30 mg/kg) and as a second method to death confirmation cardiac puncture was performed. The samples were collected from the right knees of all animals and from whole blood to obtain serum for systemic analysis of the presence of systemic reactive oxygen species, thiobarbituric acid reactive substances (TBARS), and nitric oxide (NO) and catalase enzyme activity.

Histological Processing and Morphological Analysis

Knee samples were maintained in 10% formaldehyde and processed histologically following laboratory routine (descaling, dehydration, diaphanization, and blocking). Histological sections were prepared in a microtome with 5 µM of thickness and slides were stained with hematoxylin-eosin (HE). The photo documentation was performed using a Leica DFC300 FX microscope and the images were analyzed using the Image J program (NIH/USA, free program). For morphometric evaluation, the measurements of inflammatory areas of the synovial membrane (µm^2) and the thickness of the femur articular cartilage (µm) were obtained from the collection of three digital images from each of the five sections (n = 15 images/animal) documented from the middle region of the knee joint of four animals in each experimental group.

C3 Complement Fraction Protein Expression

Protein expression of C3 complement fraction was evaluated by immunohistochemistry briefly, and slides

were submitted to antigenic recovery, anti-C3 antibody incubation (1:250, sc-28294, Santa Cruz Biotechnology) and biotinylated secondary antibodies DAKO LSAB (DAKO, Glostrup, Denmark) incubation with horseradish peroxidase. Sections were washed and then incubated with streptavidin biotin-peroxidase complex and 3,3′-Diaminobenzidine (DAB) solution. Positive immunoreactive cells (number of cells in 10^4 μm^2) were evaluated by ImageJ (NIH/USA, free program) using reaction as described before by our group.[11]

Determination of Reactive Oxygen Species and Systemic Catalase Activity

The TBARS levels were determined by adding 200 µL of serum in 2 mL of 1% thiobarbituric acid prepared in buffer solution (50mM NaOH; 10M NaOH; 20% H_3PO_4) that was heated for 10 minutes and cooled afterwards. 3mL of n-butanol was added to the reaction tubes and they were submitted to agitation and centrifuged at 1500 rpm.[12] The determination of NO was performed with 50µL of serum with an equal volume of Gries reagent.[13] Catalase activity was performed from the variation (Δ) of hydrogen peroxide consumption by the catalase enzyme (final absorbance less initial absorbance) with 500 µL of serum sample, 500 µL of phosphate buffer and 30 µL of hydrogen peroxide.[14] All analyses were performed in triplicate.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software version 5.0 with the ANOVA test with Tukey's post-test and a significance level of 5% ($P<0.05$) was set to analyze the measurements of synovial inflammation areas, C3 protein expression, femur articular cartilage thickness, TBARS, NO and catalase activity.

Results

Morphological Analysis

The analysis of the inflammatory areas after seven days in the animals of the control group showed the absence of an inflammatory process and normal joint aspect without inflammation, preserved Synoviocytes and articular cartilage without degradation (Figure 1A). In the Sham group, extensive inflammatory infiltrates, synovial

