Photodiagnosis and Photodynamic Therapy 29 (2020) 101619

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

The role of periodontal treatment associated with photodynamic therapy on the modulation of systemic inflammation in the experimental model of asthma and periodontitis

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ARTICLE INFO

Keywords:
Asthma
Periodontitis
Lasers
Periodontal treatment
Photodynamic therapy

ABSTRACT

Background: The association of Periodontitis (P) with several systemic diseases, among them asthma (A), has been previously studied. As periodontal treatment (TTO) associated with photodynamic therapy (PDT) is able to treat P, the aim of this study is to verify whether periodontitis exerts systemic effects on asthma, and whether TTO, associated or not with PDT, is capable of altering the systemic course of both pathologies.

Methods: 64 male Balb/c mice were divided into 8 groups (n = 8): Basal (B), P, P + TTO, P + TTO + PDT, Asthma, A + P, A + P + TTO, A + P + TTO + PDT. After 43 days, all animals were euthanized. The total and differential leukocyte count in serum, platelet count, alveolar bronchial lavage cell count, femoral lavage cell count in addition to the reactivity of the trachea, lung edema and gingiva cytokines were analyzed. The frequency of inflammatory cells was assessed via flow cytometry. One-way ANOVA test was used, followed by the Student-Newman-Keuls post-test.

Results: There was an increase in the number of blood circulating eosinophils in group A when compared to group B ($p < 0.01$); this characterized the asthma experimental model. P ($p < 0.05$) presented a lower amount of cytokine TNF-α in the gingiva when compared to the Asthma group. Apart from that, there was no statistical difference found for the other analyzed parameters.

Conclusion: These data contributed to elucidate that P and A, associated or not with TTO and PDT, are not able to interfere with the systemic parameters of Balb/c mice.

1. Background

Periodontitis is the most common disease worldwide and severe periodontitis affects 11.2 % of the population [1]. It´s impact continues to grow as evidenced by an increase 57,3 % [2–5] over an interval of 10 years (1990–2010).

Periodontitis is a dysbiotic disease [6] characterized by the loss of periodontal ligament insertion and the destruction of adjacent bone and connective tissues [7]. Innumerous immunopathological and inflammatory events are involved with the onset and progression of periodontal disease. As such, further studies are needed to understand the predominant inflammatory profile of periodontitis [8].

The immune response in our body is regulated by the balance between the regulatory T cells (Th1 and Th2). Discovering which profile characterizes periodontal disease is of great importance in order to guide the employment of possible therapies. In the Th1 profile, IL-2 (Interleukin-2) and IFN-γ (Interferon gama) are produced and stimulate a cellular response, mainly mediated by macrophages and neutrophils

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https://doi.org/10.1016/j.pdpdt.2019.101619
Received 22 October 2019; Received in revised form 3 December 2019; Accepted 5 December 2019
Available online 10 December 2019
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(resulting in a periodontal disease with a more stable characteristic in its early stage). However, IL-4 production suppresses this type of response, enhancing the humoral immunity characteristic of the Th2 profile (progressive periodontal disease with progressive characteristics) [8]. As the presence of bacteria is still a triggering factor of the disease, mechanisms that prevent adhesion, colonization and proliferation of these pathogens [9] are still the most effective form of treatment. In this study, the therapy of choice is scaling and root planing (periodontal treatment - TTO), considered the gold standard in the literature [9]. It may or may not be associated with antibiotic therapy (metronidazole and amoxicillin) in refractory cases [10]. However, this type of intervention should be used with caution due to bacterial resistance [11]. To overcome this problem, current Dentistry has increasingly opted for the use of Photodynamic Therapy (PDT). Although still under-explored, it has shown to be quite promising as a disinfection method [12], mainly in the treatment of chronic periodontal disease [13–16]; it is able to reach deeper areas in the periodontal pockets in addition to reaching difficult to access bone defects, without causing a systemic effect and without generating bacterial resistance [14].

The antimicrobial effect of PDT has been widely studied and recognized as effective; nonetheless, more recent studies have demonstrated a new approach to PDT, in which the immune system is recruited as the main defense mechanism. According to Gollnick et al. [18], the long-term efficacy of PDT depends on a normoreactive adaptive immune system. Particular attention should be paid to subversion of oral bacteria against the host immune response, which would allow them to remain in the inflamed subgingival tissue [19]. This condition facilitates its spread to distant sites, such as the lungs. In these sites there may be an interruption of immune surveillance and homeostasis, which then triggers or accelerates pathogenic processes [20,21].

