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Article

5-Aminolevulinic acid photoactivated over planktonic and biofilm forms of *Enterococcus faecalis* as a pharmacological therapy alternative

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The purpose of the study was to evaluate the antibacterial effect of protoporphyrin IX (PpIX) generated by the exogenous administration of 5-aminolevulinic acid or δ-ALA and activated with an argon laser over a planktonic and biofilm of *Enterococcus faecalis (E. faecalis*) as a pharmacological therapy alternative. A planktonic strain of *E. faecalis* was cultured with a solution of ∂-ALA (40 µg/mL)-thioglycolate solution for 13 min, and a biofilm of *E. faecalis* was cultured in a δ-ALA (80 µg/mL)-thioglycolate solution for 13 min. Then, both were irradiated with an argon laser. Finally, the antibacterial effect was evaluated by counting the CFU in planktonic form, and a LIVE/DEAD viability cell test. The production and accumulation of PpIX from exogenously administered δ-ALA on *E. faecalis* in planktonic and biofilm forms was confirmed by spectrofluorometry. The irradiation of PpIX with an argon laser produced an antibacterial effect on *E. faecalis* in planktonic and biofilm form, even without biofilm disruption, at a concentration of 40 µg/mL and 80 µg/mL of δ-ALA, respectively. The exogenous administration of δ-ALA in combination with laser irradiation on planktonic and biofilm forms of *E. faecalis* produces an effective antibacterial effect as complement or alternative to pharmacological therapies.

Keywords: Biofilm. Photodynamic therapy. 5-aminolevulinic acid.

INTRODUCTION

Microbial infection remains among the leading causes of death worldwide (Barra *et al*., 2015). Enterococci, important healthcare-associated pathogens, cause serious and often life-threatening diseases. The variety of infections produced by Enterococci include urinary tract infections, intra-abdominal infections, pelvic infections (Gilmore *et al*., 2014), device-associated infections (Darouiche, 2001), soft tissue infections, bacteremia and endocarditis (Gilmore *et al*., 2014).

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Pathogenic bacteria, such as *Enterococci*, can attach to biotic or abiotic surfaces (Balcázar, Subirats, Borrego, 2015), forming a microbial community (Donlan, 2011; Li *et al*., 2013) in a three-dimensional extracellular polysaccharide matrix (EPM) (Balcázar, Subirats, Borrego, 2015; Barra *et al*., 2015; Li *et al*., 2013; López-Jiménez *et al*., 2015) known as a biofilm (Balcázar, Subirats, Borrego, 2015). Additionally, some pathogenic bacteria can develop multidrug resistance due the abuse and incorrect use of pharmacological therapies (Barra *et al*., 2015).

Microorganisms in a biofilm are exposed to very different environmental conditions from those in planktonic form (López-Jiménez *et al*., 2015) and many species can change their metabolism depending on the surrounding physiological and physicochemical

conditions (Balcázar, Subirats, Borrego, 2015). One of the most important characteristics of biofilms is that the microorganisms in it are more resistant to pharmacological therapies as antimicrobials (Balcázar, Subirats, Borrego, 2015; Donlan, 2011; López-Jiménez *et al*., 2015) (up to 1000 time less susceptibility to specific antimicrobials) (Balcázar, Subirats, Borrego, 2015; Donelli, 2014; Li *et al*., 2013) and to the host immune defenses compared to their planktonic counterparts (Li *et al*., 2013), mainly because of the protection conferred by the EPM (Balcázar, Subirats, Borrego, 2015).

Mechanical disruption and pharmacological therapies are the most used methods to treat biofilms. However, the efficacy of these strategies is limited because of the persistence of biofilm and microorganisms after mechanical disruption and the appearance of antibiotic resistant strains (López-Jiménez *et al*., 2015; Rajesh *et al*., 2011). Moreover, it was reported that the drug levels necessary to kill dispersed bacteria may be ineffective against biofilm associated bacteria (Donlan, 2011).