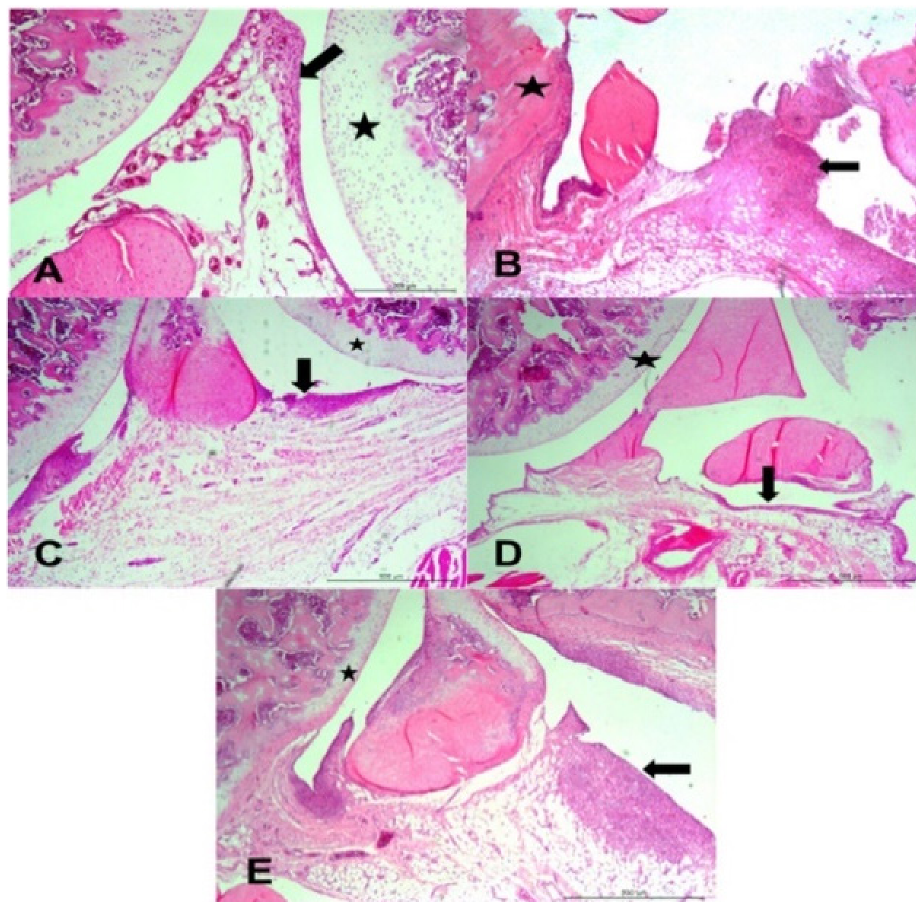

Figure 1. Analyses of the Inflammatory Areas Performed in Animals of Control (A), Sham (B), PRP (C), Laser (D) and PRP+Laser (E) Groups in the Period of 7 days. In the Sham group, the high level of inflammation is noticeable in relation to the groups of isolated/combined therapies. Arrow – Inflammatory infiltrate. Bar=100 µm. (*) – Articular cartilage.

disorganization with hyperplasia and degradation of the articular cartilage were observed (Figure 1B). In the animals of the PRP and PBM groups (Figure 1C and 1D), inflammation was observed only in the synovial membrane without joint degradation. In the group that had the combined therapies, on the other hand, the synovial region with near normality with the presence of synoviocytes, new vessels, and intact joint capsule as well as the articular cartilage were observed, although inflammation of the synovial membrane was observed (Figure 1E).

Synovial Inflammation and Cartilage Thickness

The measurements of the inflammatory areas (µm^2) were expressed as mean± standard deviation (SD) between the Control, Sham, and treated groups. Inflammation areas were present in the Sham group while in the control group they were absent, and they were smaller in the groups that underwent treatment when compared to the Sham group, close to the control group. The C3 protein expression, mean ± SD showed no differences in the number of positive cells between the experimental groups (Figure 2). The measurements of the articular cartilage of the femur (µm) were expressed as mean ± SD. Greater preservation of cartilage was observed in the control group in comparison with the Sham and the treated groups. Among the treated groups, all groups were statistically significant when compared to the Sham group, although the laser group had the best results between the groups (Table 1).

Reactive Oxygen Species and Catalase Activity

All data related to the analysis of reactive oxygen species and catalase activity are consolidated in Table 1. Oxidative stress evaluation through systemic quantification revealed higher levels of NO in the animals of the Sham group compared to the control group. The treated groups were effective in comparison to the Sham group, decreasing the levels of NO. TBARS levels did not differ significantly among the groups. Considering the consumption of catalase, significant differences were observed only between the PRP+Laser and Laser groups. The data obtained in the latter were lower than those in the PRP+Laser group.

