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Antimicrobial photodynamic therapy of *Lactobacillus acidophilus* by indocyanine green and 810-nm diode laser

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ABSTRACT

This study investigated the efficacy of photodynamic therapy (PDT) using EmunDo as a photosensitizer against *Lactobacillus acidophilus*.

A gallium aluminum arsenide diode laser was used in this experiment (810 nm, CW). Standard suspensions of *Lactobacillus acidophilus* were divided into six groups by treatment: 1) EmunDo, 2) diode laser (100 mW, 90 s), 3) diode laser (300 mW, 30 s); 4) EmunDo + diode laser (100 mW, 90 s), 5) EmunDo + diode laser (300 mW, 30 s), 6) control (no treatment). Bacterial suspensions from each group were subcultured onto the surface of MRS agar plates immediately and 24 h after treatment, and the viable microorganisms of *Lactobacillus acidophilus* were counted. The data were analyzed by ANOVA and student's *t*-test at $p < 0.05$.

There was a significant between-group difference in the number of *Lactobacillus acidophilus* colonies in cell cultures obtained at 24 h after treatment ($p < 0.001$). The viable counts were significantly lower in EmunDo and both PDT groups, as compared to the other groups ($p < 0.05$). In the control and laser-irradiated groups, the number of colonies increased significantly at 24 h compared to the immediately after treatment ($p < 0.05$), whereas in both PDT groups, the number of colonies showed a significant reduction after 24 h of therapy ($p < 0.05$).

Under the conditions used in this study, *L. acidophilus* colonies were susceptible to PDT after sensitization with EmunDo and exposure to diode laser. These findings imply that PDT is capable to reduce cariogenic bacteria, potentially leading to more conservative cavity preparation.

1. Introduction

Dental caries is the most prevalent oral disease afflicting humans throughout the world. It develops as a result of bacterial activity in supragingival plaque, resulting in the production of organic acids during the metabolism of fermentable carbohydrates. This process affects tooth mineral and leads to demineralization and finally development of caries cavity on the tooth surface. *Streptococcus mutans*, *Streptococcus sorbinus* and various *Lactobacilli* are among the main microorganisms involved in dental caries [1]. Although *S. mutans* has been regarded as the primary etiological agent of caries manifestation, but *Lactobacillus acidophilus* is believed to have a major role in progression of dentinal caries and is generally found in deep parts of caries cavities [2].

Dentin carious lesions consist of two different areas: the outer softened and wet dentine with a high degree of infection (infected dentin) and the inner layer with a low number of microorganisms (affected dentin). The concept of minimally invasive dentistry emphasizes that infected dentin should be removed during cavity preparation, whereas the affected dentin which is capable of remineralization should be maintained to preserve tooth tissue structure. However, detecting the boundary between infected and affected dentin is difficult in the clinical situation [3]. Furthermore, maintaining some degree of caries in the case of deep cavities is a conservative way to decrease the risk of pulp tissue exposure during the caries excavation process. In these circumstances, it is optimal to eradicate the remaining bacteria by antimicrobial agents to prevent pulp inflammation and to increase the

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adhesion properties of the restorative materials and thus long-term success of restorations [4]. Recently, photodynamic therapy (PDT) has been employed for disinfecting the dentine tissue.

Antimicrobial photodynamic therapy (a-PDT) is a novel, rapid, and noninvasive approach to counteract adverse microorganisms, including bacterial oral pathogens. PDT involves light to activate a photosensitizing agent in the presence of oxygen. In this process, the photosensitizer is excited to the triplet state and its energy is transferred to the molecular oxygen. Reactive oxygen species, such as singlet oxygen and free radicals are then generated, which are capable to induce lethal cell damage. A-PDT has become a viable approach for the treatment of bacterial and fungal infections [5,6]. Ease of application, few side effects, bacterial killing, topical and confined antibacterial effects, and unlikely chance for bacterial photo-resistance after multiple PDT treatments are among the particular advantages of PDT [7].

There are numerous studies regarding the efficacy of photodynamic action on a large number of gram positive and negative bacteria, using diverse photosensitizers and illumination systems [2,8–11]. PDT has been extensively used in dentistry for applications such as treatment of oral cancers, periodontitis, peri-implantitis, and fungal infections. Recently, PDT has been applied to target acidogenic bacteria in both in vitro and in vivo conditions [12–17]. EmunDo or Indocyanine green (ICG) is a photosensitizer with absorption and fluorescence maximum at 800 nm wavelength. PDT with EmunDo has been applied for treatment of pancreatic, lung, skin, colon and breast tumors, and for bactericidal purposes in the treatment of acne vulgaris and periodontitis [18–20]. According to the authors' knowledge, no study investigated the bactericidal effects of PDT with EmunDo as a photosensitizer in association with an infrared diode laser to kill *L. acidophilus*. Therefore, this study was conducted to investigate the effects of EmunDo-mediated PDT on the viability of *L. acidophilus* bacterial strain in vitro.

