UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

GIULIANA DE CAMPOS CHAVES LAMARQUE

Evaluation of the cytotoxic effect of antimicrobial photodynamic therapy on fibroblasts (NIH/3T3) and expression of Bcl-2 family genes

Efeito citotóxico da terapia fotodinâmica antimicrobiana sobre fibroblastos (NIH/3T3) e a expressão de genes da família Bcl-2

BAURU
2019

GIULIANA DE CAMPOS CHAVES LAMARQUE

Evaluation of the cytotoxic effect of antimicrobial photodynamic therapy on fibroblasts (NIH/3T3) and expression of Bcl-2 family genes

Efeito citotóxico da terapia fotodinâmica antimicrobiana sobre fibroblastos (NIH/3T3) e expressão de genes da família Bcl-2

Dissertação apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Mestre em Ciências no Programa de Ciências Odontológicas Aplicadas, área de concentração Odontopediatria.
Orientador: Prof. Dr. Thiago Cruvinel da Silva

BAURU
2019

Lamarque, Giuliana de Campos Chaves
Evaluation of the cytotoxic effect of antimicrobial photodynamic therapy on fibroblasts (NIH/3T3) and expression of Bcl-2 family genes / Giuliana de Campos Chaves Lamarque. – Bauru, 2019.
59p., il., 31cm.

Dissertação (Mestrado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientador: Prof. Dr. Thiago Cruvinel da Silva

FOLHA DE APROVAÇÃO

DEDICATÓRIA

Dedico esta dissertação a minha família, meus pais, minha irmã, meu marido e minha avó que estiveram sempre ao meu lado, me apoiando e incentivando, sendo essenciais para a conclusão desse trabalho.

AGRADECIMENTOS

A Deus, pela sua infinidade bondade, misericórdia e amor. Obrigada por me dar saúde, sabedoria e guiar meus passos, abrindo as portas quando as coisas pareciam não ter mais solução e colocando pessoas maravilhosas em minha vida. Te agradeço meu Senhor.

Ao meu marido Douglas, que foi quem mais me incentivou para iniciar essa jornada. Obrigada por acreditar em mim, enfrentar comigo todas as dificuldades que surgiram pelo caminho, me dar suporte, carinho e amor em todos os momentos e principalmente me ajudar a suportar a distância nesses dois anos. Você mais do que ninguém sabe tudo que passamos para chegar aqui, mas graças a Deus concluímos mais uma etapa e ela não seria possível sem você ao meu lado. Te amo muito!

Aos meus pais Washington e Valderez, que nunca mediram esforços para que eu tivesse uma boa formação e sempre torceram e oraram por mim. Obrigada por estarem sempre presentes. Vocês são minha base e meu orgulho. Amo vocês demais!

A minha irmã, Mariana, que sempre me apoia, me ouve em tantas conversas e me inspira pela mulher que é. Você faz parte dessa conquista sis querida! Eu te amo!

A minha avó, Cacilda, que me acolhe em sua casa há 7 anos e ameniza a saudade da família, sempre cuidando de tudo com tanto carinho. Estar com a senhora nesse tempo me fez aprender e crescer, obrigada por todo o amor que a senhora dedica a nós!

Ao meu orientador, Prof. Dr. Thiago Cruvinel, pela oportunidade de trabalhar e aprender com o senhor. Obrigada por além de orientador se tornar nosso amigo e nos ajudar a melhorar a cada dia.

A minha amiga, Daniela, que foi imprescindível para a conclusão desse trabalho. Quem teve paciência para me ensinar a rotina do laboratório, quem levantava cedinho e ficava até tarde me acompanhando em cada experimento, quem me trouxe conforto e foi instrumento de Deus nesse tempo. Você é muito especial pra mim.

A minha amiga, Bárbara, que virou uma irmã pra mim desde a graduação, sempre disponível, seja para ficar no laboratório, ir na academia ou sair pra conversar. Foi quem acompanhou os problemas que apareceram e diante deles sempre buscou me tranquilizar e a resolver cada um deles. Você torna os dias em Bauru mais amenos e eu agradeço todo o apoio e por fazer parte da minha vida.

A Adriana, por ter a paciência de me ensinar as metologias de cultura celular, pela disposição e imensa ajuda. Foi um prazer trabalhar e aprender com você.

Ao Centro Integrado de Pesquisas, Prof. Dr. Rodrigo Cardoso de Oliveira, pela doação das células para realização desse trabalho, pela enorme colaboração, pelos ensinamentos e momentos compartilhados.

Ao laboratório de farmacologia, em especial ao Thiago Dionísio, pela atenção, colaboração e disponilibidade. Seu conhecimento nos deixa admirados e a sua forma de explicar deixa as coisas muito mais simples.

Aos funcionários do Departamento de Ciências Biológicas Thelma, Larissa e Aline, que estiveram sempre à disposição.

A Profª. Drª Ana Carolina Magalhães, pela amizade e disponibilidade. A Priscila Salomão, por gentimelte ceder os primers para as análises essenciais para a conclusão desse trabalho.

Aos professores da Disciplina de Odontopediatria, Prof.ª Dr.ª Maria Aparecida de Andrade Moreira Machado, Prof.ª Dr.ª Thaís Marchini de Oliveira, Prof.ª Dr.ª Daniela Rios e Prof. Natalino Lourenço Neto, muito obrigada por ter a oportunidade de trabalhar e aprender com vocês.

A Faculdade de Odontologia de Bauru - FOB/USP, na pessoa do diretor Prof. Dr, Carlos Ferreira dos Santos, grande incentivador da pesquisa na faculdade e que não mede esforços para que a faculdade forneça todo o suporte necessário.

A todos os funcionários da FOB-USP, entre eles as secretárias da pós-graduação, pela ajuda durante esses anos.

A Comissão Nacional Pesquisa (CNPq), pela concessão da minha bolsa de mestrado e pelo apoio e incentivo que possibilitaram a realização desta pesquisa.

RESUMO

A terapia fotodinâmica antimicrobiana (aPDT) tem sido utilizada como um tratamento coadjuvante de infecções bucais em abordagens de mínima intervenção clínica. Sua efetividade antimicrobiana foi demonstrada em diversos estudos. Entretanto, há uma falta de evidência sobre seu efeito citotóxico sobre células eucarióticas. O objetivo deste estudo foi avaliar o potencial citotóxico da terapia fotodinâmica antimicrobiana mediada por dois agentes fotossensibilizantes, azul de metileno e curcumina, sobre fibroblastos de camundongos. Células foram tratadas com 0,1 ou 1,0 $mg.mL^{-1}$ de azul de metileno associado ou não a um LED 630 nm, ou 0.6 ou 6 µM de curcumina associada ou não a um LED 455 nm, com densidades de energia de 0.075 ou 7.5 $J.cm^{-2}$. A viabilidade cellular foi determinada pelos ensaios de Brometo de 3-(4,5-dimetil-2-tiazolil)-2,5-difenil-2H-tetrazólio (MTT) e cristal violeta (CV). A expressão de cDNA para os genes ligados à apoptose Bax, Bad, Bcl-2, VDAC-1, citocromo C e Fas-L foi assessada por PCR quantitative (qPCR), após 1, 3, 6 e 24 h dos tratamentos. As diferenças entre os grupos foram detectadas pelos testes de Kruskal-Wallis e post-hoc de Dunn para os ensaios de MTT e CV, e pelos testes de ANOVA e post-hoc de Tukey para qPCR (P<0.05). A combinação de azul de metileno a 1,0 $mg.mL^{-1}$ e LED a 7.5 $J.cm^{-2}$ reduziu significativamente a viabilidade celular, o mesmo sendo observado pelas combinações de curcumina a 6 µM com LED a 0,075 e 7,5 $J.cm^{-2}$, que reduziram a viabilidade celular em 47% e 99%, respectivamente. Também, a associação de curcumina a 0,6 µM com LED a 7,5 $J.cm^{-2}$ reduziu a viabilidade de fibroblastos em 34%. A aPDT mediada por azul de metileno aumentou a expressão de citocromo C e FAS-L (3 h), e Bax/Bcl-2, Bad/Bcl-2, e VDAC-1 (6 h). aPDT mediada por curcumina aumentou significativamente a expressão relativa de Bax/Bcl-2 e dos genes citocromo C, VDAC-1, e Fas-L. Portanto, aPDT mediada por azul de metileno e curcumina induziu citotoxicidade em fibroblastos de camundongo, com consequente ativação da via de sinalização apoptótica Bcl-2. Novos estudos são necessários para determinar parâmetros adequados de aPDT para inativar microrganismos com danos mínimos às células eucarióticas hospedeiras.

Palavras-chave: Terapia Fotodinâmica, Viabilidade Celular, Apoptose

ABSTRACT

Evaluation of the cytotoxic effect of photodynamic therapy on fibroblasts (NIH/3T3) and expression of Bcl-2 family genes

Antimicrobial photodynamic therapy (aPDT) has been used as an adjuvant treatment of oral infections in a minimal intervention clinical approach. Its antimicrobial efficacy was demonstrated in several studies; however, there is a lack of evidence on its cytotoxic effects on eukaryotic cells. The aim of this study was to evaluate the cytotoxicity of aPDT mediated by two photosensitizing agents, methylene blue and curcumin, on mouse fibroblasts. Cells were treated with 0.1 or 1.0 $mg.mL^{-1}$ methylene blue (MB) associated or not to LED at 630 nm, or 0.6 or 6 µM curcumin combined or not with LED at 455 nm, with densities of 0.075 or 7.5 $J.cm^{-2}$. Cellular viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet (CV) assays. The expression of cDNA for Bax, Bad, Bcl-2, VDAC-1, cytochrome C and Fas-L genes related to apoptosis was assessed by quantitative PCR (qPCR) after 1, 3, 6 and 24 h from treatments. The differences between groups were detected by Kruskal-Wallis and post-hoc Dunn's tests for MTT and CV assays, and by ANOVA and post-hoc Tukey test for qPCR ($P<0.05$). The combination of 1.0 $mg.mL^{-1}$ MB and 7.5 $J.cm^{-2}$ LED significantly reduced the cellular viability. The same was observed for the combinations of 6 µM curcumin plus 0.075 and 7.5 $J.cm^{-2}$ LED, which reduced viable cells in 47% and 99%, respectively. Also, the combination of 0.6 µM curcumin plus 7.5 $J.cm^{-2}$ LED reduced the viability of fibroblasts in 34%. MB-mediated aPDT increased the expression of cytochrome C and Fas-L after 3 h, and Bax/Bcl-2, Bad/Bcl-2, and VDAC-1 after 6 h from treatments. Curcumin-mediated aPDT increased significantly the relative expression of Bax/Bcl-2, cytochrome C, VDAC-1, and Fas-L genes. Therefore, MB- and curcumin-mediated aPDT induced cytotoxicity on mouse fibroblasts, with consequent activation of Bcl-2 apoptosis signaling pathways. Further studies are needed to determine the adequate parameters of aPDT to inactivate microorganisms without damaging eukaryotic cells.

Keywords: Photodynamic Therapy, Cell Viability, Apoptosis

TABLE OF CONTENTS

1 INTRODUCTION

1 **INTRODUCTION**

Initially, Photodynamic Therapy (PDT) has been focused on the development of effective protocols for cancer management (ALLISON et al., 2005, 2006). Recently, with growing antibiotic resistance, PDT emerges as antimicrobial treatments, particulary for superficial infections. Antimicrobial photodynamic therapy (aPDT) can be used as a complementary treatment for selective caries removal (GURSOY et al., 2013), periodontitis (ROVALDI et al., 2000; CHRISTOODOULIDES et al., 2008; NASTRI et al., 2010), and endodontic root disinfection (WILLIAMS et al., 2006; SOUKOS et al., 2006; BONSOR et al., 2006; FOSCHI et al., 2007). aPDT is based on the interaction between a photosensitizing agent (PS) absorbed by microorganisms cells, activated by a complementary light source that increases the molecular energy of PS and changes it to a triplet excited molecule (KONOPKA; GOSLINSKI, 2007). This molecule can form free radicals (photochemical reaction type I) or react with oxygen molecules, producing reactive oxygen species (ROS), such as singlet oxygen (photochemical reaction type II) (ABRAHAMSE; HAMBLIN, 2016). These reactions promote an oxidative stress, causing damages on cellular membrane, mitochondria, lysosomes or DNA (MAISCH,2007; TARASZKIEWICZ et al., 2013; VATANSEVER et al., 2013).

Methylene blue (MB) [chloride 3.7 bis(dimethylamino) phenothiazine-5-io] is a dye from the phenothiazinium class of compounds (TUITE, 1993), with favorable properties for aPDT, such as low molecular weight, classical $1O_2$ generator, hydrophilicity, cationic form at physiological pH and strong light absorption at 660 nm (OLIVEIRA et al., 2011; BACELLAR et al., 2015; ROSA et al., 2015). Briefly, molecules of MB incorporated to cellular structures of microorganisms are able to absorb photons and excite electrons. These electrons are transferred to a substrate or molecular oxygen, resulting in superoxides that can damage or kill microbial cells (CHIAVIELLO et al., 2011; GHARESI et al., 2017).

Curcumin (1,7-bis(4-hydroxy 3-methoxy phenyl)-1,6-heptadiene-3, 5-dione), a natural dye isolated from the rhizomes of *Curcuma longa*, have been purposed as a potential photosensitizing (PS) in aPDT. Traditionally, it is used for the treatment of cold, skin diseases, and inflammation (CHATTOPADHYAY et al., 2004;

AGGARWAL et al., 2007). Also, recent researches confirmed its potential as antitumor, antioxidant, antimicrobial and anti-inflammatory (AGGARWAL et al., 2003; CRIVELLO et al., 2005; DUVOIX et al., 2005). It has a maximum absorption with blue LED on wavelength of 430 nm, presenting a strong phototoxic effect in microorganisms even when used in lower concentrations (GHARESI et al., 2017; SAITAWEE et al., 2018). In tumor cells, the cytotoxicity of curcumin is due to the induction of apoptosis mediated by the direct release of cytochrome C, and the subsequent activation of caspases (STEINER-OLIVEIRA et al., 2015). In microorganism cells, the cytotoxicity occurs by the production of reactive oxygen species, such as singlet oxygen ($^1O_2$), hydrogen peroxide and superoxide (DAHL et al., 1989; CHIGNELL et al., 1994; DOVIGO et al., 2011).

