Ludmilla Mota da Silva Santos

Biocompatibilidade da terapia fotodinâmica: estudo in vitro e in vivo

ARAÇATUBA
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Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, como parte dos requisitos necessários para a obtenção do título de Doutor em Ciência Odontológica, Área de Concentração: Endodontia.

Orientador: Prof. Adj. João Eduardo Gomes Filho

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Dedicatória

À Deus, pela sua fidelidade na minha vida.

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Santos LMS. Biocompatibilidade da terapia fotodinâmica: estudo in vitro e in vivo [tese]. Araçatuba: Universidade Estadual Paulista, 2014.

RESUMO

Introdução: A terapia fotodinâmica (TFD) se baseia em um conjunto de processos biológicos, físicos e químicos que ocorre por meio da ativação de um fotossensibilizador (FS) com luz (laser ou LED) para destruir as células alvo. O objetivo deste estudo foi avaliar a citotoxicidade e a resposta tecidual da TFD incluindo a produção de citocinas (IL - 1β e IL - 6). **Métodos**: 1) Para o teste de citotoxicidade os grupos foram divididos em: hipoclorito de sódio 5%; hipoclorito de sódio 2,5%; clorexidina 2%; solução salina; TFD (curcumina + luz Led azul); e controle (meio de cultura). As soluções foram diluídas em meio de cultura DMEM ($1x10^4$ células) e colocadas em placas de 24 poços, com linhagem de fibroblastos de camundongos (L-929). Depois de 6, 24 e 48 horas, o ensaio de MTT foi utilizado para avaliar a viabilidade celular e o ensaio de ELISA foi utilizado para avaliação de citocinas no sobrenadante. 2) Para o teste de resposta tecidual, os grupos foram divididos em: controle (solução salina); hipoclorito de sódio 2,5%; hipoclorito de sódio 5%; clorexidina 2%; TFD (curcumina + luz Led Azul). As soluções foram colocadas em tubos de polietileno e foram implantadas no tecido conjuntivo dorsal de ratos Wistar por 7, 15, 30, 60, e 90 dias. Os tubos com tecido circundante foram removidos e divididos ao meio e uma das metades foi corada com hematoxilina e eosina e a outra metade utilizada para avaliação das citocinas por ELISA. A análise estatística foi realizada utilizando ANOVA, Kruskal Wallis ou teste de Tukey ($p < 0,05$). **Resultados**: Quanto ao estudo in vitro, a TFD e a solução salina apresentaram baixa citotoxicidade semelhante ao do grupo controle ($p> 0,05$), menor que o hipoclorito de sódio (2,5% e 5%) e a clorexidina 2% ($p < 0,05$) em todos os períodos de tempo. Todas as soluções liberaram citocinas em quantidade similar ao controle ($p>0,05$). Quanto ao estudo in vivo, todas as soluções provocaram reação inflamatória severa após 7 dias, que diminuiu com o tempo. Não foi observada inflamação aos 90 dias no grupo da TFD similar ao controle ($p>0,05$). Todos os materiais induziram a liberação de IL-6 e IL- 1β mas a quantidade não foi estatisticamente significativa em comparação com o grupo controle. **Conclusão**: A TFD com curcumina não foi citotóxica em cultura de fibroblastos ao contrário das soluções de hipoclorito de sódio a 2,5% e 5% e de

clorexidina 2%; a TFD foi biocompatível e liberou IL - 1β e IL - 6 à semelhança dos grupos controles.
Palavras-chave: Fotoquimioterapia; Tratamento do canal radicular, Irrigantes do canal radicular.

Santos LMS. Biocompatibility of photodynamic therapy: a study in vitro and in vivo [tese]. Araçatuba: Universidade Estadual Paulista, 2014.

ABSTRACT

Introduction: Photodynamic therapy (PDT) is based on a set of biological, physical and chemical processes that occurs through the activation of a photosensitizer (PS) with light (laser or LED) to destroy target cells. The aim of this study was to evaluate the cytotoxicity and tissue response of PDT including the production of cytokines (IL - 1β and IL – 6). **Methods**: 1) For the cytotoxicity test groups were divided into: 5% sodium hypochlorite; 2.5% sodium hypochlorite; 2% chlorhexidine; saline; PDT (curcumin + Led blue light); and control (culture medium). The solutions were diluted in DMEM culture medium ($1x10^4$ cells) and placed in 24 -well plates with mouse fibroblast line (L929). After 6, 24 and 48 hours, the MTT assay was used to assess cell viability and ELISA assay was used to assess cytokine in the supernatant. 2) To test the tissue response, the groups were divided into: control (saline); 2.5%; sodium hypochlorite, 5% sodium hypochlorite; 2% chlorhexidine; TFD (curcumin + Led blue light). The solutions were placed into polyethylene tubes and implanted in the dorsal tissue of Wistar rats for 7, 15, 30, 60, and 90 days. The tubes were removed with surrounding tissue and divided in half and one half was stained with hematoxylin and eosin and the other half used to assess cytokine by ELISA. Statistical analysis was performed using ANOVA, Kruskal Wallis or Tukey test ($p < 0.05$). **Results**: For the in vitro study, PDT and saline showed low cytotoxicity similar to the control group ($p > 0.05$) , less than sodium hypochlorite (2.5% and 5%) and 2% chlorhexidine ($p < 0.05$) at all time periods. All solutions released cytokines similar to control ($p > 0.05$) amount. As for the in vivo study, all solutions caused severe inflammatory reaction after 7 days, which decreased with time. No inflammation was observed at 90 days in the PDT group similar to the control ($p > 0.05$) . All materials induced release of IL - 6 and IL - 1β but the amount was not statistically significant compared with the control group. **Conclusion**: PDT with curcumin has not cytotoxic to cultured fibroblasts unlike solutions of sodium hypochlorite at 2.5 % and 5 % and 2% chlorhexidine; PDT was biocompatible and released IL - 1β and IL - 6 similarly to the control groups .

Keywords: Photochemotherapy; Root Canal Therapy; Root Canal Irrigants

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INTRODUÇÃO

O sucesso do tratamento endodôntico depende: da eliminação eficiente da infecção no sistema de canais radiculares e do correto selamento do canal radicular por meio da obturação (1), visando o completo reparo da região periapical (1-3).

O preparo biomecânico do canal radicular visa reduzir e/ou eliminar a população de micro-organismos e seus produtos tóxicos (endotoxinas e biofilme apical) presentes na infecção endodôntica (4). Durante o preparo biomecânico, a utilização de substâncias irrigadoras que auxiliem na descontaminação por ação química é essencial para potencializar a descontaminação (4,5).

Dentre as substâncias irrigadoras, as soluções de hipoclorito de sódio, em diferentes concentrações, são as mais empregadas e aceitas pelas suas propriedades de clarificação, dissolução de tecido orgânico, saponificação, transformação de aminas em cloraminas, desodorização e ação antimicrobiana (5-7). Uma solução irrigadora alternativa é a clorexidina, que assim como o hipoclorito de sódio, pertence ao grupo dos compostos halogenados, por apresentar cloro em sua molécula e tem se mostrado efetiva no controle microbiano (8, 9).

Embora a erradicação completa da infecção no sistema de canais radiculares seja o objetivo primordial, estudos revelaram que o preparo biomecânico utilizando soluções irrigadoras com propriedades antimicrobianas e realizado com rigor de são incapazes de deixar o sistema de canais totalmente livre das bactérias e seus produtos tóxicos residuais (10, 11). Um dos fatores que dificultam a descontaminação é a complexidade anatômica do sistema de canais radiculares, que tornam algumas áreas inacessíveis ao preparo, embora a medicação intracanal como o hidróxido de cálcio possa ajudar a atingir tais regiões (12-14).

A literatura demonstra que há micro-organismos que sobrevivem às ações da terapêutica endodôntica, como as bactérias anaeróbias Gram-negativas, que são predominantes na colonização polimicrobiana dos canais radiculares (15, 16). Estas apresentam endotoxinas, que são complexos lipopolissacarídicos (LPS) com potente ação citotóxica, que representam o principal fator de virulência dessas bactérias (17). Estudos evidenciaram que as endotoxinas são os principais fatores etiológicos envolvidos na patogênese da inflamação e infecção pulpar e periapical (18-20).

Outro fator que determina dificuldade de ação do tratamento endodôntico é a presença do biofilme apical (21, 22). O biofilme apical é um complexo aglomerado microbiano, em que as bactérias se associam e estão envolvidas por uma matriz-extracelular polissacarídia, tornando-o resistentes à ação de agentes antimicrobianos (14).

Portanto, novas modalidades terapêuticas devem ser pesquisadas com intuito de combater e erradicar as infecções endodônticas. Com o advento dos aparelhos de Laser (Light Amplification by Stimulated Emission of Radiation) e Led (light-emitting diode), surgiram novas alternativas nos tratamentos médico, odontológico, e fisioterápico (23,24). Dentre as diferentes propriedades terapêuticas destes aparelhos, deparamos com a terapia fotodinâmica (TFD), que se baseia em um conjunto de procedimentos físicos e químicos com efeitos biológicos, que ocorrem após administrar um agente fotossensibilizador (FS) exógeno ativado por meio de uma fonte de luz visível (Laser ou Led) de comprimento de onda específico para ativar o FS com a finalidade de promover necrose celular em um local específico (célula-alvo) para o tratamento contra câncer, doenças não oncológicas e redução microbiana (25, 26).

