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Effects of photodynamic therapy mediated by nanoemulsion containing chloro-aluminum phthalocyanine: a histologic and immunohistochemical study in human gingiva

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ABSTRACT

Background: Photodynamic therapy (PDT) uses photosensitizing agents, which are delivered in target cells, followed by local application of visible light in specific wavelengths. This reaction produce reactive oxygen species able to induce cell death by apoptosis or necrosis, injured to the local vasculature, and exert important effects on the immune system.
Objective: The present work evaluated the clinical findings, histomorphological alterations and immunodetection of VEGF after PDT using chloro-aluminum phthalocyanine (AlClPc) entrapped in a lipid nanoemulsion in a split-mouth clinical trial.
Material and methods: Eight healthy volunteers with clinical indication for extraction were included in the study. Seven days before the extraction 40 ul of nanoemulsion AlClPc 5 μM was injected into gingival tissue followed by irradiation with diode laser, the contralateral side was used as control. Tissue specimens were removed seven days after the PDT and divided into two groups (test and control groups) for histological and immunohistochemical analysis. Patients were monitored at days, 0, 7, 14 and 30 to assess adverse effects of the therapy.
Results: The therapy was well tolerated by all patients. Adverse effects were short-time and completely reversible. Areas of edema, vascular congestion, and intense vascularization were viewed in gingival samples that received PDT. Additionally, dystrophic calcification was observed in subepithelial region. VEGF showed moderate to strong immunostaining in specimens subjected to PDT.
Conclusions: Taken together, the results showed that the protocol used in this study mediated by nanoemulsion containing AlClPc is safe for clinical application in gingival tissue and suggests that VEGF is increased after PDT.

1. Introduction

Photodynamic therapy (PDT) is a promising therapeutic modality that has been used in research and clinical investigations for various diseases such as malignant and non-malignant tumors, periodontitis, potentially malignant disorders and other oral lesions. This non-surgical treatment has been used to be minimally invasive and easy to perform besides presenting good results in clinical studies [1]. The principle of this therapy is the delivery of a photosensitizing agent in cells and tissues followed by the application of laser irradiation (low level laser). Absorption of photons

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by the photosensitizing drug promotes its transformation into an extremely unstable molecule that reacts with the molecular oxygen and form ions peroxides, superoxide and hydroxyl radicals, and singlet oxygen who are extremely cytotoxic [2–5].

In recent years, researches involving the mechanisms of PDT have been widely exploited mainly in regard to signal transduction pathways, transcription and cell cycle regulators factors, inflammation, and apoptosis. Mechanisms associated with the destruction of cells and tissues with the use of this therapy can be mediated by multiple signaling pathways that often overlap. Cell death, changes in local vascularization, and immunomodulatory effects are involved in photosensitization and may contribute to its therapeutic action in tissues [3,5,6].

Several photosensitizers have been used in the photo-activation of biological systems. Development of new photosensitizing agents is complex and requires experimental pre-clinical testing in several steps later to enter on stage of clinical research. Studies with chloro-aluminum phthalocyanine (AlClPc) has shown that this drug keeps all its photophysical and photochemical properties when administered associated with nano-carrieir, such as nanoemulsions in both in vitro and in vivo studies [7,8]. Analysis of mitochondrial activity and cell organelles [9,10]; analysis of cell viability, cytotoxicity and genotoxic potential in cells culture [11,12]; vascular damage, tissue necrosis [13] and reduction of metastasis to cervical lymph nodes [14] were investigated in preclinical studies. Results of the studies described above demonstrate the AlClPc as an effective drug with antitumor activity.

