



Effect of Antimicrobial Photodynamic Therapy Using Indocyanine Green Doped with Chitosan Nanoparticles on Biofilm Formation-Related Gene Expression of *Aggregatibacter actinomycetemcomitans*

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ABSTRACT

Objectives: Eradication of *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), as an opportunistic periodontopathogen, and inhibition of its virulence factor expression require a new adjunctive therapeutic method. In this study, we assessed the expression level of *rcpA* gene, as a virulence factor associated with *A. actinomycetemcomitans* biofilm formation, following treatment by antimicrobial photodynamic therapy (aPDT) using indocyanine green (ICG) doped with chitosan nanoparticles (CS-NPs@ICG).

Materials and Methods: CS-NPs@ICG was synthesized and examined using scanning electron microscopy (SEM). *A. actinomycetemcomitans* ATCC 33384 strain was treated with CS-NPs@ICG, as a photosensitizer, which was excited with a diode laser at the wavelength of 810 nm with the energy density of 31.2 J/cm². Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to determine the changes in *rcpA* gene expression level.

Results: Synthesized CS-NPs@ICG was confirmed via SEM. The results revealed that CS-NPs@ICG-mediated aPDT could significantly decrease *rcpA* gene expression to 13.2-fold (P<0.05). There was a remarkable difference between aPDT using CS-NPs@ICG and ICG (P<0.05). The diode laser, ICG, and CS-NPs@ICG were unable to significantly downregulate *rcpA* gene expression (P>0.05).

Conclusion: aPDT with CS-NPs@ICG leads to a decrease of the virulence factor of *A. actinomycetemcomitans* and can be used as an adjunct to routine treatments for successful periodontal therapy in vivo.

Keywords: *Actinobacillus actinomycetemcomitans*; Gene Expression; Biofilm; Indocyanine Green

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INTRODUCTION

Periodontal infections have polymicrobial etiology where multiple microorganism species are present in a periodontal lesion [1]. Numerous studies have considered *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) as an important etiological microorganism involved in periodontitis [1-4]. This microorganism has several virulence factors including fimbriae, which are one of the important factors in the initial adhesion of *A. actinomycetemcomitans*. Rough-colony protein A (RcpA), which is found tandemly located downstream of the fimbriae, appears to play a critical role in the colonization of *A. actinomycetemcomitans* and in the formation of biofilms [3].

Traditional methods for periodontal therapy, including scaling and root planing or surgical procedures, as well as antibiotic therapy as an adjunct to the standard periodontal treatment regime, are unable to completely eliminate microorganisms [4]. Recently, antimicrobial photodynamic therapy (aPDT) has been considered as a non-invasive therapeutic approach, which allows for selective targeting of periodontal pathogens [5]. During aPDT, a photosensitizing agent, called a photosensitizer, is used with visible light at an appropriate wavelength. The photosensitizer is excited through exposure to the light and can release reactive oxygen species (ROS) in the presence of oxygen. ROS, such as superoxide, hydroxyl radicals, and hydrogen peroxide (H_2O_2), is harmful to cell membrane integrity and can cause biological death [6]. Although indocyanine green (ICG) is a photosensitizer with high in-vitro efficacy against oral microorganisms [7], it is unstable in aqueous solutions; this problem has been overcome by doping with nano-sized carriers [8]. Based on previous studies, chitosan nanoparticle (CS-NP), as a cationic aminopolysaccharide and water-soluble polymer, has ideal properties for delivery of photosensitizers during aPDT [9]. To the best of our knowledge, there are no data on the effects of aPDT with ICG doped with CS-NPs (CS-NPs@ICG). Herein, we hypothesized that CS-NPs@ICG, as a photosensitizer, may have a significant effect

on *rspA* genes expression of *A. actinomycetemcomitans* during aPDT.