2. Material and methods

2.1. Test microorganism and growth conditions

This study used *L. acidophilus* strain (ATCC523) obtained from Iranian Research organization for research and technology (IROST), Tehran, Iran). The bacteria were subcultured on MRS (medium according to de Man, Rogosa and Sharpe) agar plates (Merck, Germany) with 5% blood sheep, and incubated under anaerobic condition at 37 for 48 h. For experimental purposes, bacterial suspensions were prepared by transferring a few colonies grown on plates to microtubes. Cell number was measured in a spectrophotometer at 600 nm wavelength. Suspensions with an optical density of 0.5 McFarland were prepared, which contained approximately 1.5×10^8 colony forming unit per milliliter ($CFUmL^{-1}$) of *L. acidophilus*.

2.2. Photosensitizer and light source

EmunDo (Indocyanine green; ARC Laser GmbH, Nürnberg, Germany) was used as a photosensitizer in this study. Fresh solutions were prepared according to the manufacturer's instructions before each experiment and kept in a dark environment. A gallium aluminum arsenide (GaAlAs) diode laser (ARC Laser GmbH) was employed for PDT experiments, emitting a wavelength of 810 nm. The light was delivered in continuous wave mode (CW) by a handpiece and fiber optic cable (300 μm). The fiber optic cable was held perpendicular to the above surface of each microtube and the laser beam was directed with a sweeping motion to ensure covering the whole surface of the sample. The laser parameters were selected with respect to the manufacturer's recommendations. Two irradiation protocols were applied in this study: 100 mW for 90 s and 300 mW for 30 s. The energy delivered to individual microtube was 9 J and the energy density was 18 J/cm2, considering the surface area of 0.5 cm2 for the microtube.

2.3. Photodynamic treatment

The experiments were conducted in six groups as follows: In all the study groups, 125 μ L of *L.acidophilus* suspension (25 μ L *L.acidophilus* + 100 L sterile phosphate buffered saline (PBS)) was put into each microtube.

Group 1 (EmunDo): EmunDo was prepared according to the manufacturer's instructions. After preparation, 125 μL EmunDo was added to the bacterial suspension and the microtube was put on a shaker (100 rpm rate) for 1 min to obtain a homogenous solution. The solution was kept in a dark environment at room temperature for 5 min.

Group 2 (Diode laser/100 mW): In this group, 125 μL PBS was added to the bacterial suspension to have equal volume of solution in all groups. After storage for 5 min in a dark environment at room temperature, laser irradiation was performed using power of 100 mW for 90 s.

Group 3 (Diode laser/300 mW): In this group, 125 μL PBS was added to the bacterial suspension to have equal volume of solution in all groups. After storage for 5 min in a dark environment at room temperature, laser irradiation was performed at setting of 300 mW for 30 s.

Group 4 (PDT/100 mW): In this group, 125 μ L EmunDo was added to bacterial suspension and the microtube was mixed on a shaker for 1 min (100 rpm) to obtain a homogenous solution. The solution was then kept in a dark environment at room temperature for 5 min. Afterwards, the laser was applied using power of 100 mW for 90 s.

Group 5 (PDT/300 mW): The test condition was the same as that in group 4, but laser exposure was performed at setting of 300 mW for 30 s.

Group 6 (control group): In this group, 125 μ L PBS was added to 125 μ L bacterial suspension and the resulting solution was kept in a dark environment at room temperature for 5 min. Neither laser nor photosensitizer was applied in this group.

2.4. Microbiological assessment

After the treatments described above, the bacterial suspension in all groups was diluted 10^2 times in PBS. Immediately and 24 h after treatment, 50 μ L of diluted samples were plated on MRS agar with 5% blood sheep (Fig. 1). The streaking technique was used to spread bacterial suspension over agar plates. One agar plate was prepared from each microtube. The plates were incubated under anaerobic condition at 37 for 48 h. The viable microorganisms grown on the plates were counted with the naked eye and multiplied by the dilution factor to achieve colony forming unit (CFU) per millimeter. All experiments were repeated at least five times.