Photodynamic therapy (PDT) or photodynamic inactivation (PDI) is a clinical procedure that has been emerged as a non-invasive therapeutic modality for the treatment of infections (Dai *et al*., 2012; Rajesh *et al*., 2011; Sperandio, Huang, Hamblin, 2013). PDT was first reported by Dobson and Wilson *(*1992), and they suggest that photosensitization may be effective for eliminating periodontal pathogenic bacteria from dental plaque (Dobson, Wilson, 1992). This technique involves three components: a source of light, a photosensitizer agent (PS) and oxygen (Rajesh *et al*., 2011). One of the main advantages of PDT is the high target specificity because the PS is localized into the microorganisms without the major involvement of surrounding tissues or cells (Donelli, 2014). Additionally, with PDT, it is possible to avoid the appearance of undesired drug resistant strains (Sperandio, Huang, Hamblin, 2013).

The action of PDT is related to the activation of PS by light of a specific wavelength (Breskey *et al*., 2013; Pagonis *et al*., 2010; Rajesh *et al*., 2011; Soukos *et al*., 2006) in a non-thermal oxygen-dependent photochemical reaction (Rajesh *et al*., 2011; Sperandio, Huang, Hamblin, 2013), in which the PS undergo a transition from a low energy state to an excited state (triple state) (Homayoni *et al*., 2015; Sperandio, Huang, Hamblin, 2013). The triplet state photosensitizer can react with biomolecules by two main photochemical mechanisms called type I and type II (Rajesh *et al*., 2011; Sperandio, Huang, Hamblin, 2013), by electron transfer or by energy transfer processes, respectively (Sperandio, Huang, Hamblin, 2013). The type I pathway forms superoxide anions that lead to the formation of reactive oxygen species (ROS), such as hydroxyl radicals, whereas the type II pathway leads to singlet oxygen production (Homayoni *et al*., 2015; Sperandio, Huang, Hamblin, 2013). Both processes produce a cytotoxic effect on bacteria (López-Jiménez *et al*., 2015; Pagonis *et al*., 2010).

The PS plays a key role in the efficacy of PDT because it should have specific characteristics for clinical use (Homayoni *et al*., 2015), such as the presence of absorption bands in the optical window in the red portion of the electromagnetic spectrum (600–900 nm) when deep light penetration is required (Sperandio, Huang, Hamblin, 2013; Wachowska *et al*., 2011). The PS should also have a high yield of the excited electronic triplet state and singlet oxygen (Sperandio, Huang, Hamblin, 2013). It should be selectively accumulated in host cells (Barra *et al*., 2015), and should generate reactive oxygen species in the presence of light and oxygen (Sperandio, Huang, Hamblin, 2013). Furthermore, the PS should have low toxicity without light exposure and possess a relatively rapid clearance from normal tissues to minimize side effects (Breskey *et al*., 2013; Wachowska *et al*., 2011). Most PS agents cannot meet all these criteria; therefore, the final selection of the PS depends upon maintaining a balance between optimal photoreactivity and biological properties, which when combined with light irradiation, offers the most effective outcome with the lowest level of side effects (Breskey *et al*., 2013).

Porphyrin based photosensitizer agents have been approved clinically in the United States for PDT (Homayoni *et al*., 2015), and one of the most studied is protoporphyrin IX (PpIX) due to its properties. Unfortunately, it has some issues for clinical applications, such as hydrophobicity and low solubility in aqueous solutions (Homayoni *et al*., 2015), making it useless for deep tissues. By contrast, 5-aminolevulinic acid (5-ALA) or delta-aminolevulinic acid (δ-ALA), a natural amino acid (Hino *et al*., 2013) and an early intermediate in the heme biosynthesis pathway (Wachowska *et al*., 2011), although not a PS *per se* (Barra *et al*., 2015), is the precursor of the photosensitizer PpIX (Hino *et al*., 2013; Li *et al*., 2013). δ-ALA was used for photodynamic therapy for the first time in 1987 for the selective

elimination of erythroleukaemic cells (Malik, Lugaci, 1987), which opened the possibility of a new approach for cell and bacteria therapy due to its properties of low lipid solubility and reduced bioavailability (Wachowska *et al.*, 2011) but good solubility in water (Li *et al.*, 2013). Moreover, when δ-ALA is taken up by target cells (bacteria), δ-ALA is metabolically transformed to PpIX (Barra *et al.*, 2015). Furthermore, the accumulation of porphyrins is more pronounced in cells with high rates of metabolic activity, such as cancer cells, inflammatory cells and bacteria, resulting in high selectivity (Fotinos *et al.*, 2008).