A recent systematic review suggests a strong association between asthma and periodontal disease [22], but the lines of study are still quite divergent. Some biologically plausible mechanisms have been proposed to explain how periodontitis induces systemic changes [23] and how microorganisms residing in the oral microenvironment can access lung tissues [24]. These include: aspiration of oral and pulmonary bacteria, whose niche is the oral cavity; the remote action of inflammatory mediators and / or immune complexes of infected periodontal tissues; the hematogenous pathway, in which periodontopathogens access the circulation after the junctional epithelium has been inflamed through day-to-day procedures (such as flossing or brushing) [25] or after dental procedures [26]. It is noteworthy that in asthma, pulmonary inflammation is restricted to the airway mucosa, with little evidence of systemic inflammation [22]. This in turn would justify a more systemic approach to the disease in order to draw better parallels with other pathologies. In 2010, a study aimed at evaluating the immunoregulatory mechanism of asthma in the presence of periodontitis [27] was performed. In this study, mice infected with *P. gingivalis*, after challenge with Ovalbumin (OVA), presented increased Th2 cytokines (IL-4, IL-5 and IL-13) and an influx of inflammatory cells from the airways in relation to the control (without sensitization). Although interesting, this study did not address the periodontal treatment of mice. So, given the high prevalence of asthma and periodontitis and their strong correlation, it becomes important to understand the mechanisms of action underlying these two diseases.

2. Material and methods

The present study was conducted according to the guidelines given by the National Council for the Control of Animal Experimentation (CONCEA), as well as the standards from the Canadian Animal Care Council (1993) and the Brazilian National Congress (1995). It was submitted to the of the University of Nove de Julho's (UNINOVE) Animal Ethics Committee, with CEUA: 020.2017. 64 healthy adult male Balb / C mice, 2 months, ~ 25 g, were placed in appropriate plastic boxes at a minimum temperature of 22 °C and a maximum of 25 °C with controlled luminosity in 12 -h cycles, alternating between light and dark, with artificially controlled ventilation and 50–60 % relative humidity. Ration and filtered water was provided for the mice *ad libitum*. They were divided into 8 groups (n = 8): (1) Basal (B) - not manipulated; (2) with P; (3) with P + TTO; (4) with P + TTO + PDT, (5) Asthma, (6) A + Perio, (7) A + P + TTO, (8) A + P + TTO + PDT.

All animals in the Asthma group, A + P, A + P + TTO and A + P + TTO + PDT were first sensitized subcutaneously with 0.20 ml of a 150 µl Ova solution (Sigma®) together with 0.39 ml of 6 % aluminum hydroxide (EMS®), which works as an adjuvant (it increases the immunogenicity of the allergen [28]), diluted in 2.6 ml of 4 % PBS, on the first (sensitization) and on the seventh day (challenge). Starting on the fourteen day, asthma inductions occurred three times a week (on interspersed days) via the nebulization method (with 10 ml of 1 % Ova in 10 mice) for five weeks.

On the 21 st and 22nd day, the mice from the P, P + TTO, P + TTO + PDT, Asthma, A + P, A + P + TTO and A + P + TTO + PDT groups were submitted to anesthesia applied with an intramuscular syringe, medially, in the middle third of the thigh. For the lower molar ligature technique, materials developed for the research were used. Two metal supports served as pillars for a # 5 cotton thread that was stretched between them and used to wrap the upper central incisors of the mouse, keeping the mouth open; the mandible was supported on a flat support; the tongue was displaced with a retractor developed for the study and the interproximal space between the first and second molars was expanded with K type endodontic files in sizes # 15 and # 20. The ligature was made with very thin suture thread, inserted between the first and second lower molars, in the gingival sulcus, fixed with a node.

After 14 days of periodontitis induction, the P + TTO, P + TTO + PDT, A + P + TTO and A + P + TTO + PDT groups underwent periodontal treatment, consisting of scaling and root planing (the same procedure performed in humans) with a 5–6 mini-five curette on every gingival sulcus of the molar that received the ligature. Ten scraping movements were standardized on the buccal surface and 10 on the lingual surface. In the groups that were still given photodynamic therapy, after the gold standard periodontal treatment, 0.005 % methylene blue 0.05 mg / ml was inserted into the gingival sulcus of the molar that contained the ligature Three minutes after the insertion of the dye [29], the time needed for the PS to act, the region was irradiated with a diode laser in the red wavelength (Table 1).

After 43 days of asthma sensitization (baseline), all animals in the study were euthanized. It was performed exaguinition through the abdominal cava vein. Tail blood was collected for differential and total blood cell and platelet counts. Dissection and fixation of lungs in 10 % formalin was performed for histomorphometric analysis. The trachea was collected and weighed for the tracheal reactivity test. The femur was dissected to obtain the femoral lavage. Finally, gingiva located

Table 1
Diodo Laser Parameters.