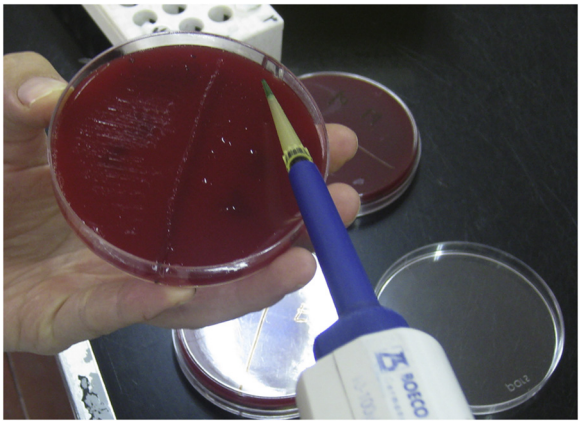

Fig. 1. Plating 50 L of bacterial suspension on MRS agar with 5% blood sheep.

Table 1
Means and standard deviations (SD) of *L. acidophilus* colonies (CFU/mL) obtained immediately and 24 h after treatment in the study groups.

	Definition	Immediately		After 24 h			p-value
		Mean	SD	Mean	SD	Pairwise comparisons*	
1	EmunDo	184600	32222	172600	121135	a	0.836
2	Diode laser/100 mW	176800	69438	843000	102200	b	< 0.001
3	Diode laser/300 mW	230800	32429	778000	157424	b	< 0.001
4	PDT/100 mW	232400	26426	58800	23181	a	< 0.001
5	PDT/300 mW	224600	67902	98000	49025	a	0.010
6	Control	147200	13608	716800	161776	b	0.001
p-value		0.084		< 0.001			

* Tukey pairwise comparison test; the groups that have been defined by different letters have statistically significant differences at $p < 0.05$, whereas those with the same letter are statistically comparable ($p > 0.05$).

2.5. Statistical analysis

The normality distribution of the data was confirmed by the Kolmogorov-Smirnov test. The difference in colony counts between groups was analyzed by one way analysis of variance (ANOVA) followed by Tukey post hoc test for pairwise comparisons. Student *t*-test was run to detect any difference in the number of viable microorganisms between the two time points in each group. The data were processed by SPSS software (Statistical Package for Social sciences, version 16.0) and the significance level was set at $p < 0.05$.

3. Results

Table 1 presents the mean and standard deviation (SD) regarding the number of *L. acidophilus* colonies (CFU/ml) obtained immediately and 24 h after treatment in the study groups. In cultures obtained immediately after treatment, no significant difference in the number of colonies was found among the study groups ($P > 0.05$). ANOVA revealed a significant between-group difference in the number of *L. acidophilus* colonies in cell cultures obtained at 24 h after treatment ($p < 0.001$). Pairwise comparison by Tukey test revealed that the viable counts (CFU/ml) were significantly lower in cultures treated with EmunDo or laser + EmunDo, as compared to the control and laser-irradiated groups ($p < 0.05$).

When the number of *L. acidophilus* colonies was compared in cell cultures obtained immediately and 24 h after treatment, it was revealed that the viable counts were significantly lower in both PDT groups at 24 h compared to immediately after treatment ($P < 0.001$), whereas in the control and laser-irradiated groups, the number of colonies showed a significant increase after 24 h of treatment ($p < 0.05$).

Fig. 2 illustrates the number of viable microorganisms in the study groups immediately and 24 h after treatment.

4. Discussion

The present study investigated the photodynamic effect of EmunDo as a photosensitizer combined with an 810 nm diode laser exposure against *L.acidophilus* grown in a planktonic culture. The outcomes of this study exhibited that exposure of bacterial cultures to laser light in the presence of EmunDo caused a significant reduction in viability of *L. acidophilus* in samples obtained after 24 h of photodynamic treatment, whereas the samples obtained immediately after treatment showed no significant difference in colony count compared to the control group. This implies that a period of time is required to observe the bactericidal effect of PDT under the conditions used in this study. It was also observed that the light source alone had no significant effect on the viability of *L. acidophilus*. The pre-irradiation period was 5 min in this study. This period of time was essential to keep the photosensitizer inside the bacteria and achieve greater light absorption.

