



Photosensitizers And Exposure Times To Light Showed Tissue Compatibility In Isogenic Mice

Fotossensibilizadores e Tempo de Exposição a Luz em Relação a Compatibilidade de Tecidos em Ratos Isogênicos

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ABSTRACT

Objective: The aim of this study was to evaluate the subcutaneous tissue response after different protocols to photodynamic therapy (PDT). In Phase 1, were tested the diode laser (used for 1min) associated to the photosensitizer phenothiazine chloride solution (PCS) in different concentrations. In Phase 2 – the diode laser and LED were tested associated to two different photosensitizers, PCS and Curcumin, in different exposure times of light application. **Material and Methods:** After 7, 21 and 63-days the animals were euthanized and the subcutaneous tissue processed to histological analysis. Qualitative and semi-quantitative descriptions of the inflammatory process and immunohistochemical technique were performed. The obtained data were analyzed by Kruskal-Wallis and Dunn's post-test ($\alpha = 0.5$). **Results:** On Phase 1, the tissue response was very similar among the groups. For the inflammatory infiltrate, PCS with concentration of 10mg/mL exhibited the most intense reaction ($p > 0.05$). On Phase 2, at 7-days period, the analyzed parameters presented small magnitude and after 21 and 63-days, all the parameters demonstrated tissue compatibility. **Conclusion:** Both photosensitizers presented proper tissue compatibility regardless the different concentrations used on Phase 1 and different durations of light exposure on Phase 2.

KEYWORDS

Photodynamic therapy; Phenothiazine chloride solution; Curcumin; Isogenic mice; Subcutaneous tissue.

RESUMO

Objetivo: Este estudo avaliou a resposta do tecido subcutâneo após terapia fotodinâmica, utilizando na Fase 1 - laser diodo por 1min e solução fotossensibilizadora de cloreto de fenotiazina (CF) em diferentes concentrações e Fase 2 - laser diodo e LED e dois fotossensibilizadores, CF e Curcumina, em diferentes tempos de exposição da aplicação de luz. **Material e Métodos:** Após 7, 21 e 63 dias, foram realizadas descrições qualitativas e semiquantitativas do processo inflamatório e técnica de imunoistoquímica. Os dados foram analisados pelo pós-teste de Kruskal-Wallis e Dunn ($\alpha = 0,5$). **Resultados:** Na Fase 1, a resposta do tecido foi muito semelhante. O infiltrado inflamatório, na concentração de 10 mg / mL, exibiu reação mais intensa ($p > 0,05$). Na Fase 2, aos 7 dias, os parâmetros analisados apresentaram pequena magnitude. Aos 21 e 63 dias, todos os parâmetros demonstraram compatibilidade com o tecido. **Conclusão:** Ambos os fotossensibilizadores apresentaram compatibilidade de tecido adequada, independentemente das diferentes concentrações utilizadas na Fase 1 e diferentes durações de exposição à luz na Fase 2.

PALAVRAS-CHAVE

Terapia fotodinâmica; Solução de cloreto de fenotiazina; Curcumina; Ratos isogênicos; Tecido subcutâneo.

INTRODUCTION

The photodynamic therapy (PDT) is a treatment in which one photoactive agent, known as photosensitizer, is activated by a light with a specific wavelength, triggering the production of singlet oxygen, superoxide and free radicals (reactive oxygen species), that are cytotoxic to target cells [1-4] leading to cellular death by oxidation of biological molecules such as proteins, nucleic acids and lipids [5].

The antimicrobial efficiency of PDT in Dentistry has been evaluated in several studies [1,3,4]. However, there are few studies presenting the microscopic biologic effects of this treatment in Dentistry, in different tissues (oral mucosa, periodontal tissues, oral mucosal ulcer and periapical tissues) after performing different protocols [2,6-15].

According to Luan et al. (2016) [6], in periodontal tissues no significant histologic differences (inflammatory infiltrate, necrosis or vascular alterations) was noted in the tissues treated with PDT. On the other hand, Garcia et al., 2014 [8] observed a small and isolated number of inflammatory cells located in dense conjunctive tissue, composed by a large amount of collagen fibers and few fibroblasts, in teeth with induced periodontitis treated by scaling and root planning followed by PDT.

Three previous studies performed by our research group in dog's teeth with experimental induced periapical lesions, evaluated the response of the apical and periapical tissues after PDT [2,10,12]. Although the PDT presented important results indicative of repair process, such as moderate collagen fiber formation and angiogenesis [2], the calcium hydroxide intracanal dressing overcome these results. Together, the results of those three papers showed the importance of the evaluation of different parameters for better results after the use of PDT, without damages for tissue repair process.

Furthermore, considering the influence of the different parameters to perform PDT,

including type of laser, light wavelength (nm), energy density (J/cm^2), intensity or potency (W), and the great variety of chemical composites that can be used as photosensitizers, in variable concentrations [1-4,6-12], biological investigations of PDT are mandatory.

Therefore, this study aimed to evaluate the tissue response in mice's dorsal subcutaneous, after performing PDT, in different conditions: Phase 1 - photosensitizer phenothiazine chloride solution in different concentrations and Phase 2 - two photosensitizers (Phenothiazine chloride solution and Curcumin), with the different laser exposure times.