Although the antimicrobial efficacy of aPDT was demonstrated in several studies, there is a lack of evidence on its cytotoxic effect on eukaryotic cells. Therefore, the purpose of this *in vitro* study was to evaluate the cytotoxicity and apoptotic effects of methylene-blue- and curcumin-mediated antimicrobial photodynamic therapy in different dye concentrations and light densities on mouse fibroblasts.

2 ARTICLES

2 ARTICLES

This dissertation is presented in a format of two manuscripts, written according to the instructions and guidelines of the *Photodiagnosis and Photodynamic Therapy* and *Journal of Photochemistry and Photobiology B: Biology*, respectively.

- ARTICLE 1 – Cytotoxic effect and apoptosis pathways activated by methylene blue-mediated photodynamic therapy in fibroblasts

- ARTICLE 2 – In vitro effect of curcumin-mediated antimicrobial photodynamic therapy on fibroblasts: viability and cell signaling for apoptosis

Cytotoxic effect and apoptosis pathways activated by methylene blue-mediated photodynamic therapy in fibroblasts

Abstract

Antimicrobial photodynamic therapy (aPDT) has been used as an adjuvant treatment of oral infections in a minimal intervention clinical approach. Its antimicrobial efficacy was demonstrated in several studies; however, there is a lack of evidence on its cytotoxic effect on eukaryotic cells. The aim of this study was to evaluate the cytotoxicity and apoptotic pathways of methylene blue-mediated aPDT on mouse fibroblasts. Cells were treated with 0.1 or 1.0 $mg.mL^{-1}$ methylene blue (MB), and 0.075 or 7.5 $J.cm^{-2}$ LED at 630 nm. Cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet (CV) assays, while cDNA expression for Bax, Bad, Bcl-2, VDAC-1, cytochrome C and Fas-L was assessed by qRT-PCR (1, 3, 6 and 24 h). The differences between groups were detected by Kruskal-Wallis and post-hoc Dunn's tests for MTT and CV assays, and by ANOVA and post-hoc Tukey test for qPCR ($P<0.05$). The combination of 1.0 $mg.mL^{-1}$ MB and 7.5 $J.cm^{-2}$ LED significantly reduced the cellular viability, whereas MB and LED alone were innocuous to fibroblasts. MB-mediated aPDT increased the expression of cytochrome C and Fas-L after 3 h, and Bax/Bcl-2, Bad/Bcl-2, and VDAC-1 after 6 h from treatment. Based on these results, MB-mediated aPDT induced cytotoxicity on mouse fibroblasts, with consequent activation of Bcl-2 apoptosis signaling pathways. Further studies are needed to determine the adequate parameters of aPDT to inactivate microorganisms without damaging eukaryotic cells.

Keywords: Photochemotherapy; Methylene Blue; Cell Viability; Apoptosis; Bcl-2

INTRODUCTION

Minimal intervention approaches have been advocated in dental procedures as a high therapeutic standard for treating oral diseases, reducing the risks of irreversible damages for tooth structure with maximum comfort and cost-effectiveness for patients. In this sense, antimicrobials are being applied into cavitated caries lesions and periodontal pockets to reduce the number of viable pathogenic microorganisms, without mechanical removal of mineralized tissues [1-5]. An option commonly employed in this field is the antimicrobial photodynamic therapy (aPDT), which promotes the inactivation and/or death of microorganisms by the association of three components, a chemical photosensitizing agent (PS), a complementary light source and molecular oxygen [4-6].

Methylene blue (MB) [chloride 3.7 bis(dimethylamino) phenothiazine-5-io] is a dye from the phenothiazinium class of compounds [9], with favorable properties for aPDT, such as low molecular weight, classical $1O_2$ generator, hydrophilicity, cationic form at physiological pH and strong light absorption at 660 nm [7-10]. Briefly, molecules of MB incorporated to cellular structures of microorganisms are able to absorb photons and excite electrons. These electrons are transferred to a substrate or molecular oxygen, resulting in superoxides that can damage or kill microbial cells [11,12].

We have previously demonstrated the efficacy of MB-mediated aPDT using LED on dentin caries microcosms [13]. However, the evidence of aPDT on eukaryotic cytotoxic effects of this therapeutic method is still scarce. A previous study showed that 10 $\mu mol.L^{-1}$ MB activated with 36 $J.cm^{-2}$ light energy did not produce a significant cytotoxicity on mouse fibroblasts (L929) [14]. Similarly, MB-mediated aPDT did not increase cytotoxicity in human fibroblasts compared to no treatment groups [15,16]. Also, 1 µM of MB combined with 53.4 $J.cm^{-2}$ light energy did not affect the mitochondrial activity of cells *in vitro* [17].

Therefore, the aim of this *in vitro* study was to evaluate the cytotoxicity and apoptotic effects of methylene blue-mediated antimicrobial photodynamic therapy, using different MB concentrations and light densities on mouse fibroblasts (NIH/3T3). The null hypotheses were that aPDT would have no cytotoxicity (H_0) and apoptotic effects (H_0') on eukaryotic cells.

MATERIALS AND METHODS

Cell culture

NIH/3T3 mouse fibroblasts cells (ATCC - American Type Culture Collection, CRL1658™) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO Laboratories, Life Technologies, Inc., New York, USA), 100 $IU.mL^{-1}$ penicillin and 0.1 $mg.mL^{-1}$ streptomycin (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were enzymatically dissociated with trypsin (0.25% trypsin, 1 $mmol.L^{-1}$ EDTA, Sigma-Aldrich) and counted under Neubauer chamber using trypan blue staining protocol.

Methylene blue-mediated antimicrobial photodynamic therapy

aPDT was conducted by using the Biotable® RGB (Institute of Physics of São Carlos, São Carlos, Brazil), an equipment that allows the simultaneous irradiation of 24 wells of a microtiter plate [13]. It was prepared a 100 $mg.mL^{-1}$ MB (Sigma-Aldrich) stock solution in phosphate buffered saline solution (PBS 1x), and stored at -20°C in dark conditions until the moment of use. Before experiments, MB was diluted in PBS 10% FBS to a final concentration of 1 and 0.1 $mg.mL^{-1}$. Cells were treated according to their experimental groups, as follows: group 1 (MB-L-, negative control), group 2 (0.001% chlorhexidine, positive control), group 3 (0.01 chlorhexidine, positive control), group 4 (0.1 MB alone), group 5 (1 MB alone), group 6 (0.075 $J.cm^{-2}$ LED alone), group 7 (7.5 $J.cm^{-2}$ LED alone), group 8 (0.1 MB + 0.075 $J.cm^{-2}$ LED), group 9 (0.1 MB + 7.5 $J.cm^{-2}$), group 10 (1 MB + 0.075 $J.cm^{-2}$ LED), and group 11 (1 MB + 7.5 $J.cm^{-2}$ LED).

Twenty-four hours after the seeding, the cells were washed with PBS 1x. Following, PBS (G1, G6 and G7), chlorhexidine (G2 and G3) or PS (G4, G5, G8-G11) were added and incubated for 2 min. Subsequently, groups 6 to 11 were irradiated with a red LED at 625±30 nm, in a continuous mode, power of 40 mW, power density of 1 $W.cm^{-2}$, distance of ± 25 mm, and energy density of 7.5 or 0.075 $J.cm^{-2}$. Power measurements were checked by an optical power meter (1916-C, Newport, Irvine, USA). When necessary, the irradiation time was corrected by the ratio of light and power densities. Finally, the wells were washed with PBS 1X, and DMEM 10% FBS was added in cellular cultures.

Cell viability

NIH/3T3 cell viability was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) and crystal violet assays, as previously described by Oliveira et al [18]. Cells were seeded in 96-well plates at a density of 3×10^3 cells in DMEM 10% FBS. After 24, 48 and 72 h of MB-aPDT treatment, the assays were performed. For MTT assays, the cells were washed with PBS 1x and incubated with a sterile MTT solution (0.5 $mg.mL^{-1}$) for 4 h at 37°C. The intracellularly reduced insoluble pigment (formazan) was extracted with DMSO for 30 min and the absorbance was determined in a spectrophotometer at 562 nm (Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader, BioTek Instruments Inc., Winooski, USA). For crystal violet assays, the wells were washed with PBS 1x and added 100% methanol for 10 min. The cells were stained with 0.2% crystal violet for 3 min followed by two washes with PBS 1x. After, the dye was extracted with 0.05 mol L^{-1} sodium citrate solution for 10 min. The absorbance was measured at 540 nm [18].

qRT-PCR

Cells were seeded in 24-well plates at a density of 5×10^4. Adherent cells were treated with the combination of 1 $mg.mL^{-1}$ MB) and 7.5 $J.cm^{-2}$ LED. 0.001% chlrhexidine was used as a positive control. After 1, 3, 6 and 24 h of treatment, the cells were harvested and the mRNA extracted using MagMAX® mirVana® Total RNA Isolation Kit (Applied Biosystems, Foster, USA), according to the manufacturer's instructions. The isolated RNA was quantified by NanoDrop®1000 (Thermo Fisher Scientific, Waltham, USA) and cDNA was synthetized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturer's recommendations. The cDNA samples were incubated with TaqMan® Gene Expression Master Mix and Taqman® Gene Expression assay (Applied Biosystems) for Bad (Mm00432042_m1), Bax (Mm00432051_m1), Bcl-2 (Mm00477631_m1), Fas-L (Mm00438864_m1), Vdac1 (Mm00834272_m1), Cytochrome C (Mm01250094_m1) genes and were read in the ViiA 7 Real-Time PCR System® (Applied Biosystems), under the following conditions: a cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, and a cycle of 60°C for 1 min. Act-b expression (Mm00607939_s1) was used as a control. All samples were analyzed in triplicate, and the relative expression was obtained according to the formula $2^{-(\Delta\Delta Ct)}$.

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 21.0 software. The non-parametric tests for independent samples, Kruskal-Wallis, and post hoc Dunn's, were used to indicate differences between groups for MTT and CV data, since they were not normally distributed. Differently, qRT-PCR values were compared using ANOVA followed by a post-hoc Tukey test. *P* values <0.05 were considered significant.

RESULTS

MB-mediated aPDT demonstrated a significant dose-dependent cytotoxicity effect in MTT assay. MB and LED alone, and 0.1% MB plus 0.075 J.cm^{-2} LED did not produce additional cytotoxicity in fibroblasts in comparison to control group. The cytotoxic effect of 0.1% MB plus 7.5 J.cm^{-2} LED was stabilized after 72 h, while 1% MB plus 7.5 J.cm^{-2} LED produced a significant reduction in the cellular viability (33.3-63.2%), in distinct times. The highest reduction of the viability of fibroblasts was observed after treatments with 0.01% chlorhexidine, with only 2% of viable cells after 72 h (Table 1, Figure 1).

In crystal violet assays, the cytotoxic effect was observed only in CHX 0.01% chlorhexidine at 24 h (1,29% of viable cells). After 72 h, it was observed an increase in the cellular viability in groups treated with MB and LED alone, 0.1% MB plus 0.075 J.cm^{-2} LED, and 1% MB plus 0.075 J.cm^{-2} LED (Table 1, Figure 2).

MB-mediated aPDT increased the expression of all genes (Figure 3). In 1 h, aPDT groups increased the expression of Bax and Bad/Bcl-2. After 3 h, cells treated with MB presented significantly higher relative expressions of Bad/Bcl-2 compared to control. After 6 h, the relative expressions of Bax/Bcl-2, Bad/Bcl-2 and VDAC-1 increased 4-, 1.82- and 2.5-fold, respectively. Cells treated with 0.001% chlorhexidine presented a significantly higher Fas-L expression.

DISCUSSION

These findings indicate that MB-mediated aPDT promoted cytotoxicity in mouse fibroblasts, reducing adherent cells with the increment of MB and light doses. This antimicrobial therapy stimulated cellular apoptosis, increasing the relative expression of Bax/Bcl-2, Bad/Bcl-2, VDAC-1 and cytochrome C. Although MB and LED alone did not produce considerable cellular cytotoxicity, the combination of 1% MB and 7.5

$J.cm^{-2}$ LED decreased the viability of fibroblasts significantly; however, these results were less aggressive to host cells compared to 0.01% chlorhexidine, which reduced viable cells in 97-98%.

The concentrations of MB employed in this study were 100 to 1,000 times lower than that most commonly used in dental studies (100 $mg.L^{-1}$), because eukaryotic cells were exposed directly to aPDT, instead of being protected by the more complexity of organized tissues found in clinical conditions, such as tridimensional cellular multilayers [19-22]. Regarding the time-course of cellular viability, our findings are in agreement with Qiao et al. [24], which showed a significant increase in the viability of human gingival fibroblasts with longer periods of time from aPDT treatments. The concentrations, agent exposure time and surface area are important factors that could vary the magnitude of these results.

qRT-PCR results suggest that apoptosis occurred by the mitochondrial mechanism pathway [25,26]. Previously, Noodt et al. [27] observed that methylene blue derivative (MBD) concentrated in mitochondria-resembling cytoplasmic organelles induced apoptosis in V79 cells (chinese hamster lung fibroblasts). MB accumulates on mitochondria reducing the potential of mitochondrial membrane, which increase the mitochondrial permeability and, consequently, the apoptosis pathway mediated by Bcl-2 family [8,25,26]. It occurs by the release of inter-membrane protein, such as cytochrome C, which activates the caspase 9 and the production of apoptosome [28-30]. The VDAC-1 releases cytochrome C into cytosol and control the flux of other metabolites [31-33].