It is expected that after 24 h of keeping bacteria in a nutrient medium, the number of colonies shows a remarkable increase. This was that occurred in some groups of this study, as the colony count was significantly greater in the control and laser irradiated groups after 24 h of storage in culture medium. In contrast, both PDT groups exhibited a significant reduction in viability of *L. acidophilus* at 24 h compared to immediately after treatment. In the EmunDo Group, the colony count was almost the same in cell cultures obtained from the two time points. It seems that the addition of EmunDo to cell culture medium inhibited bacterial growth over 24 h after treatment, but it was not effective in reducing the number of present colonies. In contrast, when EmunDo was applied in combination with laser beam (PDT groups), the number of bacterial colonies experienced a significant reduction after 24 h of treatment.

Sensitizer is an essential element in photodynamic therapy. Previous studies investigated the efficacy of various photosensitizers like photogem, curcumin, erythrosine, methylene blue (MB), radachlorin, safranin O and toluidine blue O (TBO) in elimination of cariogenic bacteria [21–26]. We studied EmunDo, which has recently been popularized in the field of dentistry. EmunDo is a photosensitizer which mainly consists of Indocyanine green (ICG) dye. Indocyanine green (ICG) is a water-soluble tricarbocyanine dye with almost no toxicity to non-target host tissue and has FDA approval for medical diagnostic applications. ICG represents high absorption in near infra-red spectrum with maximal absorption at 800 nm wavelength [27,28]. The antimicrobial mechanism of ICG is under debate. It is believed that free radical formation (photodynamic effect) and photothermal reaction (photothermal effect) may both be responsible for killing bacteria. Actually, photodynamic therapy with ICG leads to the production of reactive oxygen species (ROS), such as singlet oxygen and free radicals, which cause lethal injuries to bacterial cells. Furthermore, the photothermal effect of EmunDo can damage cells by increasing intracellular temperature, because most of the light energy absorbed by this photosensitizer is converted to heat [29,30].

The light source that is used in PDT should match the activation spectrum of the photosensitizer. Although most previous studies used visible light to activate the photosensitizer, the use of near infrared spectrum provides the additional advantage of providing more penetration depth, which is helpful for antimicrobial purposes in biological tissues such as dentinal tubules [31]. Other parameters such as the concentration of photosensitizer, the duration of irradiation, the power and energy density of the incoming light should also be optimized to gain successful PDT results [32]. In the present study, EmunDo was activated by an 810 nm diode laser. Two protocols of light irradiation were used for PDT: 100 mW for 90 s and 300 mW for 30 s. Both protocols were recommended by the manufacturer and generated the same energy and energy density. According to the outcomes of this study, the two PDT protocols were effective against *L acidophilus* with no significant difference to each other.

The outcomes of this study are consistent with several investigations that demonstrated the susceptibility of cariogenic bacteria to PDT using various light sources and different photosensitizers. Lima et al. [33] indicated that the combination of toluidine blue O (TBO) and LED