MATERIAL AND METHODS

Animals

All the procedures were submitted and approved by the Ethic Committee in the Use of Animals (CEUA), from the School of Dentistry of Ribeirão Preto, University of São Paulo (FORP/USP) (process number 2015.1.598.58.1).

Surgical procedures

The experimental procedures were based on the standards proposed by the *International Organization for Standardization* (ISO) no 7405, 2008. Two hundred and twenty-five (225) isogenic BALB/c mice, males, aged 6 to 8 weeks, weighting 15 to 20 grams were.

Animals were anesthetized with an intramuscular injection of 10% Ketamine (Agener União Química Farmacêutica Nacional S/A, Embu-Guaçu, Brazil) and 2% Xylazine (Dopaser, Laboratórios Calier, Barcelona, Spain), 35mg/kg and 7mg/kg, respectively. Next, dorsal region was shaved and cleaned with a 1% chlorhexidine gluconate solution (Assepmed -Medquímica – Juiz de Fora – MG).

Before the surgical procedure, a 2 cm^2 area on the animal dorsal region was demarcated, in order to standardized where the photosensitizer and the light were applied. An incision of 2 cm length was made on the dorsal

region followed by tissue divulsion, insertion of the photosensitizer into the connective tissue followed by the application of the light with the wavelength compatible to the photosensitizer.

The present study was divided in two experimental phases, as described:

Phase 1: 99 mice were used to evaluate different dilutions of the Phenothiazine chloride solution (Helbo Blue Photosensitizer – Helbo Photodynamic Systems GmbH & Co KG, Grieskirchen, Austria): 10mg/mL (original concentration), 1,0 mg/mL, 0,1 mg/mL, and 0,005mg/mL [2]. The photosensitizer was applied on the previously demarcated area and maintained for 1 min (pre-irradiation period), following the manufacturer's instructions. After this period, the tissue was washed with 5mL of distilled water, and the solution excess was removed using sterile gauze. Next, a diode laser was applied (Helbo Therapielaser, Helbo Photodynamic Systems GmbH & Co KG, Grieskirchen, Austria - 660nm wavelength and energy density of 3.3 J/cm²) for one min, also as recommended by the manufacturer.

Table 1 - Distribution of groups, experimental periods and number of animals used on Phase1

Experimental Periods	Groups	Number of animals
7 days	Phenothiazine chloride solution 10mg/mL	07
	Phenothiazine chloride solution 1,0mg/mL	07
	Phenothiazine chloride solution 0,1mg/mL	07
	Phenothiazine chloride solution 0,005mg/mL	07
	Control	05
21 days	Phenothiazine chloride solution 10mg/mL	07
	Phenothiazine chloride solution 1,0mg/mL	07
	Phenothiazine chloride solution 0,1mg/mL	07
	Phenothiazine chloride solution 0,005mg/mL	07
	Control	05
63 days	Phenothiazine chloride solution 10mg/mL	07
	Phenothiazine chloride solution 1,0mg/mL	07
	Phenothiazine chloride solution 0,1mg/mL	07
	Phenothiazine chloride solution 0,005mg/mL	07
	Control	05

Phase 2

126 mice were used to compare two photosensitizers, phenothiazine chloride solution (Helbo Blue Photosensitizer - Helbo Photodynamic Systems GmbH & Co KG, Grieskirchen, Austria) at 10mg/mL (based on Phase 1), and Curcumin in the concentration of 0.0074mg/mL [4]. Curcumin (Sigma Aldrich, St. Louis, MO, USA, molecular weight 368.68) was initially diluted in 10% DMSO, giving rise to a concentrated stock solution, which was adjusted in distilled water to the concentration of 0.0074mg/mL [4]. The initial dilution in DMSO was necessary to ensure the solubilization of the insoluble powder in aqueous vehicles and its stability, since it is sensitive to variations in ambient temperature and can generate free radicals [19]. The phenothiazine chloride solution was applied for a period of 1 min (pre-irradiation period), following the manufacturer's instructions. The Curcumin was applied on the demarcated area with a pre-irradiation period of 5min [4]. After this period, the tissue was washed as described on Phase 1. Also, three different exposure times of the photosensitizers to the laser (Helbo Therapielaser, Helbo Photodynamic Systems GmbH & Co KG, Grieskirchen, Austria - 660nm wavelength and energy density of 3.3 J/cm²) were used to the irradiation of the phenothiazine chloride solution, and LED (Radii-Cal, SDI Limited, Bayswater, Victoria, Austrália - 450nm wavelength and energy density of 72 J/cm²) used for the Curcumin evaluation (30 seconds, 1 min and 2 min).

In the control groups, only the incision and divulsion of the tissue was performed, in order to evaluate the inflammatory response caused by the surgical procedure (*sham animals*). The animals used as control were the same to both experimental phases.

After conclusion of the experimental procedures, the skin borders were closed with 4-0 silk sutures (Vicryl; Johnson & Johnson: Ethicon Inc., New Brunswick, USA). The animals were kept with free access to standard diet and water during the experimental periods and were

periodically observed for local, systemic and behavioral abnormalities.

After each experimental period (7, 21 e 63 days), the animals were killed by a mixture of ketamine and xylazine and a sample of the subcutaneous connective tissue and skin, involving the treated area, with the standard dimension of 6mm² from the center of the surgical wound, was removed, fixated in 10% buffered formalin for 24 hours, and then subjected to the histological processing.