On the other hand, a possible extrinsic mechanism of apoptosis was inconclusive, although we found a difference in Fas-L expression between MB-mediated aPDT and 0.001% chlorhexidine. Fas-L gene is able to induce extrinsic via of apoptosis by activation of caspases-8 and -3 [33,34]. These differences can be explained by the conection of Fas-L gene with the intrinsic apoptotic pathway [35,36]. This connection might be occurred by Bid, which is translocated to mitochondria and results in the released of apoptogenic factor cytochrome C [37].

Also, MB and LED alone promoted lower variations in gene expression involved in apoptosis, with similar results to control after 6 h from treatment, presenting no alterations on the viability of host cells. Similarly, light and photosensitizing alone showed no significant effects on the expression of Bax in human gingival fibroblast cells after using 1,000 $\mu g.mL^{-1}$ indocyanine green [38].

Although these experimental approaches were not strictly related to clinical conditions, these results are useful for demonstrating that as higher concentrations of photosensitizing agents and light doses, as higher the chance of induction of apoptosis, leading to the decrease of the viability of fibroblasts. For instance, it is important to consider a reduction of aPDT parameters in the cases of its application in deep cavities, since pulp fibroblasts might be more susceptible to damage that could lead to tissue necrosis. However, some limitations are noted in this study: i) this model was developed with mouse fibroblasts, considered the first line of cells for cytotoxic tests; ii) the cells grown in a monolayer manner, without using tridimensional *in vitro* models; iii) the potential of apoptosis provoked by MB-mediated aPDT was determined exclusively with basis on the expression of pro-apoptotic genes, without assessing the production of mitochondrial proteins.

The present study demonstrated that MB-mediated aPDT induced a significant dose-dependent cytotoxic effect on mouse fibroblasts. The apoptosis mechanisms involved in this process are related to mitochondrial photodamage and activation of the Bcl-2 family of proteins. These findings indicate the non-acceptance of hypotheses H_0 and H_0'. Further studies are needed to determine the adequate aPDT parameters to the achievement of its maximum lethal effects for microorganisms and its minimum damage for host cells.

REFERENCES

[1] Frencken JE, Imazato S, Toi C, Mulder J, Mickenautsch S, Takahashi Y, Ebisu S. Antibacterial effect of chlorhexidine-containing glass ionomer cement in vivo: a pilot study. Caries Res 2007;41(2):102–107. https://doi.org/10.1159/000098042.

[2] Silva TC, Pereira AFF, Exterkate RA, Bagnato VS, Buzalaf MAR, Andrade Moreira Machado MA, ten Cate JM, Crielaard W, Deng DM. Application of an active attachment model as a high-throughput demineralization biofilm model. J. Dent 2012;40:41–47. https://doi.org/10.1016/j.jdent.2011.09.009.

[3] Zhang J, Liang H, Zheng Y, et al. Photodynamic therapy versus systemic antibiotic for the treatment of periodontitis in a rat model. J Periodontol. 2019; 1- 10. https://doi.org/10.1002/JPER.18-0305

[4] Wilson M, Dobson J. Lethal photosensitization of oral anaerobic bacteria. Clin Infect Dis [S.l.] 1993;16:S414-5.

[5] Diniz I, Horta IV, Azevedo CS, Elmadijan TR, Matos AB, Simionato MRL, Marque MM. Antimicrobial photodynamic therapy: A promise candidate for caries lesions treatment. Photodiagnosis Photodyn Ther 2015;12:511-518.

[6] Austin E, Jagdeo J. An In Vitro Approach to Photodynamic Therapy. J. Vis. Exp. (138), e58190, doi:10.3791/58190 (2018).

[7] Wu J, Xu H, Tang W, Kopelman R, Philbert MA, Xi C. Eradication of bacteria in suspension and biofilms using methylene blue-loaded dynamic nanoplatforms, Antimicrob. Agents Chemother 2009;53:3042–3048.

[8] Oliveira CS, Turchiello R, Kowaltowski AJ, Indig GL, Baptista MS. Free Radical Biology & Medicine Major determinants of photoinduced cell death : Subcellular localization versus photosensitization efficiency. Free Radic Biol Med. 2011;51:824–33.

[9] Bacellar IOL, Tsubone TM, Pavani C, baptista MS. Photodynamic Efficiency: From Molecular Photochemistry to Cell Death. Int J Mol Sci 2015;16:20523-20559. doi:10.3390/ijms160920523

[10] Rosa LP, da Silva FC, Nader SA, Meira GA, Viana MS. Antimicrobial photodynamic inactivation of Staphylococcus aureus biofilms in bone specimens using methylene blue, toluidine blue ortho and malachite green: an in vitro study, Arch. Oral Biol 2015;60:675–680.

[11] Diniz I, Horta IV, Azevedo CS, Elmadijan TR, Matos AB, Simionato MRL, Marque MM. Antimicrobial photodynamic therapy: A promise candidate for caries lesions treatment. Photodiagnosis Photodyn Ther 2015;12:511-518.

[12] Foote CS. Mechanisms of photosensitized oxidation. Science 1968;162:963–970;.

[13] Méndez DAC, Gutierrez E, Dionísio EJ, Oliveira TM, Buzalaf MAR, Rios D, Machado MAAM, Cruvinel T. Effect of methylene blue-mediated antimicrobial photodynamic therapy on dentin caries microcosms. Lasers Med Sci 2017;October, https://doi.org/10.1007/s10103-017-2379-3

[14] George S, Kishen A. Advanced noninvasive light-activated disinfection: assessment of cytotoxicity on fibroblasts versus antimicrobial activity against Enterococcus faecalis. J Endod 2007;33:599–602. [PubMed: 17437881]

[15] Kashef N, Abadia GRS, Djavid GE. Photodynamic inactivation of primary human fibroblasts by methylene blue and toluidine blue. Photodiagnosis and Photodynamic Therapy 2012:355—358. http://dx.doi.org/10.1016/j.pdpdt.2012.05.001

[16] Darabpour E, Kashef N, Mashayekhan S. Chitosan nanoparticles enhance the efficiency of methylene blue-mediated antimicrobial photodynamic inactivation of bacterial biofilms: An in vitro study. Photodiagnosis and Photodynamic Therapy 2016;14:211–217. http://dx.doi.org/10.1016/j.pdpdt.2016.04.009

[17] Azaripour A, Azaripour M, Willershausen I, Noorden CJFV, Willershausen B. Photodynamic Therapy has no Adverse Effects In Vitro on Human Gingival Fibroblasts and Osteoblasts. Clin Lab. 2018;64(7):1225-1231. doi: 10.7754/Clin.Lab.2018.180220.

[18] Oliveira FA, Matos AA, Santesso MR, Tokuhara CK, Leite AL, Bagnato VS, Machado MAAM, Peres Buzalaf C, Oliveira RC. Low intensity lasers differently induce primary human osteoblast proliferation and differentiation, J. Photochem. Photobiol B 2016;163:14–21.

[19] Steiner-oliveira C, Longo PL, Aranha ACC, Ramalho KM, Mayer MPA, Eduardo CP. Randomized in vivo evaluation of photodynamic antimicrobial chemotherapy on deciduous carious dentin. J Biomed Opt, v. 20, n. 10, Oct 2015.

[20] Guglielmi CA, Simionato MR, Ramalho KM, Imparato JC, Pinheiro SL, Luz MA. Clinical use of photodynamic antimicrobial chemotherapy for the treatment of deep carious lesions. J Biomed Optics 2011;16(8):088003.

[21] Araújo PV, Correia-Silva Jde F, Gomez RS,Massara Mde L, Cortes ME, Poletto LT. Antimicrobial effect of photodynamic therapy in carious lesions in vivo, using culture and real-time PCR methods. Photodiagn Photody Ther 2015;12(3):401–407.

[22] Neves PA, Lima LA, Rodrigues FC, Leitão TJ, Ribeiro CC. Clinical effect of photodynamic therapy on primary carious dentin after partial caries removal. Braz Oral Res 2016;30(1):e47

[23] Lima JP, Sampaio de Mei MA, Borges FM, Teixeira AH, Steiner-Oliveira C, Nobre dos Santos M, Rodrigues Lk, Zanin IC. Evaluation of the antimicrobial effect of photodynamic antimicrobial therapy in an in situ model of dentine caries. Eur J Oral Sci 2009;117(5):568-574.

[24] Qiao J, Wang S, Wen Y, Jia H. Photodynamic effects on human periodontal-related cells in vitro. Photodiagnosis Photodyn Ther 2014 Sep;11(3):290-9 (PMID: 24751501).

[25] Chen, Y.; Zheng, W.; Li, Y.; Zhong, J.; Ji, J.; Shen, P. Apoptosis induced by methylene blue-mediated photodynamic therapy in melanomas and the involvement

of mitochondrial dysfunction revealed by proteomics. Cancer Sci. 2008, 99, 2019–2027.

[26] Chen, J.Y.; Mak, N.K.; Yow, C.M.; Fung, M.C.; Chiu, L.C.; Leung, W.N.; Cheung, N.H. The binding characteristics and intracellular localization of temoporfin (mTHPC) in myeloid leukemia cells: Phototoxicity and mitochondrial damage. Photochem. Photobiol. 2000, 72, 541–547.

[27] Noodt BB, Rodal GH, Wainwright M, Peng Q, Horobin R, Nesland JM, Berg K. Apoptosis induction by different pathways with methylene blue derivative and light from mitochondrial sites in V79 cells. Int J Cancer. 1998;75:941–948.

[28] Chiu SM, Evans HH, Lam M, Nieminen AL, Oleinick NL. Phthalocyanine 4 photodynamic therapy-induced apoptosis of mouse L5178Y-R cells results from a delayed but extensive release of cytochrome c from mitochondria. Cancer Lett 2001;165: 51–58.

[29] Chiu SM, Oleinick NL. Dissociation of mitochondrial depolarization from cytochrome c release during apoptosis induced by photodynamic therapy. Br J Cancer 2001; 84: 1099–1106.

[30] Oleinick N, Morris R, Belichenko I. Apoptosis in response to photodynamic therapy: what, where, why and how. Photochem Photobiol Sci 2002;1:1–21.

[31] Agalakova NI and Gusev GP. Molecular Mechanisms of Cytotoxicity and Apoptosis Induced by Inorganic Fluoride. ISRN Cell Biology 2012:1-16.

[32] Shoshan-Barmatz V, De S, Meir A. The Mitochondrial Voltage-Dependent Anion Channel 1, Ca(2+) Transport, Apoptosis, and Their Regulation. Front Oncol 2017;7:60.

[33] Lee JH, Jung JY, Jeong YJ, Park, JH, Yang KH, Choi NK, Kim SH, Kim WJ. Involvement of both mitochondrial- and death receptor-dependent apoptotic pathways regulated by Bcl-2 family in sodium fluoride-induced apoptosis of the human gingival fibroblasts. Toxicology 2008;243:340-347.

[34] Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495-516.

[35] Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. 1998:94:491-501.

[36] Gajate C, Gonzalez-Camacho F, Mollinedo F. Lipid raft connection between extrinsic and intrinsic apoptotic pathways, Biochem. Biophys. Res. Commun. 2009:380:780-84. https://doi.org/10.1016/j.bbrc.2009.01.147.

[37] Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death Nat. Rev. Mol. Cell Biol. 2007:8:405-13.

[38] Gharesi S, Pourhajibagher M, Chiniforush N, Raoofian R, Hashemi M, Shahabi S, Bahador A. Effect of photodynamic therapy based on indocyanine green on expression of apoptosis-related genes in human gingival fibroblast cells. Photodiagnosis and Photodynamic Therapy, 2017 April; 19:33-36.

TABLES

Table 1. Cellular viability (%) assessed by MTT and CV after 72 h of treatment. Distinct letters represent significant statistical differences between groups.

Groups	24 Hours		48 Hours		72 Hours	
	MTT (%)	*CV (%)*	*MTT (%)*	*CV (%)*	*MTT (%)*	*CV (%)*
MB-L-	100 ±11.58^{A}	100 ± 30.02^{ABC}	100 ± 16.41^{A}	100 ± 14.56^{AC}	100 ± 8.58^{A}	100 ± 11.41^{AD}
CHX 0.001%	120.77 ± 29.16^{A}	79.88 ± 18.28^{AC}	84.87 ± 11.62^{B}	112.93 ± 12.48^{C}	137.76 ± 22.52^{C}	140.94 ± 12.59^{BE}
CHX 0.01%	5.09 ± 1.48^{BC}	1.29 ± 4.51^{D}	1.73 ± 1.21^{C}	2.02 ± 7.61^{B}	3.46 ± 1.42^{B}	2.14 ± 2.31^{A}
MB 0.1	104.61 ± 12.51^{A}	132.59 ± 36.55^{B}	81.36 ± 9.58^{B}	104.30 ± 21.81^{AC}	108.47 ± 15.07^{A}	130.09 ± 16.87^{CE}
MB 1	109.85 ± 8.50^{A}	122.86 ± 29.06^{B}	83.82 ± 4.10^{B}	98.16 ± 12.61^{AC}	113.0 ± 14.87^{A}	151.97 ± 7.26^{B}
MB- L+ 0.075	98.64 ± 15.93^{A}	118.56 ± 17.46^{B}	88.92 ± 5.81^{AB}	101.79 ± 12.67^{AC}	103.78 ± 7.67^{A}	137.25 ± 15.70^{BCE}
MB- L+ 7.5	111.87 ± 6.84^{A}	119.03 ± 32.41^{AB}	85.77 ± 10.43^{AB}	89.02 ± 9.05^{C}	99.56 ± 10.56^{A}	121.82 ± 9.46^{CD}
MB0.1+ L+ 0.075	103.20 ± 15.93^{A}	107.50 ± 19.60^{AB}	88.77 ± 7.85^{AB}	81.62 ± 8.11^{DE}	107.82 ± 17.83^{A}	135.91 ± 6.51^{BCE}
MB0.1+ L+ 7.5	78.88 ± 6.27^{C}	85.50 ± 20.58^{AC}	81.07 ± 10.18^{B}	86.21 ± 6.72^{ADE}	101.19 ± 20.10^{A}	127.15 ± 21.69^{CE}
MB1+ L+ 0.075	97,75 ± 10.14^{A}	109.88 ± 30.75^{AB}	83.74 ± 2.83^{B}	81.88 ± 10.28^{DE}	104.90 ± 9.05^{A}	130.61 ± 16.01^{CE}
MB1+ L+ 7.5	58.62 ± 15.30^{C}	70.59 ± 29.85^{CD}	36.80 ± 14.27^{C}	31.54 ± 6.86^{BE}	66.68 ± 12.69^{B}	40.55 ± 8.18^{A}