The aim of the present study was to determinate the antibacterial effect of the exogenous administration of δ-ALA to produce intracellular PpIX irradiated with an argon laser (500 mW, 510 nm, 200 J/cm^2) on planktonic and biofilm forms of *E. faecalis*.

MATERIAL AND METHODS

∂-ALA solution

Previous to the experimental work, the minimal inhibitory test was performed to determinate the effective dose of the ∂-ALA solution in combination with laser irradiation. For planktonic bacteria, 40 μg/mL was the required dose to obtain an antibacterial effect and a dose of 80 μg/mL was necessary for an effective antibacterial effect on biofilm.

The ∂-ALA (40 μg/mL)-thioglycolate and ∂-ALA (80 μg/mL)-thioglycolate solution (synthesized in the Environmental Cytopathology Laboratory, IPN, Mexico) were prepared, both enriched with vitamin K. Because it was reported that ∂-ALA production is significantly enhanced when the pH is maintained at neutral range (Sasaki *et al.*, 1993), the pH of the thioglycolate solution was adjusted to 6.8-7.0 using NaOH 10 N.

Isolation of the strain and bacterial cell culture

An *Enterococcus faecalis* strain was isolated from teeth with endodontic treatment failure and a periapical lesion, according to the protocol described by Manzur (2007). To confirm isolation of the strain, the API System (BIOMÉRIEUX, USA) was used. The bacteria were cultured in 5 mL Falcon tubes with continuous shaking at 37 °C overnight. To determinate the bacterial amount, the optical density (OD) was adjusted to 1.0, which corresponds to approximately to 5X10^7 cells/mL.

∂-ALA metabolism to PpIX into bacteria

To determine the production of PpIX after ∂-ALA administration, an aliquot of *E. faecalis* was incubated (Incubator Precision Thermo Electron Corporation, USA) with 2 mL of ∂-ALA (40 μg/mL)-thioglycolate solution with continuous shaking for 13 min at 37 °C. After the incubation, the sample was centrifuged (Centra CL2 Centrifuge, Thermo Electron Corporation, USA) at 5,000 RPM for 10 min. The supernatant was removed, the cell pellets were washed with 1 mL of PBS, and the samples were centrifuged again at 5,000 RPM for 5 min. Then, the supernatant was removed, and 1 mL of a lysis solution (ethanol + dimethyl sulfoxide + acetic acid 80:20:1 V/V/V) was added to the samples that were then sonicated for 5 cycles for 5 sec. Next, the samples were centrifuged at 5,000 RPM for 3 min. Finally, the supernatant was transferred to a quartz cuvette and read with a spectrofluorometer (PTI Photon International Mod. 5020, USA). The measurements were recorded at 375 nm excitation with a wavelength range from 300 to 700 nm. All experiments were performed for triplicate.

Photosensibilitation PpIX determination

After the fluorescence of PpIX was identified by spectrofluorometry at 480 nm, the samples were irradiated with an argon laser type B, 500 mW (Leica Microsystems GmbH, Mannheim, Germany) at 510 nm (LEICA Acousto Optical Tunable Filter enable to tune the WL) for 8 min, 33 sec to reach 200 J/cm^2 (Dosage calculation, power/beam area x time = J/cm^2). Then, the samples were read by spectrofluorometry. The intensity of the fluorescence signal before and after laser irradiation was compared to determinate the photoactivation of PpIX.

Antibacterial effect of PpIX on planktonic E. faecalis

To evaluate the antibacterial effect on bacterial cells, 2 mL of culture medium (5x10^7 cells/mL) were treated as follows: group 1 (G1), a positive control group treated with 800 μL of thioglycolate + 200 μL of sodium hypochlorite (NaOCl) at 5.25%; group 2 (G2), 1 mL of thioglycolate + laser irradiation (8 min, 33 sec); group 3 (G3), 1 mL δ-ALA (40 μg/mL)-thioglycolate solution (control groups); and group 4 (G4), 1 mL of δ-ALA (40 μg/mL)-thioglycolate solution + laser irradiation for 8

min, 33 sec as the experimental group. Because there is previous evidence that there is not a significance difference between cultures treated only with laser irradiation or only δ-ALA, these groups were treated as negative control groups. A bacterial cell culture (extension technique) of each group was performed with a dilution of 1:10,000. Then, a CFU count of each group was performed at 0, 24 and 72 h.