Estudos realizados em diversos Grupos de Pesquisa mundiais, dentre eles o Laboratório de Biofotônica do Centro de Pesquisa em Óptica e Fotônica (CEPOF), do Programa CEPID-FAPESP - Grupo de Óptica, Instituto de Física de São Carlos, Universidade de São Paulo (IFSC-USP), demonstram o mecanismo de ação e as propriedades básicas e aplicadas da TFD (27-29). O mecanismo básico de ação se dá quando o FS absorve os fótons da fonte de luz e seus elétrons passam a um estado mais estimulado. Na presença de um substrato como o oxigênio, o FS transfere a energia ao substrato. Ao retornar ao seu estado natural, forma espécies altamente reativas e de vida curta, como o oxigênio singleto, que provoca sérios danos a célula alvo, como também, aos micro-organismos, via oxidação irreversível de componentes celulares (30-32).

Na Endodontia, estudos in vitro (33, 34) e in vivo (35, 36) demonstram que a TFD potencializa a desinfecção do canal radicular, tanto na ausência quanto na presença da medicação intracanal. Contudo, achados recentes (37, 38), em contraste com os demais estudos, demonstraram que a TFD não obteve completa desinfecção principalmente na ausência do emprego de medicação intracanal.

Embora haja risco de manchamento das estruturas dentais pelo FS, o azul de metileno e o azul de toluidina são comumente empregados como FS na TFD endodôntica (39). Há autores (36, 40), que recomendam novas investigações para conseguir um FS que não manche as estruturas dentais e ao mesmo tempo, aumente o potencial antimicrobiano para uso da TFD no controle e combate às infecções endodônticas, antes mesmo que seja preconizado o uso clinico na Endodontia.

A curcumina é um composto de cor amarela, extraída do rizoma da planta Curcuma longa L (açafrão) que está sendo pesquisado como FS na TFD contra o câncer (41, 42). A curcumina possui efeitos: antimicrobianos (43, 44), anti-inflamatórios (45), imunomoduladores (42), que são potencializados na presença de uma luz (Laser ou Led) de comprimento de onda específico para ativá-la.

Foi observado que as estruturas dentais radiculares podem sofrer manchamento em função do tipo e também da concentração do FS utilizado. A curcumina foi o FS que apresentou menor índice de manchamento, sem comprometer a cor da estrutura dental, quando comparado ao azul de metileno (46).

Considerando o emprego atual da TFD, novos estudos são necessários para elucidar as propriedades desta terapia. Trabalhos comparativos com análise da citotoxicidade e produção de citocinas por fibroblastos de camundongos; análise da biocompatibilidade tecidual e produção de citocinas pelo tecido subcutâneo de modelo experimental de ratos, em que comparem o emprego das diferentes soluções irrigadoras e da TFD são escassos, principalmente quando o FS é a curcumina. Assim, a analise da biocompatibilidade da TFD em diferentes modelos experimentais é oportuna.

PROPOSIÇÃO

Objetivo Geral:

O presente estudo visou avaliar a citotoxicidade e a resposta tecidual da terapia fotodinâmica comparativamente a diferentes soluções irrigadoras.

Objetivos Específicos:

1. Avaliação da citotoxicidade das soluções em cultura de fibroblastos de camundongos (L-929);
2. Avaliação da produção de citocinas (IL-1β e IL-6) por fibroblastos (L-929) estimulados pelas soluções;
3. Avaliação da resposta do tecido subcutâneo de ratos às soluções;
4. Avaliação a produção de citocinas (IL-1β e IL-6) pelo tecido subcutâneo de ratos estimulado pelas soluções.

Artigo I

Evaluation of the Effects of Photodynamic therapy and irrigating solutions on Fibroblast Viability and Cytokine Production

ABSTRACT

Introduction: Photodynamic therapy (PDT) is an aggregate of physical, chemical and biological procedures throughout the activation of the photosensitizer (PS) with light (Laser or Led) to destroy the target cells. The aim of this study was to evaluate the effects of PDT, 2.5% and 5% sodium hypochlorite, 2% chlorhexidine and saline solution on cell viability and cytokine (interleukin [IL]-1b and IL-6) production by mouse fibroblasts. Culture medium was used as control. **Methods:** The irrigating solutions and Curcumin were placed into 24-well cell culture plates with mouse fibroblasts L-929. Curcumin and Led wavelength (λ) 480 nm for 4 minutes was used for PDT. All solutions were diluted in culture medium DMEM ($1x10^4$ cells), 50 μl of the solutions to be tested. After 6, 24 and 48 hours, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was used to evaluate the cell viability and the supernatant was collected for cytokine evaluation using enzyme-linked immunosorbent assay. The data were statistically analyzed by analysis of variance and Bonferroni or Tukey´s test. **Results:** PDT and saline solution presented low cytotoxic effect similar to the control group ($p>0.05$). Sodium hypochlorite (2.5% and 5%) and 2% chlorhexidine were more cytotoxic than PDT ($p<0.05$) in all periods of time. All materials induced IL-6 and IL-1β releasing but the amount was not statistically significant compared with the control group. **Conclusions:** PDT with curcumin was not cytotoxic in fibroblast culture unlike the solutions of 2.5% and 5% sodium hypochlorite and 2% chlorhexidine. All materials were similar related to the cytokine release.

KEYWORDS: Endodontic treatment, root canal irrigants, photodynamic therapy, cell culture, cytotoxicity.

INTRODUCTION

Root canal cleaning and shaping are essential to reduce and / or eliminate the population of micro-organisms (MO) and their toxic products (endotoxins and apical biofilm) present in endodontic infection (1). The use of irrigating solution that assist in decontamination by its chemical action is essential to maximize cleaning during endodontic treatment (2).

Among the irrigation solutions, sodium hypochlorite (NaOCl) in different concentrations and 2% chlorhexidine are the most employed ones primarily for its antimicrobial action (2-3). However, despite the scientific-technical progress, authors show the persistence of MO in root canal system post-treatment (1-2, 4). Therefore, new therapeutic strategies must be investigated to potentiate the combat of endodontic infections.

Recently new methods as photodynamic therapy (PDT) are used on treatments to promote disinfection in periodontal diseases, dental caries among other dental specialties (5,6). PDT uses specific wavelength light Laser (light amplification by stimulated emission of radiation) or Led (light emitting diode) that activates the photosensitizer (PS) and produces highly reactive specie of oxygen (singlet oxygen) destroying the target cell (7,8) and assists antimicrobial action without the risk to promote microbial resistance (9).

In vitro (8, 10) and in vivo (11, 12) studies, demonstrated PDT as a new antimicrobial therapeutic modality aiming to increase the disinfection of root canal system during endodontic treatment. Studies evidenced PDT against *Enterococcus*

faecalis once PS fixes in the cell membrane and reaches the peak of absorption leading to generation of singlet oxygen, which destroys cellular wall and leads to bacterial death (8, 13).

Although PDT has been already employed (14) its cytotoxicity effect is not completely understood specially using Curcumin as a photosensitizer. Thus, the aim of this study was to determine the effects of PDT, 2.5% and 5% sodium hypochlorite, 2% chlorexidine on cell viability in fibroblasts and to assess the effects of these materials on the releasing of IL-6 and IL-1b.

MATERIALS AND METHODS

Fibroblasts Culture

L-929 mouse lineage fibroblasts were maintained in culture bottles with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD, USA), streptomycin (50 g/mL), and 1% antibiotic/antimycotic cocktail (300 units/mL penicillin, 300 μg/mL streptomycin, 5 μg/mL amphotericin B and 200 μg/mL of glutamin) (GIBCO BRL, Gaithersburg, MD, USA). The cultures were maintained under standard cell culture conditions (37°C, 100% humidity, 5% CO_2) (15)

Group Division and Photodynamic Therapy

It was used 450 μl of DMEM and 50 μl of each solution to be tested for each well. The groups were distributed: sodium hypochlorite 5%; sodium hypochlorite 2.5%; chlorhexidine 2%; saline solution; PDT; control. PDT was performed with PS curcumin 500mg/L (PDTPharma, Cravinhos, SP, Brazil), in the period of 5 minutes of pre-irradiation as recommended by Soukos et al. 2006 (13). Then the FS was activated with

blue Led for λ 480 nm for 4 minutes (16). FS and Led were designed by Physics Institute of São Carlos, University of São Paulo, São Carlos, SP, Brazil.

Cytotoxicity Testing

L929 fibroblasts were seeded into the 24-well plates ($1x10^4$ cell/well). The cells were incubated for 24 hours in a humidified air atmosphere of 5% CO2 at 37ºC. The solutions were tested for 6h, 24h and 48h. Tree wells were used for each material, and wells with DMEM were used as the control. Viable cells were stained with formazan dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) (Sigma Chemical Co, St Louis, MO). MTT was dissolved in phosphate- buffered saline at 5 mg/mL and filtered in order to sterilize and remove a small amount of insoluble residue. At the times indicated later, stock MTT solution (40 μL per 360 μL medium) was added to all wells of an assay, and plates were incubated at 37ºC for 3 hours. The medium was then removed by the inversion of the plate and the dumping of 200 mL of isopropilic alcohol, which was added to the wells and mixed during 20 minutes in order to dissolve the dark blue crystals. The blue solution was transferred to a 96-well plate, and the absorbance was read in the microplate reader by use of a test wavelength of 570 nm (17).