Table II describe the experimental and control groups, number of animals in each group and experimental periods for Phase 2.

Table II - Distribution of groups, experimental periods and number of animals on Phase 2

Experimental Periods	Groups	Number of animals
7 days	Phenothiazine chloride solution 30 s	07
	Phenothiazine chloride solution 1 min	07
	Phenothiazine chloride solution 2 min	07
	Curcumin 30 s	07
	Curcumin 1 min	07
	Curcumin 2 min	07
	Control	05
21 days	Phenothiazine chloride solution 30 s	07
	Phenothiazine chloride solution 1 min	07
	Phenothiazine chloride solution 2 min	07
	Curcumin 30 s	07
	Curcumin 1 min	07
	Curcumin 2 min	07
	Control	05
63 days	Phenothiazine chloride solution 30 s	07
	Phenothiazine chloride solution 1 min	07
	Phenothiazine chloride solution 2 min	07
	Curcumin 30 s	07
	Curcumin 1 min	07
	Curcumin 2 min	07
	Control	05

Histological Processing

After fixation, the tissue was embedded in paraffin and 5µm-thick semi serial sections were obtained, stained with hematoxylin and eosin, Picrosirius or prepared to immunohistochemical staining. Then, the slides were examined under light microscopy using the microscope Axio Imager.M1 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany), coupled to an AxioCam MRc5 camera (Carl Zeiss MicroImaging GmbH, Göttingen, Germany), by an experienced pathologist blinded to the analyzed groups.

Descriptive and Semi-Quantitative Microscopic Analysis

Sections stained with H&E were used to perform the description of the tissue reaction in relation to the photosensitizers and laser exposures, in the different experimental periods, on both Phases 1 and 2.

Also, using the same H&E sections used to the descriptive analysis, scores from 0 to 3 were attributed, to the following parameters [20]:

- collagen fiber formation - 0: absent; 1: mild; 2: moderate; 3: severe.

- tissue thickness - 0: normal; 1: slightly expanded; 2: moderately expanded; 3: severely expanded.

- inflammatory infiltrate - 0: absent; 1: mild inflammatory infiltrate; 2: moderate inflammatory infiltrate; 3: intense inflammatory infiltrate.

Immunohistochemistry for neutrophils and macrophages

In order to identify the presence of neutrophils and macrophages in the reactive tissue, immunohistochemistry was performed, on both Phases 1 and 2. Slides were deparaffinized and hydrated, antigenic recovery performed by heat and immersion in citrate buffer (pH = 6,0) using microwave oven (12 cycles of 10 second each). The slides were washed and the endogenous peroxidase was blocked using 3% hydrogen peroxide, for 20min. After washing again, the block of

unspecific ligations was performed using BSA at 1% (bovine serum albumin)/PBS for 30min. Next, the slides were incubated overnight in 4 °C with the primary antibodies diluted in BSA at 1% (anti-macrophage SC101447, dilution 1:50 and anti-neutrophil SC59338, dilution 1:100 – Santa Cruz Biotechnology Inc., Dallas, USA).

After returning to room temperature, the slides were washed and incubated with biotinylated secondary antibody diluted in BSA at 1% (for macrophage goat anti-rabbit IgG-B SC2040, dilution 1:200 and for neutrophil rabbit anti-goat IgG-B SC2774, dilution 1:200 - Santa Cruz Biotechnology Inc., Dallas, USA), for 1 hour, at room temperature. Next, the streptavidin-biotin-peroxidase complex (ABC kit, Vectastain; Vector Laboratories Inc., Burlingame, USA) was applied for 30min. Then, the reaction was performed using the diaminobenzidine solution (DAB; Sigma-Aldrich Corporation, Saint Louis, USA) and H₂O₂ at 3% in PBS for 1 min. The slides were counterstained with Harris hematoxylin for 10 seconds, washed in running water, washed in ammoniac water for 30 seconds, and then, once more, washed in running water. The slides were, then, diaphanized, dehydrated and settled. In the negative control, the primary antibody was substituted by non-immune serum.

The analysis was performed under normal light microscopy. The results were disclosed qualitative, according to be presence/absence of the immunostained cells.

Statistical Analysis

Considering the obtained data (scores), the results were expressed in median, first and second quartiles and analyzed by Kruskal-Wallis test. In the groups that significant statistical differences were found, the Dunn's post-test was applied. The results were analyzed using the Sigma Plot 12.0® program (Systat Software Inc., San Jose, USA). The significance level was 5%.

RESULTS

Phase 1

Descriptive and Semi-Quantitative Microscopic Analysis

The description of the tissue reaction after the exposure to the photosensitizer (phenothiazine chloride solution) was similar regarding the parameter tissue thickness for all tested concentrations (10, 1, 0.1 and 0.005mg/mL) and time points (7, 21 and 63 days). Regarding the fiber collagen formation, at 7 days, phenothiazine chloride solution at 0,1 and 10mg/mL presented score 0 (absent formation) with statistical difference with other groups ($p < 0.05$) After that, at 21 and 63 days, all groups presented score 1 (mild) with no statistical differences ($p = 1.0$).