Determination of the antibacterial effect of PpIX on E. faecalis biofilm

The formation of biofilm was performed following the protocols previously described by Kaplan *et al.* (2004), Djordjevic, Wiedmann and McLandsborough (2002) and Zaw Yuthika, Lakshman (2007). On a 24 well microplate with coverslips, 71 µL of the inoculum of *E. faecalis* ($5x10^7$ cell/mL) and 929 µL of 3% thioglycolate enriched with vitamin K were added. The samples were incubated for 10 days at 37 °C with continuous shaking. The medium was replaced every 24 hrs.

After, the biofilm samples were divided in 4 groups: group "a" (GA), a positive control group treated with 800 µL of thioglycolate + 200 µL of NaOCl 5.25%; group "b" (GB), 1 mL of thioglycolate + laser irradiation (8 min, 33 sec); group "c" (GC), 1 mL δ-ALA (80 µg/mL)-thioglycolate solution; and group "d" (GD), 1 mL of δ-ALA (80 µg/mL)-thioglycolate solution ($5X10^7$cells/mL) + laser irradiation to reach 200 J/cm^2.

To determinate the bacteria viability, a LIVE/DEAD Bacterial Viability Kit was used (Thermo Fisher Scientific, USA). Briefly, the culture medium from each well was removed with a Pasteur pipette to eliminate the non-adherent cells. Then, coverslips were placed in a new multiwall polystyrene plate under sterile conditions, and the biofilm was treated with 200 µL of CTC (5-cyano-2,3 ditolyl tetrazolium chloride) to indicate the cellular respiratory activity. All samples were incubated for 12 h at 37 °C, with continuous shaking. Then, 200 µL of CTC was removed and washed with 1 mL of 1X PBS. The biofilm was covered with 200 µL of propidium iodide (PI), a fluorescent molecule impermeable to the cellular membrane and generally excluded from viable cells. Finally, the sample was placed on a glass slide and examined on an inverted TCS-SPE confocal microscope (DMI4000B, Leica Microsystems GmbH, Mannheim, Germany). The window of emission ranged from 500 to 520 nm for the PI and an excitation of 488 nm for the flow cytometer laser and a window emission range of 600 to 630 nm for the CTC. This information allows the analysis of the bacterial metabolism because cells in the log phase emit red fluorescence and those in the stationary phase emit green fluorescence.

Data analysis

Descriptive analysis of the data, a multi-level analysis, ANOVA and post-hoc test (HSD Tukey) were performed to compare the groups with a 95% level of confidence. Data were analyzed using R software version 3.2.2.

RESULTS

Metabolism of ∂-ALA to PpIX into bacteria

The spectrofluorometer test (excitation of 375 nm) confirms the presence of PpIX with a wavelength of 480 nm and an intensity of $1.30X10^5$, demonstrating that PpIX was produced by *E. faecalis* after exogenous administration of δ-ALA (Figure 1, red line).

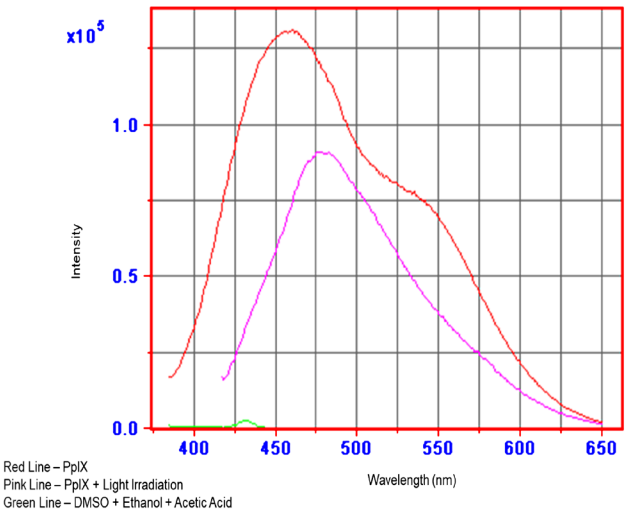

FIGURE 1 - Spectrofluorometry analysis of PpIX. The red line represents bacteria + ∂-ALA incubated for 13 min. The presence of PpIX after the exogenous administration of ∂-ALA was demonstrated. The pink line represents bacteria + ∂-ALA + laser irradiation (200 J/cm^2). A decreased of the signal of PpXI after laser activation was shown. The results are the mean of the experiments.