Parameters	Values/Treatment
Wavelength [nm]	660
Operating mode	Continuous
Radiant power [mW]	100
Irradiance [W / cm2]	3,5
Beam Area [cm2]	0,02826
Exposure time [s]	90
Radiant exposure [J / cm2]	318
Radiant energy [J]	9
Number of irradiated points	2
Application technique	Contact
Number of sessions and frequency	Single session
Total irradiated energy [J]	18

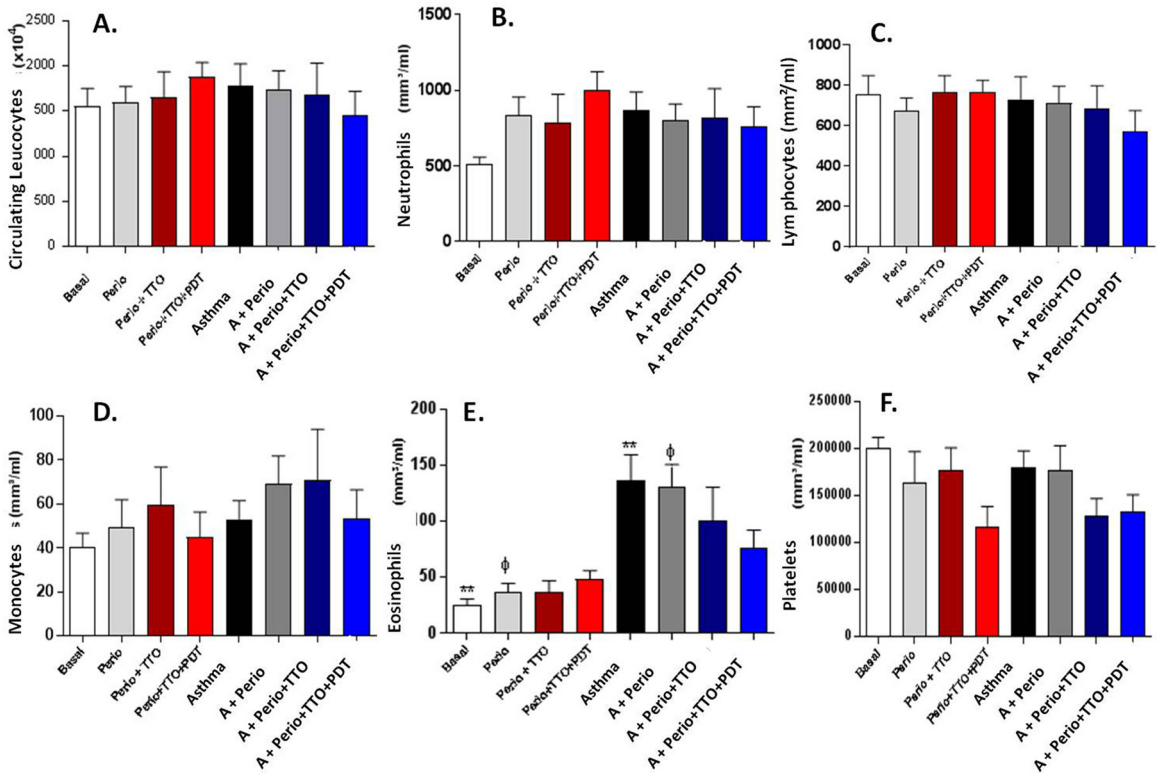

Fig. 1. Variation in the number of serum cells within the groups. A - total count of leukocytes; B- Neutrophil; C- Lymphocyte; D- Monocyte; E- Eosinophil; F- Platelets.

around the molar that contained the ligature (including its equivalence in those that did not undergo induction of periodontitis) was dissected and the lungs were stored in an oven to calculate their dry weight in relation to wet weight (edema).

Global and differential leukocyte count: In a Neubauer chamber (Turk's solution): 20 microliters of blood was collected and diluted in 380 microliters of the Turk Solution (1:20 Dilution). The leaflet was then fixed into the Neubauer chamber and filled with diluted blood, thus permitting the number of leukocytes present in the 4 outer quadrants to the central cross to be counted; the total value found was then multiplied by 50. Each quadrant has an area of 0.1 cm, 4 quadrants = 0.4 cm, thus, 0.4 cm / 20 (dilution factor) = 50

2.1. Global platelet count

Using the Giemsa-colo-phonium method, the total amount of red blood cells was first determined via the Neubauer chamber (hemocytometer method in Neubauer chamber) using 3980 µl of Hayem's solution and 20 microliters of blood. The leaflet was then fixed into the Neubauer chamber and filled with the diluted blood, thus providing the red blood cells count for the number present in the 5 quadrants. The total value found was then multiplied by 10,000. This count was performed for the sole purpose of discovering the platelet count.