FIGURES

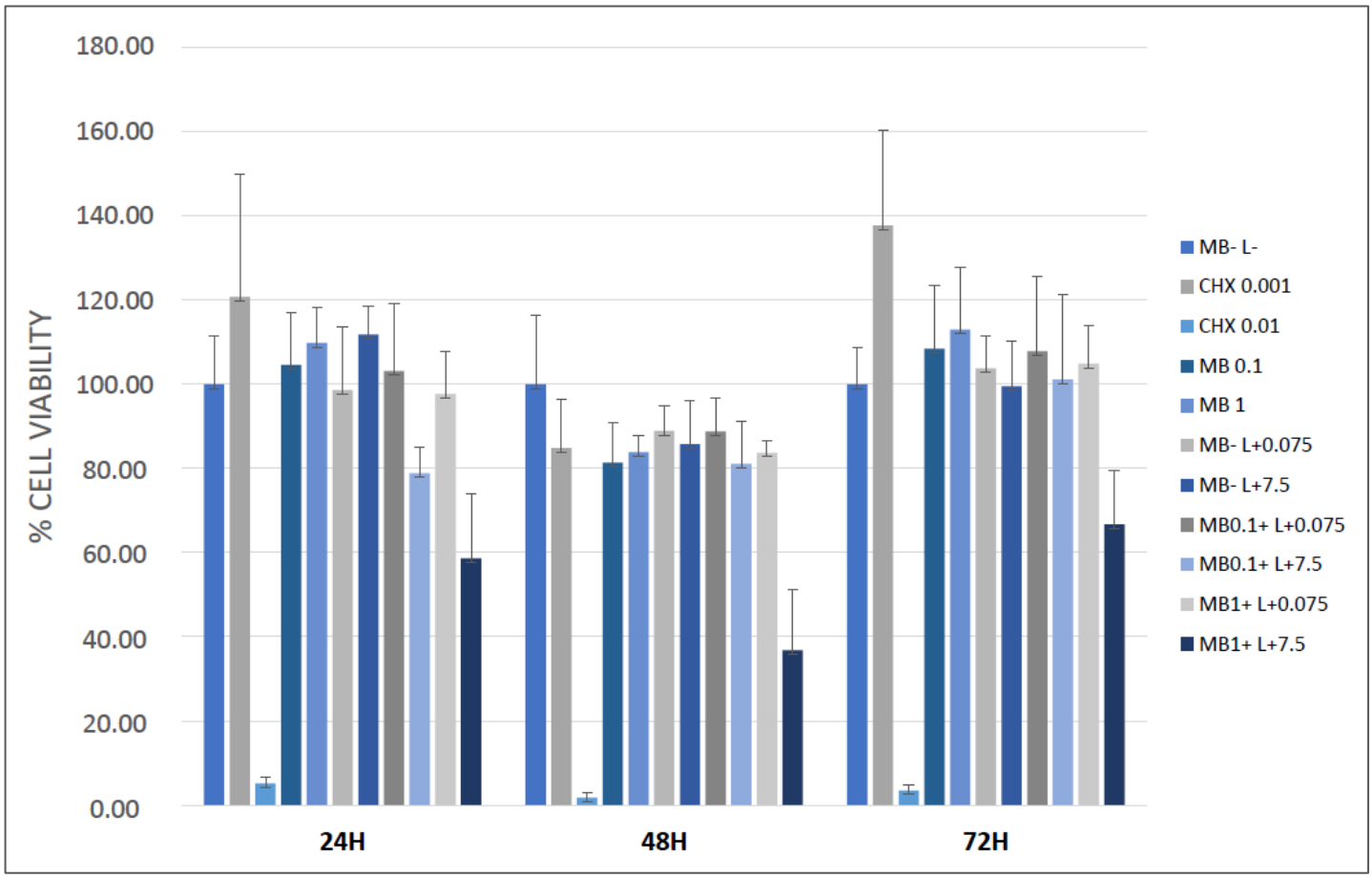

Figure 1. Percentage of viability of cells according to MTT assay after 24 h, 48 h and 72 h in relation to no treatment group (MB- L-).

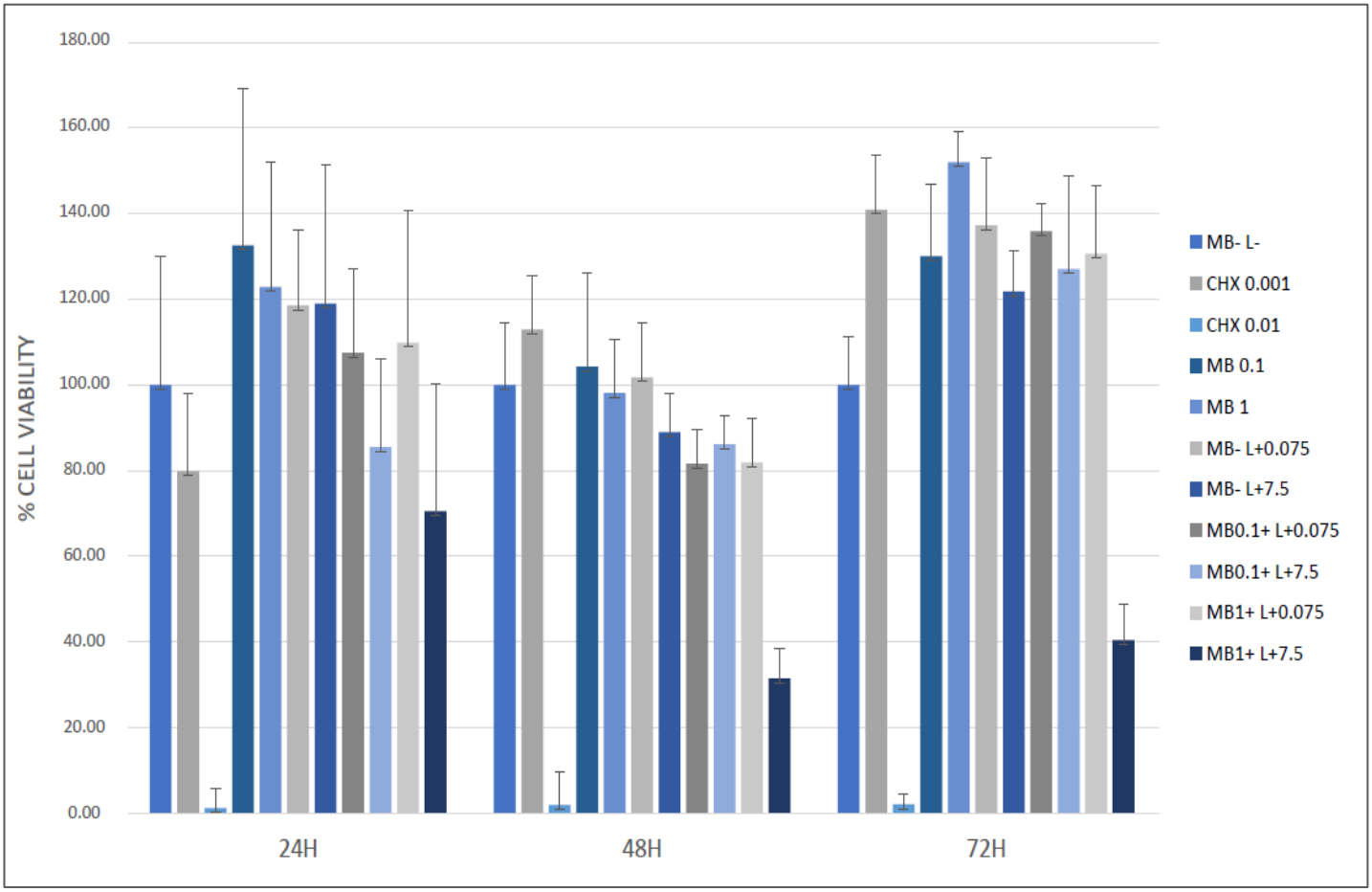

Figure 2. Percentage of viability of cells according to CV assay after 72 h in relation to no treatment group (MB- L-).

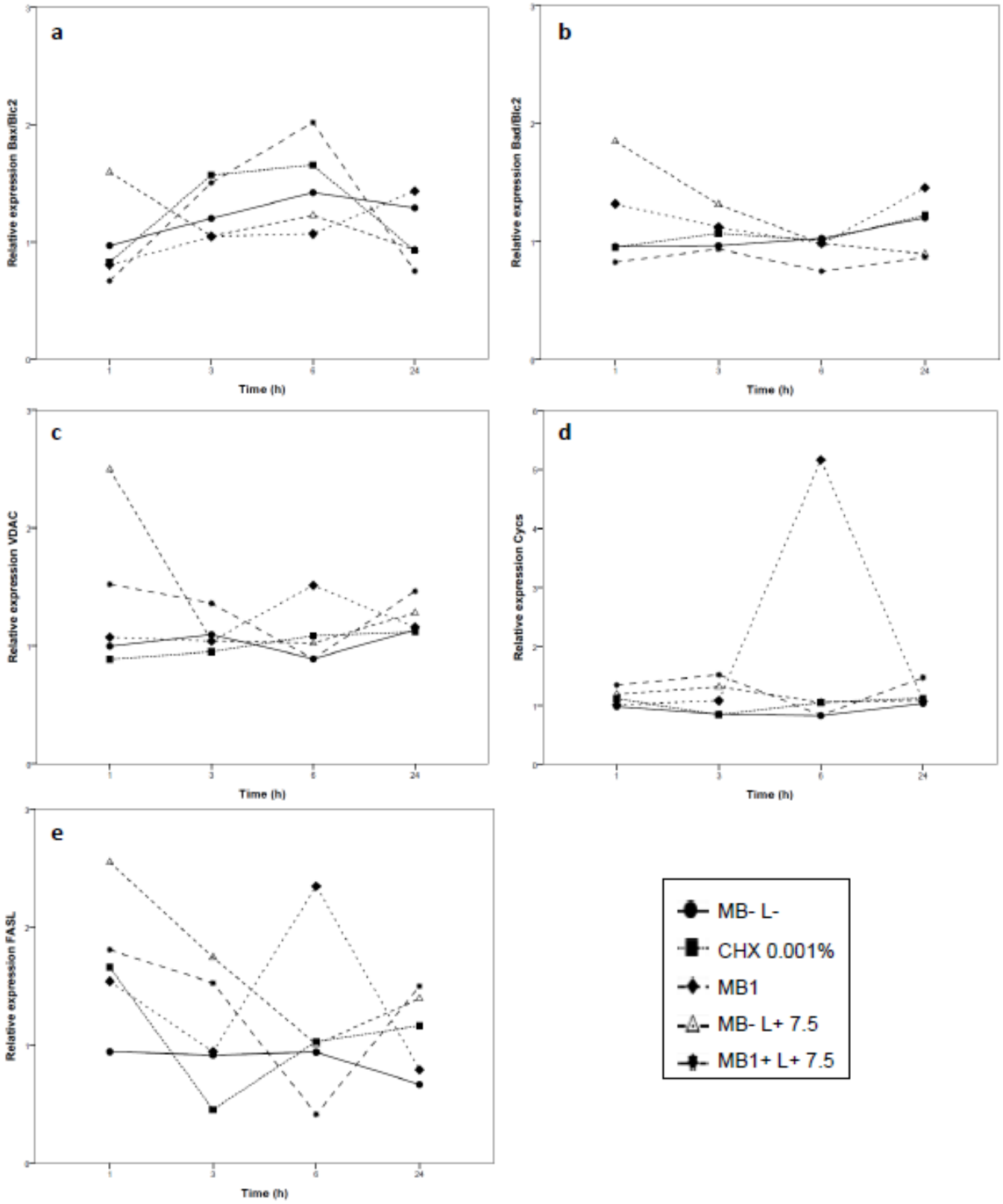

Figure 3. Relative expression of (a) Bax/Bcl-2, (b) Bad/Bcl-2, (c) VDAC-1, (d) cytochrome C and (e) Fas-L after 1 h, 3 h, 6 h and 24 h of treatment.

In vitro effect of curcumin-mediated antimicrobial photodynamic therapy on fibroblasts: viability and cell signaling for apoptosis

ABSTRACT

Although it was demonstrated that curcumin-mediated antimicrobial photodynamic therapy (aPDT) is effective for reducing the viability of microbial cells and the vitality of oral biofilms, the cytotoxicity of this therapeutic approach for host cells has not yet been elucidated. Hence, the aim of this study was to evaluate the cytotoxicity and apoptotic effects of curcumin-mediated aPDT on mouse fibroblasts. Cells were treated with 0.6 or 6 μM curcumin combined with 0.075 or 7.5 J.cm^{-2} LED at 455 nm. Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet (CV) assays, while quantitative reverse transcriptase-PCR (qRT-PCR) was used to assess the expression of Bax, Bad, Bcl-2, VDAC-1, cytochrome C, and Fas-L genes for apoptosis. The differences between groups were detected by Kruskal-Wallis and post-hoc Dunn's tests for MTT and CV assays, and by ANOVA and post-hoc Tukey test for qRT-PCR (P<0.05). The combination of 0.6 μM curcumin plus 7.5 J.cm^{-2} LED reduced viable cells in 34%, while the combinations of 6 μM curcumin plus 0.075 and 7.5 J.cm^{-2} LED reduced viable cells in 47% and 99%, respectively. qRT-PCR results demonstrated that curcumin-mediated aPDT increased significantly the relative expression of Bax/Bcl-2, cytochrome C, VDAC-1, and Fas-L genes, without influence on the ratio Bad/Bcl-2. Therefore, curcumin-mediated aPDT activated Bcl-2 apoptosis signaling pathways in mouse fibroblasts, reducing the viability of cells with the increase of concentrations of curcumin and light energies.