Photosensibilitation of PpIX

Bacterial samples were incubated with ∂-ALA, the precursor of PpIX. After the PpIX was identified by spectrofluorometry, the samples were irradiated with an argon laser at 510 nm (Leica Microsystems GmbH, Mannheim, Germany) for 8 min, 33 sec to reach a 200 J/cm^2 dose. Then, the samples were evaluated by spectrofluorometry to assess the signal of PpIX after irradiation. A decrease in the signal was found, (pink line), which could be interpreted as less availability of PpIX due to its photoactivation (Figure 1).

Determination of the antibacterial effect of PpIX over planktonic E. faecalis

The multilevel model included the follow terms: groups (G1, G2, G3 and G4), time and the interaction term for both. According to the data, G4 show a lower number of CFUs compared with G2 (P<0.001) and G3 (P=0.0167) at 0, 24 and 72 h. For G4 and G1 at 0, 24 and 72 hours, there is no significant difference between groups (P=0.9685), indicating that the δ-ALA (40 µg/mL) activated with the argon lase has an antibacterial effect similar to 5.25%NaOCl. For G2 and G3 at 0, 24 and 72 h, there is no significant difference between groups (P<0.001), indicating that the application of either δ-ALA or laser alone has no antibacterial effect (Figures 2, 3).

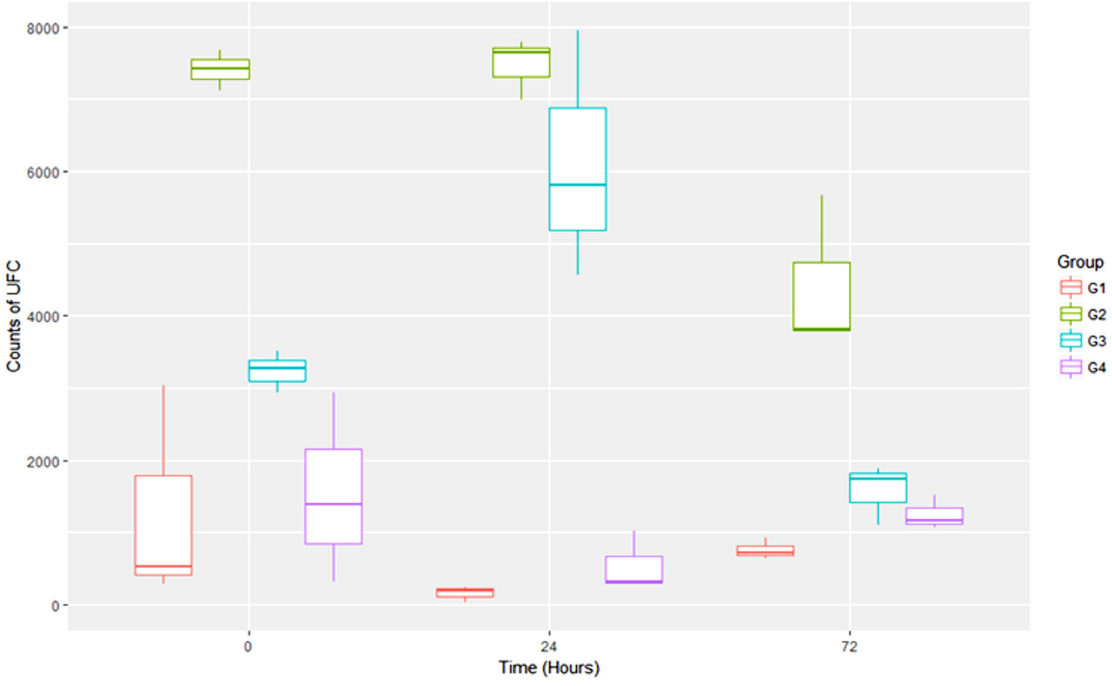

FIGURE 2 - Box-Plot: UFC vs Time for Group. Group 1 (G1), A positive control group treated with 800 µL of thioglycolate + 200 µL of NaOCl 5.25%; group 2 (G2), 1 mL of thioglycolate + laser irradiation (8 min, 33 sec); group 3 (G3), 1 mL δ-ALA (40 µg/mL)-thioglycolate solution; and Group 4 (G4), 1 mL of δ-ALA (80 µg/mL)-thioglycolate solution + laser irradiation (8 min, 33 sec). There was no significant difference between G4 and G1 at 0, 24 and 72 h. There is a statically significant for G4 vs G2 and G3, at 0, 24 and 72 h. There was no significant difference between G2 and G3.

