Journal of Lasers in Medical Sciences

J Lasers Med Sci 2020 Spring;11(2):187-192

Original Article

http://journals.sbmu.ac.ir/jlms

doi 10.34172/jlms.2020.31

Photoelimination Potential of Chitosan Nanoparticles-Indocyanine Green Complex Against the Biological Activities of *Acinetobacter baumannii* Strains: A Preliminary *In Vitro* Study in Burn Wound Infections

Maryam Pourhajibagher[1], Nava Hosseini[2], Ebrahim Boluki[3], Nasim Chiniforush[4], Abbas Bahador[5*]

[1]Dental Research Center, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Microbiology, Faculty of Biology, College of Science, University of Tehran, Tehran, Iran
[3]Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[4]Laser Research Center of Dentistry, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran
[5]Oral Microbiology Laboratory, Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

***Correspondence to**
Abbas Bahador, Ph.D., Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Keshavarz Blvd, 100 Poursina Ave., Tehran, Iran. 14167-53955
Tel/Fax: +98-21- 889-55810;
Email: abahador@sina.tums.ac.ir, ab.bahador@gmail.com

Published online March 15, 2020

Abstract

Introduction: *Acinetobacter baumannii* strains are important agents causing serious nosocomial infections including soft-tissue and skin infections in patients with burn wounds which have become resistant to several classes of antibiotics. Antimicrobial photodynamic therapy (aPDT) as an alternative antimicrobial procedure is suggested for the treatment of these kinds of infections. The aim of the current study is to evaluate the antibacterial and anti-biofilm efficiency of aPDT by the utilization of an improved form of indocyanine green (ICG) which is encapsulated in chitosan nanoparticles (NCs@ICG).

Methods: NCs@ICG were synthesized and confirmed by the scanning electron microscope (SEM). aPDT was performed using NCs@ICG with an 810 nm wavelength of the diode laser at the fluency of 31.2 J/cm^2 on 50 *A. baumannii* strains isolated from burn wounds. The antibacterial and anti-biofilm potential of NCs@ICG-aPDT was determined via the colony forming unit (CFU)/mL and crystal violet assays, respectively. In addition, microbial biofilm degradation was evaluated by the SEM.

Results: According to the results, NCs@ICG-aPDT showed a significant reduction of 93.2% on the CFU/mL of planktonic *A. baumannii* strains compared to the control group (untreated group; $P < 0.05$). In addition, the biofilm formation of *A. baumannii* strains was significantly reduced by 55.3% when the bacteria were exposed to NCs@ICG-aPDT ($P < 0.05$). In contrast, NCs@ICG, ICG, and the diode laser alone were not able to inhibit the CFU/mL and biofilm of *A. baumannii* strains ($P > 0.05$). Based on the results of SEM images, NCs@ICG-aPDT disrupted the biofilm structure of *A. baumannii* strains more than other groups.

Conclusion: NCs@ICG-aPDT demonstrates a promising treatment candidate for exploitation in wound infections against both planktonic and biofilm forms of *A. baumannii* strains.

Keywords: Antimicrobial photodynamic therapy; Biofilms; Chitosan nanoparticles; Indocyanine green; *Acinetobacter baumannii*.

Introduction

Acinetobacter baumannii is a gram-negative, opportunistic pathogen and the emergence of extensively drug-resistant (XDR) and multidrug-resistant (MDR) *A. baumannii* strains which take part in a variety of infections has caused serious concerns.[1] Also, *A. baumannii* is able to form a biofilm which is one of the important virulence factors found in pathogenic bacteria, because biofilms are resistant to antibiotics.[2-5]

The outbreak of resistant forms of *A. baumannii* has led researchers to look for alternative ways to solve the problem, one of which is antimicrobial photodynamic therapy (aPDT).[6] During this technique, after the activation of a photosensitizer (PS) by irradiation of light with a specific wavelength, according to the type of photosensitizer, reactive oxygen species (ROS) are generated. ROS are highly reactive and can interact with important cellular molecules and consequently kill the bacteria or alter the antimicrobial susceptibility of bacterial cells.[7,8] According to power and the better penetration of an 810 nm diode laser in deep tissues and the consequence of better repair of damaged host cells, indocyanine green (ICG) as a PS is an appropriate choice for aPDT.[9] ICG is a non-toxic anionic cyanine type of photosensitizer with the disadvantage of instability, which is highly soluble in water with a high absorption of 810 nm. According to

