

## Rat tissue reaction and cytokine production induced by antimicrobial photodynamic therapy



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### ABSTRACT

**Background:** The antimicrobial photodynamic therapy (aPDT) inactivates the target cell via reactions among the photosensitizer (PS), Laser or Led and O<sub>2</sub>. The aim of this study was to evaluate the tissue reaction and cytokine production promoted by aPDT with curcumin photosensitizer.

**Methods:** Polyethylene tubes containing saline solution (control), 5% sodium hypochlorite (NaOCl) and aPDT with curcumin PS 500 mg/L, were implanted into dorsal connective tissue of Wistar rats. After 7, 15, 30, 60 and 90 days of implantation, the animals were euthanized and the tubes with surrounding tissues were removed. The specimens were divided in two part, one half was processed, fixed and prepared for histological analysis by staining with hematoxylin and eosin. The other half was collected for IL-1β and IL-6 cytokine production using ELISA assay. The results were statistically analyzed by Kruskal-Wallis test followed by Dunn test ( $p < 0.05$ ) for tissue reaction and ANOVA followed by Bonferroni's correction ( $p < 0.05$ ) for ELISA.

**Results:** All groups showed severe tissue reactions at 7 days, whilst a significantly decrease by time was observed. Regarding to cytokine production, aPDT increases the IL-1β levels in all periods of time ( $p < 0.05$ ). However, for IL-6 levels, the highest value was observed with aPDT on the 90th day ( $p < 0.05$ ).

**Conclusions:** aPDT with curcumin PS 500 mg/L demonstrated biocompatibility similar to saline solution and induced the IL-1β and IL-6 cytokines production.

### 1. Introduction

The success of endodontic therapy depends on the efficiency of cleaning, shaping and fillings of the root canal system with biocompatible filling materials [1,2]. Irrigating solutions are used in endodontic therapy to reduce the number of microorganisms and their toxic by-products (endotoxins) [3], and their need to have appropriate biological and physical-chemical properties [4].

Sodium hypochlorite (NaOCl) has been widely used in endodontic therapy due to its antibacterial action and its ability to dissolve organic matter [5]. However, NaOCl is known to be a potential irritant of periapical tissues and often causes inflammatory reactions at high concentrations. Though, in low concentrations is ineffective against certain microorganisms [6]. Moreover, complete disinfection is not

achieved when using NaOCl as an irrigation solution in routine endodontic therapy [2]. For this reason, further investigations have been conducted to identify therapeutic strategies that can enhance endodontic infection control.

The antimicrobial photodynamic therapy (aPDT) inactivates the target cells [7] and/or enhances antimicrobial activity without generating microbial resistance [8]. It uses a specific wavelength of light ( $\lambda$ ), Laser or Led that activates a photosensitizer (PS). Activated PS, in the presence of O<sub>2</sub>, produces highly reactive species that inactivate the target cell [7]. Recently, aPDT was employed as a new therapeutic adjunct modality to enhance the disinfection of the root canal system [9] in endodontic treatment, and showed to be effective against *Enterococcus faecalis* [10].

Different PS can be used with aPDT: toluidine blue, methylene blue,

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chlorophyll and curcumin [11]. Curcumin, a compound from *Curcuma longa* L., has a variety of applications and has no cytotoxic effects in L-929 fibroblasts [12], or in periodontal ligament cells [13], moreover, it has antimicrobial effects [14]. But, the use of curcumin as PS has rarely been reported in root canal treatment [12,14].

The importance of sub-cutaneous implantation of the polyethylene tube in rats demonstrate of assessing *in vivo* biocompatibility and your biological responses, moreover, allows the material being tested to be in direct contact with tissue. [15]. The production of cytokines is complex system and the reason of their expression create very factors that include other cells and mediators [12].

However, no previous studies have investigated the use of aPDT with curcumin PS, its biocompatibility, or effect on *in vivo* cytokine production. The aim of this study was to evaluate the tissue reaction and cytokines production induced by aPDT with curcumin PS and to compare its effects to those of saline solution (control) and 5% NaOCl.

## 2. Materials and methods

### 2.1. Animals

Thirty male 4- to 6-month-old Wistar Albino rats, weighing 250–280 g, were used. The animals were housed in temperature-controlled rooms and received water and food *ad libitum*. The care of the animals complied with the guidelines of the Araçatuba School of Dentistry-UNESP Ethical Committee, which approved the project (Process: 00682-12).

### 2.2. Subcutaneous implant, group division and antimicrobial photodynamic therapy

Polyethylene tubes (Delton, Johnson & Johnson, São José dos Campos, SP, Brazil) with a 1.1-mm internal diameter, 1.6-mm external diameter, and 10-mm length, were filled with fibrin sponges (Lasbrasil, São Paulo, SP, Brazil) soaked with 0.1 mL of the test solutions, according to Gomes-Filho et al., 2008 [4] and Gomes-Filho et al. [15].

