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Introduction of 5-aminolevulinic acid as a theranostics agent in dentistry

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ABSTRACT

Objective: The aim of this paper is to study the theranostic potential of 5-Aminolevulinic Acid (5-ALA)in dentistry.
Methods: Photodynamic inactivation (PDI) and fluorescence spectroscopy of *Streptococcus sanguis*, and laser induced fluorescence (LIF) of several decayed teeth were performed using 5-ALA.
Results: In the absence of 5-ALA, 15 min illumination of the bacteria by the means of an LED light source led to only 1.16% viability reduction. On the other hand, 5-ALA revealed remarkable dark toxicity at concentrations above 20 μM. Furthermore, the synergistic effects of 10 μM 5-ALA and illumination by the light source for 5 and 15 min intervals led respectivelyto0.74log10 and 1.69log10 reduction of viability. Also, fluorescence spectroscopy of the bacteria showed a direct relationship between emission line intensity at 620 nm and the concentration of 5-ALA. In dental experiments, following exposing tooth with 40 mM 5-ALA, a significant autofluorescence growth was observed just in the decayed parts.
Conclusion: Based on the strong dual modality of 5-ALA to annihilate cariogenic bacteria through photodynamic inactivation and enhancing LIF intensity for identification of dental caries, 5-ALA is proposed as a theranostic agent in dentistry.

1. Introduction

At the present time, early stage diagnosis and consequently inactivation of cariogenic bacteria are greatly focused upon, due to their great impact on prevention and control of dental caries. Therefore, enormous attention is given to introduce modern theranostics methods in dentistry. Among modern methods, laser induced fluorescence (LIF) has played a promising role for several diagnostic purposes [1–3]. In LIF, light excitation of a fluorescent material at the main absorption peak generates a unique emission spectrum. Therefore, fluorescence spectrum, as the fingerprint of the dye, is extensively applied for the identification of biological targets such as cancerous cells and microorganisms [4–6]. This method is known as a developing harmless and rapid approach. Although LIF has been used for diagnosis of dental caries [7–10], identification of small size caries has remained an important subject of research. In order to overcome LIF failure to identify small caries, we propose the application of a nontoxic fluorescent dye to exclusively increase fluorescence intensity just in decayed parts of tooth.

On the other hand, photodynamic inactivation (PDI) reveals hopeful results for destruction of cariogenic bacteria with fewer side effects [11–17]. In photodynamic inactivation, synergistic application of photosensitizer, molecular oxygen and a suitable light source leads to the production of reactive oxygen species (ROS) [18]. Consequently, this process will cause destruction of the neighboring microorganisms. Among photosensitizers, porphyrin derivatives have certain appeal because of their biocompatibility and high production efficiency of singlet oxygen. Various prior experimental studies about the application of porphyrin derivatives for photodynamic inactivation of gram positive and gram negative bacteria have led to significant annihilation of both bacterial groups [19,20]. For example, Oriel et al. demonstrated the application of three porphyrins (cationic, anionic or neutral) and blue light for PDI of *Candida albicans* [21]. Also, porphyrins have been utilized for identification of dental caries based on laser induced fluorescence method [22–25]. According to these studies, dental caries can be detected based on red autofluorescence emission from natural porphyrins of oral bacteria. On the other hand, studies have shown a direct relationship between the LIF intensity and porphyrin concentration [26]. We think the introduction of an external source to produce porphyrin into the cariogenic bacteria probably may lead to a

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significant growth in the autofluorescence intensity. Despite porphyrin attractions, its applications in PDI and LIF are restricted due to its high price [27]. Fortunately, bacteria can turn 5-ALA, an inexpensive chemical component, into porphyrin derivatives through biological processes [28–30]. Aminolevulinic acid is approved to be used in photodynamic therapy to treat Actinic keratosis and glioma. Aminolevulinic acid is also being studied in the treatment of other conditions and types of cancer [31–35]. In addition, there are several reports about fluorescence guided surgery with Aminolevulinic acid in oncology [36–38].

Based on our study, theranostic potentials of 5-ALA in dental research have not been well investigated. To address this issue, this paper attempts to present a primary model of fluorescence diagnosis and photodynamic inactivation of *Streptococcus sanguis* using 5-ALA. *Streptococcus sanguis* is considered as one of the causes of dental caries [39]. The results of this research will be considered as a basis for future studies about simultaneous early stage diagnosis and inactivation of tooth decays.