Please cite this article as follows: Pourhajibagher M, Hosseini N, Boluki E, Chiniforush N, Bahador A. Photoelimination potential of chitosan nanoparticles-indocyanine green complex against the biological activities of *Acinetobacter baumannii* strains: a preliminary *in vitro* study in burn wound infections. *J Lasers Med Sci.* 2020;11(2):187-192. doi:10.34172/jlms.2020.31.

the negative charge of the bacterial outer membrane, ICG can contract slightly and this slight contraction leads to the least attachment of ICG to microorganisms which affect the proficiency of ICG as a photosensitizer during aPDT.[10] Nowadays by conjugation of photosensitizers with a variety of nanoparticles, the efficiency of photosensitizers for the aim of microbial reduction or elimination during aPDT has increased.[11-13] Chitosan nanoparticles (NCs) have some advantages like being non-toxic, low immunogenic and biodegradable. The positive charge of NCs makes this opportunity to reduce the negative charge of ICG, which leads to the better contraction of the photosensitizer with microorganisms.[12] This phenomenon enhances the antimicrobial effect of ICG during the aPDT process. Therefore, the aim of this preliminary in-vitro study is to measure the antibacterial efficiency of NCs@ICG as a photosensitizer, instead of ICG photosensitizer alone, during aPDT (NCs@ICG-aPDT) of planktonic and biofilm forms of *A. baumannii* strains isolated from burn wounds.

Materials and Methods

To evaluate the effect of aPDT based on NCs@ICG versus planktonic and biofilm forms of *A. baumannii* strains, the test groups were subjected to:

A: ICG group
B: NCs@ICG group
C: Diode laser group
D: ICG + Diode laser group
E: NCs@ICG + Diode laser group
F: Control group

Specimen Collection From Burn Patients and *Acinetobacter baumannii* Isolation

A total of 85 microbiological wound swabs from the patients with burn wounds were collected from Motahari hospital in Tehran, Iran, from Oct 2017 to Jun 2018. Based on the previous study, the identification of *A. baumannii* strains was performed by standard microbiological methods and was confirmed by polymerase chain reaction.[14]

Bacterial Strain and Culture Condition

Acinetobacter baumannii strains were incubated in fresh brain heart infusion (BHI) broth (Merk, Darmstadt, Germany) in an aerobic atmosphere at 37°C in the logarithmic growth phase to provide a concentration of 1.0×10^6 colony forming units (CFU)/mL, as verified by both spectrophotometry (optical density [OD] 600 nm: 0.01-0.02) and colony counting.

Preparation of NCs@ICG

According to the study by Govindarajan et al., 15 NCs were synthesized. In short, 50 mg of Cs powder (ACROS Organic, UK) was dissolved in 50 mL deionized distilled water. 1% acetic acid solution (Merck KGaA, Darmstadt, Germany) was then added dropwise under the magnetic stirring process to create the homogeneous NCs solution. Then, 2.0 mg/mL of ICG (Santa Cruz Biotechnology Co., Ltd; Shanghai, China) was added and this mixture was centrifuged at 10 000 rpm (Eppendorf, Germany) for 30 minutes. The collected sediment NCs@ICG was lyophilized and used for further experiments. The morphological analysis of NCs@ICG was performed using the scanning electron microscope (SEM).

Photosensitizers and Light Source

The stock solution of NCs@ICG as a photosensitizer was dissolved in fresh water and kept in a dark condition in 4°C before use. Also, ICG at a final concentration of 1 mg/mL was dissolved in distilled water. For aPDT with ICG and NCs@ICG, a diode laser (DenLase, diode laser therapy system (Daheng Group Inc., China) was used as a light source in a wavelength of 810 nm in the output of 250 mW with the energy density of 31.2 J/cm^2.

aPDT Effect Against *Acinetobacter baumannii* Strains in Planktonic Form

In groups A and B, 150 µL of 1.5×106 CFU/mL bacterial suspension was added to each well of the 96–well flat-bottomed microtiter plate (TPP, Trasadingen, Switzerland). Next, 150 µL of the NCs@ICG and 150 µL of the ICG were added separately to bacterial suspension for groups A and B respectively. In order to the absorbance of photosensitizers by bacterial cells, the mixture was incubated for 5 minutes in a dark condition at room temperature (25°C ± 2).

In group C, the bacterial suspension was exposed to the light source in a wavelength of 810 nm at the fluency of 31.2 J/cm^2 alone. The bacterial suspension exposed to photosensitizers was prepared for groups D and E similar to groups A and B. After incubation time, the suspension was illuminated by a diode laser with an energy density of 31.2 J/cm^2. For the aim of the prevention of light reflection during laser irradiation, a black flat paper was used under the microtiter plate. During the process, the distance between the well surface and the laser tip was fixed at one mm.