It is well known that PDT application produces a series of tissue effects that include inflammation and vascular shutdown in regions close to the target area. In general, these vascular effects are followed by an important tissue hypoxia that promotes the release of a series of molecular signals to recovery the normal conditions of the tissues. One of the central molecular pathways involved in this process is mediated by the Vascular Endothelial Growth Factor (VEGF), an essential mediator during the process of angiogenesis, which can be produced by different cell types in hypoxia conditions. Numerous authors describe an up-regulation of VEGF after PDT in tumor cells [15–18], and this mediator is related to tissue recovery post-treatment. Thus, the objectives of this study is to investigate the tissue and clinical effects, as well as the presence of VEGF, of normal gingival tissue after application of PDT mediate by AlClPC entrapped in a nanoemulsion in a split-mouth experimental clinical trial involving human volunteers.

2. Material and methods

2.1. Subjects and study design

Experimental protocol was reviewed and approved by the Institution's Human Research Committee and the protocol was approved on April 20, 2012 (protocol 0156.0.051.000-11, Natal, Federal University of Rio Grande do Norte, Brazil) and the experiments were undertaken with the understanding and written consent of each subject. Eight patients (5 females, 3 males, aged 23–63) presenting two teeth with a clinical indication for extraction were selected for the study. Absence of gingival bleeding on periodontal probing was the inclusion criteria for both teeth included in samples. Additionally, the patients had neither other oral or systemic diseases, nor any overt immunological abnormalities and did not take any preoperatory medication.

The study was performed using the split-mouth design in a non-randomized manner. A total of 8 pairs of contralateral maxillary or mandibulary teeth were included. In each contralateral pair, one tooth was assigned as control whereas the other tooth was treated with photodynamic therapy (PDT). All patients were treated by the same operator and the extractions of both teeth (control and treated) were performed 7 days after treatment by PDT. Gingival tissue samples, which otherwise would have been discarded, were obtained during surgery under local anaesthesia, avoiding local anaesthetic infiltration into the biopsy site, and deformation or compression of the samples. Gingival samples of control and treated teeth were obtained from the mesial and distal buccal marginal gingiva.

2.2. Photosensitizer solution and laser

Aluminum-chloride-phthalocyanine (AlClPc) was synthesized, purified, and provided by Aldrich Chemical Company (St. Louis, MO, USA). Incorporation of aluminum-chloride-phthalocyanine (AlClPc) into the nanoparticles was performed according to the method described by Muehlmann et al. [12]. A continuous red laser (660 nm, Bio Wave Dual LLLT Kondortech, São Carlos, São Paulo, Brazil) was used in this study. For the clinical PDT application, 4 min of laser irradiation with 30 mW of potency was applied in gingival tissue. The equipment had a spot laser of 0.03 cm^2, which confers an energy fluence of 7 J/cm^2 and a total irradiance of 30 mW/cm^2.

2.3. Treatment

Seven days prior to the extraction procedure, drug application was performed. Treatment consisted of injecting of 40 μl nanoemulsion containing chloro-aluminum phthalocyanine in the interdental papilla region (mesial or distal marginal gingiva—approximate length of a tooth face), without local anestesia (with a sterile insulin syringe and a minimum time 5 min of drug infusion). Fifteen minutes later, diode laser irradiation for 4 min (7 J/cm^2). Analgesics (acetaminophen, 750 mg/tablet, one tablet three or four times a day) were prescribed to the patients after PDT.

2.4. Clinical assessment

At baseline, 1 week, 2 week and 1 month after treatment, the adverse effects of the therapy were evaluated. Patient and investigator pain assessments were recorded on a 4-point pain scale (0 = no, 1 = slight, 2 = mild, 3 = moderate, and 4 = severe). Adverse effects including stinging, burning, erythema, purpura, blistering, or crusting were recorded on patient and investigator assessment scales (0–4, as above) [19]. Clinical photographs were taken at each patient's visit to evaluate the clinical outcome of PDT.

Patients were followed up at 7, 14 and 30 days. Were assessed: adverse effects, reports of complications, post-PDT pain; the effectiveness of the drug for the purpose of analgesia protocol; well as reports of the patient toward the therapy used.