2.2. Lung samples

Pulmonary tissue samples were stored in 10 % formalin. For histomorphological analysis, the slides were stained with Hematoxylin and Eosin (HE) (tissue histology and remodeling - peri-bronchial space cells and alveolar distension) and picrosirius (reorganization of collagen fibers). The morphometric technique allowed quantitative analysis and morphological parameters to be analyzed using Image Pro Plus software (version 4.5, NIH, Maryland, USA). For the photographic analysis of the slides, the Olympus BX43 microscope and the Cellscens Standard program were used. Cell differentiation and collagen reorganization were analyzed via Image J pro plus software version 7.0. 3.11.

2.3. In vitro tracheal reactivity

The isometric force of the airway contraction was quantified using tracheal rings mounted on a 15 ml insulated organ bath with two steel hooks. Maximum contraction was determined using a displacement force transducer coupled to a data acquisition system (Powerlab®, LabChart, AD Instruments). For this purpose, tracheal rings were suspended in continuously aerated Krebs-Hanseleit (KH) solution.

2.4. Analysis of lung edema

After the separation of tissues from the euthanized animal, the lower posterior lobe of the lung was harvested and weighed immediately on a precision scale (BEL®). After this was done, the fragments were left in an oven (ECB 1.1 digital®), at a controlled and constant temperature of 37 °C and new measurements were taken at 0, 4 and 24 h.

2.5. Evaluation of pulmonary inflammation in BAL

After exposure to the trachea, a cannula was gently placed inside it so that the lung could be washed with PBS for cell collection. The volume of the recovered lavage was centrifuged at 1500 rpm for 10 min at 4 °C. For analysis of the cytokines the supernatant was transferred to another microcentrifuge tube and stored at -80 °C. Using the ELISA method, the inflammatory and regulatory cytokines were evaluated. The cell button was resuspended in 1 ml of PBS and used for counting the total number of cells in Neubauer's chamber. The LBA differential cell count was made via flow cytometry

2.6. Collection of mandibles

A flap of vestibular gingival tissue was removed and stored at

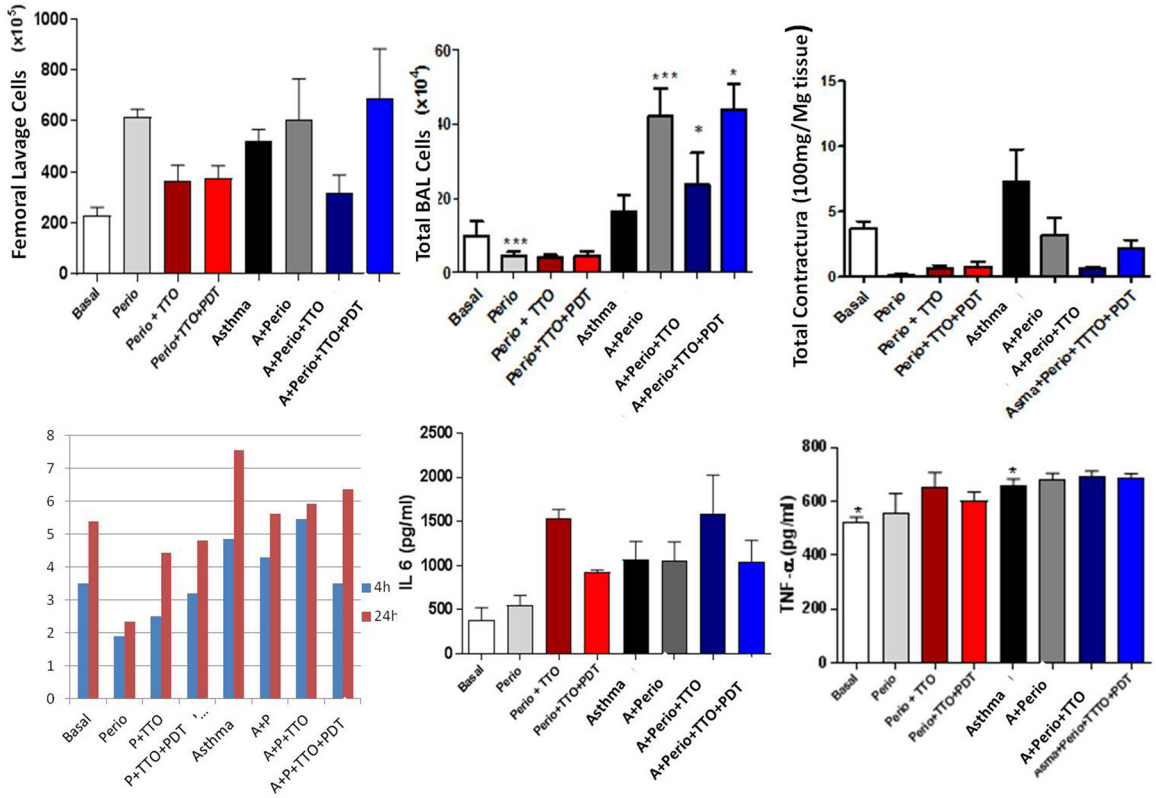

Fig. 2. Variation in the number of serum cells within the groups.
G- *femoral lavage cells; H- Total cells in the BAL; I- Tracheal Reactivity; J- Pulmonary edema; K- IL-6 in the gingiva;* L-*TNF-α in the gingival.*