MATERIALS AND METHODS

Study design:

To evaluate the effect of CS-NPs@ICG-mediated aPDT on gene expression of *A. actinomycetemcomitans*, the samples were allocated to the following groups:

A: ICG group

B: CS-NPs@ICG group

C: Diode laser group

D: ICG + Diode laser group

E: CS-NPs@ICG + Diode laser group

F: Control group

Preparation of CS-NPs@ICG:

According to the study by Govindarajan et al [10], CS-NPs were synthesized. In short, 50 mg of CS powder (Acros Organics; Fisher Scientific UK Ltd., Fair Lawn, UK) was dissolved in 50 ml of deionized distilled water. 1% acetic acid solution (Merck KGaA, Darmstadt, Germany) was then added dropwise under magnetic stirring process to create a homogeneous CS solution. Then, 2.0 mg/ml of ICG (Santa Cruz Biotechnology Co., Ltd; Shanghai, China) was added and mixed at 1000 rpm (revolutions per minute) for 10 minutes using a magnetic mixer. This mixture was centrifuged at 10,000 rpm (Eppendorf, Germany) for 30 minutes. The collected sediment CS-NPs@ICG was lyophilized and used for further experiments. The morphological analysis of CS-NPs@ICG was performed using scanning electron microscopy (SEM).

Bacterial strain and growth conditions:

A. actinomycetemcomitans ATCC 33384 strain (ETH Zurich, Switzerland) was cultured in brain heart infusion (BHI) broth (Merck KGaA, Darmstadt, Germany) containing 5 g/l of yeast extract (Merck KGaA, Darmstadt, Germany), 5 mg/l of hemin (Sigma-Aldrich Co., Ltd., Dorset, UK), and 1 mg/l of menadione (Sigma-Aldrich Co., Ltd., Dorset, UK) at 37°C in a microaerophilic atmosphere to adjust the cell density to 1.5×10^8 cells/ml.

Effect of aPDT using CS-NPs@ICG on biofilm formation-related gene expression of *A. actinomycetemcomitans*:

The wells of a 96-well round bottomed sterile

polystyrene microplate (TPP; Trasadingen, Switzerland) were filled with 100 μ l of *A. actinomycetemcomitans* suspension at the concentration of 1.5×10^8 colony-forming units (CFU)/ml. In groups A and B, 100 μ l of ICG and 100 μ l of CS-NPs@ICG were added to the wells, respectively. The microplate was incubated in the dark at room temperature for 5 minutes in a microaerophilic atmosphere. In group C, *A. actinomycetemcomitans* suspensions were exposed to DenLase diode laser therapy system (China Daheng Group, Inc., Beijing, China) irradiation at the wavelength of 810 nm with an output power of 250 mW and the energy density of 31.2 J/cm². The output power of the diode laser was measured by a power meter (Laser Point s.r.l., Milano, Italy). The probe of the laser was fixed at 1 mm and 3 mm above the top surfaces of the microplate and the bacterial suspension, respectively, by a microphone stand (Fig. 1).



Fig. 1. Laser irradiation of the surface of microplate wells during the treatment of bacteria with antimicrobial photodynamic therapy (aPDT).

The diameter of the irradiated area was the same as the diameter of the laser probe (6.39 mm). Groups D and E were treated with ICG and CS-NPs@ICG, respectively, similar to groups A and B. Then, the wells were exposed to the diode laser, similar to group C. *A. actinomycetemcomitans* suspension without any treatment (photosensitizers and light)

served as a control group.

Preparation of total RNA and complementary DNA (cDNA) synthesis of treated *A. actinomycetemcomitans*:

The contents of each group in microplate wells were collected separately in sterile tubes. Total RNAs were extracted from all treated and untreated (control) *A. actinomycetemcomitans* samples using Geneall Hybrid-R™ RNA purification kit (Geneall Biotechnology Co., Ltd., Seoul, South Korea), according to the manufacturer's recommendations. The total RNA samples were treated with DNase I (Thermo Fisher Scientific, Darmstadt, Germany) after assessment with NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Before reverse transcription, RNA samples were treated with DNase I to digest genomic DNAs. The RNA was used as a template to synthesize the primary cDNA chain by reverse transcription with a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR) assay:

The resulting cDNAs were used for the quantification of virulence gene mRNA levels by qRT-PCR with LightCycler® 96 Real-Time PCR System (Roche Applied Science, Mannheim, Germany). All primers utilized in this study were designed using the Primer 3 Plus web tool (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>). The PCR primer sequences were *rcpA*-F, 5'-TGGGCATTA ACTGGAGCCAC-3'; *rcpA*-R, 5'-ATCCACCTCCGAAACCGAAG-3'; *16S rRNA*-F, 5'-AAGCACCGGCTAACTCCGT-3'; *16S rRNA*-R, 5'-TTCCGATTAACGCTCGCAC-3'. The 20- μ l reaction volume contained 10 μ l of SYBR Premix Ex Taq II (Takara Bio Inc., Kyoto, Japan) under the following conditions: 95°C for 5 minutes, amplification for 35 cycles with denaturation at 95°C for 15 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 30 seconds. The specificity of the primers was evaluated using melt curves.

Statistical analysis:

Statistical analysis was performed using one-way repeated-measures analysis of variance (ANOVA) and Bonferroni post-hoc test. The mRNA expression levels were shown as n-fold differences relative to the calibrator. Changes in expression levels of the target gene were interpreted as significant if the variation was >2-fold.

RESULTS**SEM experiment:**

As shown in Figure 2, the CS-NPs@ICGs are nano-sized particles, approximately 60-100 nm in diameter, with uniform shapes.

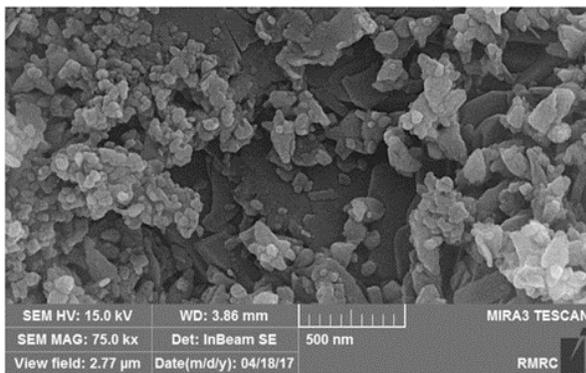


Fig. 2. Scanning electron microscopic image of synthesized indocyanine green doped with chitosan nanoparticle (CS-NPs@ICG)

Effects of aPDT using CS-NPs@ICG on *rcpA* gene expression:

The melting curve profiles generated by real-time amplification confirmed the non-specific PCR product. According to Figure 3, the transcription of *rcpA* gene decreased by 6.4- and 13.2-fold with aPDT using ICG and CS-NP@ICG, respectively ($P < 0.05$). In contrast, there was no significant change in the expression of data obtained from the results of the analysis of *rcpA* gene in the diode laser, ICG, and CS-NPs@ICG groups alone (0.7-, 1.0-, and 1.7-fold, respectively, $P > 0.05$; Fig. 3). On the other hand, there was a significant difference between aPDT groups; CS-NPs@ICG-aPDT could downregulate the expression of *rcpA* gene by 6.8-fold compared to ICG-aPDT ($P < 0.05$).

DISCUSSION

Recently, aPDT has attracted a lot of attention

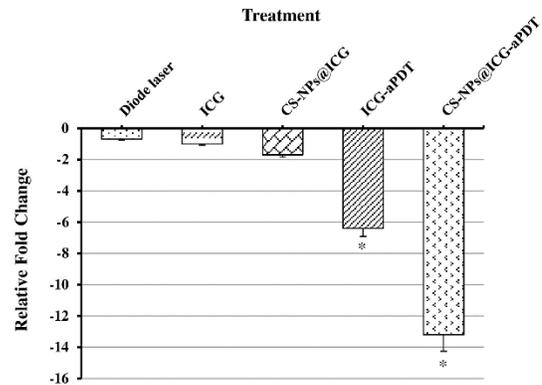


Fig. 3. Effect of antimicrobial photodynamic therapy (aPDT) on gene expression of *Aggregatibacter actinomycetemcomitans*; *Significant variation >2-fold.

among clinicians for treatment of microbial pathogenic biofilms [11,12]. *A. actinomycetemcomitans* is considered as the main agent in periodontitis and periimplantitis [1]. *A. actinomycetemcomitans* has numerous virulence factors that are associated with colonization, biofilm formation, and invasion of host tissue, such as fimbriae, adherence proteins, biofilm polysaccharides, leukotoxin, chemotactic inhibitors, cytolethal distending toxin, and bacteriocins [13]. Fimbria, as the virulence factor that attaches to the host cells via channels formed by RcpA, is involved in biofilm formation [14]. Therefore, prevention of *A. actinomycetemcomitans* dispersion from the oral cavity and initiation of systemic infections has led to a new therapeutic approach.