2.5. Histology and immunohistochemistry

Seven days after the treatment by PDT, for each patient, the teeth were removed and gingival tissues were immediately fixed in 4% phosphate buffered formalin, pH 7.4 and processed by routine laboratory techniques for paraffin embedding Tissue specimens divided into two groups (test and control groups). Serial sections (5 μm thick) were obtained from all specimens. For all specimens was performed staining with hematoxylin-eosin and slides of all samples were reviewed to evaluate the magnitude of inflammation at the histologic level according to adaptation of Tsai et al. [20]. Each specimen was graded at 400× magnification as: grade 1 (slight), inflammatory cells less than 1/3; grade 2 (moderate), inflammatory cells between 1/3 and 2/3; and grade 3 (severe), inflammatory cells higher than 2/3. Gradation of each specimen was

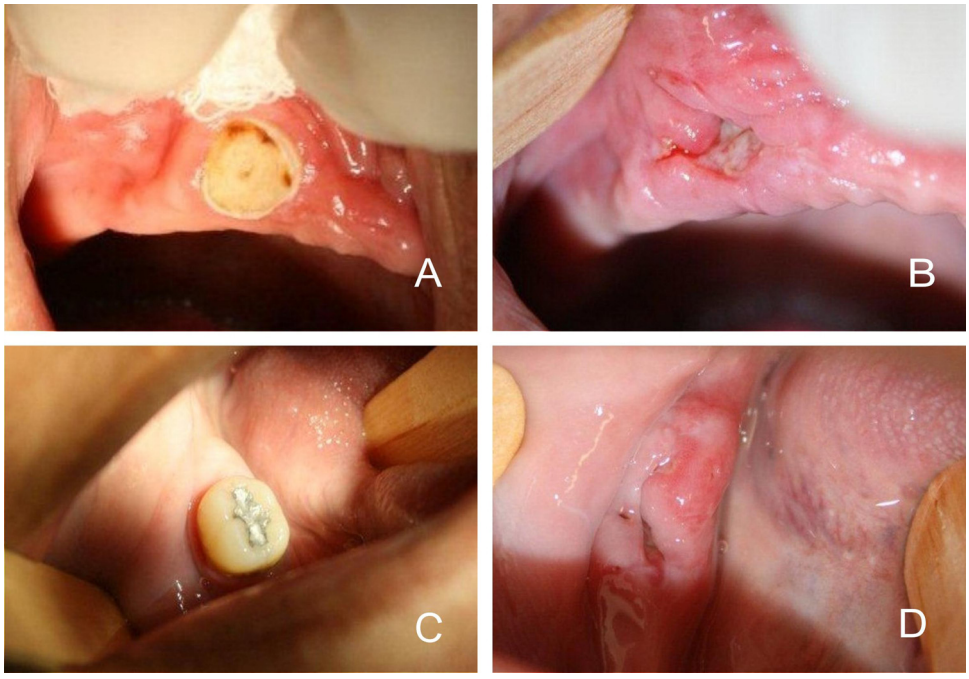

Fig. 1. Clinical aspect of regions after photodynamic therapy. (A,C) On day of delivered of the drug and irradiation; (B, C) 14 days after PDT.

based on the inflammatory condition starting from the epithelial-conective tissue border and proceeding gradually deeper into lamina propria. For immunohistochemistry, a policlonal antibodie anti-VEGF-A (C1, dilution 1:600, Santa Cruz Biotechnology) was used in avidin–biotin–immunoperoxidase (LSAB—Labeled Streptavidin Biotin), technique as previously described in the literature [21].

2.6. Evaluation method

Analysis of VEGF immunostaining was performed according to (i) the presence and (ii) the intensity of the staining (1 = weak, 2 = moderate, 3 = strong), as performed by Moriyama et al. [22]. Intensity of VEGF staining was evaluated according to the color of diaminobenzidine (DAB) under light microscopy. When the intensity of staining observed was identical to the endothelial cells of the specimen was assigned a grade 2. For the marking weaker or stronger than the endothelial cells in the sample was given a score of 1 or 3, respectively. Slideshow without immunostaining were marked with 0 (no immunostaining). Immunostained slides were scanned using the Pannoramic 3DHISTECH MIDI equipment (3DHISTECH Ltd., Hungary). Thereafter, they were analyzed, quantified and photographed with the virtual microscopy software Pannoramic Viewer, version 1.15.2 (© Copyright 2013 3DHISTECH Ltd., Hungary).