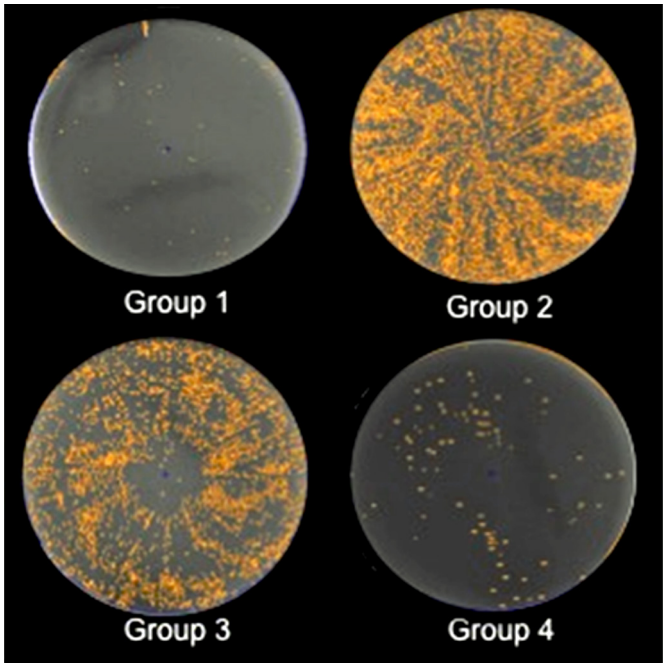

FIGURE 3 - The image shows the bacterial cell culture at 72 hrs. observed in a photodocument system (Bio-Rad of Quantity One). Group 1 (G1), a positive control group treated with 800 µL of thioglycolate + 200 µL of NaOCl 5.25%; group 2 (G2), 1 mL of thioglycolate + laser irradiation (8 min, 33 sec); group 3 (G3), 1 mL δ-ALA (40 µg/mL)-thioglycolate solution; and group 4 (G4), 1 mL of δ-ALA (80 µg/mL)-thioglycolate solution + laser irradiation (8 min, 33 sec). Yellow shows the UFCs of each of the groups, and there is a lower CFU amount in G4 compared to G2 and G3.

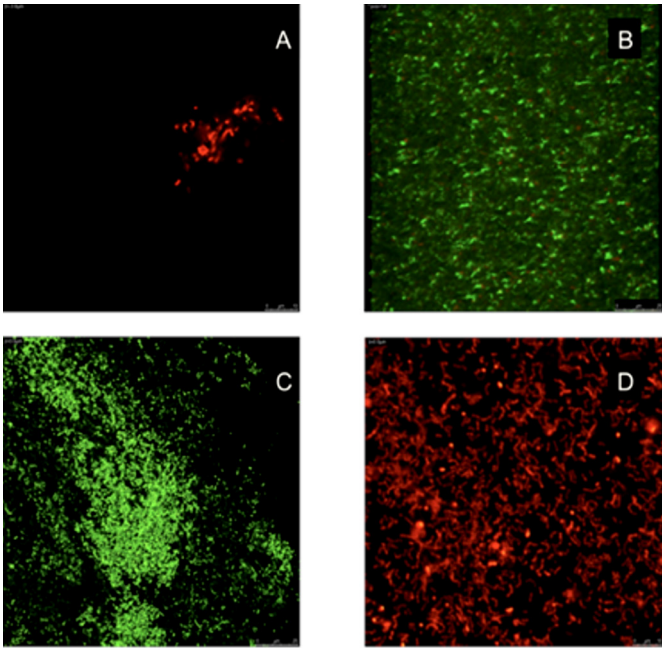

FIGURE 4 - LIVE/DEAD Test. Group "a" (GA), a positive control group treated with 800 µL of thioglycolate + 200 µL of NaOCl 5.25%; group "b" (GB), 1 mL of thioglycolate + laser irradiation (8 min, 33 sec), group "c" (GC), 1 mL δ-ALA (80 µg/mL)-thioglycolate solution; group "d" (GD), 1 mL of δ-ALA (80 µg/mL)-thioglycolate solution ($5X10^7$cells/mL) + laser irradiation (8 min, 33 sec). Group "a" show decomposition and an antibacterial effect on the biofilm treated with NaOCl 5.25%. Group "b" and group "c" do not show antibacterial effects or decomposition of the biofilm. Group "d" shows an antibacterial effect without decomposition of biofilm when exogenously administered δ-ALA and irradiated with an argon laser at 510 nm and 200 J/cm^2.