Regarding the inflammatory infiltrate, the concentration of 10mg/mL induced a more intense reaction ($p < 0,001$) that persists over time and maintained until the later one (score 1). Regarding the other tested concentrations (1, 0.1 and 0.005mg/mL), inflammatory infiltrate magnitude diminished over time turning to absent in the later time point (score 0). The control group presented difference for the inflammatory infiltrate when compared to the 10mg/mL concentration group in all three evaluated time-points ($p < 0,001$).

In general, the tissue reactional showed mild fiber collagen formation, moderate infiltrated of macrophages and numerous fibroblastic and endothelial cells, forming a fine and delicate collagen capsular structure. At the end of the experimental periods, all the tested concentrations of the phenothiazine chloride solution presented tissue compatibility (Figure 1) (Table III). The control group presented a thin capsular structure, with few collagen fibers, however, well organized and permeated by macrophages and fibroblasts. The absence of neutrophils was characteristic. The organization, thickness and structural composition of the reactive tissue were very similar to the experimental groups.

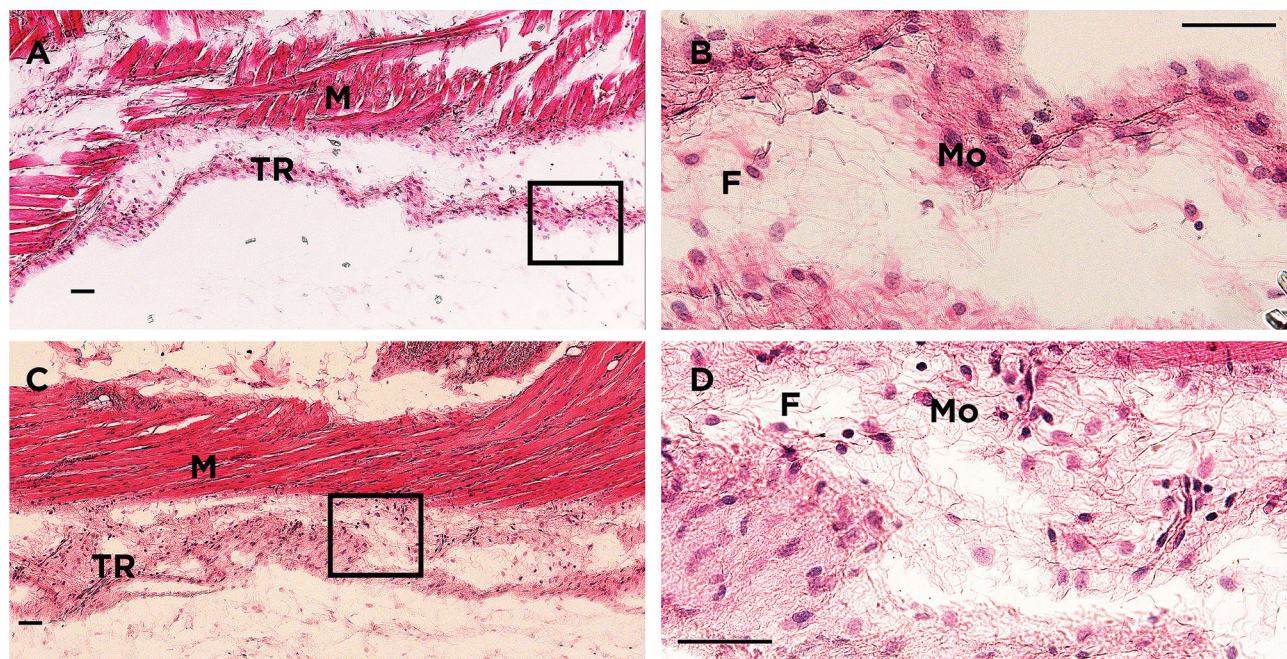


Figure 1 - Microscopic aspects representing the inflammatory reaction in the subcutaneous tissue after the use of phenothiazine chloride solution, in different concentrations (Phase 1). M: muscle, TR: reactive tissue, Mo: macrophage, F: fibroblast (Hematoxylin and Eosin – scale bar = 50µm).

Table III - Statistical analyses of median values obtained on Phase 1 related to collagen fiber formation, tissue thickness and inflammatory infiltrate, after using the photosensitizer Phenothiazine chloride solution in all concentrations in the periods of 7, 21 and 63 days (1Q: 1° quartile; 3Q: 3° quartile)