Evidence from several systematic reviews and clinical trials suggests that aPDT is a useful adjunct to mechanical debridement for nonsurgical management of chronic and aggressive periodontitis and periimplantitis [15-18]. In order for aPDT to be efficient, the use of an appropriate photosensitizer is an essential factor for damages experienced by the cytoplasmic membrane and DNA. Therefore, an ideal photosensitizer must have the following properties: availability in pure form, high singlet oxygen quantum yield, strong absorption in the visible spectrum, stability and solubility in aqueous media, easy

delivery to the target site, and non-toxicity [19]. In this regard, ICG would be an acceptable candidate as a photosensitizer for aPDT. ICG has a very interesting photochemical property with strong absorption at 700-800 nm and optimal peak absorption at 800-810 nm. Nagahara et al [20] stated that ICG is a potential photosensitizer for aPDT to achieve clearance of periodontal pathogens. Despite the potential effects of ICG in inhibition of proliferation and formation of microbial biofilm, the disadvantages of ICG can reduce its efficiency. The major disadvantage of using ICG in PDT is the aqueous instability, photodegradation, and thermal degradation in vitro and in vivo [21]. ICG undergoes physicochemical variations such as aggregation and irreversible degradation in aqueous solutions, which can lead to discoloration, decreased light absorption, decreased fluorescence, and a shift in the wavelength of absorption [22,23].

In recent years, polymeric nanoparticles, such as CS-NPs, have provided efficient aqueous, photo, and thermal stability for ICG [24]. CS is both biodegradable and biocompatible and widely used in various pharmaceutical nano-formulations [25]. Therefore, in this study, we made a nano-carrier by doping ICG to a natural polymer (low molecular-weight CS). These facts encouraged us to evaluate the efficacy of aPDT using CS-NPs@ICG against biofilm formation-related gene expression of *A. actinomycetemcomitans*.

As previously mentioned, quantitative data showed that *rcpA* gene in *A. actinomycetemcomitans* was significantly decreased following aPDT using CS-NPs@ICG. In contrast, diode laser alone without previous incubation with CS-NPs@ICG could not lead to a significant reduction of *A. actinomycetemcomitans* gene expression.

The results of this study are consistent with the study conducted by Pourhajibagher et al [26]. They investigated the potential of aPDT using curcumin (CUR) against *A. actinomycetemcomitans*. The results of their study showed that the respective dye used in combination with a light source resulted in a substantial reduction of the number of

surviving *A. actinomycetemcomitans* or of the growing *A. actinomycetemcomitans* compared to the control groups [26]. Several studies have been performed by Pourhajibagher et al [26-28] on gene expression of *A. actinomycetemcomitans rcpA* based on aPDT with different photosensitizers. The results of their investigations showed that *rcpA* gene expression was downregulated by 4-, 6-, and 8.5-fold following treatment by methylene blue (MB), ICG, and CUR, respectively [26-28]. The findings of these studies are consistent with our results.

It is noteworthy that the main finding of this study, compared to other studies, is that aPDT using CS-NPs/ICG could reduce the expression of biofilm formation-related gene far more than ICG. Despite all the valuable findings of this study, additional in situ and in vivo studies are needed for definitive establishment of an effective protocol for periodontitis and periimplantitis treatment.

CONCLUSION

As revealed by the results of this study, aPDT treatment using CS-NPs@ICG significantly downregulated the expression of *rcpA* gene which is involved in biofilm formation of *A. actinomycetemcomitans*. Thus, aPDT using CS-NPs@ICG, described herein, may demonstrate a versatile method to control periopathogens.

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CONFLICT OF INTEREST STATEMENT

None declared.

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