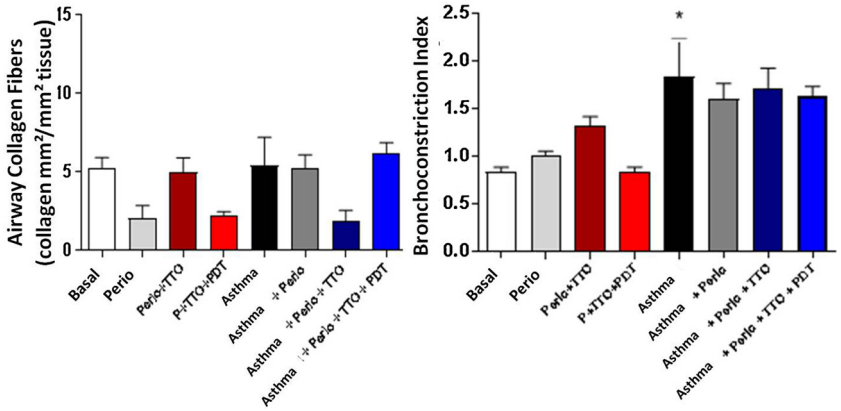

Fig. 3. Pulmonary evaluation. *M- Collagen quantification in the airways; N-Bronchoconstriction Index.*

−80 °C for quantification of local cytokines. The pieces were then fixed in 10 % buffered formalin solution. After this, the samples underwent a process of demineralization and inclusion in paraffin. The sections were then dewaxed, hydrated in ethanol, washed in distilled water and stained with hematoxylin-eosin. Next the slides were dehydrated using ethanol, then diaphanized with xylol and mounted with Permount. The microscope slide containing the most detailed cut was selected. The slides were stained for HE (histological analysis), Picrossírius (evaluation of collagen fibers) and von Kossa (quantification of osteoclasts).

3. Results

Among these analyses, the only one presenting a statistical difference between the groups was eosinophils (E.), where the Asthma group had a higher circulating eosinophil count in the blood compared to the Basal group ($p < 0.01$). And the A + Perio group had higher numbers of circulating eosinophils when compared to the Perio group ($p < 0.05$) (Fig. 1)

Additionally, we only found a statistical difference for BAL cells (H.), where ***p was < 0.005 of the Perio group in relation to the A + Perio group; and *p was < 0.05 of the A + P + TTO group compared to the A + P + TTO + PDT group. In addition, there was a difference in TNF-α cytokine in the gingival (L.), where the Perio group had a higher amount of cytokine when compared to the Basal group ($p < 0.05$). On the other hand, the Asthma group presented a greater amount of TNF-α when compared to the basal group. (Fig. 2)

The Asthma group also presented a greater amount of bronchoconstriction in the airways (N.) than the Basal group ($p < 0.05$) (Fig. 3)