Keywords: Antimicrobial photodynamic therapy; Curcumin; Photochemotherapy; Cell viability; Bcl-2 family

INTRODUCTION

Photodynamic therapy (PDT) has been widely used in different treatments over the last years, with promising results for treating cancer, bacterial and fungal infections [1-6] The mechanisms of PDT are characterized by the use of a combination of a photosensitizing agent (PS) and a complimentary visible light in an oxygen rich environment [7,8]. PS molecules absorb photons, leading to the excitation of electrons and production of reactive oxygen species (ROS) [7-10]. These products result in a cascade of oxidative events that are highly toxic for pathogenic cells, causing their damage and death by targeting membrane lipids, proteins, nucleic acids and other cellular components [11,12]. In this sense, antimicrobial PDT (aPDT) has been indicated as an alternative treatment for the elimination of microorganisms into the oral cavity, due to the convenient application of photosensitizing agents and light sources [5,13].

Curcumin (1,7-bis(4-hydroxy 3-methoxy phenyl)-1,6-heptadiene-3, 5-dione), a natural dye isolated from the rhizomes of *Curcuma longa*, have been purposed as a potential photosensitizing (PS) in aPDT. Traditionally, it is used for the treatment of cold, skin diseases, and inflammation [14,15]. Also, recent researches confirmed its potential as antitumor, antioxidant, antimicrobial and anti-inflammatory [16-18]. It has a maximum absorption with blue LED on wavelength of 430 nm, presenting a strong phototoxic effect in microorganisms even when used in lower concentrations [19,20]. In tumor cells, the cytotoxicity of curcumin is due to the induction of apoptosis mediated by the direct release of cytochrome C, and the subsequent activation of caspases [21,22]. In microorganism cells, the cytotoxicity occurs by the production of reactive oxygen species, such as singlet oxygen ($^1O_2$), hydrogen peroxide and superoxide [23-25].

Several dental studies have already been demonstrated that curcumin-mediated aPDT was effective against *Candida albicans*, *Enterococcus faecalis*, cariogenic planktonic cells, mono-species biofilms and dentin microcosm biofilms [26-32]; however, there is a scarcity of evidence about the cytotoxicity of therapeutic doses of photosensitizing agents and light source on host cells. Bulit et al. [33] showed that curcumin-mediated PDT was able to inactivate lactobacilli at lower concentrations than that affected mitochondrial activity of odontoblast-like cells (MDPC-23), undifferentiated pulp cells (OD21) and human embryonic stem cells (hESC H1).

The aim of this *in vitro* study was to evaluate the cytotoxicity and apoptotic effects of curcumin-mediated antimicrobial photodynamic therapy on mouse fibroblasts (NIH/3T3). The null hypotheses were that aPDT would have no cytotoxicity (H_0) and apoptotic effects (H_0^1) on eukaryotic cells.

MATERIALS AND METHODS

Cell culture

Mouse fibroblasts (NIH/3T3) cells (ATCC - American Type Culture Collection, CRL1658™) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, USA) containing 10% (v/v) fetal bovine serum (FBS, GIBCO Laboratories, Life Technologies, Inc., New York, USA), 100 IU.mL^{-1} penicillin and 0.1 mg.mL^{-1} streptomycin (Sigma-Aldrich). The cellular cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air. After reaching confluency, the cells were enzymatically dissociated with 0.25% trypsin and 1 mmol.L^{-1} EDTA (Sigma-Aldrich), and counted under Neubauer chamber using trypan blue staining protocol.

Curcumin-mediated antimicrobial photodynamic therapy

A stock solution of 30 mM curcumin (Sigma-Aldrich) was prepared by dilution in dimethylsulfoxide (DMSO, Sigma-Aldrich), and stored at -20°C in dark conditions. At the moment of use, the stock solution was diluted in PBS 10% FBS, to final concentrations of 6 and 0.6 μM curcumin. Biotable® RGB (Institute of Physics of São Carlos, São Carlos, Brazil) was used as a light source, with parameters of irradiation of visible blue light (455 ± 30 nm), irradiance of 40 mW.cm^{-2}, distance of ± 25 mm and two energy densities (7.5 or 0.075 J.cm^{-2} LED).

After 24 h, cells were washed with PBS 1x and treated according to their experimental groups, as follows: group 1 [MB-L-, negative control exposed to 0.1% DMSO concentration (v/v)], group 2 (0.001% chlorhexidine, positive control), group 3 (0.01% chlorhexidine, positive control), group 4 [0.001% sodium hypochlorite (NaOCl), positive control], group 5 (0.01% NaOCl, positive control), group 6 (0.6 mM curcumin alone), group 7 (6 mM curcumin alone), group 8 (0.075 J.cm^{-2} LED alone), group 9 (7.5 J.cm^{-2} LED alone), group 10 (0.6 mM curcumin + 0.075 J.cm^{-2} LED), group 11 (0.6 mM curcumin + 7.5 J.cm^{-2}), group 12 (6 mM curcumin + 0.075 J.cm^{-2} LED), and group 13 (6 mM curcumin + 7.5 J.cm^{-2} LED). The agents were added to

cells and incubated for 2 min in dark conditions. The PDT groups were subsequently exposed to a blue LED, with the time of light exposure of 2 s (0.075 $J.cm^{-2}$) or 360 s (7.5 $J.cm^{-2}$). The measurements of power densities of light were checked by using an optical power meter 1916-C (Newport, Irvine, USA), adjusting time of irradiation by the ratio between energy and power densities of light. After irradiation, cells were washed with PBS 1x, and DMEM 10% FBS was added.

Cell Viability

Cells were plated in 96-well microplates at a density of $3x10^3$ in DMEM 10% FBS. The cell viability was evaluated by crystal violet (CV) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Sigma-Aldrich) assays, after 24, 48 and 72 h from treatments [34]. For CV assays, the medium above the cells was removed and the wells were washed with PBS 1x and added 100% methanol for 10 min. Then, cells were stained with 0.2% crystal violet for 3 min and washed twice with PBS 1x. The dye was extracted with 0.05 $mol.L^{-1}$ sodium citrate solution for 10 min. The absorbance was measured at 540 nm. For MTT assays, the medium was removed and cells were washed with PBS 1x and incubated with a sterile MTT solution (0.5 $mg.mL^{-1}$) for 4 h at 37°C. The intracellularly reduced insoluble pigment (formazan) was extracted with DMSO for 30 min, and the absorbance was determined in a spectrophotometer at 562 nm (Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader, BioTek Instruments Inc.).

qRT-PCR

Cells were cultivated in 24-well microtiter plates at a density of $5 x10^4$ cells for 24 h, and then were treated with 0.6 or 6 µM curcumin combined or not with 0.075 $J.cm^{-2}$ LED. A concentration of 0.001% chlorhexidine was used as a positive control. After 1, 3, 6 and 24 h, the cells were submitted to mRNA extracttion using MagMAX® mirVana® Total RNA Isolation Kit (Applied Biosystems, Foster, USA), according to the manufacturer's instructions. The isolated RNA was quantified by NanoDrop®1000 (Thermo Fisher Scientific, Waltham, USA) and the cDNA was obtained using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), also following the manufacturer's recommendations. The cDNA samples were incubated with TaqMan® Gene Expression Master Mix and Taqman® Gene Expression assay (Applied Biosystems) for Bad (Mm00432042_m1), Bax

(Mm00432051_m1), Bcl-2 (Mm00477631_m1), VDAC-1 (Mm00834272_m1), cytochrome C (Mm01250094_m1) and Fas-L (Mm00438864_m1) genes. The reading was done in the ViiA 7 Real-Time PCR System® (Applied Biosystems), under the following conditions: a cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, and a cycle of 60°C for 1 min. Act-b expression (Mm00607939_s1) was used as a constitutive gene (control). The samples were analyzed in triplicate, and the relative expression was obtained according to the formula $2^{-(\Delta\Delta Ct)}$.

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 21.0 software. Kruskal-Wallis, and post hoc Dunn's were used to indicate differences between groups for MTT and CV data, since they were not normally distributed. Distinctly, qRT-PCR values were compared using ANOVA followed by a post-hoc Tukey test, since these data presented a normal and homogeneous distribution, identified by Shapiro-Wilk and Levene tests, respectively. *P* values <0.05 were considered significant.

RESULTS

The viability tests demonstrated that aPDT promoted dose-dependent reductions of viability of mouse fibroblasts. The reductions on viable cells were 47% in MTT and 78% in CV for the combination of 6 μM curcumin with 0.075 $J.cm^{-2}$ LED, and 99% in MTT and 81.5% in CV for the combination of 6 μM curcumin with 7.5 $J.cm^{-2}$ LED. Also, it was observed a significant decrease of viable cells (34% at 24 h) only when 0.6 μM curcumin was associated with 7.5 $J.cm^{-2}$ LED (Table 1, and Figures 1 and 2).

A concentration of chlorhexidine solution at 0.01% presented a significant cytotoxicity for fibroblasts, reducing their viability in 96%. An interesting result was that 0.01% NaOCl reduced the percentage of viable cells only in the first 24 h, with a subsequent recovery after 72 h (Table 1, and Figures 1 and 2).

The results of qRT-PCR demonstrated that 6 μM curcumin-mediated aPDT increased significantly the expression of apoptotic genes in fibroblasts NIH/3T3. After 3 h, the expression of cytochrome C genes was higher in cells treated with 0.6 μM curcumin plus 0.075 $J.cm^{-2}$ LED (Figure 3). After 6 h, the ratio Bax/Bcl-2 and the expressions of Fas-L, VDAC-1 and cytochrome C increased 2.5-, 6.8-, 1.5- and 5.6-fold, respectively (Figure 3). Fibroblasts treated with 6 μM curcumin presented a

significant lower Bax/Bcl-2 ratio expression after 24 h. The ratio Bad/Bcl-2 was similar among different treatment and control groups (Figure 3).

DISCUSSION

The present study demonstrated the cytotoxic potential of curcumin-mediated PDT on cellular cultures of fibroblast NIH/3T3 *in vitro* conditions, specially when it was associated with 7.5 $J.cm^{-2}$ LED. qRT-PCR results were in agreement with these findings, with a significant increase of the relative expression of Bax/Bcl-2, VDAC-1 and cytochrome C genes after aPDT. Digluconate chlorhexidine and 0.01% sodium hypochlorite presented highly cytotoxicity for these eukaryotic cells.

Previously, contradictory cytotoxic effects of curcumin-mediated aPDT on fibroblasts were reported. Gomes-Filho [40] showed that 5% NaOCl inhibited the viability of mouse fibroblasts in 75%, while their viability was similar to control group after 500$mg.L^{-1}$ curcumin plus 72 $J.cm^2$ LED. Differently, Ribeiro et al. [38] demonstrated that 5, 10 and 20 μM curcumin associated with 37.5 $J.cm^{-2}$ LED decreased the metabolic activity of L929 cells in 68.1, 75.1 and 80%, respectively. These differences could be explained by the differences in curcumin concentrations, pre-irradiation times, oil-solvent reagent, treatment protocol, and origin of cells [41-43]. In this sense, our results were closer to those described by Ribeiro et al. [38], even using curcumin 3-30 times more concentrated than previous authors. This similarity could be occurred by the use of same type of cells, with the simultaneous application of lower pre-irradiation time and light energies in the present study, which might compensate the differences in PS concentrations.

Apoptosis is a process that includes an ordered cascade enzymatic events, leading to the production of unique and biochemical features. The most important regulators of this process are members of the Bcl-2 family of proteins, controlling the intrinsic apoptotic pathway. VDAC-1 releases molecules from mitochondria into cytosol, such as cytochrome C. Then, this apoptogenic factor activates caspases-9 and -3 that promote apoptosis. Bax and Bad are pro-apoptotic, whereas Bcl-2 is anti-apoptotic [44-47]. Studies have showed that the apoptotic effect of photodynamic therapy initiated from its intrinsic via by a substantial loss of Bcl-2 [48-50]. Woo et al. (2003) demonstrated that curcumin causes oxidative stress, down-regulating Bcl-2 levels in renal carcinoma cells [51]. These findings corroborate with our results, which suggest

that Bcl-2 family activates the mechanisms of apoptosis underlying curcumin-mediated aPDT.

Fas-L is a death-receptor that acts in the extrinsic pathway of apoptosis. It is localized on cellular membranes as a ligand able to induce a sequence of apoptotic events, for example, by the activation of caspases -8 and -3 [46,47]. Curcumin induces apoptosis in several types of human melanoma cells through Fas receptor and caspase-8 [52]. However, in some other cellular lines, such as human gingival fibroblast, the cytotoxic activity of curcumin was attributed to generation of reactive oxygen species (ROS) [53,54]. Some studies suggested that Fas-L is connected to the intrinsic apoptosis pathway through Bid [55,56]. Caspase-8 cleaves Bid, and translocates its C-terminal to mitochondria, also resulting in the release of cytochrome C [56,57].

This study presents some limitations. First, the cytotoxic tests were performed on cells originated from mice, i.e., the cytotoxicity of aPDT on human fibroblasts might differ considerably. Second, this *in vitro* analysis did not consider the application of models involving multilayer cellular assays, theoretically favoring the increase of cytotoxic effects of therapies in comparison to clinical conditions. Third, the role of the intrinsic via of apoptosis in the reduction of the viability of fibroblasts was defined only by the expression of apoptotic genes. However, from these findings is possible to infer that the application of curcumin-mediated aPDT on unprotected soft tissues might induce irreversible damages, depending on specific therapeutic parameters.