Enzyme-linked Immunosorbent Assay

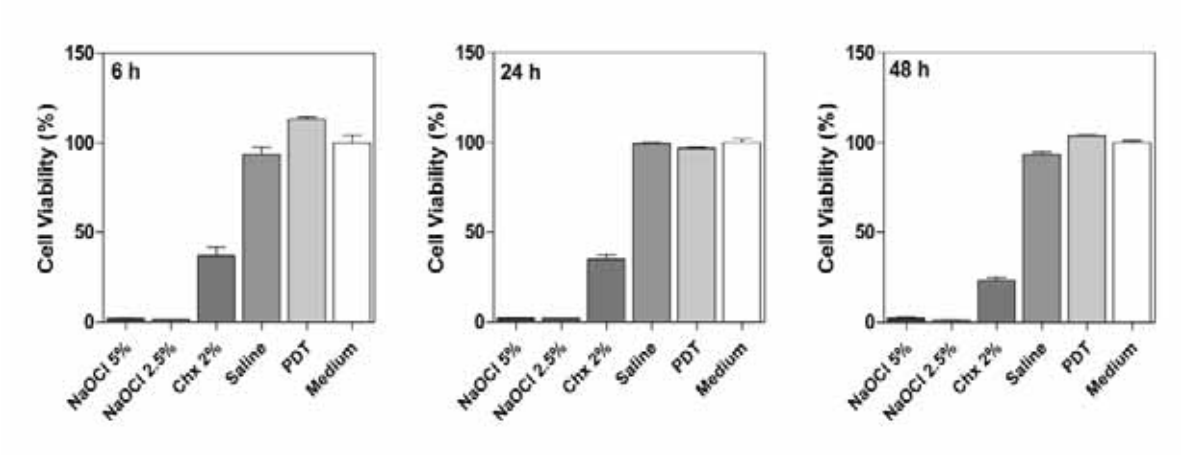
IL-1b and IL-6 levels in supernatants were measured using Peprotech Murine IL-1b and IL-6 mini ELISA development kits (Peprotech) according to the manufacturer's instructions. All of the experiments were performed 3 times.

Statistical Analysis

The results were statistically analyzed by ANOVA test with BonFerroni correction ($p<0,05$) for MTT and by ANOVA with Tukey´s Test ($p<0,05$) for ELISA.

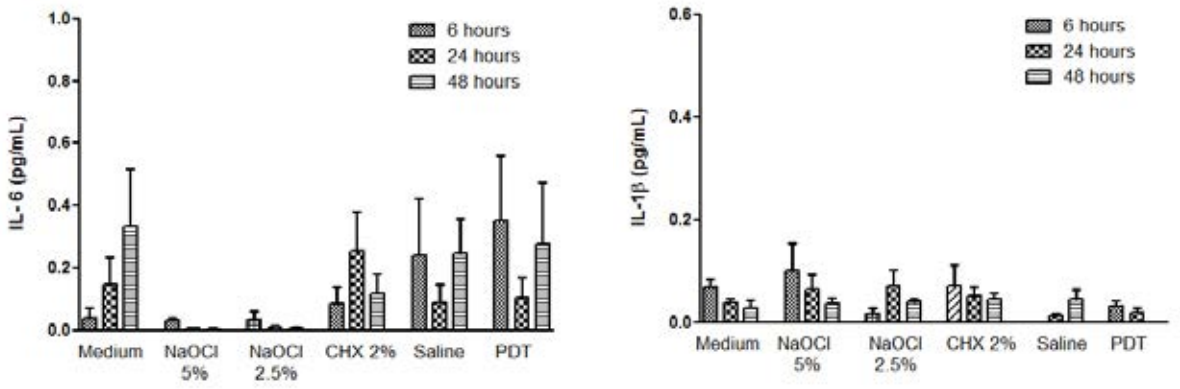
RESULTS

The results of MTT test in the periods of 6h, 24h and 48h revealed that saline solution and PDT presented similar mild cytotoxic effect ($p>0.05$) when compared to control. However, sodium hypochlorite 5%, sodium hypochlorite 2.5% and chlorhexidine 2% was more cytotoxic than PDT ($p<0.05$) (Fig. 1)



Figures 1: Viability of fibroblasts in the presence of PDT and irrigating solutions, the periods 6h, 24h and 48h. NaOCl – sodium hypochlorite; Chx – chlorhexidine; PDT – photodynamic therapy.

ELISA assay revealed that the average of IL-6 IL-1β (pg/mL) releasing was similar among the groups ($p>0.05$) (Figure 2).



Figures 2: Mean levels of IL-6 and IL-1b were raised when the cells were grown in the presence of the materials. There was no statistically significant difference (P > 0.05) between the experimental materials and the control group. NaOCl – sodium hypochlorite; Chx – chlorhexidine; PDT – photodynamic therapy

DISCUSSION

The cytotoxicity analysis allows us to verify the effects of substances or procedures in cellular level, besides to enable the evaluation of cells behavior in controlled ambient, once it is a simple, rapid and precision method (18).

Sodium hypochlorite is the most popular and widely substance used for irrigation of root canals, due to its properties such as the ability to dissolve organic matter, neutralizing toxic contents and broad antimicrobial spectrum (19). Although it is an effective antibacterial agent, sodium hypochlorite is harmful when spilled out of the root canals, causing injuries when in contact with the periapical tissues (20, 21). In recent years chlorhexidine has also been noted in irrigation of root canals, as an alternative to the use of sodium hypochlorite as an irrigating solution, because it has broad-spectrum antimicrobial activity, beyond having residual effect called substantivity of 48h to 72h (20, 22), which favors the decontamination of channels infected root.

In vitro cytotoxicity studies of irrigating solutions sodium hypochlorite (2.5% and 5%) and 2% chlorhexidine (23, 24) showed that all solutions were toxic. Although

the concentration used, the time of exposure to the agent and the surface exposure are important factors that vary the magnitude of the resulting effect. In this study, it was demonstrated by MTT assay that PDT with curcumin PS and Blue Light in the concentration and parameters used was less cytotoxic than sodium hypochlorite (2.5% and 5%) and 2% chlorhexidine at all the periods tested (6h, 24h, 48h) and similar to saline and control. Similar results were found by other authors (23), however with methylene blue PS and red light. Another study concluded that mitochondrial activity of cultured cells remained unaffected when exposed to blue light and curcumin, suggesting a good therapeutic index in vitro (25). These results are contrary to others studies that showed such combination promoted reduction in fibroblast, macrophage and human head and neck cancer cell line cell culture (26,27,28).

Although sodium hypochlorite and chlorhexidine had been more cytotoxic, all irrigants showed similar Il-6 and IL-1β release. A proinflammatory cytokine cascade is induced in response to bacterial infection of the dental pulp or even material toxicity. Some of these mediators stimulate bone resorption, in particular, interleukin-1α (IL-1α) and IL-1β, which have been shown to be key mediators of periapical bone destruction in vivo (29). IL-1 expression is induced by exposure of host cells to lipopolysaccharide (LPS) and other bacterial cell wall components (30). Interleukin-6 (IL-6) is a cytokine that plays an important role in immune responses. IL-6 is considered both a pro- and anti-inflammatory cytokine; it is produced during inflammation and after interleukin- 1 (IL-1) secretion. IL-6 subsequently inhibits the secretion of IL-1 (31). Our results with PDT are in agreement with this statement.

The results obtained can be related to the PS and the protocol of light stimulation used. Curcumin is a yellow-color compound, extracted from Curcuma longa L plant

rhizome. It has antimicrobial, anti-inflammatory and immunomodulators effects that can be potentiated in the presence of blue light (Laser or Led), considering that this PS is very employed in the treatment against cancer (32). Recent study showed curcumin prevents PDT-induced cell death (33). It was already shown that curcumin significantly reduced IL-1β (34).

In this study, we employed the same concentration of curcumin PS and parameters of Blue Light of previous report used in the treatment of inflammatory intestine diseases, pancreatitis and cancer (16). It was used 5 minutes pre irradiation and 4 minutes of light stimulation. Such protocol agree with other studies (12, 14, 35), however using methylene blue PS. For curcumin PS we find a study that used 15 minutes pre irradiation and 4 minutes of light stimulation and obtained similar results to ours (25). The same cannot be said when comparing our results with the studies that recommended pre irradiation and concentrations higher than our (26, 27, 28).

According to the methodology employed, it was possible to conclude that PDT with curcumin PS in the used concentration and blue Led in the parameters employed was not cytotoxic and did not inhibited the L-929 fibroblasts viability, demonstrating distinct results of 2,5% and 5% sodium hypochlorite and 2% chlorhexidine solutions.

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Conflict of Interest: The authors declare that they have no conflict of interest

REFERENCES

1. Vera J, Siqueira JF Jr, Ricucci D, et al. One- versus two-visit endodontic treatment of teeth with apical periodontitis: a histobacteriologic study. J Endod 2012; 38: 1040-52.
2. Rôças IN, Siqueira JF Jr. Characterization of microbiota of root canal-treated teeth with posttreatment disease. J Clin Microbiol 2012; 50: 1721-4.
3. Givol N, Rosen E, Taicher S, et al. Risk management in endodontics. J Endod 2010; 36: 982-4.
4. Nair PN, Henry S, Cano V, et al. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one-visit" endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2005; 99 :231-52.
5. Theodoro LH, Silva SP, Pires JR, et al. Clinical and microbiological effects of photodynamic therapy associated with nonsurgical periodontal treatment. A 6-month follow-up. Lasers Med Sci 2012; 27: 687-93.
6. Araujo NC, Fontana CR, Bagnato VS, et al. Photodynamic effects of curcumin against cariogenic pathogens. Photomed Laser Surg 2012; 30: 393-9.
7. Wilson BC, Patterson MS. The physics, biophysics and technology of photodynamic therapy. Phys Med Biol 2008; 53: 61-109.
8. Komine C, Tsujimoto Y. A small amount of singlet oxygen generated via excited methylene blue by photodynamic therapy induces the sterilization of Enterococcus faecalis. J Endod 2013; 39: 411-4.
9. Gonzales FP, Felgenträger A, Bäumler W, et al. Fungicidal photodynamic effect of a twofold positively charged porphyrin against Candida albicans planktonic cells and biofilms. Future Microbiol 2013; 8 :785-97.