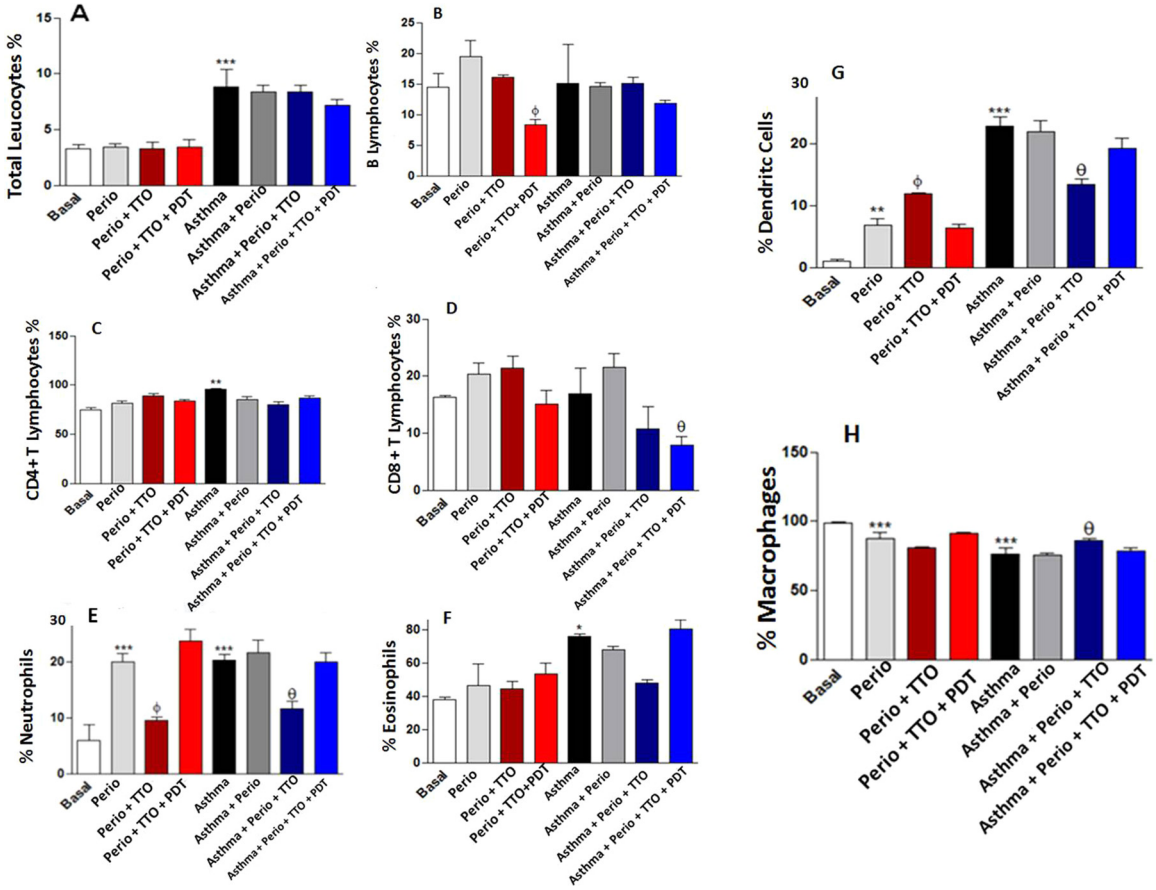

Fig. 4. Attachment 1 - Flow cytometry of the total number of cells recovered from BAL. A- % Total leukocytes; B- % B lymphocytes; C- % CD4 + T lymphocytes; D- % CD8 + T lymphocytes; E- % Neutrophil; F- % eosinophil; G- % Dendritic Cells; H- % Macrophages.

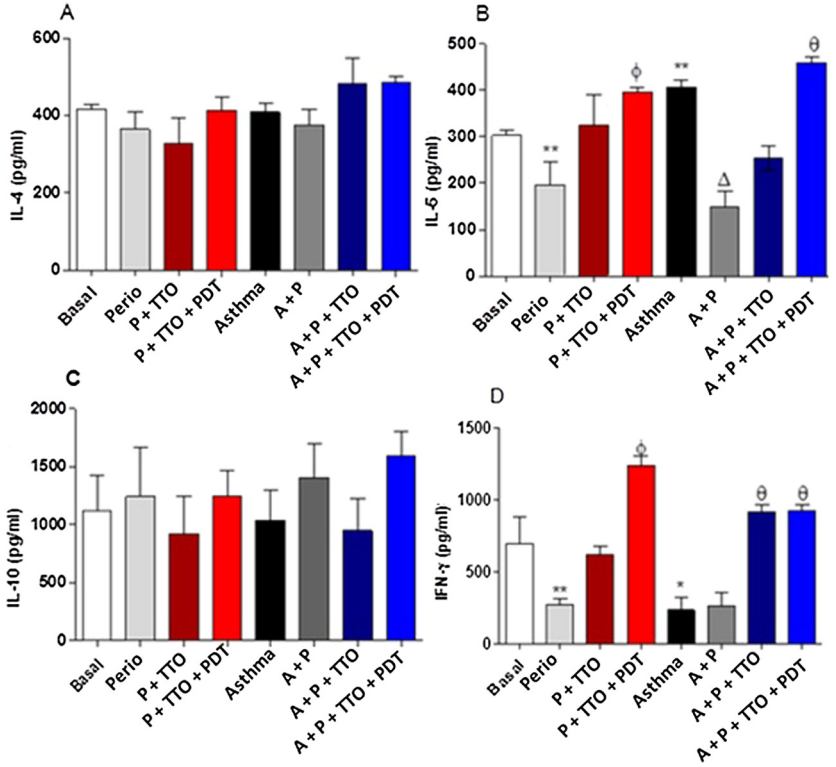

Fig. 5. Attachment 2 - **Analysis of cytokines IL-4 (A), IL-5 (B), IL-10 (C) and IFN-$_{\gamma}$ in serum.**