In conclusion, curcumin-mediated aPDT was significantly cytotoxic to mouse fibroblasts, proportionally to the increase of curcumin concentration and light doses. The reduction of viability of cells was related to the intrinsic apoptosis pathway, activated by the Bcl-2 family genes. Hence, these results indicate the rejection of hypotheses H_0 and H_0^1.

REFERENCES

[1] R.R. Allison, et al. Clinical photodynamic therapy of head and neck cancers—a review of applications and outcomes, Photodiagn. Photodyn. Ther. 2 (2005) 205–22.

[2] R.R. Allison, et al., The future of photodynamic therapy in oncology, Future Oncol. 2 (2006) 53–71.

[3] M.C. Teichert, M.D. Jones, M.N. Usacheva, M.A. Biel, Treatment of oral candidiasis with methylene blue-mediated photodynamic therapy in an

immunodeficient murine model, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 93 (2002) 155-60.

[4] J.A. Williams, G.J. Pearson, M.J. Colles, Antibacterial action of photoactivated disinfection (PAD) used on endodontic bacteria in planktonic suspension and in artificial and human root canals, J. Dent. 34 (2006) 363–71.

[5] R.F. Donnelly, P.A. McCarron, M.M. Tunney, A. Woolfson, Potential of photodynamic therapy in treatment of fungal infections of the mouth. Design and characterisation of a mucoadhesive patch containing toluidine blue , J. Photochem. Photobio.l B 86 (2007) 59–69.

[6] H. Gursoy, C. Ozcakir-Tomruk, J. Tanalp, S. Yilmaz, Photodynamic therapy in dentistry: a literature review, Clin. Oral Invest. 17 (4) (2013) 1113–1125, https://doi.org/10.1007/s00784-012-0845-7.

[7] M. Wilson, J. Dobson, Lethal photosensitization of oral anaerobic bacteria. Clin. Infect. Dis. [S.l.], 16 (4) (1993) S414-5.

[8] I.O.L. Bacellar, T.M. Tsubone, C. Pavani, M. Baptista. Photodynamic Efficiency: from molecular photochemistry to cell death. Int. J. Mol. Sci. 16 (2015) 20523-20559; doi:10.3390/ijms160920523.

[9] M. Bhatti, et al., Effect of dosimetric and physiological factors on the lethal photosensitization of Porphyromonas gingivalis in vitro. Photochem. Photob. [S.l.] 65 (6) (1997) 1026-1031.

[10] A.A. Takasaki, et al., Application of antimicrobial photodynamic therapy in periodontal and peri-implant disease, Periodontol. 2000. 51 (2009) 109-40.

[11] M.R. Hamblin, T. Hasan, Photodynamic therapy: a new antimicrobial approach to infectious disease, Photochem. Photobiol. Sci. 3 (2004) 436–450.

[12] M. Wainwright, Photodynamic antimicrobial chemotherapy (PACT). J Antimicrob Chemother 42 (1998) 13–28.

[13] R.F. Donnelly, P.A. McCarron, M.M. Tunney, Antifungal photodynamic therapy, Microbiol. Res. 163 (2008) 1–12.

[14] S. Aggarwal, S. Young-Joon, S. Shishodia, The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease, Springer, NY USA (2007) 1–76.

[15] I. Chattopadhyay, K. Biswas, U. Bandyopadhyay, R.K. Banerjee, Turmeric and curcumin: biological actions and medicinal applications, Curr. Sci. Assoc. 87 (2004) 44–53.

[16] A. Duvoix, et al., Diederich, Chemopreventive and therapeutic effects of curcumin, Cancer Lett. 223 (2) (2005) 181–190, https://doi.org/10.1016/j.canlet.2004.09.041.

[17] J.V. Crivello, U. Bulut, Curcumin: a naturally occurring long-wavelength photosensitizer for diaryliodonium salts, J. Polym. Sci. Part A: Polym. Chem. 43 (21) (2005) 5217–5231, https://doi.org/10.1002/pola.21017.

[18] B.B. Aggarwal, A. Kumar, A.C. Bharti, Anticancer potential of curcumin: preclinical and clinical studies, Anticancer Res. 23 (2003) 363–398.

[19] S. Gharesi, et al., Effect of photodynamic therapy based on indocyanine green on expression of apoptosis-related genes in human gingival fibroblast cells. Photodiagn. Photodyn. Ther. 19 (2017) 33-36.

[20] D. Saitawee, et al., Photodynamic therapy of Curcuma longa extract stimulated with blue light against Aggregatibacter actinomycetemcomitans, Photodiagn. Photodyn. Ther. 22 (2018) 101–105.

[21] K. Park, J.H. Lee, Photosensitizer effect of curcumin on UVB-irradiated HaCaT cells through activation of caspase pathways, Oncol. Rep. 17 (3) (2007) 537–540.

[22] C. Steiner-Oliveira, et al., Randomized in vivo evaluation of phtodynamic antimicrobial chemotherapy on deciduous carious dentin, J. Biomed. Opt. 20 (10) (2015).

[23] T.A. Dahl, W.M. McGowan, M.A. Shand, V.S. Srinivasan, Photokilling of bacteria by the natural dye curcumin, Arch Microbiol. 151 (2) (1989) 183-5.

[24] C.F. Chignell, et al., Spectral and photochemical properties of curcumin. Photochem. Photobiol. 59 (3) (1994) 295-302.

[25] L.N. Dovigo, et al., Investigation of the photodynamic effects of curcumin against Candida albicans. Photochem Photobiol. 87 (4) (2011) 895-903.

[26] M.C. Andrade, et al., Effect of different pre-irradiation times on curcumin mediated photodynamic therapy against planktonic cultures and biofilms of Candida spp. Arch. Oral Biol 58(2) (2012) 200-10.

[27] L Dovigo, et al., Curcumin-mediated photodynamic inactivation of Candida albicans in a murine model of oral candidiasis. Med Mycol 51 (3) (2013) 243-51.

[28] G. Pileggi, et al., Blue light-mediated inactivation of Enterococcus faecalis in vitro. Photodiagnosis Photodyn Ther 10 (2) (2013)134-40.

[29] N. Araújo, et al., Overall-mouth disinfection by photodynamic therapy using curcumin. Photomed Laser Surg 30 (2) (2012) 96-101.

[30] M.A. Paschoal, et al., Photodynammic potential of curcumin and blue LED agaisnt Streptococcus mutans in a planktonic culture. Photodiag Photodyn Ther 10 (3) (2013)313-319.

[31] N. Araújo, et al., Photodynamic antimicrobial therapy of curcumin in biofilms and carious dentine. Lasers Med Sci 29 (2014) 629-35.

[32] D.A.C. Méndez, et al., Curcumin-mediated antimicrobial photodynamic therapy reduces the viability and vitality of infected dentin caries microcosms. Photodiagn Photodyn Ther 24 (2018) 102–108, https://doi.org/10.1016/j.pdpdt.2018.09.007.

[33] F. Bulit, et al., Antimicrobial activity and cytotoxicity of 3 photosensitizers activated with blue light. J Endod. 40 (2014) 427-31.

[34] F.A. Oliveira, et al., Low intensity lasers differently induce primary human osteoblast proliferation and differentiation, J. Photochem. Photobiol. B 163 (2016) 14–21.

[35] D. Manoil, et al., Flow cytometric assessment of Streptococcus mutans viability after exposure to blue light-activated curcumin, Photodiagn Photodyn Ther 11(3) (2014) 372–379.

[36] C.C. Tonon, et al., Comparative effects of photodynamic therapy mediated by curcumin on standard and clinical isolate of Streptococcus mutans, J Contemp Dent Pract 16(1) (2015) 1–6.

[37] C. Santezi, et al., Potential of curcumin-mediated photodynamic inactivation to reduce oral colonization, Photodiagn. Photodyn Ther 15 (2016) 46-52.

[38] A.P.D. Ribeiro, et al., Phototoxic effect of curcumin on methicillin-resistant Staphylococcus aureus and L929 fibroblasts, Lasers Med Sci 28 (2013) 391–398, doi 10.1007/s10103-012-1064-9.

[39] M. Pourhajibagher, et al., Evaluation of antimicrobial photodynamic therapy with indocyanine green and curcumin on human gingival fibroblast cells: An in vitro photocytotoxicity investigation, Photodiagn. Photodyn Ther 15 (2016) 13-18.

[40] J.E. Gomes-Filho, et al., Evaluation of photodynamic therapy on fibroblast viability and cytokine production. Photodiagnosis Photodyn Ther (2016) 97-100.

[41] E.M. Bruzell, E. Morisbak, H.H. Tønnesen, Studies on curcumin and curcuminoids. XXIX. Photoinduced cytotoxicity of curcumin in selected aqueous preparations, Photochem. Photobiol. Sci. 4 (2005) 523–530

[42] T. Haukvik, E. Bruzell, S. Kristensen, H.H. Tønnesen, Photokilling of bacteria by curcumin in different aqueous preparations. Studies on curcumin and curcuminoids XXXVII, Pharmazie 64 (2009) 666–673.

[43] J. Epstein, I.R. Sanderson, T.T. Macdonald, Curcumin as a therapeutic agent: the evidence from in vitro, animal and human studies, Br. J. Nutr. 103 (2010) 1545–1557.

[44] N.I. Agalakova, G.P. Gusev, Molecular Mechanisms of Cytotoxicity and Apoptosis Induced by Inorganic Fluoride, Cell Biology (2012) 1-16.

[45] V. Shoshan-Barmatz, S. De, A. Meir, The Mitochondrial Voltage-Dependent Anion Channel 1, Ca(2+) Transport, Apoptosis, and Their Regulation, Front Oncol 7 (2017) 60.

[46] J.H. Lee, et al., Involvement of both mitochondrial- and death receptor-dependent apoptotic pathways regulated by Bcl-2 family in sodium fluoride-induced apoptosis of the human gingival fibroblasts. Toxicology 243 (2008) 340-347.

[47] S. Elmore, Apoptosis: a review of programmed cell death. Toxicol Pathol. 35 (2007) 495-516.

[48] D. Kessel, M. Castelli, J.J. Reiners, Ruthenium red-mediated suppression of Bcl-2 loss and Ca2 release initiated by photodamage to the endoplasmic reticulum: Scavenging of reactive oxygen species. Cell Death Differ. 12 (2005) 502–511.

[49] L. Duprez, E. Wirawan, T.V. Berghe, P. Vandenabeele, Major cell death pathways at a glance. Microbes Infect. 11 (2009) 1050–1062.

[50] P. Mroz, A. Yaroslavsky, G.B. Kharkwal, M.R. Hamblin, Cell death pathways in photodynamic therapy of cancer. Cancers (Basel) 3 (2011) 2516–2539.

[51] JH. Woo, et al., Molecular mechanisms of curcumin-induced cytotoxicity: induction of apoptosis through generation of reactive oxygen species, down-regulation of Bcl-X L and IAP, the release of cytochrome c and inhibition of Akt , Carcinogenesis 24 (7) (2003) 1199–1208, https://doi.org/10.1093/carcin/bgg082

[52] J.A. Bush, K.J. Cheung Jr, G. Li, Curcumin induces apoptosis in human melanoma cells through a Fas receptor/caspase-8 pathway independent of p53 Exp Cell Res, 271 (2) (2001) 305-314.

[53] K. Elizabeth, M.N.A. Rao Oxygen radical scavenging activity of curcumin Int J Pharmacol, 58 (1990) 237-240.

[54] T. Atsumi, K. Tonosaki, S. Fujisawa, Induction of early apoptosis and ROS-generation activity in human gingival fibroblasts (HGF) and human submandibular

gland carcinoma (HSG) cells treated with curcumin Arch Oral Biol, 51 (10) (2006) 913-921.

[55] H. Li, H. Zhu, C.J. Xu, J. Yuan, Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis Cell, 94 (1998) 491-501.

[56] C. Gajate, F. Gonzalez-Camacho, F. Mollinedo, Lipid raft connection between extrinsic and intrinsic apoptotic pathways, Biochem. Biophys. Res. Commun. 380(4) (2009) 780-784, https://doi.org/10.1016/j.bbrc.2009.01.147.

[57] S.J. Riedl, G.S. Salvesen The apoptosome: signalling platform of cell death Nat. Rev. Mol. Cell Biol. 8 (2007) 405-413.

TABLES

Table 1. Cellular viability (%) assessed by MTT and CV after 72 h of treatment. Distinct letters represent significant statistical differences between groups.

