



Evaluation of Indocyanine-Mediated Photodynamic Therapy Cytotoxicity in Human Osteoblast-Like Cells: an In Vitro Study

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Abstract

Introduction: Antimicrobial photodynamic therapy (aPDT) is an adjunctive non-invasive procedure for the management of periodontal tissue infection and deep periodontal pocket decontamination. However, the effects of this procedure on periodontal cells like osteoblasts that play a role in periodontal tissue repair and regeneration is not yet clear.

Aim: This study aimed to investigate aPDT based on indocyanine green (ICG) on MG-63 osteoblast-like cells in vitro.

Materials and methods: MG-63 cells were treated in 9 different groups: 1) Control (untreated cells), 2) ICG alone at a concentration of 1000 µg/mL, 3) ICG alone at a concentration of 2000 µg/mL, 4) diode laser irradiation alone for 30 s, 5) laser irradiation for 30 s combined with ICG with a concentration of 1000 µg/mL, 6) laser irradiation for 30 s combined with ICG at a concentration of 2000 µg/mL, 7) laser irradiation alone for 60 s, 8) laser irradiation for 60 s combined with ICG at a concentration of 1000 µg/mL, and 9) laser irradiation for 60 s combined with ICG at a concentration of 2000 µg/mL. Cell viability was assessed by MTT assay in different groups.

Results: In groups that were treated with 2000 µg/mL of ICG or diode laser irradiation at fluency of 39 J/cm² for 60 s alone or in combination during ICG-aPDT, osteoblast-like cells viability decreased, remarkably.

Conclusions: ICG-mediated aPDT with 1000 µg/mL of ICG combined with diode laser irradiation at fluency of 39 J/cm² for 30 s is safe for MG-63 human osteoblast-like cells; however, higher concentration of ICG or laser irradiation time will increase cells death. There is still a need for more in vivo studies.

Keywords

antimicrobial photodynamic therapy, cell viability, cytotoxicity, diode laser, indocyanine green, MG-63 cells, osteoblast cells

INTRODUCTION

Periodontal disease is known as a common oral inflammatory pathological condition in response to a chronic infection that engages the soft tissue and hard tissue that support the teeth and its prognosis depends on factors such as bacterial biofilm, patient's response to inflammation and some other risk factors.^[1] The complex anatomy of the multi-rooted tooth especially in the furcation area is difficult to access for removing microbial plaque and tartar deposits by conventional scaling and root planning without periodontal surgeries.^[2] Periodontal surgical treatments result in significant long-term improvement, but they are aggressive procedures and cause some morbidity, including post-treatment pain and edema.^[2] Another procedure such as the antimicrobial photodynamic therapy (aPDT) is mentioned as an alternative method for eliminating microorganisms in deep pockets that are created following advanced periodontal diseases.^[3]

aPDT is a non-invasive technique that produces reactive oxygen radicals to damage living agents such as bacteria or microbes. A light-activated molecule or photosensitizer (PS) has a major role during this process. After being exposed to light, it changes to an excited state and causes the release of reactive oxygen species when it decays. An adequate combination of light and PS as well as PS properties such as high absorption coefficient, long triplet state of appropriate energy and high photostability take part in aPDT results.^[3,4]

Low power lasers have biological effects including accelerating wound healing, collagen synthesis and neovascularization in periodontal tissue. Diode laser is known to enhance the periodontal ligament fibroblasts survival and has been successfully used for the treatment of periodontal diseases; it also has been used as a light source during aPDT.^[5-8]

Indocyanine green (ICG) is a water-soluble dye with a strong absorption band around 800 nm near the infrared region that has been approved by the United States Food and Drug Administration (US FDA) for some medical diagnostic procedures.^[9] Despite very low toxic effects, it could be used as a PS during aPDT for producing reactive oxygen species as near infrared light penetrates deep into tissue without causing significant heating.^[9-11]

There are still many questions about aPDT parameters including type of PS, PS concentration, light dose, time and energy and its effect on periodontal cells survival, especially the osteoblast cells that play a role in bone regenerative therapies for replacing damaged and lost bone tissue with functional bone, so understanding the effect of ICG-mediated aPDT on osteoblast proliferation or differentiation would be beneficial.^[8,12-14]

AIM

The present study aimed at investigating the effect of aPDT with ICG and diode laser with a wavelength of 808 nm on

MG-63 osteoblast-like cells viability and to find optimal ICG concentration and laser beam irradiation time in vitro.