10. Ng R, Singh F, Papamanou DA, et al. Endodontic photodynamic therapy ex vivo. J Endod 2011; 37: 217-22.
11. Garcez AS, Nuñez SC, Hamblin MR, et al. Antimicrobial effects of photodynamic therapy on patients with necrotic pulps and periapical lesion. J Endod 2008; 34: 138-42.
12. Silva LA, Novaes AB Jr, Oliveira RR, et al. Antimicrobial photodynamic therapy for the treatment of teeth with apical periodontitis: a histopathological evaluation. J Endod 2012; 38: 360-6.
13. Soukos NS, Chen PS, Morris JT, et al. Photodynamic therapy for endodontic disinfection. J Endod 2006; 32: 979-84.
14. Garcez AS, Fregnani ER, Rodriguez HM, Nunez SC, Sabino CP, Suzuki H, Ribeiro MS. The use of optical fiber in endodontic photodynamic therapy. Is it really relevant? Lasers Med Sci. 2013; 28: 79-85.
15. Gomes-Filho JE, Watanabe S, Gomes AC, et al. Evaluation of the effects of endodontic materials on fibroblast viability and cytokine production. J Endod 2009; 35: 1577-9.
16. Jurenka JS. Anti-inflammatory properties of curcumin, a major constituent of Curcuma longa: a review of preclinical and clinical research. Altern Med Rev 2009; 14: 141-53.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65 :55–63.
18. Malheiros CF, Marques MM, Gavini G. In vitro evaluation of the cytotoxic effects of acid solutions used as canal irrigants. J Endod 2005; 31: 746-8.

19. Guerreiro-Tanomaru JM, Morgental RD, Flumignan DL, Gasparini F, Oliveira JE, Tanomaru-Filho M. Evaluation of pH, available chlorine content, and antibacterial activity of endodontic irrigants and their combinations against Enterococcus faecalis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011; 112: 132-5.
20. Gomes-Filho JE, Aurélio KG, Costa MM, Bernabé PF. Comparison of the biocompatibility of different root canal irrigants. J Appl Oral Sci 2008; 16: 137-44.
21. Berber VB, Gomes BPFA, Sena NT, Vianna ME, Ferraz CCR, Zaia AA, Souza-Filho FJ. Efficacy of various concentrations of NaOCl and instrumentation techniques in reducing Enterococcus faecalis within root canal and dentinal tubules. Int Endod J 2006; 39: 10-7.
22. Gomes BP, Vianna ME, Zaia AA, Almeida JF, Souza-Filho FJ, Ferraz CC. Chlorhexidine in endodontics. Braz Dent J 2013; 24: 89-102
23. George S, Kishen A. Advanced noninvasive light-activated disinfection: assessment of cytotoxicity on fibroblast versus antimicrobial activity against Enterococcus faecalis. J Endod 2007; 33: 599-602.
24. Chang YC, Huang FM, Tai KW, Chou MY. The effect of sodium hypochlorite and chlorhexidine on cultured human periodontal ligament cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001; 92: 446-50.
25. Bulit F, Grad I, Manoil D, Simon S, Wataha JC, Filieri A, Feki A, Schrenzel J, Lange N, Bouillaguet S. Antimicrobial activity and cytotoxicity of 3 photosensitizers activated with blue light. J Endod. 2014; 40: 427-31.

26. Ribeiro AP, Pavarina AC, Dovigo LN, Brunetti IL, Bagnato VS, Vergani CE, Costa CA. Phototoxic effect of curcumin on methicillin-resistant Staphylococcus aureus and L929 fibroblasts. Lasers Med Sci. 2013; 28: 391-8.
27. Dovigo LN, Pavarina AC, Ribeiro AP, Brunetti IL, Costa CA, Jacomassi DP, Bagnato VS, Kurachi C. Investigation of the photodynamic effects of curcumin against Candida albicans. Photochem Photobiol. 2011; 87: 895-903.
28. Ahn JC, Kang JW, Shin JI, Chung PS. Combination treatment with photodynamic therapy and curcumin induces mitochondria-dependent apoptosis in AMC-HN3 cells. Int J Oncol. 2012; 41: 2184-90
29. Kawashima N., Stashenko P. Expression of bone resorptive and regulatory cytokines in infraosseus inflammation. Arch. Oral Biol. 1998; 44: 55–66.
30. Burysek L., Houstek J. Multifactorial induction of gene expression and nuclear localization of mouse interleukin 1 alpha. Cytokine 1996; 8: 460–467.
31. Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood. 1990 Jan 1;75(1):40-7.
32. Ali S, Ahmad A, Banerjee S, et al. Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF. Cancer Res 2010;70:3606-17.
33. Chan WH1, Wu HJ. Anti-apoptotic effects of curcumin on photosensitized human epidermal carcinoma A431 cells. J Cell Biochem. 2004 May 1;92(1):200-12.
34. Clutterbuck AL1, Allaway D2, Harris P2, Mobasheri A3. Curcumin reduces prostaglandin E2, matrix metalloproteinase-3 and proteoglycan release in the

secretome of interleukin 1β-treated articular cartilage. F1000Research 2013;2:147.

35. Souza LC, Brito PR, de Oliveira JC, et al. Photodynamic therapy with two different photosensitizers as a supplement to instrumentation/irrigation procedures in promoting intracanal reduction of Enterococcus faecalis. J Endod 2010;36:292-6.

Artigo II

Rat tissue reaction and cytokine production induced by photodynamic therapy and irrigating solutions

Abstract

Introduction: Photodynamic therapy (PDT) aims to destroy the target cell through the reaction between Laser or LED, photosensitizer (PS) and O2. This study evaluated the rat subcutaneous tissue reaction and cytokine (interleukin IL-1β and IL-6) production to PDT, 2% chlorhexidine, 2.5% and 5% sodium hypochlorite. **Methods**: The solutions were placed in polyethylene tubes and implanted into the dorsal connective tissue of Wistar rats for 7, 15, 30, 60, and 90 days. One half of the specimens were stained with hematoxylin and eosin. The other half was collected for cytokine evaluation by using an enzyme-linked immunosorbent assay. Statistical analysis was performed using ANOVA, Kruskal Wallis and Tukey´s tests ($p < 0.05$). **Results**: All solutions caused severe reactions after 7 days that decreased with time. All groups presented moderate reaction except chlorhexidine that remained severe at the 15th day. Control group and 2.5% sodium hypochlorite caused mild reactions after 30 days. Chlorhexidine and PDT caused moderate and mild inflammation at the 60th day, respectively. PDT caused no inflammation on 90th day. PDT produced more IL-1β at 7, 15, and 90 days. At the 30th day, the highest average was 2.5% hypochlorite and at 60th day, chlorhexidine. For IL-6 the highest production on 7 and 60 days was the chlorhexidine, on 15 days was 5% hypochlorite, on 30 days was 2.5% hypochlorite and on 90 days was PDT. **Conclusions**: PDT was biocompatible and expressed IL - 1β and IL – 6 similarly to the other solutions.

Introduction

Endodontic treatment is essential to deal with the root canal system infection when biomechanical treatment tries to eliminate bacteria as well as deprives the canal of nutrients favoring the tissue healing (1). Treatment success depends on the efficient cleaning, shaping and obturation of the root canal system (2).

Endodontic treatment using irrigating solutions such as sodium hypochlorite reduces the number of microorganisms and their toxic by-products (endotoxins and

apical biofilm), especially when associated calcium hydroxide intracanal medication (3). However, endodontic treatment failures may occur regarding the persistence of microorganisms and its by-products (4) due to: anatomical complexity of the root canal system and coronary infiltration, apical infiltration or combinations that leads to endodontic failures (5).

Therefore, constant investigations must be conducted in the search for therapeutic strategies to enhance the endodontic infection control. Photodynamic therapy (PDT), seeks to destroy the target cell (6) and/or assist in antimicrobial activity without generating microbial resistance (7). It uses a specific wavelength light (λ) (laser or LED) that activates the photosensitizer (PS). Activated PS, in the presence of oxygen, produces highly reactive species that destroys the target cell (6).

Recently, PDT was employed as a new therapeutic modality adjunct to endodontic treatment, to enhance the disinfection of the root canal system (8), including against Enterococcus faecalis (9). Considering the current use of PDT, studies about the tissue reaction and cytokines production induced by PDT are scarce and needed to be clarified. The aim of this study was to evaluate the tissue reaction and production of cytokines by PDT comparing it to different irrigating solutions.

Material and Methods

Thirty male 4- to 6-month-old Wistar Albino rats, weighing 250–280 g, were used in the study. The animals were housed in temperature-controlled rooms and received water and food ad libitum. The care of the animals was performed according to the Araçatuba School of Dentistry-UNESP Ethical Committee, which approved the project before the beginning of the experiment.