3. Results

3.1. Clinical application of PDT

During the infusion of AlClPc was observed that the sensitizer is easy to apply and handling. The drug was applied with no complications, no clinical signs of deterioration of the mucosa; and 2 in 8 patients ($n = 2$, 25%) complained of pain scored as 1 (slight). In 1 patient, edema was observed at the time of drug infusion ($n = 1$, 12.5%). During exposure to light source, 2 patients complained of a mild burning. Fig. 1 illustrates the region on day application of PDT and 14 days after application.

3.2. Adverse effects of PDT

Therapy was well tolerated by most patients. In all participants, 14 days after the PDT was shown edema and erythema. It was observed all of these events were of short duration and completely reversible. Safety assessments demonstrated that stinging/burning sensation after AlClPc application was scored as 0 (no) in 6 patients (75%) and 1 (slight) in 2 patients (25%) in day 7. There was a significant increase in erythema after laser irradiation and extraction. After 14 days, erythema was scored as 1 in 5 patients (62,5%) and 2 in 3 patients (37,5%). No purpura, crusting, or scarring occurred in any patient. Dysphagia and allergic reactions were not observed.

3.3. Histological findings

Specimens of the treated teeth showed collections of inflammatory cells, predominantly mononuclear, and areas of edema and vascular congestion (Fig. 2A–C), marking an exudative reaction. No correlations was found between the VEGF immunostaining and degree of inflammation in groups ($p > .05$, data not shown). Vascular modifications were observed in the gingival samples that received PDT. In two cases it can be observed the presence of numerous tiny blood vessels in the subepithelial region (Fig. 2D), and histological appearance of numerous vascular sprouts (Fig. 2C) characteristic of the process of neoangiogenesis. It is further highlight the presence of intense extravasation of plasma and interstitial fluid precipitated inside the blood vessels. In three cases were visualized the presence of an amorphous, acellular material, well-defined, basophilic (Fig. 2E, and F) and in proximity to blood vessels compatible with dystrophic calcification process. These findings were in the vicinity of areas with intense hemorrhagic extravasation and vascular congestion and in subepithelial region. In specimens of the control group was detected epithelial and connective tissue within the normal pattern.