The groups were distributed as follow: G1-saline solution (control); G2-5% NaOCl; and G3-aPDT with curcumin PS 500 mg/L.

The test solution curcumin PS at 500 mg/L (PDT Pharma, Cravinhos, SP, Brazil), was used with the Led (Physics Institute of São Carlos-IFSC-USP, São Carlos, SP, Brazil) under 60 s of pre-irradiation as recommended by Gomes-Filho et al. [12]. Then, aPDT was performed with blue Led at  $\lambda$  480 nm, 400 mW, with a fluence of 72 J/cm<sup>2</sup>, for 240 s, point diameter  $\phi$  = 2 mm, real diameter of the irradiated area  $\phi$  = 3.5 mm, continuous wave. The lighting was turned off and the ambient light in the laboratory was reduced to avoid PS degradation as described by Gomes-Filho et al. [12]. The depth of treating point of the irradiation = 0 cm.

The animals were anesthetized with Xylazine (10 mg/kg) and Ketamine (25 mg/kg), then were disinfected with 5% iodine solution and shaved. A 2-cm incision was made in a head-ail orientation on the shaved back of each animal, using a number 15 Bard-Parker™ blade (Franklin Lakes, NJ, USA). The skin was reflected to create – three pockets and the prepared polyethylene tubes were implanted into the pockets and the skin was closed with 4/0 silk sutures.

### 2.3. Histological processing

After 7, 15, 30, 60, and 90 days from implantation, the animals were euthanized by an overdose of anesthetic. The tubes with the surrounding tissues were removed and fixed in 10% buffered formalin at pH 7.0 [16]. The tubes were then bisected transversely. One half of the specimens were processed for glycol methacrylate embedding, serially sectioned into 3- $\mu$ m slices, and stained with hematoxylin and eosin [17].

Inflammatory reactions in the tissue that was in contact with the

material on the open end of the tube were scored as described in previous studies [15–17], as follows: 0, none or few inflammatory cells and no reaction; 1, less than 25 cells and a mild reaction; 2, between 25 and 125 cells and a moderate reaction; and 3, 125 and more cells and a severe reaction. Fibrous capsules were considered thin when they were < 150  $\mu$ m and considered thick at > 150  $\mu$ m respectively. The average number of cells for each group was obtained from 10 separate areas (400 X). Analyses were performed by a single calibrated operator in a blinded manner.

### 2.4. ELISA assay

The other half of the specimens were processed for ELISA assay, when the surrounding tube tissues were collected, weighed, and kept in frozen liquid nitrogen in order to measure the concentrations of IL-1 $\beta$  and IL-6. One day before the experiment, the samples were homogenized in phosphate-buffered saline plus protease-inhibitor tablets. After centrifugation, the supernatant was collected and kept at –80 °C until use. A 96-well plate was coated using the Rat IL-1 $\beta$  Platinum ELISA and Rat IL-6 Platinum ELISA reagents (eBioscience, Vienna, Austria). Color changes proportional to the amount of IL-1 $\beta$  and IL-6 were quantified by comparing the absorbance of the samples with that of known dilutions using a plate reader at 450 nm. The concentrations of IL-1 $\beta$  and IL-6 were calculated (pg/mL) by comparison with a standard curve.

### 2.5. Statistical analysis

The results were statistically analyzed by Graph Prism (version 5.0) software program. For nonparametric data, Kruskal-Wallis test was used followed by Dunn test for tissue reaction. For parametric data, ANOVA followed by Bonferroni's correction for ELISA. The p value was considered significant at 5%.