Hundred and fifty polyethylene tubes (Delton, Johnson & Johnson, São José dos Campos, SP, Brazil) with a 1.1-mm internal diameter, 1.6-mm external diameter, and 10.0-mm length were filled with fibrin sponges (Lasbrasil, São Paulo,SP, Brazil) soaked with 0,1ml of test solutions: saline solution (used as control), 2,5% and 5% sodium hypochlorite, 2% chlorhexidine (Apothicario, Araçatuba, São Paulo, Brazil) and the photosensitizer curcumin 500 mg/L (PDT Pharma, Cravinhos, SP, Brazil) for 5 minutes pre-irradiation in dark room, than PDT was performed with Blue Light (Instituto de

Física de São Carlos - IFSC-USP, São Carlos, SP, Brazil) λ 480 nm, fluence of 75 J/cm2, for 4 minutes.

The animals were disinfected with 5% iodine solution, after that they were shaved under xylazine (10 mg/kg) and ketamine (25 mg/kg) anesthesia. A 2-cm incision was made in a head–tail orientation on the shaved back of each animal with a number 15 Bard-ParkerTM blade (Franklin Lakes, NJ, USA). The skin was reflected to create five pockets, two in the cranial portion and three in the caudal portion. The tubes were implanted into the pockets and the skin was closed with 4/0 silk sutures.

After 7, 15, 30, 60, and 90 days from the implantation time, the animals were killed by overdose of an anesthetic solution. The tubes with surrounding tissues were removed and fixed in 10% buffered formalin at pH 7.0 (10). The tubes were then bisected transversely. One half of the specimens were processed for glycol methacrylate embedding, serially sectioned into 3 μm slices, and stained with hematoxylin-eosin (11).

Inflammatory reactions in the tissue in contact with the material on the open end of the tube were scored according to previous studies (11,12,13) as follows: 0, none or few inflammatory cells and no reaction; 1, <25 cells and mild reaction; 2, between 25 and 125 cells and moderate reaction; and 3, 125 or more cells and severe reaction. Fibrous capsules were considered thin when it is <150 μm and considered thick at >150 μm. An average number of cells for each group was obtained from 10 separate areas (x400). Analyses were performed by a single calibrated operator in a blinded manner. Results were statistically analyzed by ANOVA and Kruskal–Wallis tests.

The other half of the specimens was processed for ELISA assay, when the surrounding tube tissues were collected, weighed and kept in frozen liquid nitrogen in order to measure IL-6 and IL-1β. 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 -80ºC until use. The 96-well plate was coated using Rat IL-6 Platinum ELISA and Rat IL-1 beta Platinum ELISA (eBioscience, Vienna, Austria). A color change proportional to the amount of IL-6 and IL-1β was quantified by comparing the absorbance of the samples with those of known dilutions using a plate reader at 450 nm. The concentrations of IL-6 and IL-1β were

calculated (in pg/mL) by comparison with a standard curve. The results were statistically analyzed by ANOVA and Tukeys's test ($p<0.05$).

Results

Test materials

Control (saline solution)

At the 7th day, severe inflammatory cell infiltration with prevalence of lymphocytes macrophages, focal areas with polymorphonuclear cells sparse areas of necrosis was observed. At the 15th day, moderate inflammatory cell infiltration with presence of angiogenesis and granulation tissue was noted. On days 30 and 60, mild inflammatory cell infiltration with formation of reactive tissue, but at the 90th days, absence of inflammation, remodeling and presence of a thin capsule were observed.

Sodium Hypochlorite (2,5%)

At the 7th day, severe inflammatory cell infiltrated with prevalence of lymphocytes, macrophages and areas of collagen organization was observed. However, at the 15th day, moderate inflammatory cell infiltration with angiogenesis and granulation tissue was noted. The intensity of inflammation was reduced on days 30, 60 and 90 with mild inflammatory cell infiltration and formation of reactive tissue.

Sodium Hypochlorite (5%)

At the 7th day, severe inflammatory cell infiltration with prevalence of lymphocytes, macrophages, focal areas with polymorphonuclear cells and early capsule formation was observed. On days 15, 30 and 60 moderate inflammatory cell infiltration with angiogenesis and granulation tissue was noted. At the 90th day, mild inflammatory cell infiltration was present in a thin capsule.

Chlorhexidine 2%

On the days 7, 15 and 30, severe inflammatory cell infiltration with prevalence of lymphocytes, plasma cells and organization of some foreign body giant cells and sparse areas of necrosis was observed. On days 60 and 90, moderate inflammatory cell infiltration with neovascularization was present in a thick capsule.

PDT (Curcumin and Blue Light)

At the 7th day, severe inflammatory cell infiltration with prevalence of lymphocytes, macrophages, focal areas with polymorphonuclear cells and sparse areas of necrosis was observed. On days 15 and 30, moderate inflammatory cell infiltration with angiogenesis and granulation tissue was noted. At the 60th day, mild inflammatory cell infiltration was observed with formation of reactive tissue. At the 90th day, it was noted absence of inflammation, remodeling and presence of thin capsule.

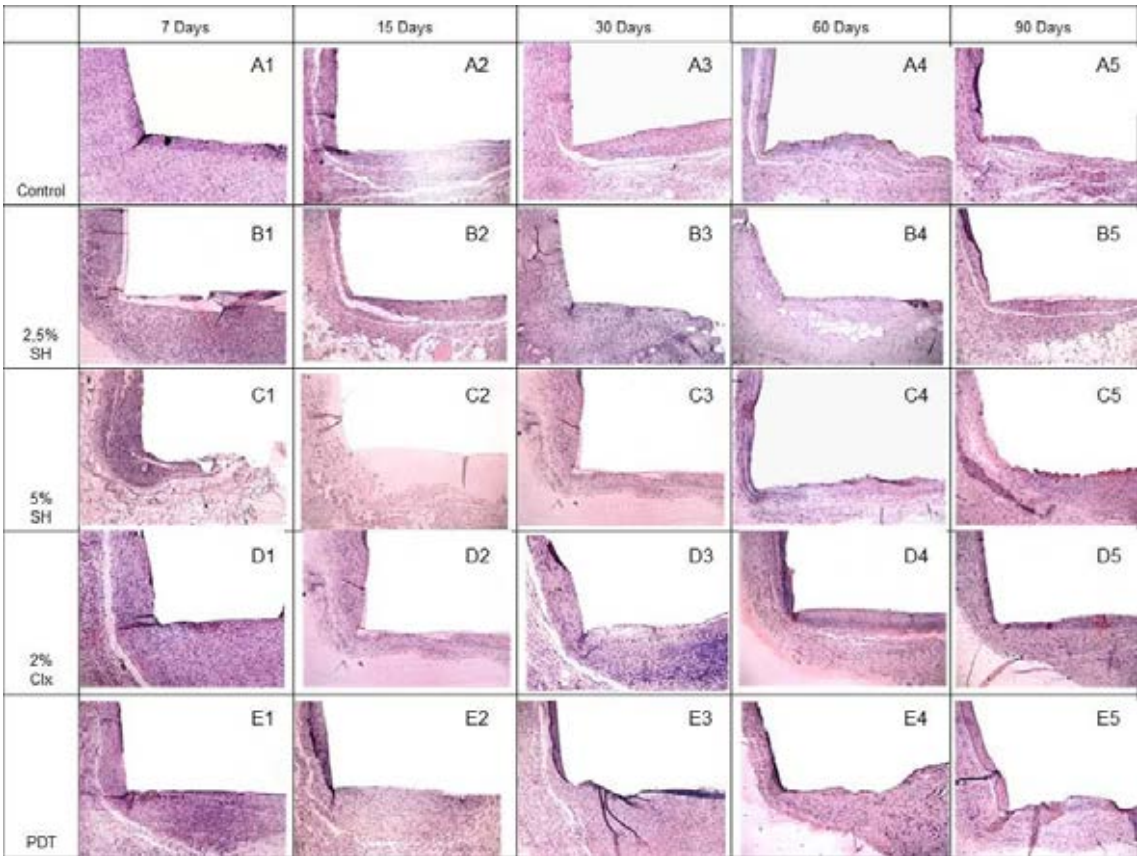


Figure 1: After 7 days, thick fibrous capsule formation and severe inflammatory cell infiltration were observed with 2.5% and 5% hypochlorite (B1 and C1), 2% chlorhexidine (D1) and PDT (E1). After 15 days, note that fibrous capsule remained thick with moderate inflammatory cell infiltration (A2, B2, C2 and E2) while 2% chlorhexidine remained severe (D2). After 30 days, thin fibrous capsule were observed with control (A3) and PDT (E3). The same thin fibrous capsule was observed with 2,5% hypochlorite (B4) after 60 days and 5% hypochlorite (C5) after 90 days. Hematoxilin and eosin 10x.