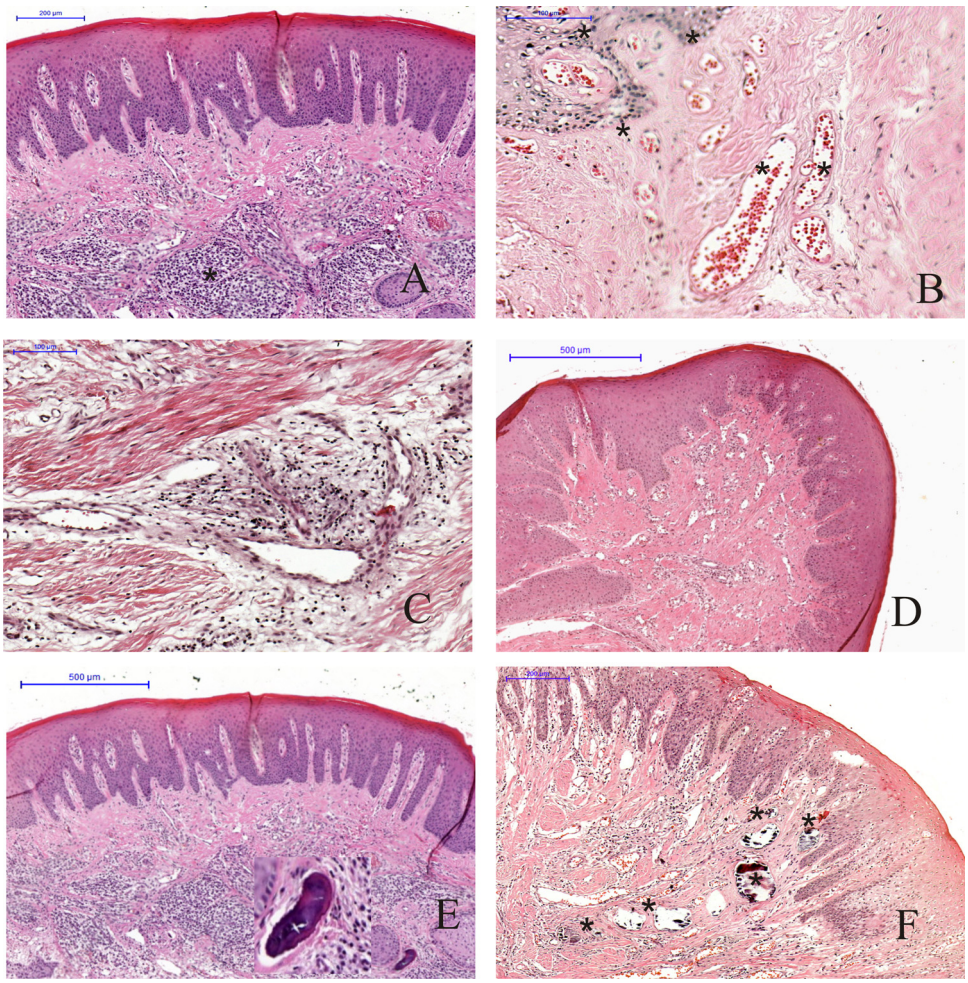

Fig. 2. Phtotomicrography of clinically healthy gingiva in test group. (A) The highlight shows collections of inflammatory cells in fibrovascular tissue (Pannoramic Viewer, HE, 200 µm). (B) Detail of numerous congested blood vessels in the subepithelial region (Pannoramic Viewer, HE, 100 µm). (C) Myxomatous connective tissue exhibiting edema, mononuclear cell infiltration, and numerous blood vessels with some vascular sprouts (Pannoramic Viewer, HE, 100 µm). (D) Large number of blood vessels in subepithelial region proliferation contributes to chronic inflammation. (Pannoramic Viewer, H.E., 200 µm). (E) Basophilic deposits in sub-epithelial region, consistent with dystrophic calcification process (Pannoramic Viewer, HE, 500 µm). Detail of calcification. (F) Basophilic deposits in subepithelial region, consistent with dystrophic calcification process. Asterisks highlight the vascular calcifications (Pannoramic Viewer, HE, 200 µm).

3.4. Immunohistochemical findings

VEGF showed a nuclear and cytoplasmic pattern of immunoreactivity. Immunostaining was visualized in endothelial cells, epithelial cells, fibroblasts and mononuclear inflammatory cells. Was observed a predominance of nuclear staining in basal and suprabasal layer of the epithelium, whereas at connective tissue cytoplasmic staining (Fig. 3). When present, muscles and blood vessels associated, also showed positive staining. No statistical difference was observed when compared VEGF in test and control group. Some cases of test group showed a tendency to present a strong pattern of expression for VEGF compared to control group. No positive staining was observed when primary antibodies were omitted. Positive control samples showed strong reactivity.