Acinetobacter baumannii suspension without any treatment (photosensitizers and light) was considered as a control group. After the aPDT treatment of suspension, in order to assess the bacterial viability, after the preparation of serial dilution from the contents of each well, 10 µL aliquots of each dilution were plated in BHI agar (Merck, Darmstadt, Germany) and incubated for 24 hours at 37°C. After incubation, the number of CFU/mL was determined based on the previous study.[16]

aPDT Effect Against the Biofilm Form of *Acinetobacter baumannii* Strains

In order to form a biofilm, 200 µL of 1.5×10^8 CFU/mL bacterial suspension was distributed in the 96–well flat-

bottomed microtiter plate and placed in a 37°C incubator for 24 hours. Next, the biofilm was treated in accordance with the test groups. To measure the effect of aPDT on the biofilm formation of *A. baumannii* strains, crystal violet assay was performed according to the previous study.[14] Briefly, the content of each well was decanted and the wells were gently washed with PBS (2 mM NaH_2PO_4, 10 mM Na2HPO4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) to remove planktonic and loosely-bound cells. At the next step, the attached cells were fixed by 200 µL of 96% ethanol for 10 min. After emptying the wells and letting wells be air-dried, the adherent bacteria were stained with 200 µL of 0.1% (wt/vol) crystal violet solution for 15 min at room temperature and then the wells were slightly rinsed three times by PBS. Then 150 µL of 33% (wt/vol) acetic acid was added to each well. Biofilm formation was then quantified by measuring the absorbance of the solution at 570 nm using a microplate reader (Thermo Fisher Scientific, US). In addition, the SEM was used to confirm the presence of *A. baumannii* biofilms in each treated group.

Statistical Analysis

The results were expressed as mean values ± standard deviations (mean ± SD) and analyzed using one-way repeated measures ANOVA and Bonferroni post hoc tests in SPSS statistical software version 22.0. The level of significance for all analyses was set at $P < 0.05$.

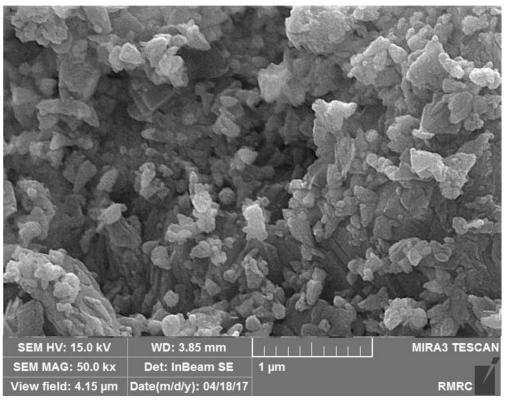

Figure 1. Scanning Electron Microscope Image of Synthesized NCs@ICG (scale bar represents 1 µm).

Results

Identification of *Acinetobacter baumannii* Strains

In this study, 85 clinical isolates suspected to *A. baumannii* were collected, 50 of which (58.8%) were identified as *A. baumannii* by biochemical and molecular assessments.

aPDT Inhibition Effect on *Acinetobacter baumannii* Planktonic Strains

The SEM image of synthesized NCs@ICG is shown in Figure 1. On the basis of the results shown in Table 1, the count of bacteria decreased by 93.2% after aPDT with NCs@ICG ($P < 0.05$). The antimicrobial effect of NCs@ICG-aPDT compared to ICG-aPDT relatively increased (about 15.1%). ICG-aPDT could only eliminate almost 78.1% of bacteria ($P < 0.05$; Figure 2). In the treated groups with the photosensitizers only (NCs@ICG and ICG) or irradiation only, no significant reduction of CFU/mL was observed ($P > 0.05$; Figure 3).

aPDT Inhibition Effect of *Acinetobacter baumannii* Biofilm Form

According to Table 1, after NCs@ICG-aPDT, the biofilm form of *A. baumannii* decreased significantly by about 55.3% in comparison with the control group ($P < 0.05$), whereas the ICG-aPDT group could only decrease *A. baumannii* biofilm by about 46.2%. As it is shown in Figure 4, NCs@ICG-aPDT could enhance the anti-biofilm efficiency by about 9.1% in comparison with ICG-aPDT. According to the above results, NCs@ICG, ICG, and the diode laser alone were not able to inhibit the biofilm form of *A. baumannii* ($P > 0.05$; Figure 4). These data were confirmed with the SEM images in Figure 5.