Determination of the antibacterial effect of PpIX over E. faecalis biofilm

The cell viability test evaluated by CTC of the biofilms treated with δ-ALA (80 µg/mL) and irradiated with an argon laser, 510 nm, 200 J/cm^2 (group D), show a small number of bacteria compared with the groups not treated (group "b" and group "c"), demonstrating that the activation of PpIX by a laser has an antibacterial effect, even without biofilm disruption (Figure 4).

DISCUSSION

PDT is a clinical procedure that has emerged as a non-invasive therapy for the treatment of infections (Dai *et al.*, 2012; Rajesh *et al.*, 2011) and represents an alternative to conventional pharmacological therapies. This therapy is a promising strategy to eliminate pathogenic microorganisms, including Gram-positive and Gram-negative bacteria, viruses, yeasts and fungi (Rajesh *et al.*, 2011; Sperandio, Huang, Hamblin, 2013), and its application could be particularly useful against resistant microorganisms or when traditional treatment does not completely eliminate microorganisms (Soukos *et al.*, 2006). Additionally, it was shown that PDT not only kills the microorganism, but may also lead to the detoxification of endotoxins,

such as lipopolysaccharides (Rajesh *et al*., 2011). Another advantage of PDT over traditional antibiotic treatments is that with PDT, is possible avoid the appearance of undesired drug-resistant bacteria strains (Sperandio, Huang, Hamblin, 2013).

In the present study, the antibacterial effect of PDT on *E. faecalis* in planktonic and biofilm forms was evaluated using δ-ALA as a pre-photosensitizer in combination with an argon laser. The results obtained by spectrofluorometry verified that the δ-ALA administered to the microorganism is metabolically transformed in PpIX. When the PpIX was irradiated by an argon laser, the amount of PpIX was reduced, thereby decreasing the intensity of PpIX, indicating that the PpIX was activated by laser irradiation. This outcome also corroborated the antibacterial effect on microorganisms in planktonic and biofilm forms, even without biofilm disruption, comparable with the effect of sodium hypochlorite (NaOCl).

Biofilms on a variety of medical devices have been studied extensively over the past 25 years, and research on some of devices has demonstrated the susceptibility of most materials to microbial adherence and biofilm formation (Talsma, 2007). The use of δ-ALA may become an alternative or complement to solve this clinical issue of medical devices, even without biofilm mechanical disruption.

The results obtained in this study are consistent with data reported previously about the use of PDT to treat bacterial infections (Kharkwal *et al*., 2011), which concluded that although PDT has some limitations that need to be overcome, it has the potential to be used to treat a number of infectious diseases.

The present results are similar to data obtained by López-Jiménez *et al.* (2015), who evaluated the effect of toluidine blue and methylene blue as PS agents in combination with a light-emitting diode, at 628 nm, 30 J, on a biofilm of an ATCC 29212 of *E. faecalis.* They reported that this protocol has effective antibacterial activity, and they also evaluated the surface of the biofilm treated with PDT by atomic force microscopy, reporting that the topography of the biofilm was modified after PDT treatment with an increase in the roughness of the surface of the biofilm. This effect could be beneficial because this could expose the bacteria of biofilms to the PS agent and light, producing a better antibacterial effect. It is important to highlight that toluidine blue and methylene do not a have wide range of activation such as PpIX, limiting its application to certain tissues.

In this study, a dose of 200 J/cm^2 was used based on the results reported by Li *et al.* (2013) who evaluated the use of δ-ALA on biofilm of methicillin-resistant strains (*S. aureus* and *S. epidermidis*), and reported that δ-ALA was absorbed and converted to PpIX by both types of bacteria strains. After the samples were irradiated with a semiconductor laser at different potencies (0, 100, 200, and 300 J/cm^2), an antibacterial effect was shown directly proportional to the dose of J/cm^2 administered with a max response at 200-300 J/cm^2. The present results are consistent with these data, showing the presence of PpIX after the exogenous administration of δ-ALA and a reduction in the amount of PpIX after laser irradiation. The results also showed an antibacterial effect at a laser dose of 200 J/cm^2.