## 3. Results

### 3.1. Tissue reaction

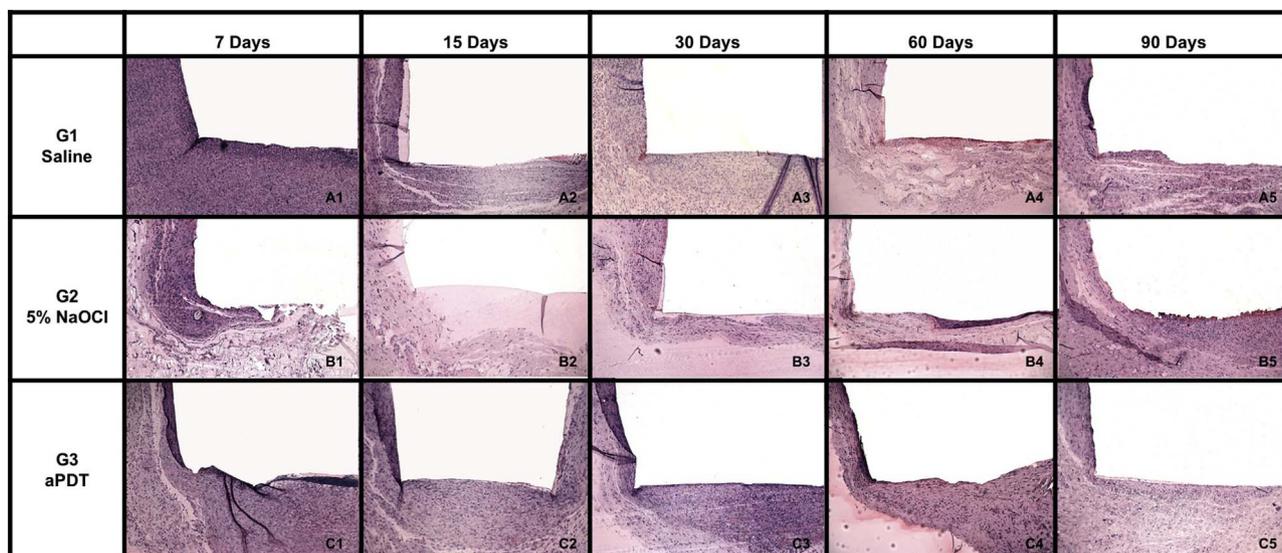
On the 7th day, severe inflammatory cell infiltration was observed involving predominantly lymphocytes and macrophages and focal areas with polymorphonuclear cells in all groups evaluated (Fig. 1 – A1; B1; C1). A mild inflammatory cell infiltration with presence of angiogenesis and granulation tissue at 15th day were detected in the saline solution and aPDT groups (Fig. 1 – A2; C2); moreover, a moderate inflammatory cell infiltration was identified in 5% NaOCl group (Fig. 1 – B2). At the 30th day, mild inflammatory cell infiltration was detected in all groups (Fig. 1 – A3; B3; C3). On the 60th day and 90th day the absence of inflammation was observed in all groups (Fig. 1 – A4; B4; C4; A5; B5; C5). Necrosis were absent in all groups, independently of the time-point.

### 3.2. Cytokine production

The mean concentration levels of IL-1 $\beta$  and IL-6 produced in the presence of aPDT and irrigating solutions are showed in Fig. 2a and 2b. The aPDT group produced more IL-1 $\beta$  at 7, 15, 30, 60 and 90 days than the saline solution and NaOCl groups ( $p < 0.05$ ) (Fig. 2a). For IL-6, increased production was observed on the 7th and 30th day for the saline solution ( $p < 0.05$ ). On the 15th day, the highest cytokine production was evident for the 5% NaOCl group ( $p < 0.05$ ). On the 90th day, the highest concentration level was measured in the aPDT group ( $p < 0.05$ ) (Fig. 2b).

## 4. Discussion

In this study, we aimed to evaluate and compare the tissue response



**Fig. 1.** After 7 days, thick, fibrous capsule formation was seen in the 5% NaOCl and aPDT groups (B1 and C1, respectively), but was absent in the saline solution group (A1); additionally, a severe inflammatory cell infiltration was seen in all groups (A1, B1, and C1). After 15 days, it was noted that the fibrous capsule remained thick, with moderate inflammatory cell infiltration for 5% NaOCl group (B2), whereas a thin fibrous capsule with mild inflammatory cell infiltration was evident in the control (A2) and in the aPDT group (C2). After 30 days, a thin fibrous capsule was observed for the control (A3) and the aPDT group (C3), and a thick, fibrous capsule was observed for the 5% NaOCl group (B3), with mild inflammatory cell infiltration present in all groups. The same fibrous capsule was observed after 60 days in all groups, presenting few inflammatory cell infiltration (A4, B4, and C4). After 90 days, a thin fibrous capsule was seen in all groups, with none or few inflammatory cells evident (A5, B5, and C5). Samples were stained with hematoxylin and eosin (400x).

and cytokine production induced by new adjuvant endodontic therapy aPDT using curcumin PS 500 mg/L with the solutions saline and 5% NaOCl.

The aPDT with curcumin 500 mg/L was demonstrated a better response when compared to the 5% NaOCl and similar response to saline. However, reports of this equivalence in the reaction of connective tissue to the implantation of aPDT with curcumin have not been established and clarified and the pertinent literature is scarce. Nevertheless, only studies in dogs have assessed the response its application in teeth with apical periodontitis [19,20].

In other study, higher cell viability was detected using a curcumin PS with pre-irradiation and light stimulation for 60 s and 240s, respectively, similar to ours results [21]. However, others investigations have found a low cell viability when using longer pre-irradiation and higher concentrations [22–24].