Discussion

The aim of the present *in vitro* study was to monitor the efficacy of a proficient procedure against *A. baumannii* which is isolated from burn wounds and is one of the opportunistic pathogens in infected wounds. There have been a lot of studies which have measured the efficiency of ICG-aPDT in its bactericidal aspect.[17-27] In an investigation by Omar et al, photo-activated ICG with light from a NIR laser was able to eradicate common burn wound pathogens.[17] In fact, this method has been

Table 1. Effects of Different Treatments Against Microbial Viability and Biofilm of *Acinetobacter baumannii* Strains

Groups	Mean of CFU/mL ± SD [a]	% Of Reduction	*P* Value	Mean of OD [b] ± SD	% Of Reduction	*P* Value
Control	3.20 ± 0.09 ×10^5	-	-	3.20 ± 0.06	-	-
Diode laser	2.24 ± 0.11 ×10^5	27	> 0.05	3.02 ± 0.08	5.62	> 0.05
ICG	2.08 ± 0.13 ×10^5	33	> 0.05	2.88 ± 0.06	10.00	> 0.05
NCs@ICG	1.88 ± 0.09 ×10^5	42.5	> 0.05	2.55 ± 0.07	20.31	> 0.05
ICG-aPDT	0.78 ± 0.14 ×10^5	78.1	< 0.05	1.72 ± 0.08	46.25	< 0.05
NCs@ICG-aPDT	0.24 ± 0.07 ×10^5	93.2	< 0.05	1.43 ± 0.10	55.31	< 0.05

[a] Standard deviation; [b] Optical density.

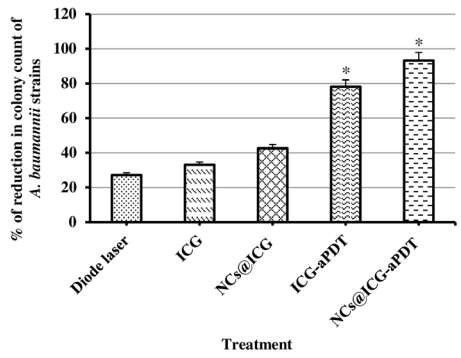

Figure 2. Effect of Different Test Groups on the Colony Count of *Acinetobacter baumannii* Strain. *Significantly different from the control, $P < 0.05$.

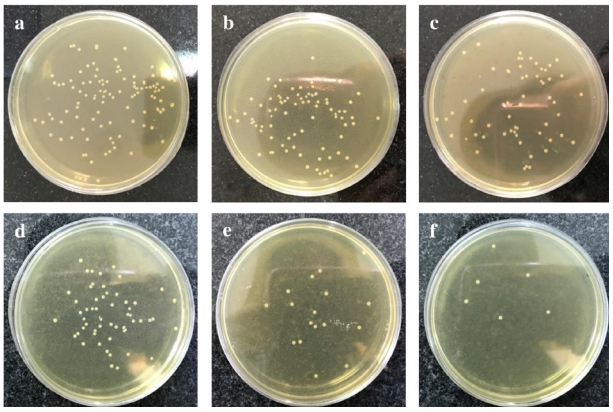

Figure 3. The Images of Plates in Each Treated Group. (a) Control, (b) Diode laser, (c) ICG, (d) NCs@ICG, (e) ICG-aPDT, (f) NCs@ICG-aPDT.

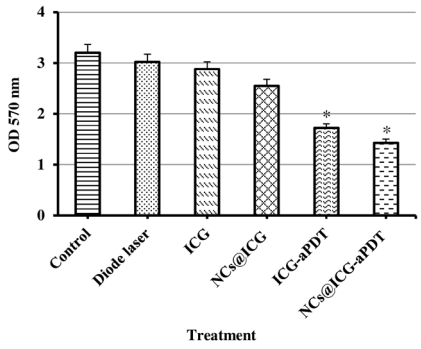

Figure 4. Effect of Different Test Groups on the Biofilm Form of *Acinetobacter baumannii*. *Significantly different from the control, $P < 0.05$.

estimated for different kinds of bacterial species, from periodontal pathogens to nosocomial ones, and in all of them, this method has been reported as an effective antibacterial method.[13,18,19] According to Boehm et al only less than 10% of *Porphyromonas gingivalis* could survive from ICG-aPDT,[20] or Fekrazad et al monitored the bactericidal effect of ICG-aPDT up to 96.68% on this bacterium.[21] In this way, Pourhajibagher et al suggested