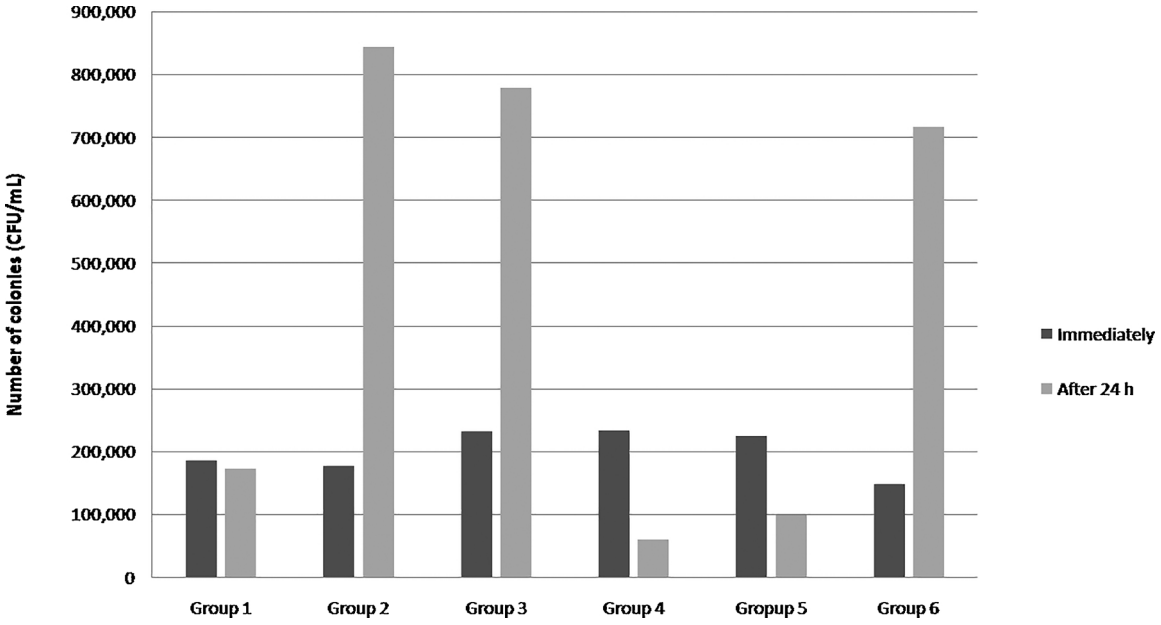

Fig. 2. The number of *L. acidophilus* colonies (CFU/ml) in the study groups immediately and 24 h after treatment (group 1: EmunDo, group 2: Diode laser/100 mW, group 3: Diode laser/300 mW, group 4: PDT/100 mW, group 5: PDT/300 mW, group 6: control.

resulted in a significant reduction in cariogenic species present in dentin caries in situ. Arauje et al. [34] demonstrated a significant reduction in *S. mutans* and *L. acidophilus* colonies using curcumin in combination with 450 nm blue light. Longo et al. [35] showed that photodynamic therapy mediated by aluminum chloride phtalocyanine and a 660 nm laser was efficient in the reduction of microbial load from bacterial cultures and produced significant disinfection in carious cavities in vivo. Melo et al [7] found that a single application of photodynamic antimicrobial chemotherapy using a light-emitting diode associated with toluidine blue O (TBO) caused a significant reduction in all analyzed bacterial group species in contaminated dentin during deep caries management in adult patients. Ricatto et al [36] demonstrated that PDT combined with laser or LED and methylene blue had significant antimicrobial effect on *S. mutans* and *L. casei*. George et al. [37] demonstrated the bactericidal activity of ICG on gram-positive and gram-negative bacteria. Topaloglu et al [38] indicated that PDT with the combination of ICG and an 809 nm laser can inactivate *P. aeruginosa*. Another study reported a significant reduction of *S. mutans colonies* after photothermal therapy with EmunDo and 810 nm diode laser [9].

In contrast to the outcomes of this study, Neves et al [39] indicated that PDT using methylene blue dye at dosimetry of 120 J/cm2 was not a reliable clinical alternative to reduce bacterial contamination in deep caries. Monzavi et al. [19] demonstrated that the application of ICG with an 810 nm diode laser and scaling and root planning (SRP) did not have any additional advantage in terms of clinical attachment gain and plaque score over conventional SRP. Fekrazad et al. [40] demonstrated that the antibacterial effect of 2% chlorohexidine gel on *P. gingivalis* was significantly greater than that of photodynamic therapy with EmunDo.

It is worth mentioning that comparison between the results of the present study with the above-mentioned studies is difficult due to the different light sources and type of photosensitizers used in these studies. The present study stands as the first one to demonstrate promising results regarding the antibacterial feature of EmunDo-mediated PDT against *L. acidophilus*. Therefore, PDT can be employed as a novel approach to reduce the chance of pulp exposure while maximizing tooth tissue preservation by reducing the volume of infected dentine removed during excavation. In this way and by reducing the bacterial load before placing restorative materials, the success of deep caries management would increase. The outcomes of this study, however, should be interpreted with considering the limitation of transferring laboratory results into clinical reality, because the remaining bacteria in the bottom of deep cavities are penetrated to dentinal tubules and may be in accessible in this specific target area. The relatively high price of EmunDo presents an obstacle to routine use of this photosensitizer for PDT. Still, the lack of randomized clinical trials to provide reliable evidence for effectiveness of PDT in caries management prevents from using this approach as a routine strategy in prevention, control and treatment of dental caries. Further laboratory and clinical studies are warranted to explore the anti-cariogenic potential of PDT using various photosensitizers and light sources under the optimum irradiation parameters and drug concentration. The alteration in pre-irradiation time may also be proposed as a strategy to improve PDT performance.

5. Conclusion

Under the conditions used in this study, *L. acidophilus* colonies were susceptible to photodynamic therapy after sensitization with ICG and exposure to 810 nm diode laser. Therefore, PDT could be considered as a promising adjunctive to caries management, aiming to decrease cavity contamination prior to restorative procedures, while contemplating conservative cavity preparation.

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Ethical approval

None.

Informed consent

None.

Conflictof interest

The authors declare that they have no competing interest.

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