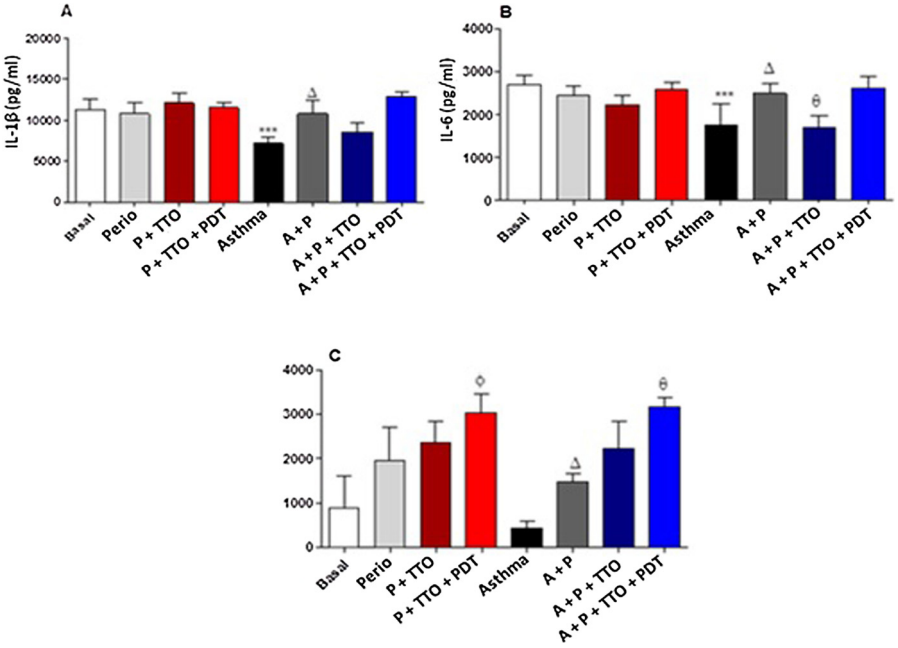

Fig. 6. Attachment 3 - **Analysis of cytokines IL-1β (A), IL-6 (B), TNF-α (C) in serum.**

Attachment 1 - There was an increase in leukocytes ($p < 0.001$) only for the Asthma group in relation to the Basal group. Decreased B lymphocyte ($p < 0.01$) for the group Perio + TTO + PDT in relation to the group Perio. Increased CD4 + T lymphocytes ($p < 0.01$) for the Asthma group in relation to the Basal group. Reduction of TCD8 + lymphocytes ($p < 0.001$) for the Asthma + P + TTO + PDT group compared to the Asthma + Perio group. Neutrophil increase ($p < 0.001$) for the group Asthma + Perio in relation to the Basal group; and reduction ($p < 0.01$) for the Perio + TTO group in relation to the Perio group; Θp < 0.1 for the A + P + TTO group in relation to the A + Perio group. Increase in eosinophils ($p < 0.05$) for the Asthma group in relation to the Basal group. Dendritic cell increase ($p < 0.01$) for the Perio group compared to the Basal group; ($p < 0.001$) for the Asthma group in relation to the Basal group; ($p < 0.01$) for the Perio + TTO group in relation to the Perio group; ($p < 0.001$) for the Asthma + Perio + TTO group compared to the Asthma + Perio group. We observed macrophage increase (figure 20 H) ($p < 0.001$) for the group Asthma + Perio in relation to the Basal group; Θp 0 < 0.05 for the Asthma + Perio + TTO group in relation to the Asthma + Perio group (Fig. 4).

Attachment 2 – Analysis of cytokines IL-4 (A), IL-5 (B), IL-10 (C) and IFN-$_\gamma$ in serum. The groups consisted of: Basal (unmanaged animals), Perio (periodontitis), Perio + TTO (periodontitis + periodontal treatment), Perio + TTO + PDT (periodontitis + periodontal treatment + photodynamic therapy), Asthma (animals sensitized and challenged with OVA), A + Perio (asthmatic animals + periodontitis), A + Perio + TTO (asthmatic animals + periodontitis + periodontal treatment), A + Perio + TTO + PDT (asthmatic animals + periodontitis + periodontal treatment + photodynamic therapy). The results represent mean SEM ± of two experiments (n = 4–5). (A) There is no difference for any of the values analyzed ($p > 0,05$). (B) ** $p < 0,01$ relative to the Basal group; φp < 0001 compare to Perio group; Δ $p < 0001$ relative to the Asthma group; θ $p < 0001$ compared to the A + Perio group; (C) There are no difference for any of the analyzed values ($p > 0,05$). (D) *$p < 0,05$; ** $p < 0,01$ in relation to the Basal group; φp < 0001 compared to the Perio group; ϕp < 0001 compared to the A + Perio group for the A + P + TTO + PDT group and θ $p < 0,01$ compared to the A + Perio group for the A + P + TTO group (Fig. 5).