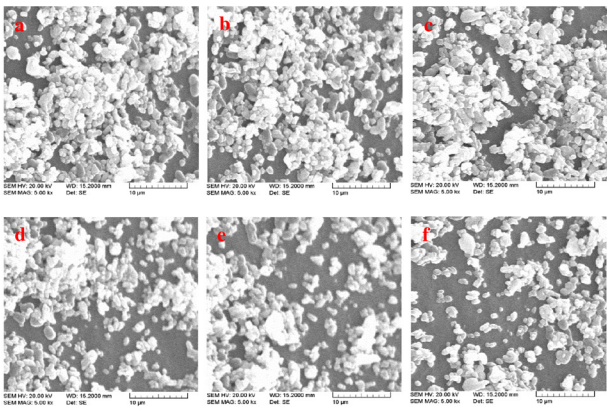

Figure 5. Scanning Electron Microscope Images of *Acinetobacter baumannii* Biofilms in Each Treated Group. (a) Control, (b) Diode laser, (c) ICG, (d) NCs@ICG, (e) ICG-aPDT, (f) NCs@ICG-aPDT.

that the higher concentration of ICG could improve the bactericidal and anti-biofilm effect of this method (ICG-aPDT) on *P. gingivalis,* which led to decreasing cell survival up to 30.4% in CFU/mL and 25.1% in biofilm formation.[26]

However, in a way of making the procedure more superior, some modifications have been made in this case. Adding Torolox, which is a water-soluble vitamin E analogue, was done by Kranz et al for improvement of ICG-aPDT efficiency on the elimination of Gram-negative periodontal bacteria,[22] but for improvement of the photosensitizer itself, it was first accomplished by Nagahara et al reporting that using ICG-loaded nanospheres coated with chitosan as a photosensitizer can be effective in decreasing *P. gingivalis* number (approximately 2-$\log_{10}$) with low-level diode laser irradiation in comparison to the group which was incubated by the ICG photosensitizer alone because ICG alone could not decrease bacterial number and ICG-loaded Nanospheres has shown to be able to adhere more efficiently to *P. gingivalis* cell surface as we have alleged here.[23] Furthermore, another report from Akbari et al showed that antibacterial activity of even a low concentration (200 µL) of ICG, which is loaded in Nanostructured graphene oxide during aPDT, was 47% more than a higher concentration (1 mg/mL) of ICG during aPDT against Gram-positive bacteria like *Enterococcus faecalis.* Also, Sasaki et al estimated the efficiency of using ICG-loaded Nanospheres coated with chitosan as a photosensitizer with an 810 nm diode laser and confirmed the improved result by this method.[27]

As mentioned before, the reason of this evidence is that the ICG photosensitizer has a negative charge, so being encapsulated in Nanoparticles like chitosan, which is an inexpensive, non-toxic, biodegradable, naturally occurring cationic polysaccharide, would help ICG to attach easily to the bacterial cell membrane and increase the bactericidal effect of this photosensitizer.[24,25]

There have been piles of studies on the bactericidal

effect of ICG, ICG-aPDT or NCs@ICG-aPDT separately, but during our study, for the first time, the bactericidal effect of the ICG-aPDT group was compared with the NCs@ICG-aPDT group and interestingly, we figured out that NCs@ICG-aPDT was relatively more efficient. NCs@ICG-aPDT was able to decrease the bacteria number for about 15.1% more than the ICG-aPDT treatment group with the same laser irradiation time and energy density (31.2 J/cm^2). Adjusting the ICG concentration and the light irradiation power should be considered in order to get the most efficient bactericidal effect and not to damage the host tissue because of the high temperature.
Although the results of the current study are promising, its limitations include the lack of molecular investigation into the effect of NCs@ICG-aPDT procedure on biofilm formation key genes in *A. baumannii*, which is strongly suggested to be accomplished in future investigations.

Conclusion

The outcomes declared in this paper mainly indicate the promising role of aPDT using NCs@ICG as a photosensitizer and the more bactericidal efficiency of this procedure compared to ICG-aPDT, and through this way more attention to the improvement of the procedure is needed to be paid.

Ethical Considerations

This study was approved by the Ethics Committee of Tehran University of Medical Sciences (application number 92-03-30-23186).

Conflict of Interests

The authors declare no conflict of interest.

Acknowledgment

This research has been supported by Tehran University of Medical Sciences.

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