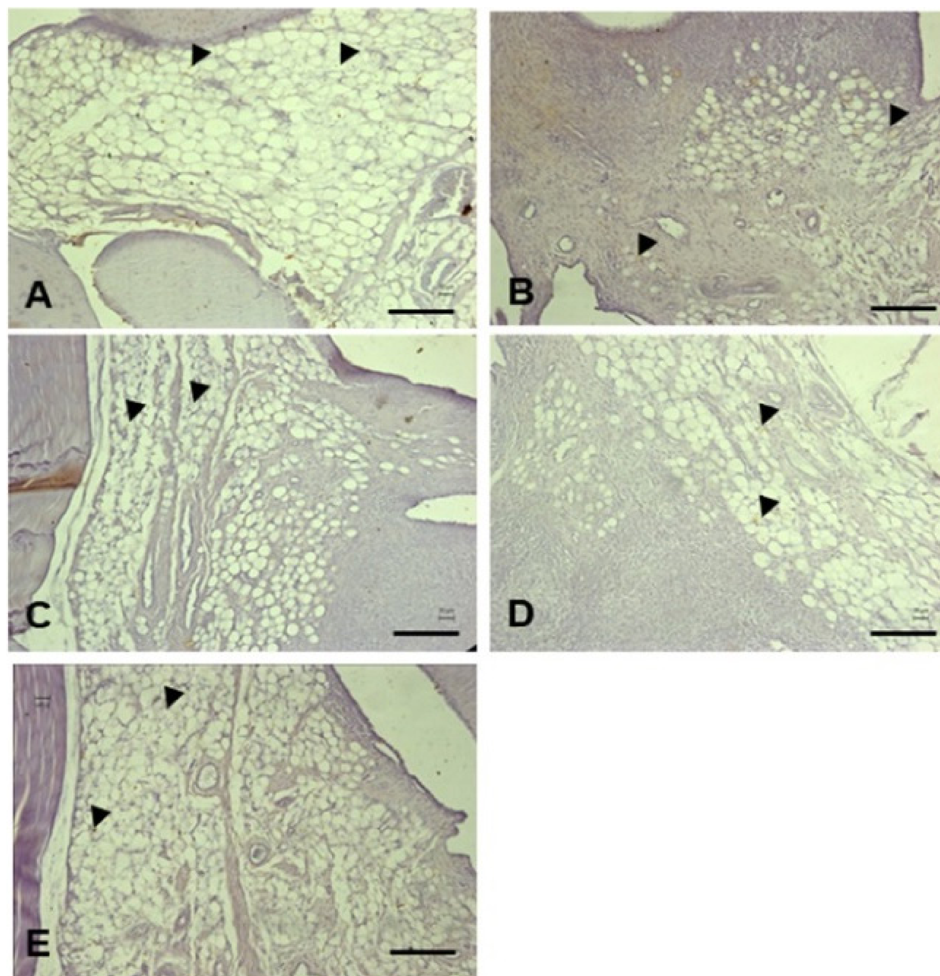

Figure 2. Immunohistochemical Localization of C3-epitope in Synovial Membrane From Animals of Control (A), Sham (B), PRP (C), Laser (D) and PRP+Laser (E) Groups in the Period of 7 Days. Arrowhead – positive cells. Bar=100 µm.

Table 1. Morphometric and Biochemical Parameters Evaluated in Articular Tissues and Blood Serum From Animals of Different Experimental Groups

Group/Parameters	Inflammation Areas (μM)	C3 Expression (Positive Cells)	Cartilage Thickness (Femur μM)	Nitric Oxide (μg/mL)	TBARS (μmol/L)	Catalase (Δ Consuption)
Control	9.55±1.675	0.4667±0.6399	53.61±2.819[b*]	3.40±1.287	128.0±22.38	0.058±0.0179
Sham	187.8±56.95[a*]	0.4667±0.5164	24.89±2.810[b*]	7.73±1.314[c*]	108.3±31.50	0.0672±0.0071
PRP	59.89±8.042[a*]	0.2667±0.4577	39.21±0.8576[b*]	3.06±0.938[d*]	126.7±34.96	0.0535±0.0070
Laser	15.15±3.554[a*]	0.4000±0.5071	43.41±1.533[b*]	3.06±1.722[e*]	121.2±36.34	0.0586±0.0046
PRP+Laser	13.55±1.067[a*]	0.2667±0.4577	26.83±1.432[b*]	2.55±0.727[f*]	116.5±24.49	0.0754±0.0093[g*]

[*]**Significant difference**. [a]ControlXSham; ShamXPRP; ShamXLaser; ShamXPRP+Laser $P<0.0001$. [b]ControlXSham/PRP/Laser/PRP+Laser; ShamXPRP; ShamXLaser; PRP+LaserXPRP; PRP+LaserXLaser $P<0.0001$. [c]ShamXControl $P=0.0043$; [d]PRPXSham $P=0.0043$; [e]LaserXSham $P=0.0087$; [f]PRP+LaserXSham $P=0.0079$. [g]PRP+LaserXPRP $P=0.0225$.