4. Discussion

Aluminum-phthalocyanine chloride is a highly hydrophobic photosensitizer that strongly aggregate in aqueous solutions, feature that decreases their photodynamic effect, and impairs its use in aqueous solutions alone. One well know strategy to prevent this aggregation and keep all the photophysical proprieties is the association of the photosensitizer with nano-carriers, such as the nanoemulsions used in this study. The nanoemulsion used in this study was previously developed showed that this nanocarrier kept, and sometimes improved the AlClPc proprieties [23] and several studies have demonstrated effective properties against tumors [11,13,14,24]. Currently only two studies in humans [8,18] have evaluated the effects of AlClPc in association with laser therapy. No side effects were reported in the eight volunteers included in this study when compared to other PDT protocols, especially those using aminolevulinic acid (ALA) which is painful requiring the use of local anesthetics [25–27]. The lack of side effects can be an advantage for using AlClPc-PDT. However, the repeated use of a nanoemulsion of AlClPc still needs further investigation since single doses have only showed short term and reversible adverse effects (erythema and edema). Erythema and edema 14 days after therapy can be the result of the local inflammatory response normally associated with the surgical extraction procedure.

Microscopic observations of primary vascular reactions on the specimens subjected to PDT, including vascular congestion, thrombose and edema were also observed by other authors [13,28–30]. Although some authors [11,13,24,28] have shown areas of necrosis in tumors after treatment with PDT, this finding was not observed in the samples treated with PDT, these results may be due to the drug's high affinity to cancer cells [11–13]. Since, this study utilized

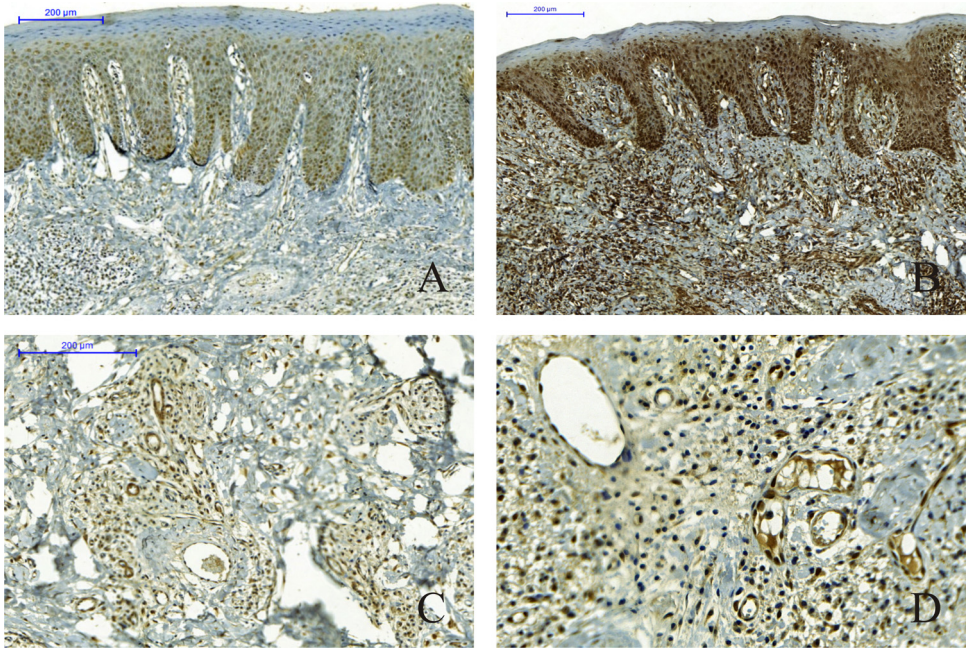

Fig. 3. (A) Photomicrograph showing moderate immunoreactivity for VEGF in basal and suprabasal layer of the epithelium and connective tissue of clinically healthy gingiva (control group). (Pannoramic Viewer, LSAB, 100 μm); (B) Photomicrograph showing strong staining for VEGF in the basal layer and suprabasal epithelial and connective tissue clinically healthy gingiva (test group). (Pannoramic Viewer, LSAB, 200 μm); (C, D) Photomicrograph showing moderate immunoreactivity of VEGF in endothelial and inflammatory cells (mononuclear type) of clinically healthy gingiva (test group). (Pannoramic Viewer, LSAB, 200 μm).

healthy tissues no tissue selectivity to the drug occurred which explains the lack of tissue necrosis.