Groups	24 Hours		48 Hours		72 Hours	
	MTT (%)	*CV (%)*	*MTT (%)*	*CV (%)*	*MTT (%)*	*CV (%)*
C-L-	100 ±19.49^{AC}	100 ± 30.02^{A}	100 ± 18.20^{A}	100 ± 14.56^{A}	100 ± 17.46^{AD}	100 ± 11.41^{A}
CHX 0.001%	78.52 ± 18.96^{AC}	79.88 ± 18.28^{ADF}	86.10 ± 11.79^{A}	112.93 ± 12.48^{A}	119.80 ± 17.95^{B}	140.94 ± 12.59^{AD}
CHX 0.01%	3.31 ± 0.96^{B}	6.59 ± 15.68^{BE}	1.75 ± 1.23^{B}	4.86 ± 11.05^{BC}	2.76 ± 1.13^{C}	2.14 ± 2.31^{BC}
HIP 0.001%	91.41 ± 17.12^{ACG}	76.19 ± 34.02^{A}	88.07 ± 10.53^{A}	109.04 ± 24.73^{A}	120.03 ± 15.92^{AB}	119.52 ± 8.76^{A}
HIP 0.01%	33.28 ± 14.47^{B}	24.22 ± 12.53^{BCD}	50.86 ± 7.91^{BC}	48.26 ± 23.27^{B}	83.83 ± 19.36^{CD}	62.19 ± 11.73^{CD}
C0.6	86.44 ± 14.16^{AEF}	76.91 ± 24.43^{A}	86.64 ± 8.60^{AC}	92.99 ± 18.07^{A}	106.27 ± 21.76^{B}	136.54 ± 15.30^{A}
C 6	89.22 ± 19.53^{AD}	63.03 ± 30.39^{A}	97.14 ± 12.35^{A}	97.93 ± 23.56^{A}	117.62 ± 18.93^{ADE}	73.30 ± 5.81^{A}
C- L+ 0.075	85.98 ± 12.66^{AE}	99.16 ± 13.77^{A}	88.27 ± 10.38^{A}	104.89 ± 20.60^{A}	125.39 ± 27.75^{BE}	143.27 ± 15.87^{A}
C- L+ 7.5	59.36 ± 14.24^{AC}	86.25 ± 20.70^{CF}	85.9 ± 12.03^{A}	120.20 ± 34.41^{A}	102.99 ± 14.84^{B}	146.06 ± 20.44^{A}
C0.6+ L+ 0.075	86.52 ± 10.20^{CE}	109.59 ± 23.98^{A}	100.74 ± 18,19^{AC}	94.19 ± 6.94^{A}	122.35 ± 15.69^{AB}	117.93 ± 11.63^{A}
C0.6+ L+ 7.5	34.46 ± 7.72^{BDG}	34.5 ± 16.91^{BC}	34.45 ± 8.54^{B}	16.12 ± 2.46^{BC}	55.16 ± 18.78^{CD}	34.95 ± 8.82^{BC}
C6+ L+ 0.075	47.60 ± 8.44^{BDF}	29.53 ± 15.71^{CE}	42.98 ± 27.99^{B}	25.74 ± 13.64^{BC}	68.10 ± 13.94^{CE}	8.74 ± 5.37^{CB}
C6+ L+ 7.5	0.13 ± 0.36^{BDG}	38.09 ± 11.83 ^{B}	-0.20 ± 0.78^{B}	6.19 ± 3.22^{C}	-0.19 ± 1.46^{C}	1.65 ± 9.12^{B}

FIGURES

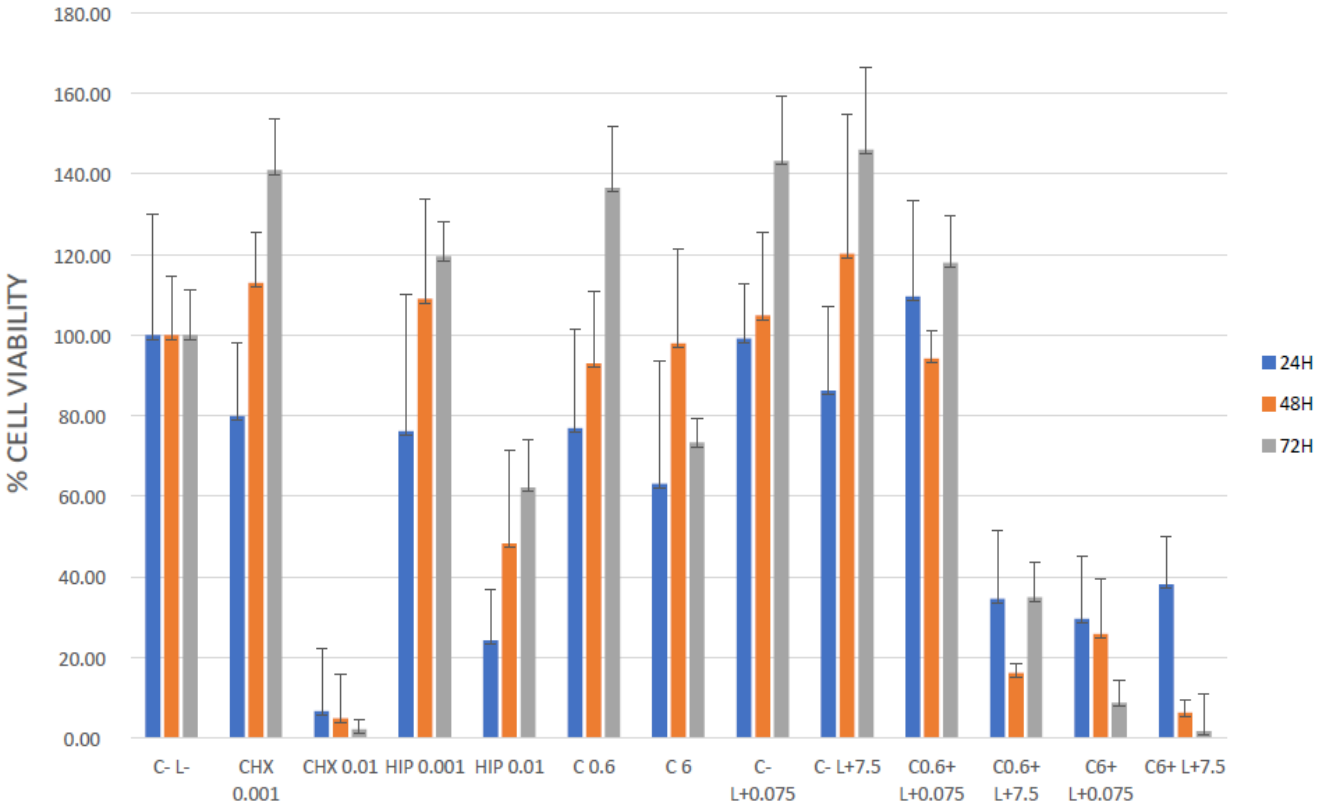

Figure 1. Percentage of viability of cells according to CV assay after 72 h in relation to no treatment group (C- L-).

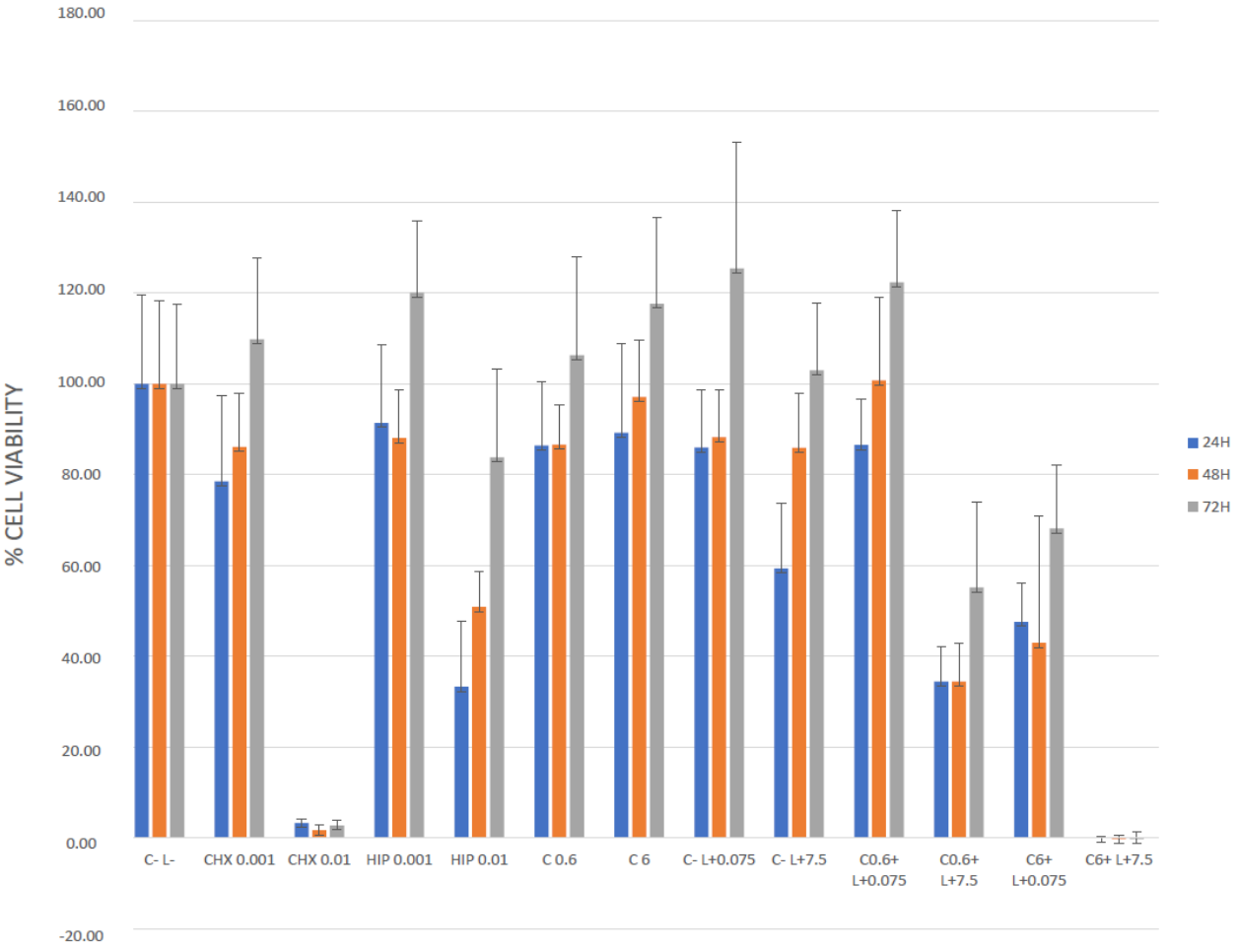

Figure 2. Percentage of viability of cells according to MTT assay after 24 h, 48 h, and 72 h in relation to no treatment group (C- L-).

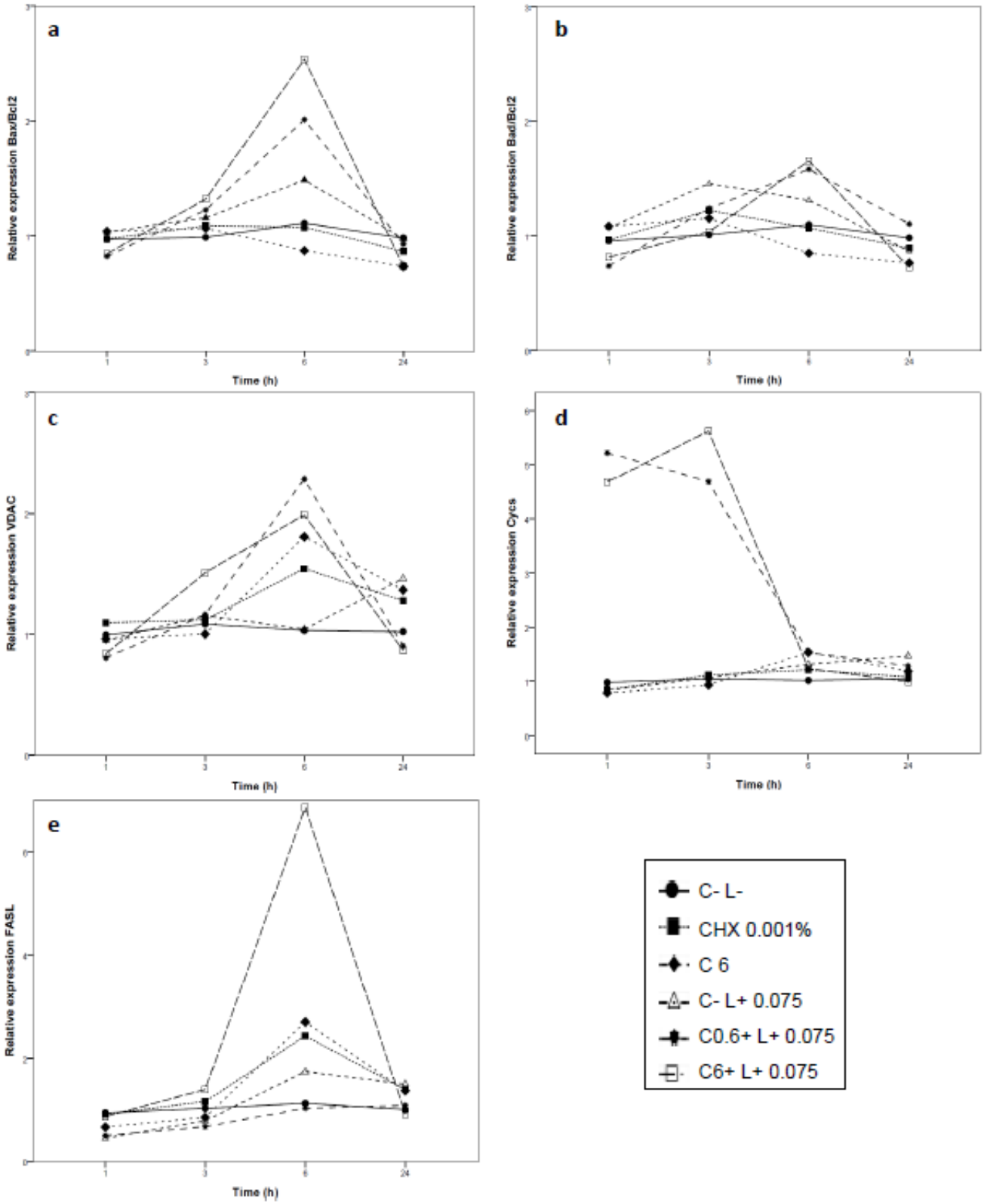

Figure 3. The relative expression of (a) Bax/Bcl-2, (b) Bad/Bcl-2, (c) VDAC-1, (d) Cytochrome C and (e) Fas-L genes in 1, 3, 6 and 24 h after treatment

3 DISCUSSION

3 DISCUSSION

The photodynamic action of any photosensitizer in biological systems still unclear, because it may be affected by several variables including photochemical mechanism, photosensitizer localization, the reactivity of biological targets and cell signaling network (TARDIVO et al., 2005). Targeting mitochondria are very important in PDT research, since it is known that mitochondria damage may induce the apoptotic cascade (BUNTING, 1992; KESSEL; LUO, 1998). It is relevant investigate whether parameters used on aPDT could result in cytotoxic effect to the host cells.