MATERIALS AND METHODS

Cell culture

MG-63 human osteoblast-like cells (code NCBI: C555) were obtained from the National Cell Bank of Iran, Pasteur Institute (Iran, Tehran). Cells were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (Gibco, Grand Island, USA) without phenol red supplemented by 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic solution (Sigma, St. Louis, MO, USA) in the standard atmosphere (5% CO₂, 95% humidity, 37°C). Culture media was changed every 3 days. Trypsinization was done with 0.25% trypsin-EDTA solution (Sigma, St. Louis, MO, USA) after cells reached confluence. Cell densities were 5×10³ cells per well in 24-well microplates (Greiner Bio-One, Germany). After seeding, cells were maintained for 24 hours in the incubator for adhesion before any treatment.^[15]

ICG solution preparation

ICG powder (Green +I, Novateb Pars, Iran) was dissolved in 0.5 mL sterile PBS and to obtain a fresh stock solution (2000 µg/mL). It was kept in the dark and was diluted to the desired concentrations (1000 µg/mL) just before performing the experiments.^[16]

Light source

An 808-nm diode laser (DX82, Konftec, Taiwan) with an output of 250 mW in continuous mode was used in this experiment. Laser power was measured and checked with a power meter (Coherent, USA) before and after each treatment during the study, instantly. The laser probe was moved 1 mm above the microplate and light was distributed homogeneously on four wells of a 24-well plate at a time. Power density throughout the target area measured using a power meter (Coherent, USA). The tests wells were separated from each other by wells that were filled by methylene blue. Black paper sheet was placed under the plates to reduce laser beam scattering. The plates were protected from room light during all procedures.

Photodynamic therapy of cells

The culture medium was aspirated and cells were washed with buffered PBS (Ph=7.4) twice, then 100 µL ICG solution with desired concentration was added to microplate and cells were incubated for 5 min in incubator (37°C, 95% humidity and 5% CO₂) in dark conditions before laser application. At the end of incubation period, the cells in the presence of ICG solution in microplates were immediately

exposed to the diode laser at 250 mW for 30 s (19.5 J/cm²) or 60 s (39 J/cm²) at room temperature (25±2°C).

Experimental groups

Following nine groups were tested:

1. Control (no exposure to either ICG or irradiation)
2. ICG alone at a concentration of 1000 µg/mL
3. ICG alone at a concentration of 2000 µg/mL
4. Laser irradiation for 30 s without ICG
5. Laser irradiation for 30 s with ICG at a concentration of 1000 µg/mL
6. Laser irradiation for 30 s with ICG at a concentration of 2000 µg/mL
7. Laser irradiation for 60 s without ICG
8. Laser irradiation for 60 s with ICG at a concentration of 1000 µg/mL
9. Laser irradiation for 60 s with ICG at a concentration of 2000 µg/mL

These energy doses of irradiation were chosen based on our previous studies and literature.^[16-18]

MTT test

MTT assay (Sigma, St. Louis, MO, USA) was employed to calculate cells viability rate. MTT assay is a colorimetric test that is based on the ability of mitochondrial dehydrogenase enzyme from vital cells to metabolize yellow MTT solution to violet formazan crystals. 10 gL of MTT solution (5 mg/ml) was added to each well containing 100 gL medium. After 4 hours of incubation (37°C, 95% humidity and 5% CO₂) in darkness, the formazan crystals were dissolved with 150 gL of DMSO, and the absorbance was measured at 570 nm with microplate reader (iMark, Bio-Rad Lab., USA).^[19] The MTT assay for all groups was performed at day 7 for all groups.

Statistical analysis

All of the experiments were carried out in triplicate. The results are represented as mean values ± standard deviations (means ± SD). Statistical analysis was done using the Statistical Package for the Social Sciences (SPSS) v. 22.0 (SPSS Inc., Chicago, IL). Data were found to be normally distributed based on the Shapiro-Wilk test. To determine the significant differences between the groups and possible interaction between ICG and laser irradiation two-way analysis of variance (two way ANOVA) followed by the Tukey test was performed. Differences were considered to be significant at $p < 0.001$, $p < 0.05$, respectively.