Comparisons among the groups

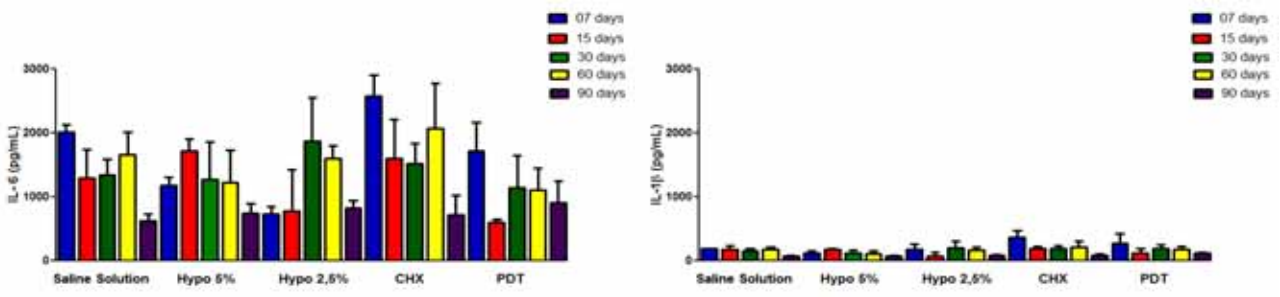
The data were compared for each time point as shown in Table 1. At the 7^{th} day, all groups showed severe inflammatory reaction ($p>0.05$). At 15^{th} day, all groups presented moderate reaction ($p>0.05$), except for chlorhexidine that remained severe ($p<0.05$). On 30 days, control and 2.5% sodium hypochlorite caused mild reactions, 5% sodium hypochlorite and PDT caused moderate reaction, and 2% chlorhexidine caused severe reaction ($p<0.05$). At the 60^{th} day, PDT, saline and 2.5% sodium hypochlorite caused mild reaction, but 5% sodium hypochlorite and 2% chlorhexidine remained moderately inflamed ($p<0.05$). PDT and saline caused no inflammation on 90^{th} day, 2.5% and 5% sodium hypochlorite remained mildly inflamed, and 2% chlorhexidine remained moderately inflamed ($p<0.05$). Calcification and necrosis were absent in all groups independently of the period.

	SCORE						
MATERIAL	**0**	**1**	**2**	**3**	**Calcificacion**	**Necrosis**	**Capsule**
7 days							
Saline Solution	0	0	0	100	Absent	Present	Absent
HS 2,5%	0	0	0	100	Absent	Present	Thick
HS 5%	0	0	0	100	Absent	Present	Thick
Clx	0	0	0	100	Absent	Present	Thick
PDT	0	0	0	100	Absent	Present	Thick
15 days							
Saline Solution	0	0	100	0	Absent	Absent	Thick
HS 2,5%	0	0	100	0	Absent	Absent	Thick
HS 5%	0	0	100	0	Absent	Absent	Thick
Clx	0	0	0	100	Absent	Absent	Thick
PDT	0	0	100	0	Absent	Absent	Thick
30 days							
Saline Solution	0	100	0	0	Absent	Absent	Thin
HS 2,5%	0	100	0	0	Absent	Absent	Thick
HS 5%	0	0	100	0	Absent	Absent	Thick
Clx	0	0	0	100	Absent	Absent	Thick
PDT	0	0	100	0	Absent	Absent	Thin
60 days							
Saline Solution	0	100	0	0	Ausente	Ausente	Thin
HS 2,5%	0	100	0	0	Absent	Absent	Thin
HS 5%	0	0	100	0	Absent	Absent	Thick
Clx	0	0	100	0	Absent	Absent	Thick
PDT	0	100	0	0	Absent	Absent	Thin
90 days							
Saline Solution	100	0	0	0	Absent	Absent	Thin
HS 2,5%	0	100	0	0	Absent	Absent	Thin
HS 5%	0	100	0	0	Absent	Absent	Thin
Clx	0	0	100	0	Absent	Absent	Thick
PDT	100	0	0	0	Absent	Absent	Thin

Table 1- Score: 0 – none or few inflammatory cells and no reaction; 1 – <25 cells and mild reaction; 2 – between 25 and 125 cells and moderate reaction; 3 – 125 or more cells and severe reaction.
Same letters indicate no statistical difference among the materials and same numbers indicate no statistical difference of each material in different experimental periods of time.

Cytokine production

The results of ELISA showed that the average IL-1β was higher for PDT on days 7, 15 and 90, compared to the other groups. In 30 days 2,5% sodium hypochlorite showed greater media and on 60 days the highest level found was 2% chlorhexidine. For IL-6 2% chlorhexidine expressed higher after 7 and 60 days. In 15 days, 5% sodium hypochlorite provided higher average and at 30 days was higher level 2,5% sodium hypochlorite. The PDT showed a higher level at 90 days (Figure 2).



Figures 2: Mean levels of IL-6 and IL-1b were raised when the cells were grown in the presence of the solutions. For IL-6 there was a statistically significant difference ($P < 0.05$) between 2.5% hypochlorite and control, 5% hypochlorite and 2% chlorhexidine, and 2.5% and 2% chlorhexidine. For IL-1b there was no statistically significant difference ($P > 0.05$) between the experimental materials and the control group.

Discussion

Various irrigants have been investigated for use in canal preparation. Each product has different properties, and several studies have compared their antimicrobial and chemical activities and biocompatibility in an effort to develop the ideal solution to be used as an adjuvant to root canal treatment. Sodium hypochlorite has been widely used in endodontic therapy due to its antibacterial action and ability to dissolve organic matter (14). However, NaOCl is known to be a potential irritant to the periapical tissues and often causes inflammatory reactions, which mainly occur at high concentrations; in low concentrations, however, it is ineffective against certain microorganisms (15). Furthermore, its use poses additional potential problems, including odor and discoloration of operatory items (16).

Chlorhexidine is a broad-spectrum antimicrobial agent and is mainly used as a mouth rinse in the prevention and treatment of periodontal diseases and dental caries (17). When used as a root canal irrigant and intracanal medication, it has an antibacterial efficacy comparable to that of the NaOCl (18) while being effective against certain NaOCl-resistant bacteria (19).

Which of these irrigants is preferable with respect to clinically important properties such as antibacterial activity and tissue dissolution is still an open question (20). The results of the present study demonstrated that PDT in concentration and parameters used had similar tissue response to control (saline solution) exhibiting moderate inflammatory infiltration on days 15, 30 and 60, and absence of inflammation, with the presence of thick fibrous capsule on 90 days. These results agree with previous reports that demonstrated saline as biocompatible solution (21).

In the present study, it was employed the same concentration of curcumin PS and parameters of Blue Light of previous report used in the treatment of inflammatory intestine diseases, pancreatitis and cancer (22). It was used 5 minutes pre irradiation and 4 minutes of light stimulation. Such protocol agree with other studies (8, 10), however using methylene blue PS. Curcumin PS was previous used in a different protocol of pre irradiation and light stimulation during 15 minutes and 4 minutes respectively producing a high cell viability similar to the results of the present study (23). However other papers found low cell viability when higher pre irradiation and concentrations were used (24-26) evidencing that the best protocol is not completely achieved.

By the other hand, 5% sodium hypochlorite showed moderate to severe inflammatory infiltration between 30 and 60 days. It has been reported that 5.25% NaOCl solution promotes an irritating effect on the periapical tissues and that, at the end of the second week of evaluation, foreign body granuloma formation occurred (27). In the same way as observed in the present investigation, other studies have reported that the number of inflammatory cells remained high at the sites treated with 5.25% NaOCl even after 14 days, and that complete healing was not observed (27-29). Also evaluating the toxic effect of 5.25% NaOCl, a previous study showed that in sites injected with this solution, tissue regeneration occurred at a slower rate when compared to the sites injected with 2.0% chlorhexidine gluconate. It has also been reported that 2.0% chlorhexidine gluconate displayed residual antibacterial activity and was more powerful and less toxic than 5.25% NaOCl (28).

In the present study, chlorhexidine also showed moderate to severe inflammatory infiltration between 30 and 60 days. A similar outcome was previously observed at 14 days, when 0.12% chlorhexidine gluconate was evaluated as an irrigating solution (29). In these cases, the inflammatory response was at the highest level 48 h later, whereas the level of inflammation dropped by the end of the second week (29). It has also been demonstrated that chlorhexidine gluconate had no less antibacterial effect than NaOCl and that, because of its lower toxicity, should be preferred in root canal therapy, especially in cases of immature teeth (16).

Although sodium hypochlorite and chlorhexidine had evoked a more intense inflammatory response, all irrigants showed similar Il-6 and IL-1β release. A proinflammatory cytokine cascade is induced in response to bacterial infection of the dental pulp or even material toxicity. Some of these mediators stimulate bone resorption, in particular, interleukin-1α (IL-1α) and IL-1β, which have been shown to be key mediators of periapical bone destruction in vivo (30). IL-1 expression is induced by exposure of host cells to lipopolysaccharide (LPS) and other bacterial cell wall components (31). IL-6 is a pleiotropic cytokine that possesses activities that may enhance or suppress inflammatory bone destruction (32). IL-6 is produced locally in bone following stimulation by IL-1 and tumor necrosis factor (TNF) (33). IL-6 stimulates the formation of osteoclast precursors from colony-forming unit–granulocyte-macrophage (34) and increases osteoclast numbers in vivo, leading to

systemic increases in bone resorption (35). However, emerging data suggest that IL-6 also has significant anti-inflammatory activities (32).

The ideal solution and protocol was not achieved yet but based on the methods used, it was concluded that PDT was biocompatible and expressed IL - 1β and IL – 6 similarly to the other solutions.

References

1. Sundqvist G. Ecology of the root canal flora. J Endod 1992; 18:427-30.
2. Nair PN. On the causes of persistent apical periodontitis: a review. Int Endod J 2006; 39:249-81
3. Mohammadi Z, Dummer PM. Properties and applications of calcium hydroxide in endodontics and dental traumatology. Int Endod J 2011; 44:697-730.
4. Nair PN, Henry S, Cano V, et al. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one-visit" endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2005; 99:231-52.
5. Givol N, Rosen E, Taicher S, et al. Risk management in endodontics. J Endod 2010; 36:982-4.
6. Wilson BC, Patterson MS. The physics, biophysics and technology of photodynamic therapy. Phys Med Biol 2008; 53:R61-109.
7. Gonzales FP, Felgenträger A, Bäumler W, et al. Fungicidal photodynamic effect of a twofold positively charged porphyrin against Candida albicans planktonic cells and biofilms. Future Microbiol 2013; 8:785-97.
8. Garcez AS, Nuñez SC, Hamblin MR, et al. Antimicrobial effects of photodynamic therapy on patients with necrotic pulps and periapical lesion. J Endod 2008; 34:138-42.