Discussion

This study aimed to evaluate the treatments with PRP, PBM with low-level laser therapy and combined therapies to control the inflammatory process and systemic levels of oxidative stress in female animals, a fact related to a higher incidence of RA in the female population.[15] In our study, acute inflammation and oxidative stress were analyzed in an experimental model of arthritis by Zymosan, whose intra-articular application promotes arthritis induction through the stimulation of nociceptors.[16,17]

Oxidative stress has a direct relationship with diseases of acute and chronic progression, such as RA.[18] In our study, one of the parameters to evaluate the oxidative stress was NO which showed high levels in the Sham group compared to the control and treated groups. These results are related to the presence of inflammation in the Sham group. On the other hand, the treated groups with a low-level laser can be stimulated by the cytochrome c oxidase pathway in mitochondria, which results in lower levels of NO by an increase of the antioxidant system.[19]

There are several markers of oxidative stress such as nitrotyrosine, NO, and TBARS, and these are related to deleterious actions of the joint system, such as senescence and cartilage aging that can be a consequence of the synovial inflammatory process.[20]

Systemic oxidative stress is closely related to the activation of inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor (TNF-α) and complement pathways after NF-KB activation. In our findings, synovial inflammation was controlled in the animals that underwent PBM and both treatments, and NO levels were reduced in all proposed treatments.[21]

In a study evaluating the systemic levels of oxidative stress in patients with RA, no differences were observed in the levels of TBARS while in the analysis of the levels of superoxide dismutase (SOD), a chain precursor of Catalase activation, the levels were lower in healthy patients; these data corroborate those found in our study in which treatments were effective in controlling oxidative stress by catalase levels and synovial and joint preservation.[22]

The groups treated with both therapies showed a catalase increase that demonstrates a positive effect once this enzyme works as an antioxidant complex, playing a balancing role in oxidative stress.[23] Catalase acts as a second enzymatic response in the degradation of the superoxide anion after conversion by SOD to hydrogen peroxide, with the laser attenuating these effects.[24]

PBM with a low-level laser has been used to promote healing by its anti-inflammatory potential. PBM decreases pro-inflammatory markers and edema and the local repair is accelerated.[25] These effects were evidenced in the animals treated with PRP and low-level laser, and significant differences were observed in morphological aspects when compared with the Sham group.

Therapy based on the use of PRP is extremely controversial. A study evaluating injured muscles showed an increase in the levels of antioxidant enzymes, including SOD and catalase acting as an antioxidant,[26] whereas in injured tendons, PRP treatment increased the levels of iNOS[27] that is a precursor and can increase NO levels. In our study, NO showed lower values when using isolated and combined therapies that leads us to a possible mechanism of secondary activation of antioxidant enzymes. In the treatment of arthritis and osteoarthritis, PRP is important in controlling pain and joint function when the intraarticular injection is used, although the method for producing PRP and number of doses is controversial in the literature; in our study, only one dose of PRP was used.[28-30]

Oxidative stress can be a pathway to start the joint inflammatory process, and the C3 fraction of the complement is an extremely important marker to understand the inflammatory dynamics, although it did not reveal differences among the experimental groups, which was an unexpected response in our study. C3 release products release vasoactive amines and leukotrienes leading to increased capillary permeability and influx of cells, that is, stimulation of the inflammatory process. This fact can be related to the phase in which our study was carried out, seven days' post-induction (acute phase of inflammation), once the expression and release of C3 may have occurred in the previous phase, so the evaluation of super-acute periods is important.[31]

Due to the local improvement, the laser can be associated with the use of PRP. The exact mechanism is

not yet elucidated, yet laser has angiogenic properties that can be related to the release of PRP growth factors. In our study, the decrease of synovial inflammation was observed and morphology was preserved. Furthermore, neoangiogenesis was observed when both treatments were proposed.

Related to the synovial inflammatory process, a previous study conducted by our group using an induced arthritis model treated with PBM showed that at 7 days, the irradiated laser group had a similar number of granulocytes in relation to the induced and untreated group; these cells are related to the inflammatory process.[32] Our results for inflammatory regions showed an improvement in the morphological aspects with PRP and the laser. Both treatments were used in relation to Sham, and the best results were observed in both treatments.