Experimental Periods	Groups	Collagen Fiber Formation (1Q-3Q)	Tissue Thickness (1Q-3Q)	Inflammatory Infiltrate. (1Q-3Q)
7 days	Phenothiazine chloride solution 10mg/mL	0 ^{ab} (0-0)	2 ^a (1-2)	2 ^a (2-2)
	Phenothiazine chloride solution 1,0mg/mL	1 ^{ab} (1-1)	1 ^a (1-2)	1 ^{ab} (1-2)
	Phenothiazine chloride solution 0,1mg/mL	0 ^a (0-0)	1 ^a (1-1)	1 ^{ab} (1-1)
	Phenothiazine chloride solution 0,005mg/mL	1 ^a (1-1)	1 ^a (1-1)	1 ^a (0-1)
	Control	1 ^{ab} (1-1)	1 ^a (1-1)	0 ^b (0-0)
	p value (Kruskal-Wallis test)	p < 0,001	p = 0,005	p < 0,001
21 days	Phenothiazine chloride solution 10mg/mL	0 ^a (0-0)	1 ^a (1-1)	1 ^a (1-1)
	Phenothiazine chloride solution 1,0mg/mL	1 ^a (1-1)	1 ^a (1-1)	0 ^b (0-0)
	Phenothiazine chloride solution 0,1mg/mL	0 ^a (0-0)	1 ^a (1-1)	0 ^b (0-0)
	Phenothiazine chloride solution 0,005mg/mL	1 ^a (1-1)	1 ^a (1-1)	0 ^b (0-0)
	Control	1 ^a (1-1)	1 ^a (1-1)	0 ^b (0-0)
	p value (Kruskal-Wallis test)	p = 1,0	p = 0,446	p < 0,001
63 days	Phenothiazine chloride solution 10mg/mL	0 ^a (0-0)	1 ^a (1-1)	1 ^a (1-1)
	Phenothiazine chloride solution 1,0mg/mL	1 ^a (1-1)	1 ^a (1-1)	0 ^b (0-0)
	Phenothiazine chloride solution 0,1mg/mL	0 ^a (0-0)	1 ^a (1-1)	0 ^b (0-0)
	Phenothiazine chloride solution 0,005mg/mL	1 ^a (1-1)	1 ^a (1-1)	0 ^b (0-0)
	Control	1 ^a (1-1)	1 ^a (1-1)	0 ^b (0-0)
	p value (Kruskal-Wallis test)	p = 1,0	p = 1,0	p < 0,001

* Different superscript letters indicate statistically significant differences (Dunn's Post-test; p < 0,05).

Phase 2

Descriptive and Semi-Quantitative Microscopic Analysis

Regarding phenothiazine chloride solution, at 7 and 21 days, the reactive capsule presented thin thickness characterized by a discreet collagen formation. In some specimens, there was a greater collagen fiber formation, with a moderate degree. There was a moderate inflammatory infiltrate with sparsely distributed inflammatory cells, in a diffuse format. At 63 days, the fibrotic capsule tissue exhibited remodeling characteristics, such as new fibroblasts cells and collagen fibers. From 21 to 63 days, this inflammatory infiltrate became progressively discreet, until

total absence in most of the specimens at 63 days (Figures 2 A and B).

Similarly, regarding curcumin, at 7 and 21 days, the reactive capsule presented thin and slightly collagenized, with collagen formation varying from mild to moderate. At 63 days, in the group which received the laser exposure for 2min, the fibrous capsule presented very heterogenic with variable degrees of collagen fiber formation. At 7 days, after 2min laser exposure, the inflammatory infiltrate was characterized as moderate. In the subsequent periods, the observed inflammatory infiltrate was mild, diffuse and disorganized, with some leukocytes randomly distributed, and, in some cases, absent (Figure 2 C and D).

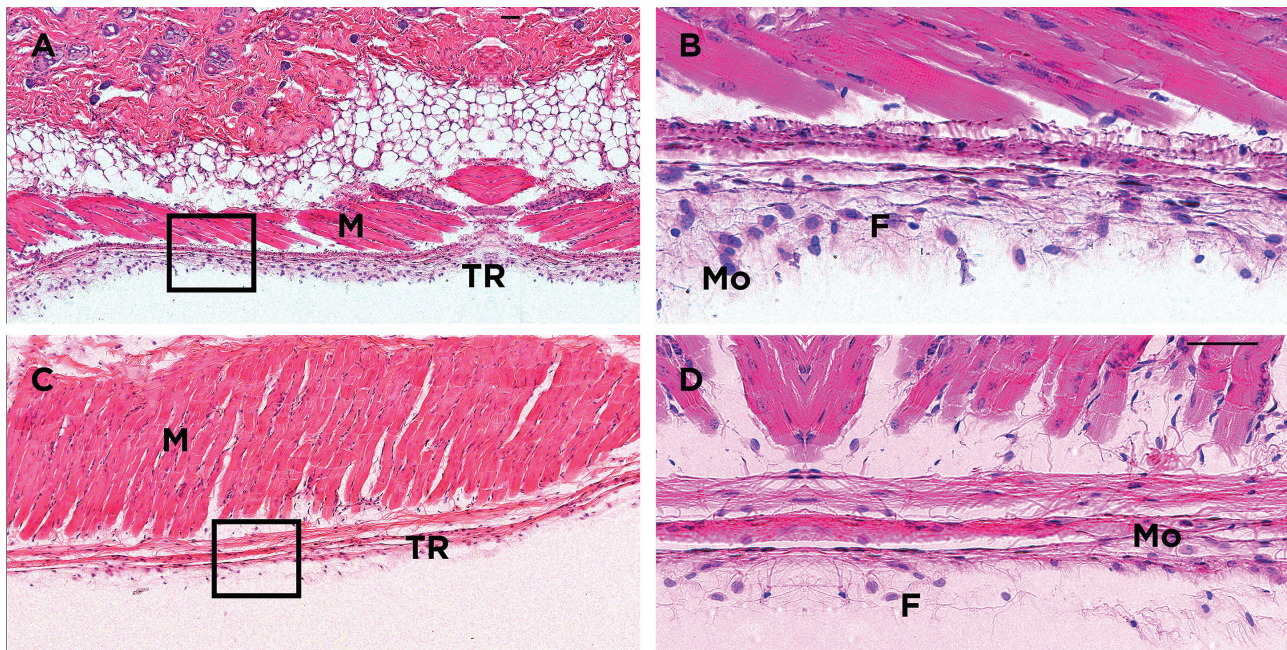


Figure 2 - Microscopic aspects representing the inflammatory reaction in the subcutaneous tissue after the use of phenothiazine chloride solution (A and B) and curcumin (C and D), in different laser application duration (Phase 2). M: muscle, TR: reactive tissue Mo: macrophage, F: fibroblast (Hematoxylin and Eosin – scale bar = 50µm).