9. Soukos NS, Chen PS, Morris JT, et al. Photodynamic therapy for endodontic disinfection. J Endod 2006; 32:979-84.

10. American National Standards Institute/Revised American National Standards Institute American Dental Association. Document No. 41. For recommended standard practices for biological evaluation of dental materials. New York, NY: American National Standards Institute; 1979.

11. Gomes Filho JE, Gomes BPFA, Zaia AA, Novaes PD, Souza Filho FJ. Glycol Methacrylate: an alternative method for embedding subcutaneous implants. J Endod 2001; 27:266–8.

12. Gomes-Filho JE, Watanabe S, Lodi CS, Cintra LT, Nery MJ, Filho JA, Dezan E Jr, Bernabé PF. Rat tissue reaction to MTA FILLAPEX®. Dent Traumatol. 2012 Dec; 28(6):452-6.

13. Gomes-Filho JE, Duarte PC, de Oliveira CB, et al. Tissue reaction to a triantibiotic paste used for endodontic tissue self-regeneration of nonvital immature permanent teeth. J Endod. 2012; 38:91-4.

14. Zehnder M Root canal irrigants. J Endod 2006 32:389–398.

15. Leonardo MR, Tanomaru Filho M, Silva LA, Nelson Filho P, Bonifacio KC, Ito IY. In vivo antimicrobial activity of 2% chlorhexidine used as a root canal irrigating solution. J Endod 199 25:167–177.

16. Jeansonne MJ, White RR. A comparison of 2.0% chlorhexidine gluconate and 5.25% sodium hypochlorite as antimicrobial endodontic irrigants. J Endod 1994; 20:276–278.

17. Cervone F, Tronstad L, Hammond B. Antimicrobial effect of chlorhexidine in a controlled release delivery system. Endod Dent Traumatol 1990; 6:33–36.

18. Siqueira JF, Batista MM, Fraga RC, de Uzeda M. Antibacterial effects of endodontic irrigants on black-pigmented gram-negative anaerobes and facultative bacteria. J Endod 1998; 24:414–416.

19. White RR, Hays GL, Janer LR. Residual antimicrobial activity after canal irrigation with chlorhexidine. J Endod 1997; 23:229–231.

20. Ercan E, Ozekinci T, Atakul F, Gul K. Antibacterial Activity of 2% Chlorhexidine Gluconate and 5.25% Sodium Hypochlorite in Infected Root Canal: In Vivo Study. J Endod 2004 30:30–32.
21. Gomes-Filho JE, Aurélio KG, Costa MM, et al. Comparison of the biocompatibility of different root canal irrigants. J Appl Oral Sci 2008; 16:137-44. 9.
22. Jurenka JS. Anti-inflammatory properties of curcumin, a major constituent of Curcuma longa: a review of preclinical and clinical research. Altern Med Rev 2009; 14:141-53.
23. Bulit F, Grad I, Manoil D, Simon S, Wataha JC, Filieri A, Feki A, Schrenzel J, Lange N, Bouillaguet S. Antimicrobial activity and cytotoxicity of 3 photosensitizers activated with blue light. J Endod. 2014 Mar;40(3):427-31.
24. Ribeiro AP, Pavarina AC, Dovigo LN, Brunetti IL, Bagnato VS, Vergani CE, Costa CA. Phototoxic effect of curcumin on methicillin-resistant Staphylococcus aureus and L929 fibroblasts. Lasers Med Sci. 2013 Feb;28(2):391-8.
25. Dovigo LN, Pavarina AC, Ribeiro AP, Brunetti IL, Costa CA, Jacomassi DP, Bagnato VS, Kurachi C. Investigation of the photodynamic effects of curcumin against Candida albicans.. Photochem Photobiol. 2011 Jul-Aug;87(4):895-903.
26. Ahn JC, Kang JW, Shin JI, Chung PS. Combination treatment with photodynamic therapy and curcumin induces mitochondria-dependent apoptosis in AMC-HN3 cells. Int J Oncol. 2012 Dec;41(6):2184-90
27. Yesilsoy C, Whitaker E, Cleveland D, et al. Antimicrobial and toxic effects of established and potential root canal irrigant. J Endod 1995;21:513-5.
28. Önçag O, Hosgör M, Hilmioglo S, Zekioglu O, Eronat C, Burhanoglu D. Comparison of antibacterial and toxic effects of various root canal irrigants. Int Endod J. 2003; 36:423-32.
29. Türkün M, Gökay N, Özdemir N. Comparative investigation of the toxic and necrotic tissue dissolving effects of different endodontic irrigants. Istanbul Univ Dishekim Fak Derg. 1998; 32:87-94.
30. Kawashima N., Stashenko P. Expression of bone resorptive and regulatory cytokines in infraosseus inflammation. Arch. Oral Biol. 1998;44:55–66

31. Burysek L., Houstek J. Multifactorial induction of gene expression and nuclear localization of mouse interleukin 1 alpha. Cytokine 1996; 8:460–467.
32. Balto K, Sasaki H, Stashenko P. Interleukin-6 deficiency increases inflammatory bone destruction. Infect Immun. 2001 Feb;69(2):744-50.
33. Feyen J. H. M., Elford P., Dipadova F. E., Trechsel U. Interleukin-6 is produced by bone and modulated by parathyroid hormone. J. Bone Miner. Res. 1989; 4:633–638.
34. Kurihara N., Bertolini D., Akiyam Y., Roodman G. D. IL-6 stimulates osteoclast like multinucleated cell formation in long term human marrow cultures by inducing IL-1 release. J. Immunol. 1990; 144:4226–4230.
35. Jordan M., Otterness I. G., Ng R., Gessner A., Rollinghoff M., Beuscher H. U. Neutralization of endogenous IL-6 suppresses induction of IL-1 receptor antagonist. J. Immunol. 1995; 154:4081–4090.

CONCLUSÃO

Com relação ao teste de citotoxicidade a TFD não foi citotóxica à linhagem celular de fibroblastos L929, tendo resultado semelhante ao grupo controle, enquanto que as soluções de hipoclorito de sódio 5%, 2,5% e clorexidina 2% foram citotóxicas; a TFD induziu liberação de IL-1 β e IL-6 semelhante ao grupo controle.

Com relação à resposta tecidual, a TFD apresentou resposta semelhante ao grupo controle; a TFD induziu a liberação de IL-1 β e IL-6 semelhante ao grupo controle.

REFERÊNCIAS

1. Gomes-Filho JE, Silva FO, Watanabe S, Cintra LT, Tendoro KV, Dalto LG, Pacanaro SV, Lodi CS, de Melo FF. Tissue reaction to silver nanoparticles dispersion as an alternative irrigating solution. J Endod 2010;36:1698-702.

2. Holland R, Otoboni Filho JA, de Souza V, Nery MJ, Bernabé PF, Dezan E Jr. A comparison of one versus two appointment endodontic therapy in dogs' teeth with apical periodontitis. J Endod 2003; 29: 121-4.

3. Gomes-Filho JE, Watanabe S, Gomes AC, Faria MD, Lodi CS, Penha Oliveira SH. Evaluation of the effects of endodontic materials on fibroblast viability and cytokine production. J Endod 2009;35:1577-9.

4. Gomes-Filho JE, Aurélio KG, Costa MM, Bernabé PF. Comparison of the biocompatibility of different root canal irrigants. J Appl Oral Sci 2008;16:137-44.

5. Cintra LTA. Análise microscópica da influência das soluções químicas auxiliares e do uso de medicação intracanal no processo de reparo de dentes de cães portadores de lesão periapical [tese Doutorado]. Piracicaba: Faculdade de Odontologia de Piracicaba, UNICAMP; 2008. p.277.

6. Tanomaru Filho M, Leonardo MR, Silva LA. Effect of irrigation solution and calcium hydroxide root canal dressing on the repair of apical and periapical tissues of teeth with periapical lesion. J Endod 2002; 28: 295-9.

7. Estrela C, Estrela CR, Decurcio DA, Hollanda AC, Silva JA. Antimicrobial efficacy of ozonated water, gaseous ozone, sodium hypochlorite and chlorhexidine in infected human root canals. Int Endod J 2007; 40: 85-93.

8. Leonardo MR, Tanomaru-Filho M, Silva LA, Nelson Filho P, Bonifacio KC, Ito IY. In vivo antimicrobial activity of 2% chlorhexidine used as a root canal irrigating solution. J Endod 1999; 25: 167-71.

9. Gomes BP, Vianna ME, Zaia AA, Almeida JF, Souza-Filho FJ, Ferraz CC. Chlorhexidine in endodontics. Braz Dent J 2013;24:89-102.

10. Iqbal M, Kurtz E, Kohli M. Incidence and factors related to flare-ups in a graduate endodontic programme. Int Endod J 2009;42:99-104.

11. McGurkin-Smith R, Trope M, Caplan D, Sigurdsson A. Reduction of intracanal bacteria using GT rotary instrumentation, 5.25% NaOCl, EDTA, and Ca(OH)2. J Endod 2005;31:359-63.