Fotinos *et al.* (2008) demonstrated the ability to inactivate Gram-positive (methicillin-resistant *S. aureus*) and Gram-negative (two *E. coli* strains and a *P. aeruginosa* strain) bacteria by photodynamic therapy after incubation with δ-ALA. An inactivation rate of 99.9% was reported for both *E. coli* strains and *P. aeruginosa. S. aureus* was significantly more sensitive to δ-ALA porphyrins, with a survival rate even lower than the Gram-negative strains, demonstrating the antibacterial effect of porphyrins metabolized from δ-ALA on Gram-positive and Gram-negative bacteria. Similar results were obtained in this study, in which Gram-positive bacteria (*E. faecalis*) in planktonic and biofilm forms were used, demonstrating that activation of PpIX by an argon laser has an antibacterial effect even in the presence of a biofilm.

As previously discussed, the microorganisms in a biofilm are more resistant to antimicrobials (Balcázar, Subirats, Borrego, 2015; Donelli, 2014; Donlan, 2011; Li *et al*., 2013; López-Jiménez *et al*., 2015), and the most common methods to treat and eliminate biofilms are mechanical disruption and antimicrobial therapy; however, the efficacy of these strategies is limited (López-Jiménez *et al*., 2015; Rajesh *et al*., 2011). In this study, it was shown that the disruption of the biofilm is not necessary to inactivate the bacteria with the use of exogenously administered δ-ALA to produce intracellular PpIX. Barra *et al.* (2015) evaluated the application of δ-ALA and light-emitting diode irradiation on bacteria biofilms (*S. aureus, S. epidermidis, and S. haemolyticus*) using a viability cell test. It was shown that the viability of the three bacteria in the biofilm was reduced to 20% of the original values, but it was necessary to increase the light dose administered to

reach adequate bacterial inactivation, indicating that the light dose is a critical factor to achieve the desire effect. In the present study, it was necessary to increase the concentration of ∂-ALA to achieve an antibacterial effect on the biofilm. This may be because the biofilm acts a semi-barrier that limits the penetration of ∂-ALA to the bacteria cells.

One of the main disadvantages related to the clinical application of PDT is that the PS agents have specific absorption peaks, limiting the use of certain types of light sources, and in some cases the wavelength used cannot penetrate deep tissues. With δ-ALA as a pre-photosensitizer, it is possible to overcome this clinical situation because PpIX has different absorption peaks (410 nm, 510 nm, 545 nm, 580 nm, and 635 nm) (Hino *et al.*, 2013), offering the ability to be activated by different sources of light at different wavelengths, thus making it useful to treat bacterial infections on dermis and epidermis tissues (Sperandio, Huang, Hamblin, 2013; Wachowska *et al.*, 2011). Furthermore, δ-ALA has several advantages over other photosensitizers. It has a rapid metabolism and high selectivity for malignant lesions (Wachowska *et al.*, 2011) and bacteria (Barra *et al.*, 2015). In this study, all treatment groups incubated with δ-ALA show an antibacterial effect, including the G3 (δ-ALA, without laser irradiation). Finally, the rapid systemic clearance of ALA-induced PpIX within 24 h eliminates prolonged photosensitivity and allows treatment to be repeated at regular intervals without cumulative effects and the risk of damage to tissues (Wachowska *et al.*, 2011).

The production and accumulation of PpIX induced by an exogenously administered δ-ALA on *E. faecalis* biofilms was shown. The photoactivation of PpIX with an argon laser produced an antibacterial effect even without disruption of the biofilm. From our results, this therapy could become an alternative to conventional mechanical disruption or pharmacological therapies for the treatment of bacterial infections related to the presence of biofilm on biomedical devices that cannot be used treated with sodium hypochlorite. Exhaustive *in vitro* and *in vivo* studies are required to fully support the use of this methodology as an alternative or complementary method to treat bacterial infections.

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COMPLIANCE WITH ETHICAL STANDARDS

All actions performed in studies including human participants were in accordance with the 1964 Helsinki declaration and with the ethical standards of the institutional and/or national research committee.

ETHICAL APPROVAL

This study was approved by the Institutional Research Ethic Committee of the Faculty of Dentistry, Sinaloa University with the register code CEYS 2011-11.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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