Regarding the parameters collagen fiber formation, tissue thickness and inflammatory infiltrate microscopy results showed that, in the initial period of 7 days, tissue alterations were of mild magnitude. Regarding the parameter tissue thickness, the two photosensitizers were statistically different, especially when the light was applied for twomin ($p < 0,005$) and the inflammatory infiltrate was more intense to the Phenothiazine chloride solution after 30 seconds and twomin of light exposure, when compared to the control ($p < 0,05$). At 21 days, only the

inflammatory infiltrate parameter exhibited small variation between groups, where it was found to be more severe for Curcumin, after 30 seconds and one min of exposure to the light, with significant differences when compared to the control ($p < 0,05$). At the final period of 63 days, the tissue compatibility was observed to the two photosensitizers, which did not present significant differences for the evaluated parameters, regardless the duration of time of the laser application (Table IV).

Table IV - Statistical analyses of median values obtained on Phase 2 related to collagen fiber formation, tissue thickness and inflammatory infiltrate, after using the photosensitizers Phenothiazine chloride solution and Curcumin in the periods of 7, 21 and 63 days (1Q: 1° quartile; 3Q: 3° quartile)

Experimental Periods	Groups	Collagen Fiber Formation (1Q-3Q)	Tissue Thickness (1Q-3Q)	Inflammatory Infiltrate. (1Q-3Q)
7 days	Phenothiazine chloride solution 30 s	1 ^a (1-1)	1 ^a (1-1)	2 ^a (2-2)
	Phenothiazine chloride solution 1 min	1 ^a (1-2)	1 ^a (1-1)	1 ^{ab} (1-2)
	Phenothiazine chloride solution 2 min	1 ^a (1-2)	2 ^a (1-2)	2 ^a (2-2,25)
	Curcumin 30 s	1 ^a (1-2)	1 ^a (1-1)	1 ^{ab} (1-1)
	Curcumin 1 min	2 ^a (1-2)	1 ^a (1-1)	1 ^{ab} (1-2)
	Curcumin 2 min	1 ^a (1-2)	2 ^a (1-2)	2 ^{ab} (1-2)
	Control	1 ^a (1-1)	1 ^a (1-1)	0 ^b (0-0)
	p value (Kruskal-Wallis test)	p = 0,104	p = 0,003	p < 0,001
21 days	Phenothiazine chloride solution 30 s	2 ^a (1-2)	1 ^a (1-1)	1 ^a (1-1)
	Phenothiazine chloride solution 1 min	1 ^a (1-2)	1 ^a (1-2)	1 ^{ab} (0-1)
	Phenothiazine chloride solution 2 min	1 ^a (1-2)	1 ^a (1-1)	1 ^{ab} (0-1)
	Curcumin 30 s	1 ^a (1-2)	1 ^a (1-2)	0 ^{ab} (0-1)
	Curcumin 1 min	1 ^a (1-1)	1 ^a (1-2)	1 ^a (1-1)
	Curcumin 2 min	2 ^a (1-2)	1 ^a (1-1)	1 ^{ab} (0-1)
	Control	1 ^a (1-1)	1 ^a (1-1)	0 ^b (0-0)
	p value (Kruskal-Wallis test)	p = 0,139	p = 0,256	p = 0,008
63 days	Phenothiazine chloride solution 30 s	1 ^a (1-2)	2 ^a (1-2)	0 ^a (0-0)
	Phenothiazine chloride solution 1 min	1,5 ^a (1-2)	1 ^a (1-2)	0 ^a (0-0,25)
	Phenothiazine chloride solution 2 min	2 ^a (1-2)	1 ^a (1-2)	0 ^a (0-0)
	Curcumin 30 s	2 ^a (1-2)	1 ^a (1-1)	0 ^a (0-1)
	Curcumin 1 min	1 ^a (1-2)	1 ^a (1-1)	0 ^a (0-0)
	Curcumin 2 min	2 ^a (1,75-2)	1,5 ^a (1-2)	0,5 ^a (0-1,25)
	Control	1 ^a (1-1)	1 ^a (1-1)	0 ^a (0-0)
	p value (Kruskal-Wallis test)	p = 0,212	p = 0,132	p = 0,142

s = seconds; min =min.

* Different superscript letters indicate statistically significant differences (Dunn's Post-test; $p < 0,05$).

30 s, 1 min and 2 min are regarding the application time refer to the different exposure times of the photosensitizers to the laser or LED.