Attachment 3 - Analysis of cytokines IL-1β (A), IL-6 (B), TNF-α (C) in serum. The groups consisted of: Basal (unmanaged animals), Perio (periodontitis), Perio + TTO (periodontitis + periodontal treatment), Perio + TTO + PDT (periodontitis + periodontal treatment + photodynamic therapy), Asthma (animals sensitized and challenged with OVA), A + Perio (asthmatic animals + periodontitis), A + Perio + TTO (asthmatic animals + periodontitis + periodontal treatment), A + Perio + TTO + PDT (asthmatic animals + periodontitis + periodontal treatment + photodynamic therapy). The results represent mean SEM ± of 2 experiments (n = 4–5); (A) * $p < 0,001$ relative to the Basal group; Δp < 0,01 in relation to the Asthma group. (B)*** $p < 0,001$ in relation to the Basal group; Δ $p < 0,05$ in relation to the Asthma group; Θp < 0,05 in relation to the group A + Perio. (C) φp < 0.05 in relation to the Perio group; Δp < 0,05 compared to the Asthma group; θ $p < 0,01$in relation to the A + Perio group (Fig. 6).

4. Discussion

Periodontitis is traditionally considered a chronic oral inflammatory infection [30]. It is known that controlling this infection can reduce serum pro-inflammatory markers [31,32]. Some studies have correlated periodontitis with the development and exacerbation of lung diseases [22,33]. However, in asthma, pulmonary inflammation is restricted to the airway mucosa, with little evidence of systemic inflammation [34]. Clinically, the association of asthma and periodontitis does not always occur [35–38] and only two studies have evaluated its mechanism of action in vivo [27,39]. To date, no study has evaluated systemic parameters in the presence of both diseases nor the role of periodontal treatment, associated or not with photodynamic therapy, in the possible remission of these parameters. To study the systemic factors that influence periodontitis and asthma, we evaluated total and differential counts of the cells recovered in the serum (neutrophils, monocytes, lymphocytes and eosinophils). We observed that there was no change in any of these cells. There was only an increase in eosinophils for the A + Perio compared to the Asthma group, returning to basal levels after conventional periodontal treatment either alone or with PDT. There was also no change in total platelet count or in femoral lavage cells. As for the lung, there were no signs of edema or increases in collagen fibers in the airways.

Cells derived from bone marrow are considered an important source of mesenchymal stem cells in adults and are known to be effective in tissue regeneration through periodontal graft. A recent study [21] has demonstrated that bone marrow cells are mobilized for periodontal defects. In our study, we observed a slight increase in the number of marrow cells in the Perio group, Asthma group and in the A + Perio group. These results demonstrate the recruitment of mesenchymal cells in the presence of both periodontitis and asthma, and that periodontal treatment is able to suppress this process. The only group in which there was no remission was the A + P group which was treated with PDT, but there were no differences between these groups. More studies are needed to understand if there is any regulatory mechanisms in PDT [39]. Some studies have shown an increase in total platelet count in subjects with periodontitis [40,41] associated with systemic inflammation. It is interesting to note that in animal models of allergic airway inflammation, platelet depletion causes a profound reduction in the recruitment of eosinophils in the lung. Studies have suggested that eosinophil activation may be influenced by this alteration [42]. In our study, there was no difference in the total platelet count for any of the groups analyzed.

For TNF-α, a greater increase in circulating cytokine has been found in asthmatic patients than in patients with other chronic lung diseases [43]. However, in our study there was no systemic increase of this cytokine in the Asthma group. However it was interesting to observe that locally, in the Asthma group, there was an increase in TNF-α, unlike the other groups studied..

5. Conclusion

Therefore, it can be concluded that, in general, periodontitis was not able to cause systemic alterations after its installation in the mice periodontium. Although not very significant, PDT caused a slight increase in the systemic inflammatory response in mice with asthma. Notwithstanding this, it remains to be discovered which cells are involved in this process. This study has contributed to elucidate, in part, the relationship between periodontitis and asthma. Additional analysis are still needed to better understand the effects of PDT in the treatment of periodontitis in mice with asthma as it is not clear how periodontal treatment plays a role in adverse respiratory events [44]. It is believed that secondary factors linked to asthma treatment (such as medication use) may influence periodontitis progression [45,46]. Other studies have also suggested that periodontal treatment may decrease the frequency of adverse respiratory events [44] by improve lung activity [47], thus decreasing mortality [44]. Although a number of studies have reported the association between asthma and periodontitis [44–47], the role of periodontal treatment in human patients with asthma is not, as of yet, very well explored. For these reasons further studies are needed to more fully evaluate how periodontal treatment can influence the course of asthma.

Acknowledgments

This work has been supported in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)1690822, and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) 2017/14139-1 and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)2015/20535-1.

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