Through MTT and CV assays, significant cytotoxicity of aPDT mediated by MB and curcumin was observed. This is in agreement to Kashef (2012), who evaluated *in vitro* cytotoxicity of this therapeutic approach on primary human fibroblasts. At 24 h, they demonstrated the mitochondrial activity of MB-mediated aPDT (using 50 $\mu g.mL^{-1}$ MB and 163.8 $J.cm^{-2}$ light) was 27% lower than control. Also, Kunwar (2008) studied the cytotoxicity of curcumin at 20 and 40 $nmol.mL^{-1}$ on NIH/3T3 cells. The results demonstrated a cytotoxicity of 41% when using the highest concentration of the dye. Both studies also found a dose-dependent cytotoxicity of aPDT on host cells (KUNWAR et al., 2008; KASHEF et al; 2012).

Our qPCR results are in accordance with the viability tests, observing an increase in the relative expression of Bax/Bcl-2 ratio, VDAC-1 and cytochrome C in both conditions of aPDT, which indicated the role of the intrinsic apoptotic pathway of aPDT on cellular cytotoxicity (ANTO et al., 2002; WOO et al., 2003; dos SANTOS et al., 2017). Apoptosis is a process that includes an ordered cascade enzymatic events, leading to the production of unique and biochemical features. The most important regulators of this process are members of the Bcl-2 family of proteins, controlling the intrinsic apoptotic pathway. VDAC-1 releases molecules from mitochondria into cytosol, such as cytochrome C. Then, this apoptogenic factor activates caspases-9 and -3 that promote apoptosis. Bax and Bad are pro-apoptotic, whereas Bcl-2 is anti-apoptotic (ELMORE, 2007; LEE et al., 2008; AGALAKOVA et al., 2012; SHOSHAN-BARMATZ et al., 2017). Fas-L is a death-receptor and acts on extrinsic pathway. Localized on the cells membrane is a ligand able to induce a sequence of apoptotic events such as the caspases -8 and -3 activation The

differences observed on Fas-L expression can be explained by the conection of the intrinsic apoptosis pathway through Bid (LI et al., 1998; GAJATE, GONZALEZ-CAMACHO, MOLLINEDO, 2009). Bid was translocated to mitochondria, resulting in the release of apoptogenic factor cytochrome C (RIEDL, SALVESEN, 2007).

4 CONCLUSIONS

4 CONCLUSIONS

Based on the results, we can conclude that:

- High concentrations of aPDT mediated by methylene blue and curcumin promoted significantly reduction of viable fibroblasts when compared to control;
- In lower concentrations, MB- and curcumin-mediated aPDT did not promoted significant cellular cytotoxicity to mouse fibroblasts;
- MB- and curcumin-mediated aPDT increased the expression of Bax/Bcl-2 ratio, VDAC-1 and cytochrome C genes, suggesting the activation of an intrinsic apoptotic pathway.

REFERENCES

REFERENCES

ABRAHAMSE H, HAMBLIN MR. New photosensitizers for photodynamic therapy. Biochem. J. v. 473, p. 347–64, 2016.

AGGARWAL BB, KUMAR A, BHARTI AC. Anticancer potential of curcumin: preclinical and clinical studies, Anticancer Res. v. 23, p.363–98, 2003.

ANDRADE M et al. Effect of different pre-irradiation times on curcumin-mediated photodynamic therapy against planktonic cultures and biofilms of Candida spp. Arch Oral Biol. v. 58, n. 2, p. 200-10, 2012.

ANTO R et al. Curcumin (diferuloylmethane) induces apoptosis through activation of caspase-8, BID cleavage and cytochrome c release: Its suppression by ectopic expression of Bcl-2 and Bcl-xl. Carcinogenesis v. 23, p. 143–150, 2002.

ARAÚJO N et al. Photodynamic antimicrobial therapy of curcumin in biofilms and carious dentine. Lasers Med Sci. v. 29, p. 629-35, 2014.

ARAÚJO N et al. Photodynamic effects of curcumin against cariogenic pathogens. Photomed Laser Surg. v. 30, n. 7, p. 393-9, 2012.

BACELLAR IOL et al. Photodynamic Efficiency: From Molecular Photochemistry to Cell Death. Int. J. Mol. Sci. 2015, 16, 20523-20559; doi:10.3390/ijms160920523

BONSOR SJ et al. Microbiological evaluation of photo-activated disinfection in endodontics (an in vivo study). Br. Dent. J. v. 25, p. 337-41, 2006. [PubMed: 16568063]

BULIT F et al. Antimicrobial activity and cytotoxicity of 3 photosensitizers activated with blue light. J Endod. v. 40, p. 427-31, 2014.

BUNTING JR. A test of the singlet oxygen mechanism of cationic dye photosensitization of mitochondrial damage. Photochem Photobiol.v. 55, p. 81-7, 1992.

CHIAVIELLO A et al. Targets and mechanisms of photodynamic therapy in lung cancer cells: a brief overview, Cancers 3 p. 1014–41, 2011.

CHIGNELL CF et al. Spectral and photochemical properties of curcumin. Photochem Photobiol. v. 59, n. 3, p. 295-302, 1994.

CHRISTODOULIDES N et al. Photodynamic therapy as an adjunct to non-surgical periodontal treatment: a randomized, controlled clinical trial. J Periodontol. v. 79, n. 9, p. 1638-44, 2008.

CRIVELLO JV, BULUT U. Curcumin: a naturally occurring long-wavelength photosensitizer for diaryliodonium salts, J. Polym. Sci. Part A: Polym. Chem. v. 43, n. 21, p. 5217-31, 2005. https://doi.org/10.1002/pola.21017.

DAHL TA et al. Photokilling of bacteria by the natural dye curcumin. Arch Microbiol. v. 151, n. 2, p. 183-5,1989.

dos SANTOS AF et al. Methylene blue photodynamic therapy induces selective and massive cell death in human breast cancer cells. BMC Cancer. v. 17, n.1, 194, 2017. doi:10.1186/s12885-017-3179-7

DOVIGO L et al. Curcumin-mediated photodynamic inactivation of Candida albicans in a murine model of oral candidiasis. Med Mycol. v. 51, n. 3, p. 243-51, 2013.

DOVIGO L.N et al. Investigation of the photodynamic effects of curcumin against Candida albicans. Photochem Photobiol. v. 87, n. 4, p. 895-903, 2011.

DOVIGO, LN et al. Susceptibility of clinical isolates of Candida to photodynamic effects of curcumin. Lasers Surg Med. v. 43, n. 9, p. 927-34, 2011a.

DUVOIX A et al. Chemopreventive and therapeutic effects of curcumin, Cancer Lett. v. 223, n. 2, p. 181-90, 2005. https://doi.org/10.1016/j.canlet.2004.09.041.

ELMORE S. Apoptosis: a review of programmed cell death. Toxicol Pathol. v. 35, n. 4, p, 495-516, 2007.

FOSCHI F et al. Photodynamic inactivation of Enterococcus faecalis in dental root canals invitro. Lasers Surg Med. v. 39, p.782-7, 2007. [PubMed: 18081066]

GAJATE C, GONZALEZ-CAMACHO F, MOLLINEDO F. Lipid raft connection between extrinsic and intrinsic apoptotic pathways, Biochem. Biophys. Res. Commun. v. 380, n. 4, p. 780-84, 2009. https://doi.org/10.1016/j.bbrc.2009.01.147.

GEORGE S, KISHEN A. Advanced noninvasive light-activated disinfection: assessment of cytotoxicity on fibroblasts versus antimicrobial activity against Enterococcus faecalis. J Endod v. 33, p. 599-602, 2007. [PubMed: 17437881]

GHARESI S et al. Effect of photodynamic therapy based on indocyanine green on expression of apoptosis-related genes in human gingival fibroblast cells. Photodiagn. Photodyn. Ther. v. 19, p, 33-6, 2017.

GURSOY H et al. Photodynamic therapy in dentistry: a literature review. Clin. Oral Investig. v. 17, p, 1113–25, 2013. doi: 10.1007/s00784-012-0845-7

KASHEF N, ABADI GRS, DJAVID GE. Photodynamic inactivation of primary human fibroblasts by methylene blue and toluidine blue O. Photodiag Photodyn Ther. v. 9, p. 355-58, 2012. doi: 10.1016/j.pdpdt.2012.05.001.10.1016/j.pdpdt.2012.05.001

KESSEL D, LUO Y. Mitochondrial photodamage and PDTinduced apoptosis. J Photochem Photobiol B: Bio. v. 42, p. 89-95, 1998.

KONOPKA K, GOSLINSKI T. Photodynamic therapy in dentistry. J Dent Res. v. 86, n. 8, p. 694-704, 2007.

KUNWAR A et al. Quantitative cellular uptake, localization and cytotoxicity of curcumin in normal and tumor cells. Biochim Biophys Acta. v.1780, p. 673–79, 2008.

LEE JH et al. Involvement of both mitochondrial- and death receptor-dependent apoptotic pathways regulated by Bcl-2 family in sodium fluoride-induced apoptosis of the human gingival fibroblasts. Toxicology v. 243, p. 340-47, 2008.

LI H et al. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. v. 94, n. 4, p. 491-501, 1998.

MAISCH, T. "Anti-microbial photodynamic therapy: useful in the future?," Lasers Med. Sci. v. 22, n. 2, p. 83-91, 2007.

NASTRI L et al. Effects of toluidineblue-mediated photodynamic therapy on periopathogens andperiodontal biofilm: in vitro evaluation. Int J. Immuno Pathol. Pharmacol. v. 23, n. 4, p. 1125-32, 2010.

NIKAIDO H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. v. 67, n. 4, p. 593–656, 2003.

NOODT BB et al. Apoptosis induction by different pathways with methylene blue derivative and light from mitochondrial sites in V79 cells. Int J Cancer. v.75, p. 941-48, 1998.

OLIVEIRA CS et al. Major determinants of photoinduced cell death: Subcellular localization versus photosensitization efficiency. Free Radic Biol Med. v. 51, n.4, p. 824–33, 2011.

OLIVEIRA FA et al. Low intensity lasers differently induce primary human osteoblast proliferation and differentiation, J. Photochem. Photobiol. B v. 163, p. 14–21, 2016.

PASCHOAL MA et al. Photodynammic potential of curcumin and blue LED agaisnt Streptococcus mutans in a planktonic culture. Photodiag Photodyn Ther. v. 10, n. 3, p. 313-19, 2013.

PILEGGI, G. et al. Blue light-mediated inactivation of Enterococcus faecalis in vitro. Photodiagnosis Photodyn Ther. v. 10, n. 2, p. 134-40, 2013.

QIAO J et al. Photodynamic effects on human periodontal-related cells in vitro, Photodiag. Photodyn. Ther. v. 11, n. 3, p. 290-99, 2014 https://doi.org/10.1016/j.pdpdt.2014.04.001.

RIBEIRO APD et al. Photodynamic therapy associating Photogem® and blue LED on L929 and MDPC-23 cell culture. Cell Biol. Int. v. 34, p. 343-351, 2010.

RIBEIRO APD et al. Phototoxic effect of curcumin on methicillin-resistant Staphylococcus aureus and L929 fibroblasts, Lasers Med. Sci. v. 28, p. 391-98, 2013. doi 10.1007/s10103-012-1064-9.

RIEDL SJ, SALVESEN GS. The apoptosome: signalling platform of cell death. Nat. Rev. Mol. Cell Biol. v. 8, p. 405-13, 2007.

ROSA LP et al. Antimicrobial photodynamic inactivation of Staphylococcus aureus biofilms in bone specimens using methylene blue, toluidine blue ortho and malachite green: an in vitro study. Arch. Oral Biol. v. 60, p. 675–80, 2015.

ROVALDI CR et al. Photoactive porphyrin derivative with broad-spectrum activity against oral pathogens in vitro. Antimicrob. Agents Chemother. v. 44, n. 12, p. 3364-7, 2000.

SHOSHAN-BARMATZ V, DE S, MEIR A. 2017. The Mitochondrial Voltage-Dependent Anion Channel 1, Ca(2+) Transport, Apoptosis, and Their Regulation. Front Oncol v. 7, p. 60, 2017

SOUKOS NS et al. Photodynamic therapy for endodontic disinfection. J. Endod. v.32, p. 979-84, 2006. [PubMed: 16982278]

STEINER-OLIVEIRA CS et al. Randomized in vivo evaluation of photodynamic antimicrobial chemotherapy on deciduous carious dentin. J. Biomed. Optics. v. 20, n. 10, p. 108003, 2015.

TARASZKIEWICZ A et al. Innovative strategies to overcome biofilm resistance. Biomed. Res. Int. v. 2013, ID 150653, 13 pages, 2013.

TARDIVO JP et al. Methylene blue in photodynamic therapy: From basic mechanisms to clinical applications. Photodiagnosis Photodyn Ther. v. 2, p. 175–91, 2005.

THOMAS CD et al. Cellular density, a major factor involved in PDT cytotoxic responses: study on three different lines of human retinoblastoma grafted on nude mice. Photodiag. Photodyn. Ther. v. 12, p. 267-75, 2015.

TUITE EM, KELLY JM. Photochemical interactions of methylene blue and analogues with DNA and other biological substrates. J Photochem Photobiol B. v. 21, p. 103-24, 1993.

VATANSEVER F et al. Antimicrobial strategies centered around reactive oxygen species- bactericidal antibiotics, photodynamic therapy, and beyon. FEMS Microbiol. Rev. v. 37, n. 6, p. 955-89, 2013. doi: 10.1111/1574-6976.12026

WOO J et al. Molecular mechanisms of curcumin-induced cytotoxicity: Induction of apoptosis through generation of reactive oxygen species, down-regulation of Bcl-XL and IAP, the release of cytochrome c and inhibition of Akt. Carcinogenesis v. 24, p. 1199-1208, 2003.