The histological finding of a basophilic, amorphous and acellular material compatible with dystrophic calcification was noted on test group specimens. We believe that oxidative stress, induced by PDT, may have caused the abnormal tissue calcification. These calcifications often occur in tissues undergoing necrosis or degeneration [31]. and should be carefully evaluated since, the drug under investigation has anti-tumor properties, amongst them cell death by necrosis [11,13,14,24].

Vascular calcification is a process of active mineralization which occurs in both pathological and physiological conditions. This process is cell-mediated and is a result of the imbalance between factors that stimulate or inhibit mineralization. Under light microscopy, samples show initially acidophilic characteristics, followed by the presence of irregular basophilic granules (sometimes mistaken for bacteria) which converge or grow into larger granules. These granules may, sometimes, be fragmented during histological processing, similar to that observed in our specimens. Dystrophic or local calcifications do not cause signs or symptoms, however they may occasionally produce edema and tissue ulceration [32–34]. Thus, it can be suggested that PDT applied to the gingiva promoted injury to the mucosa, reaching the endothelial cells in the subepithelial layer, which may explain the alterations found in our specimens.

Oxidative stress is known as one of the main contributors to the immune response observed in PDT [6]. Release of pro-inflammatory cytokines after PDT activates transcriptional factors such as nuclear factor Kappa-B [5], as well as other growth factors such as VEGF. Oxygen reactive species lead to oxidative stress, tissue hypoxia and activation of hypoxia inducible factor (HIF-1), which in turn induces VEGF expression [15,35,36]. The drop in the circulating levels of oxygen, at the initial stages of PDT, acts as a stimulus for endothelial cells to release HIF-1 which in turn induces the expression of VEGF, a potent stimulator of mitosis for endothelial cells [15,35,36]. Some authors [15,16,35–39] have shown upregulation of VEGF after PDT. Our samples were evaluated 7 days after PDT by histology and immunohistochemistry and showed an increase in VEGF after PDT which is in agreement with other studies. However, the length of time between treatment and specimen harvesting was not disclosed in some of these studies.

Studies indicate that VEGF can promote calcification either by neovascularization or by directly affecting bone cells. In bone formation and regeneration, angiogenesis and bone resorption are two essential and intimately associated processes, which suggests a common mediator for both events. In this context, VEGF appears as a possible stimulator via secretion of growth factors and cytokines by endothelial cells, which can direct mesenchymal cell differentiation towards the osteogenic pathway and osteogenesis [36,40]. Increased VEGF levels in the areas treated with PDT may favor a phenotypic change of endothelial cells into osteoblastic cells, thus promoting mineral deposition and calcification build-up. Previous studies have suggested a possible role of oxidative stress in mediating differentiation of various cells such as peripheral blood dendritic cells and osteoclasts [41,42]. Mody et al. [33] showed that endothelial cells, when stimulated by oxidative agents, such as xanthine oxidase, produced alkaline phosphatase, and with these results have suggested that oxidative stress favors osteoblastic differentiation.

The relationship between the therapeutic effects of PDT and cytokine expression, such as VEGF, is still controversial. Several PDT protocols have been studied (different photosensitizing agents, concentrations and length of application; light sources with different wavelengths, power, fluency and length of application) which makes it difficult to compare the results. Nevertheless, clinical studies have allowed for a better understanding of the role of tissue microenvironment, and how the body responds to the treatment with PDT. This study consisted of clinical observations on the response of gingival tissues to the application of the investigated drug, which allowed for observations that would not be possible in an in vitro or animal study. When taking into consideration the limitations of this study, the application of the nanoemulsion of AlClPc in the current protocol demonstrated to be safe for use in humans. It is also suggested to increase the expression of VEGF after PDT.

However, further clinical trials are required to validate the use of this photosensitizing agent and recognize it as a therapy of choice with anti-tumor, angiogenic and osteogenic properties.

Conflict of interest

None of the authors have any conflicts of interests regarding this manuscript to declare.

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