RESULTS

The ICG toxicity alone without laser irradiation or dark toxicity was assessed with a concentration of 1000 µg/mL and 2000 µg/mL. Higher ICG concentration caused more cell death, significantly ($p = 0.004$).

Laser irradiation with 808 nm wavelength, 250 mW power was evaluated at 30 s and 60 s. Higher laser irradiation time caused more cell death, significantly ($p = 0.008$).

For light-combined experiments, cells were incubated for 5 min with different ICG concentrations (0, 1000, 2000 µg/mL) and then irradiated with laser light (**Fig. 1**). According to the MTT results, cell viability was significantly decreased following laser irradiation for 60 s. It appears that both increasing laser irradiation time and ICG concentration significantly decreased cell viability; however, these two parameters did not have interaction or synergic effect on each other base on two way ANOVA analysis ($p = 0.116$).

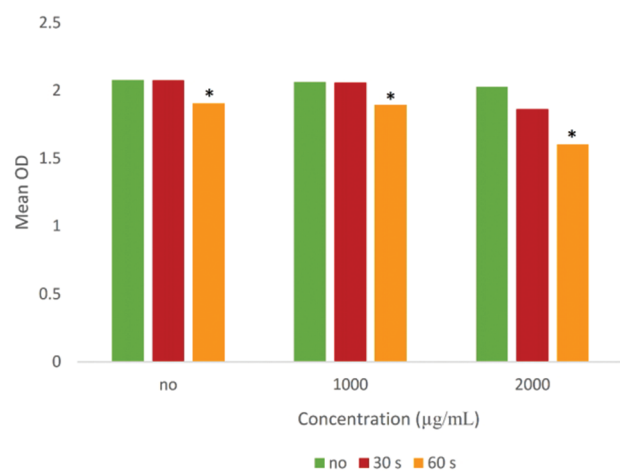


Figure 1. MG-63 cells viability in different groups. * $p < 0.005$, significantly different from control group.

Based on Tukey analysis, the highest cytotoxicity was seen in the group with 2000 µg/mL concentration of ICG combined with 60 s laser irradiation time.

DISCUSSION

aPDT has been developed as a promising, non-invasive treatment method in treating some skin-related cancers, age-related macular degeneration and infectious diseases.^[19,20] aPDT also is mentioned as an adjunct for periodontal therapy as it kills periodontal pathogens or might decrease their virulence, so help clinicians to improve treatment outcomes.^[21] Gharesi et al.^[22] investigated the effect of aPDT based on ICG with an 808-nm diode laser on apoptosis-related genes expression in human gingival fibroblasts (HGF) as they play a role in the periodontal tissues repair and regeneration. Expression of BAX and BCL-2 genes that are involved in cell apoptosis were assessed and compared by real-time quantitative reverse transcription PCR in 4 different groups of monolayers of HGF cells including ICG alone (1000 µg/mL), 60 s laser irradiation alone, combined ICG, and laser irradiation and control. They concluded that ICG-aPDT with a 1000 µg/mL con-

centration of ICG and 60 s exposure time for diode laser would induce HGF apoptosis as it caused the significant expression of BAX; however, treatment with ICG or laser irradiation alone did not change BCL-2 and BAX genes expression significantly and appeared to be safe for HGF cells. In our study, ICG with a 1000 µg/mL concentration and laser irradiation for 30 s alone both were safe for MG-63 cells, too; however, increasing ICG concentration to 2000 µg/mL or laser irradiation time for 60 s alone or combination of them during aPDT caused cytotoxicity.

Mokhtari et al.^[23] studied the expression of basic fibroblast growth factor (bFGF) gene after aPDT in human gingival fibroblasts by real-time quantitative reverse transcription PCR (qRT-PCR). The cells were treated in 4 different groups: 1) ICG alone, 2) diode laser irradiation alone, 3) combined ICG and laser irradiation (aPDT), and 4) control. They concluded that ICG-PTD treatment at a concentration of 1000-2000 µg/mL ICG combined with laser irradiation at fluency of 31.25 J/cm² for 60 s and laser irradiation for 60 s alone, both increase the expression of bFGF gene which can reflect gingival fibroblast higher metabolism that play a role in the periodontal tissue regeneration; however, gene expression following ICG treatment with different concentration of 500 to 2000 µg/mL was not changed, remarkably. In our study, laser radiation with the same duration (60 s) but different intensity (39 J/cm²) alone or combined with ICG caused more cell death, which may be due to higher laser energy dose or different cells type. Also in our study, ICG with 2000 µg/mL concentration caused more cell death that may be a result of different cells type in our study.