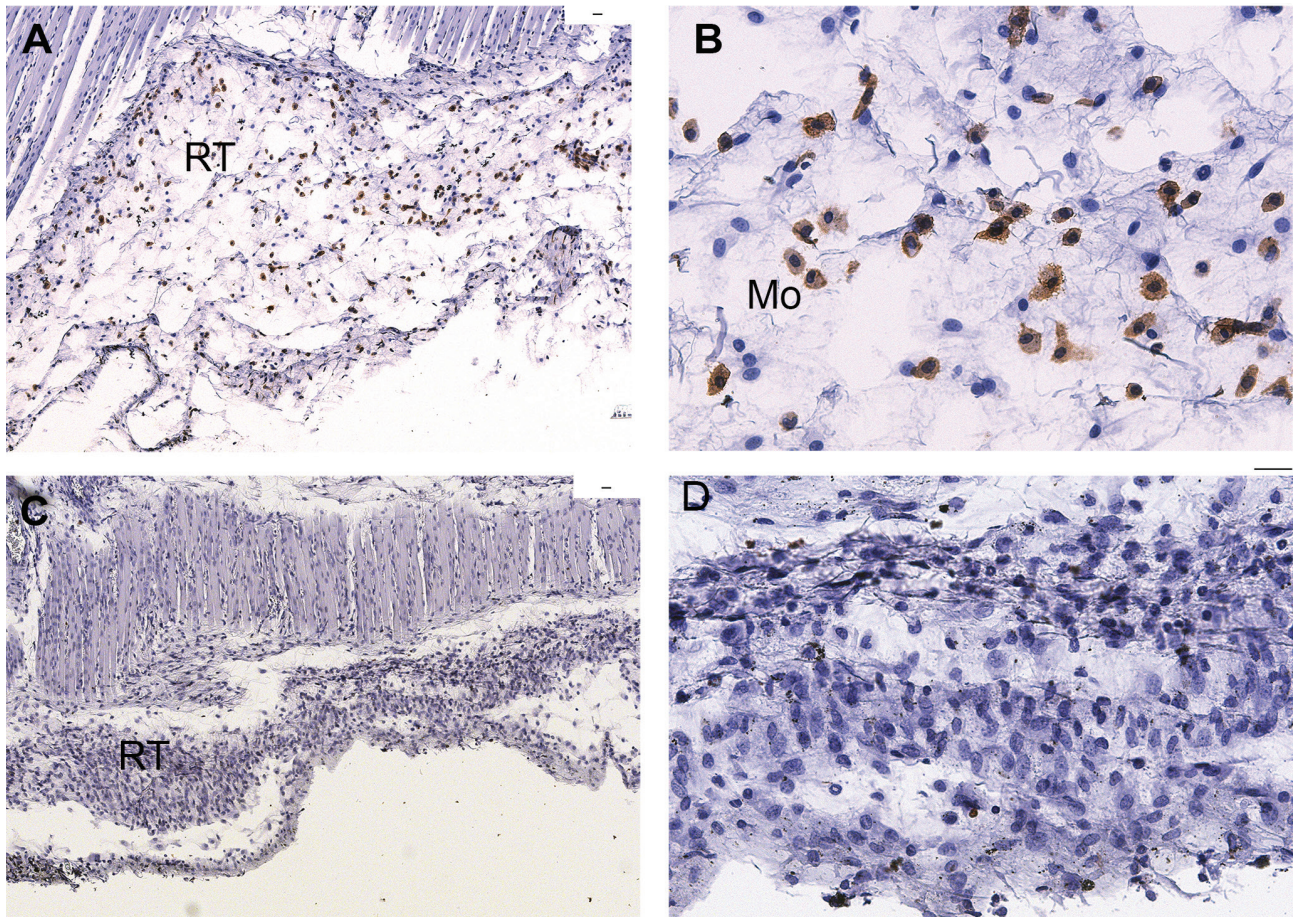


Figure 3 - Microscopic aspects representing immunohistochemical staining for neutrophils (A and B) and macrophages (C and D), in the subcutaneous conjunctive tissue after PDT. Mo: macrophage, TR: reactive tissue (scale bar = 50µm).

DISCUSSION

This study evaluated the inflammatory response of the subcutaneous tissue of isogenic mice after PDT application, using two photosensitizers activated by laser and LED in three experimental periods. The contact of different materials with the connective tissues triggers an inflammatory response from the interaction between the components of the material and the vascular and cellular responses to its aggressive potential [21]. The criteria used to evaluate the tissue response in this study included events related to the body response to the injury caused by the tested materials, such as collagen fiber formation, tissue thickness and inflammatory infiltrate in the affected area [20]. According to Taha et al., (2016) the analysis of the inflammatory process is criteria of great importance to evaluate the biologic compatibility of material, and should

include parameters going beyond the number of inflammatory cells [22].

Also, the evaluation of materials in subcutaneous tissues is highly adopted in Endodontics [22,23]. Furthermore, there is a direct correlation between the formation of the reactional fibrotic capsule and the capacity of the tissue to be tolerant to the tested material, once that this reflects an immune reaction to a foreign body recognized by the biologic system [22]. Variations in inflammatory response between the specimens in 7 to 21 days possibly occurred due to the simultaneity of the events triggered by the reaction of the material with the surgical act for its placement, in addition to the characteristics inherent to the inflammatory process in this period. In the final experimental period, at 60 days, tissue compatibility was observed, suggesting the body's resolution for injury caused by the material, and it

is unlikely that the inflammatory process will recur again unless there is bacterial contamination [24].

Although the events related to the inflammatory process are known to be of fundamental importance to determine the aggressive potential of materials and treatments, when discussing the PDT, there is not enough studies in Dentistry showing the microscopic alterations in different tissues (oral mucosa, periodontal tissues, cutaneous burn and dorsal wounds in mice) after using this type of treatment [6-12].