PBM with the low-level laser has the ability to decrease the flow of inflammatory cells, production of pro-inflammatory cytokines, and the activity of metalloproteinase matrix in the synovial region, which is especially responsible for synovial degradation and can also contribute to articular cartilage degradation. These side effects on the cell influx are linked to the absorption of light by photoreceptors within subcellular components resulting in the activation of respiratory chain enzymes, mainly cytochrome C oxidase inside the mitochondria and the sodium-potassium pump.[33,34]

The control of synovial inflammation is crucial to articular cartilage maintenance and our results showed greater cartilage thickness in all treatments, and laser treatment showed the best values. The results corroborate previous findings of our group after laser treatment in a model of microcrystalline arthritis that showed values next to normal cartilage.[35]

On the other hand, the application of PRP is still unclear; it was observed that when applied to the inflamed tissue, it is activated by endogenous thrombin or collagen, quickly activating growth factors such as PDGF and VEGF which help in stimulating the platelet, responsible for the synthesis of fibroblasts, which results in the repair of injured tissue.[36]

At the moment, when PRP is injected into the injured site, platelets are activated by endogenous thrombin or like in our study by intra-articular collagen. Once activated, growth factors are secreted through the degranulation of these compounds. Secreted substances include PDGF, interleukin-1 receptor antagonist (IL-1RA), soluble tumor necrosis factor (TNF-RI) receptors, TGF, platelet factor 4 (PF4), VEGF, Epidermal growth factor (EGF) and insulin-like growth factor.[36-38]

In a systematic review of clinical studies, the benefit of PRP treatment for cartilage injury in knee and hip osteoarthritis was evidenced, temporarily decreasing pain and improving the function of the involved joints, with positive results in comparison with several alternative treatments.[39]

The synovial inflammatory process is a key process within joint degradation, and in the PRP group, there was a decrease in the inflammatory process compared to the Sham group, although lasers or combined therapies have led to more positive results. This may be associated with increased vascularization and hyperemia that PRP promotes, and when injected, it stimulates the VEGF secretion.[40] In synovial cells isolated from humans it has been observed that PRP provides a complex combination of molecules that can induce positive cellular responses within the joint.[41]

In a study for the treatment of arthritis induced in rabbits, PRP was effective in inhibiting the development of synovitis and loss of the cartilaginous matrix, especially in the initial phase. These data are in line with our study in which the effects of PRP and the association with the laser were analyzed after seven days in the acute phase of the disease.[42]

Joint degradation is a secondary effect of synovial changes caused by arthritis. In this context, the decrease of synovial inflammation observed in our study and the greater cartilage thickness in the groups treated in relation to the Sham group corroborate the increase in chondrocyte differentiation and maturation in rabbits[43,44] and the decrease of COX-2 and TNF-α expression[45] with PRP therapy, while the maintenance of the collagen matrix that allows collagen recovery can be observed with low-level laser therapy.[46]

In another study with a low-level laser ($\lambda = 4$J and 50 mW) in experimental RA, the laser showed a positive effect on reducing inflammatory mediators and cells.[47] Evaluating the effects of the laser in the acute phase, we observed a reduction in inflammatory areas and better tissue organization, and the literature describes better synovial organization and reorganization of adipocytes after 7 days of arthritis induction.[48]

The effects of the association of PBM with a laser ($\lambda = 810$ nm and 100 mW) and PRP were observed in the reduction of pain in patients with knee inflammation in our study; the pain measurement (nociception) of the animals was not performed, but the reduction of the inflammatory process was observed. We can suggest that with less inflammatory process, lower levels of leukotrienes and prostaglandins responsible for pain during inflammation are released.[49]

To the best of our knowledge, this is the first study that seeks to elucidate mechanisms related to treatment with PBM and PRP in the control of acute inflammation and oxidative parameters.

Conclusion

PBM associated with PRP has anti-inflammatory effects and promotes joint preservation. Morphological aspects were better with the proposed therapies, decreased or

maintained constant levels of oxidative stress, although they did not play a regulatory role in the C3 expression pathway in the studied period. Studies must be developed to demonstrate the efficiency of non-invasive therapies such as PBM with laser and PRP, doses, and parameters since these therapies showed biostimulant effects on arthritis inflammation, which leads us to the possible improvement of pain and the quality of life with potential clinical application.

Ethical Considerations

All experimental procedures were conducted under the ethical guidelines of National Commission for Animal Welfare (COBEA), Brazil, submitted and approved by Animal Research Ethical Committee of Centro Universitário da Fundação Hermínio Ometto, protocol number 077/2017.

Conflict of Interests

The authors declare no conflicts of interest.

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