Pourhajibagher et al.^[24] conducted a study to evaluate the effect of aPDT based on ICG combined with curcumin on human gingival fibroblast cell viability by neutral red assay. Cells were incubated in different experimental groups with a concentration of ICG ranged from 500 to 2000 µg/mL and curcumin amount ranged from 3 to 20 mM and then exposed to diode laser in three different modes (30 s, 60 s and 2×30 s irradiation at an interval of 1 min) and one group exposed to LED light for 5 min. The cells were incubated with chlorhexidine (CHX) - a gold standard antimicrobial agent for periodontal disease - assumed as a control group. Despite CHX not caused a remarkable change in the cell viability; but a combination of ICG and curcumin reduced HGF cells viability at concentrations below 1000 µg/mL and 10 mM, respectively. Cells viability was lower in the groups that were exposed to a diode laser for 2×30 s in comparison to other groups. They suggest low doses of ICG and curcumin combination with the lowest exposure time of light source as an effective way for aPDT. In our study, higher laser exposure time decreased cell viability, too. However, 1000 µg/mL of ICG was not toxic for cells that may be due to the difference in the cells type being studied.

There is still a need for an in vivo study to evaluate aPDT's applicability in clinical periodontics and to find the ideal ICG concentration and laser dose.

CONCLUSIONS

aPDT with ICG concentration of 1000 µg/mL and an 808-nm diode laser for 30 seconds was safe for MG-63 osteoblast-like cells. However, higher ICG concentrations like 2000 µg/mL or 808-nm diode laser radiation for 60 s would increase cell death. As a result, aPDT is suggested based on ICG at a concentration of 1000 µg/mL with 808 nm low power laser radiation for 30 s.

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Оценка цитотоксичности индоцианин-опосредованной фотодинамической терапии в остеобластоподобных клетках человека: исследование *in vitro*

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Резюме

Введение: Антимикробная фотодинамическая терапия (аФДТ) является дополнительной неинвазивной процедурой лечения инфекции тканей пародонта и обеззараживания глубоких пародонтальных карманов. Однако влияние этой процедуры на клетки пародонта, такие как остеобласты, которые играют роль в восстановлении и регенерации тканей пародонта, ещё не выяснено.

Цель: Это исследование было направлено на изучение аФДТ на основе индоцианина зелёного (ICG) на остеобластоподобных клетках MG-63 *in vitro*.

Материалы и методы: Клетки MG-63 обрабатывали в 9 различных группах: 1) контроль (необработанные клетки), 2) только ICG в концентрации 1000 µg/mL, 3) только ICG в концентрации 2000 µg/mL, 4) только диодное лазерное облучение в течение 30 сек., 5) лазерное облучение в течение 30 сек. в сочетании с ICG в концентрации 1000 µg/mL, 6) лазерное облучение в течение 30 сек. в сочетании с ICG в концентрации 2000 µg/mL, 7) только лазерное облучение в течение 60 сек., 8) лазерное облучение в течение 60 сек. в сочетании с ICG в концентрации 1000 µg/mL и 9) лазерное облучение в течение 60 сек. в сочетании с ICG в концентрации 2000 µg/mL. Жизнеспособность клеток оценивали с помощью МТТ-анализа в разных группах.

Результаты: В группах, получавших 2000 µg/mL ICG или облучение диодным лазером с плотностью потока 39 J/cm² в течение 60 сек. отдельно или в комбинации во время ICG-aPDT, жизнеспособность остеобластоподобных клеток значительно снизилась.

Заключение: ICG-опосредованная аФДТ с 1000 µg/mL ICG в сочетании с облучением диодным лазером с плотностью потока 39 J/cm² в течение 30 сек. безопасна для остеобластоподобных клеток MG-63 человека; однако более высокая концентрация ICG или время лазерного облучения увеличивают гибель клеток. По-прежнему существует потребность в дополнительных исследованиях *in vivo*.

Ключевые слова

антимикробная фотодинамическая терапия, жизнеспособность клеток, цитотоксичность, диодный лазер, индоцианин зелёный, клетки MG-63, клетки остеобласты