The 5% NaOCl demonstrated more intense tissue reaction when compared with saline solution and aPDT. Conversely, 5% NaOCl was shown to result in a mild to absent inflammatory infiltration between 30 and 60 days, corroborating with a previous report that showed that 5% NaOCl was the most toxic irrigant and the saline solution could be used as a biocompatible irrigant solution [4].

In this study, 5% NaOCl was considered cytotoxic with inflammatory cells even after 14 days. Other studies also have reported that the number of inflammatory cells remained high at sites treated with 5.25%

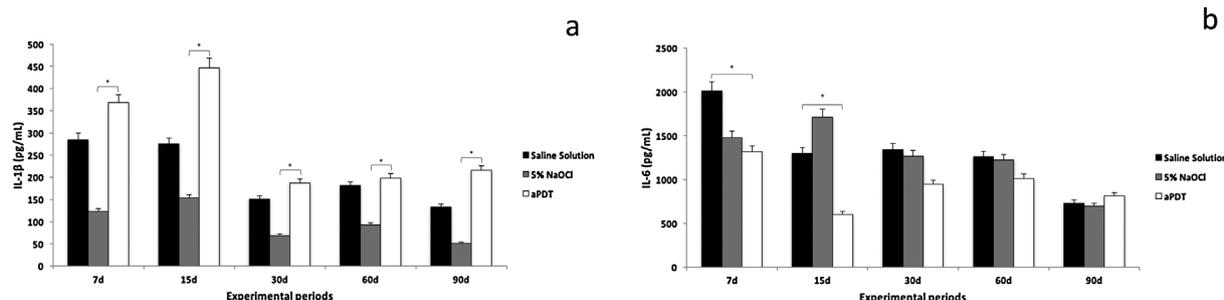
NaOCl even after 14 days [4,25], and according to Mohri et al., this cytotoxicity resulted in the production of cytokines [26].

In the inflammatory process, pro-inflammatory and anti-inflammatory cytokines, such as IL-1 $\beta$  [27] and IL-6, are produced [28,29]. In this study, the aPDT group produced more IL-1 $\beta$  than did the other saline and 5% NaOCl groups, at all time points. Nevertheless, showed the lowest release of IL-6, when compared to the other groups, in almost time points, except at 90 days, however, no similar studies were found in the literature.

The according to Kawashima et al., 1999 [30], the cytokine production by human after aPDT, in the short term, did not have an impact on the IL-1 $\beta$  production of the keratocytes, in contrast to our observations in the present study, the differences can be due to the photosensitizer, treatment protocol. On the other hand, similar to the present study, aPDT inhibited IL-6 secretion by keratocytes at 5 h.

Thus, it was evident in this study, that aPDT with curcumin PS 500 mg/L was mainly involved in IL-1 $\beta$  up-regulation and IL-6 down-regulation, highlighting the potential that equilibrium could be achieved.

In this study, rats were used as a model animal, because are extensively used and well known scientifically [31,32]. The experimental solutions (saline, 5% NaOCl and curcumin PS 500 mg/L), were insert into polyethylene tubes and implanted immediately into the animals, which allowed direct contact between the material and the



**Fig. 2.** Mean concentration levels of IL-1 $\beta$  (2a) and IL-6 (2b) were produced in the presence of aPDT and irrigating solutions. There was a statistically significant difference ( $P < 0.05$ ) between NaOCl and aPDT groups for the levels of IL-1 $\beta$  (2a). There was a statistically significant difference ( $P < 0.05$ ) between the groups for the levels of IL-6 (2b). \*( $p < 0.05$ ) indicates significant statistical difference.

connective tissue, promoting the tissue reaction [4,16–18].

The present study used a blue Led 480 nm, according with Paschoal et al. [33], because curcumin PS has an absorption peak ranging between 300 and 500 nm. The protocol used curcumin PS under 60 s of pre-irradiation followed by blue Led at  $\lambda$  480 nm, 400 mW, with a fluence of 72 J/cm<sup>2</sup>, for 240 s, point diameter  $\varnothing = 2$  mm, real diameter of the irradiated area  $\varnothing = 3.5$  mm, continuous wave, according to our previous study [12].

Therefore, the according to Krammer et al. [34], the different of cellular responses depend mainly on the aPDT-protocol, photosensitizer localization and available intracellular energy. This may contribute to tissue repair and suggests the use of aPDT in Endodontics.

## 5. Conclusion

According to the methodology employed, antimicrobial photodynamic therapy with curcumin had biocompatibility similar to saline solution and induced the IL-1 $\beta$  and IL-6 cytokines production.

## Conflict of interest

The authors do not have any conflicts of interest related to this study.

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