Specifically, in Endodontics, which is one of the areas of application of PDT in Dentistry, only few studies evaluated tissue reactions to this therapy. Three of these studies [2,10,12,25], performed by our research group, evaluated the histopathologic characteristics of the periapical tissue in dog's teeth, after endodontic treatment using PDT protocols. In the present study, the evaluation of the subcutaneous tissue in mice, aimed to evaluate the tissue compatibility, without the presence of bacteria or pre-existent pathologic processes. In the studies mentioned above, the experimental models used were teeth with experimental induced periapical lesion, besides the bacteria presence, which influenced the characteristics of the inflammatory infiltrate and the repair process. Even so, favorable results after PDT were found by the authors.

Both Silva et al. (2012) [2] and Borsatto et al. (2016) [10] highlighted that the PDT parameters could have influenced their results. Silva et al. (2019) [25] suggested that the used aPDT protocol stimulated the repair process, however the CH-based dressing promoted better apical periodontitis repair. Therefore, new studies must establish a safe protocol, enabling the inactivation of bacteria without causing aggression to the host apical and periapical tissues. For this reason, performing studies evaluating the different parameters used of the PDT are justified.

Two photosensitizers were selected in this study. The photosensitizers have their anti-inflammatory activity related to their ability to inhibit the action of mediators involved in the inflammatory process, such as cyclooxygenase, interleukins, leukotrienes, prostaglandins, nitric oxide and tumor necrosis factor alpha (TNF- α) [26]. This anti-inflammatory potential probably

can be attributed to the low intensity tissue reaction obtained in the groups treated in the present study.

The first photosensitizer, the Helbo Blue Photosensitizer, is a Phenothiazine chloride solution. The use of this type of photosensitizers is established in the literature by many studies [1,18]. In particular, the Helbo Blue Photosensitizer, used in this study already had its efficiency evaluated, in PDT [2,27]. Also, the concentrations showed antimicrobial activity on previous studies [18,27] and satisfactory tissue response in dog's teeth with periapical lesion [2]. Similarly, in our study, it was showed a mild to moderate fiber collagen formation process and absent/mild inflammatory infiltrate in the later period.

Despite the fact that Curcumin have been used in diverse therapeutic modalities for a long time [28], its action as photosensitizer agent with antimicrobial activity is relatively recent [4,7]. Unlike the Phenothiazine chloride solution, there is not a commercial product, ready to be used. Therefore, in this study, the Curcumin was used in a concentration of 0,0074 mg/mL, reported by Frota et al., (2015) to show antimicrobial activity [4]. In the present study, the Curcumin also presented a favorable tissue response at the final period of 63 days. This can be attributed to the fact that, as described above, the Curcumin is able to regulate an amount of transcription factors, cytokines, kinases, adhesion molecules, and enzymes related to inflammation process [29].

The action of light over tissues is as important in the PDT results as the photosensitizers' effect. The application with the correct parameters, can promote healing of wounds, proliferation of fibroblasts, collagen synthesis and deposition, growth factors and ATP production, proliferation of undifferentiated epidermal cells, as well as the decrease of the number of inflammatory cells [30]. The results of the present study showed that in the final periods (21 and 63 days) the three exposure durations to the light (30 seconds, 1 min or 2 min) exhibited tissue compatibility. However, in Dentistry, there are no reports in the literature evaluating the compatibility of tissue after a variation of the exposure duration with laser irradiation in PDT.

Considering the relation between cellular types and the stages of inflammatory process, the

absence of staining for neutrophils, obtained in the present study, is justified, since this is a short life cell and only present during the initial stages of the process of inflammation. Also, it suggests that the possible initial negative effect caused by PDT was not persistent. For the same reasons, in the present study, the macrophages were positively stained, since they are long-life cells and present in every stage of the inflammation process [20]. According to Wataha et al., (2012), the monocyte-macrophage-mediated collagen fibrous capsule acts as a protective barrier between the material and the tissues [31]. Thus, the similarity of the histopathological findings between the treated groups and the controls, in the present study, shows that the application of aPDT was not aggressive to the tissues.

Furthermore, in the present study, the evaluation in subcutaneous tissue had as objective to evaluate the tissue compatibility, without the presence of bacteria or of preexisting pathological processes. The presence of bacteria influences the characteristics of the inflammatory infiltrate and the course of the repair.

Therefore, it is evident that direct comparisons of the present study with the other findings in the literature is difficult, since there is a lack of studies evaluating PDT protocols in subcutaneous tissues. Even if results of studies evaluating other tissues response to this therapy were used to our findings, it is not possible to assure that this response represents exactly the same inflammatory reactions. Such considerations are important, once the characteristics of the target tissue, as well as the photosensitizer and light source parameters have great influence in the effects of PDT [3].

The present study proved that, at the final experimental period (63 days), the two photosensitizers used in the two phases, Phenothiazine chloride solution and Curcumin, presented tissue compatibility. Clinical studies evaluating this protocol are still needed, in order to provide further support for indication and use of PDT by clinicians in Endodontics.

CONCLUSION

Based on the results obtained with the experimental conditions of this study, it was

possible to conclude that the two photosensitizers presented proper tissue compatibility, at the final experimental period, regardless the duration of time of the laser or LED application.

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