12. Silva LA, Nelson-Filho P, Leonardo MR, Rossi MA, Pansani CA. Effect of calcium hydroxide on bacterial endotoxin in vivo. J Endod 2002;28:94-8.

13. Siqueira JF, Rôças IN, Paiva SSM, Guimarães-Pinto T, Magalhães KM, Lima KC. Bacteriologic investigation of the effects of sodium hypochlorite and chlorhexidine during the endodontic treatment of teeth with apical periodontitis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;104:122-30.

14. Karim IE, Kennedy J, Hussey D. The antimicrobial effects of root canal irrigation and medication. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;103:560-9.

15. Figdor D, Sundqvist G. A big role for the very small-understanding the endodontic microbial flora. Aust Dent J 2007;52(1 Suppl):S38-51.

16. Mohammadi Z, Dummer PM. Properties and applications of calcium hydroxide in endodontics and dental traumatology. Int Endod J. 2011;44:697-730.

17. Mohammadi Z. Endotoxin in endodontic infections: a review. J Calif Dent Assoc 2011;39:152-5, 158-61.

18. Stashenko P. Role of immune cytokines in the pathogenesis of periapical lesions. Endod Dent Traumatol 1990;6:89-96.

19. Murakami Y, Hanazawa S, Tanaka S, Iwahashi H, Yamamoto Y, Fujisawa S. A possible mechanism of maxillofacial abscess formation: involvement of Porphyromonas endodontalis lipopolusaccharide via the expression of inflammatory cytokines. Oral Microbiol Immunol 2001;16:321-5.

20. Tanomaru JMG, Leonardo MR, Tanomaru-Filho M, Bonetti-Filho I, Silva LA. Effect of different irrigation solutions and calcium hydroxide on bacterial LPS. Int Endod J 2003;36:733-9.

21. Wang Z, Shen Y, Haapasalo M. Effectiveness of endodontic disinfecting solutions against young and old Enterococcus faecalis biofilms in dentin canals. J Endod 2012;38(10):1376-9.

22. Garcez AS, Ribeiro MS, Tegos GP, Núñez SC, Jorge AOC, Hamblin MR. Antimicrobial photodynamic therapy combined with conventional endodontic treatment to eliminate root canal biofilm infection. Lasers Surg Med 2007;39:59-66.

23. Berbert FL, Sivieri-Araujo G, Ramalho LT, Pereira SA, Rodrigues DB, de Araújo MS. Quantification of fibrosis and mast cells in the tissue response of endodontic sealer irradiated by low-level laser therapy. Lasers Med Sci 2011;26:741-7.

24. Sivieri-Araujo G, Berbert FLCV, Ramalho LTO, Rastelli ANS, Crisci FS, Bonetti-Filho I, Tanomaru-Filho M. Effect of red and infrared low-level laser therapy in endodontic sealer on subcutaneous tissue. Laser Phys 2011; 21:1-7.

25. Solban N, Rizvi I, Hasan T. Targeted photodynamic therapy. Lasers Surg Med 2006;38:522-31.

26. Calzavara-Pinton PG, Venturini M, Sala R. Photodynamic therapy: update 2006. Part 1: Photochemistry and photobiology. J Eur Acad Dermatol Venereol 2007;21:293-302.

27. Vollet-Filho JD, Menezes PFC, Moriyama LT, Grecco C, Sibata C, Allison RR, Castro-E Silva O, Bagnato VS. Possibility for a full optical determination of photodynamic therapy outcome. J Appl Phys 2009;105:1020381-7.

28. Ferraz RCMC, Ferreira J, Menezes PFC, Sibata CH, Castro-E-Silva O, Bagnato VS. Determination of Threshold Dose of photodynamic Therapy to Measure Superficial Necrosis. Photomed Laser Surg 2009;27:93-9.

29. Nicolodelli G, Angarita DP, Inada NM, Tirapelli LF, Bagnato VS. Effect of photodynamic therapy on the skin using the ultrashort laser ablation. J Biophotonics. 2013 Apr 11. [Epub ahead of print].

30. Giusti JS, Fontana CR, Guimarães OC, Pinchemel LC, Bagnato VS. Single equipment combines simultaneous application of mechanical ultrasound and photodynamic action for microbial control. Photodiagnosis Photodyn Ther 2010;7:137-8.

31. Allison RR, Moghissi K. Photodynamic Therapy (PDT): PDT Mechanisms. Clin Endosc 2013;46:24-9.

32. Allison RR, Mota HC, Bagnato VS, Sibata CH. Bio-nanotechnology and photodynamic therapy-state of the art review. Photodiagnosis Photodyn Ther 2008;5:19-28.

33. Komine C, Tsujimoto Y. A small amount of singlet oxygen generated via excited methylene blue by photodynamic therapy induces the sterilization of Enterococcus faecalis. J Endod 2013;39:411-4.

34. Klepac-Ceraj V, Patel N, Song X, Holewa C, Patel C, Kent R, Amiji MM, Soukos NS. Photodynamic effects of methylene blue-loaded polymeric nanoparticles on dental plaque bacteria. Lasers Surg Med 2011;43:600-6.

35. Garcez AS, Nuñez SC, Hamblim MR, Suzuki H, Ribeiro MS. Photodynamic therapy associated with conventional endodontic treatment in patients with antibiotic-resistant microflora: a preliminary report. J Endod 2010;36:1463-6.

36. Silva LA, Novaes AB Jr, Oliveira RR, et al. Antimicrobial photodynamic therapy for the treatment of teeth with apical periodontitis: a histopathological evaluation. J Endod 2012;38:360-6.

37. Souza LC, Brito PR, de Oliveira JC, Alves FR, Moreira EJ, Sampaio-Filho HR, Rôças IN, Siqueira JF Jr. Photodynamic therapy with two different photosensitizers as a supplement to instrumentation/irrigation procedures in promoting intracanal reduction of Enterococcus faecalis. J Endod 2010;36:292-6.

38. Siqueira JF Jr, Rôças IN. Optimising single-visit disinfection with supplementary approaches: A quest for predictability. Aust Endod J 2011;37:92-8.

39. Sivieri-Araujo G, Guerreiro-Tamomaru JM, Tanomaru-Filho M. Terapia Fotodinâmica Antimicrobiana na Endodontia (TFDA). Estado Atual e Perspectivas. In: Vanderlei Salvador Bagnato. Controle Microbiológico com Ação Fotodinâmica. São Carlos: Compacta Gráfica e Editora; 2011. p.89-104.

40. Fimple JL, Fontana CR, Foschi F, Ruggiero K, Song X, Pagonis TC, Tanner AC, Kent R, Doukas AG, Stashenko PP, Soukos NS. Photodynamic treatment of endodontic polymicrobial infection in vitro. J Endod 2008;34:728-34.

41. Ferrari E, Pignedoli F, Imbriano C, Marverti G, Basile V, Venturi E, Saladini M. Newly Synthesized Curcumin Derivatives: Crosstalk between Chemico-physical Properties and Biological Activity. J Med Chem 2011;54:8066-77.

42. Norris L, Karmokar A, Howells L, Steward WP, Gescher A, Brown K. The role of cancer stem cells in the anti-carcinogenicity of curcumin. Mol Nutr Food Res 2013 Jul 31. [Epub ahead of print].

43. Chang YF, Chuang HY, Hsu CH, Liu RS, Gambhir SS, Hwang JJ. Immunomodulation of Curcumin on Adoptive Therapy with T Cell Functional Imaging in Mice. Cancer Prev Res (Phila). 2011 Dec 1. [Epub ahead of print].

44. Haukvik T, Bruzell E, Kristensen S, Tønnesen HH. Photokilling of bacteria by curcumin in different aqueous preparations. Studies on curcumin and curcuminoids XXXVII. Pharmazie 2009;64:666-73.

45. Sun J, Zhao Y, Hu J. Curcumin Inhibits Imiquimod-Induced Psoriasis-Like Inflammation by Inhibiting IL-1beta and IL-6 Production in Mice. PLoS One 2013 Jun 25. [Epub ahead of print].

46. Sivieri-Araujo G, Rastelli ANS, Jacomassi DP, Tanomaru-Filho M, Guerreiro-Tamomaru JM, Kurachi C, Bagnato VS. Efeito da terapia fotodinâmica (PDT) em Endodontia: avaliação da capacidade de manchamento da dentina radicular. Estudo piloto. Braz Oral Res 2011; 25:28-28.

ANEXO I

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

Comitê de Ética no Uso de Animais (CEUA)
Committee for Ethical Use of Animals (CEUA)

CERTIFICADO

Certificamos que o Projeto **"Influência de diferentes soluções irrigadoras associadas à terapia fotodinâmica: estudo em ratos"** sob responsabilidade do Pesquisador **JOÃO EDUARDO GOMES FILHO** e colaboração de Gustavo Sivieri de Araujo, Ludmila Santos, Simone Watanabe e Paulo Carvalho Tobias Duarte está de acordo com os Princípios Éticos da Experimentação Animal (COBEA) e foi aprovado pelo CEUA, de acordo com o processo **00682-2012**.

CERTIFICATE

We certify that the research **"Influence of diferente irrigating solutions associated to photodinamic therapy: study in rats"**, process number **00682-2012**, under responsibility of **EDUARDO GOMES FILHO** and with collaboration of Gustavo Sivieri de Araujo, Ludmila Santos, Simone Watanabe and Paulo Carvalho Tobias Duarte agree with Ethical Principles in Animal Research (COBEA) and was approved by CEUA.

Prof. Dr. Edilson Ervolino
CEUA Vice-Coordinador

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