2. Materials and methods

2.1. Ethics and dissemination

Ethical approval was provided by the ethics committee of the Babol University of Medical Sciences. Ethical approval number is IR-MUBABOL-REC-1397-030.

2.2. Preparation of 5-aminolevulinic acid hydrochloride solution

5-Aminolevulinic Acid hydrochloride (CAS Number 5451-09-2) with ≥98% purity was purchased from Sigma-Aldrich (Germany). The substance with the chemical formula $CH_2CH_2COOH_2NH_4$ has a molar weight equal to 167.59 g/mol. Reference solution with a concentration of 80mMwas obtained through diluting 0.134 g of powdered 5-Aminolevulinic Acid hydrochloride in 10 ml phosphate buffer saline solution. This solution was passed through a 0.25 μm filter. Then, different concentrations were obtained by diluting the reference 5-ALA solution with phosphate buffer saline.

2.3. Light exposure system

In our photodynamic inactivation experiments, illumination setup was the same as previously reported [40]. The system containing 6 high power red LEDs (XLamp XP-E, Cree, Shenzhen, China) was used to have a uniform illumination of samples. LEDs were set in two rows and three columns serially. The spectral distribution of LEDs was obtained by a spectrometer (Ava Spec 2048, Avantes, Apeldoorn, Netherlands). A 300 lines/mm spectrometer with 0.4 nm resolution power and NA = 0.2.A power supply (GPS-x303, Goodwill Instek CO., New Taipei City, Taiwan) was used in order to provide a constant current for the exposure system. A cooling system including a heat sink and a fan was installed to maintain the LEDs temperature constant. A power meter (PM160 T Thorlab CO., Dortmund, Germany) was also used to control the intensity of light during irradiation. The spatial distribution of light intensity at a surface set 4 cm away from the illumination system showed there was a uniformed area (2×2 cm^2) in the pattern. Therefore, for each of our vitro PDI experiments, 4 wells (2×2 wells) of a 96 well plate were placed right in this area (Fig. 1).

2.4. Culture of bacteria

The *Streptococcus sanguis* (ATCC 10556) was purchased from Iranian Research Organization for Science and Technology (Tehran, Iran). A ring of bacteria grown on blood agar medium was added to 100 ml of Brain Heart Infusion broth (BHI broth) into an Erlenmeyer flask. The flask was then placed in a shaker incubator (Pars Azma co. Model F.F-81, Tehran, Iran) at 150 rpm and 37 °C for 18 h. After that, the solution was centrifuged at 5000 rpm for 10 m and the supernatant was removed. Then, residual biomass was diluted into 10 ml phosphate buffer saline solution. At the end, the reference solution of *Streptococcus sanguis* (ATCC 10556) was prepared at the concentration of 1.72×10^8 CFU/ml. In order to prepare fresh reference samples, absorption of the bacterial solutions at 600 nm was compared to their references using a UV–vis spectrophotometer (Spekol 2000, Analytic jena Co., Jena, Germany).

2.5. Photodynamic inactivation experiments

In this section, individual or synergistic effects of the light and 5-ALA on the viability of the bacteria were investigated. For this purpose, four groups were introduced.

2.5.1. Main control group ($L^- A^-$)

This group contains reference bacteria without the presence of light and 5-ALA. The reference bacterial samples were kept in a dark shaker incubator at 150 rpm and 37 °C for 4 h. Next, the samples were diluted serially and then were distributed on blood agar medium. Then, the plates were incubated overnight at 37 °C for 24 h. Finally, a number of colonies grown on the surface of plates was counted.

2.5.2. Light control group (L^+A^-)

In this group, reference bacteria were exposed by the 30 mW/cm^2 LED light source for 15 min in the absence of 5-ALA. Then, the number of remaining bacteria was obtained based on the aforementioned method.

2.5.3. Drug control group (L^-A^+)

In this group, we added 2, 20, and 80 mM as well as 6, 10, 14, 20, 40, 80 and 200 μM 5-ALA samples into the same volume as the reference bacterial solutions. Resulting solutions were kept in a dark shaker incubator at 150 rpm for 4 h. After that, colony counting was done based on the serial dilution method as described for the main control group.

2.5.4. Photodynamic inactivation group (L^+A^+)

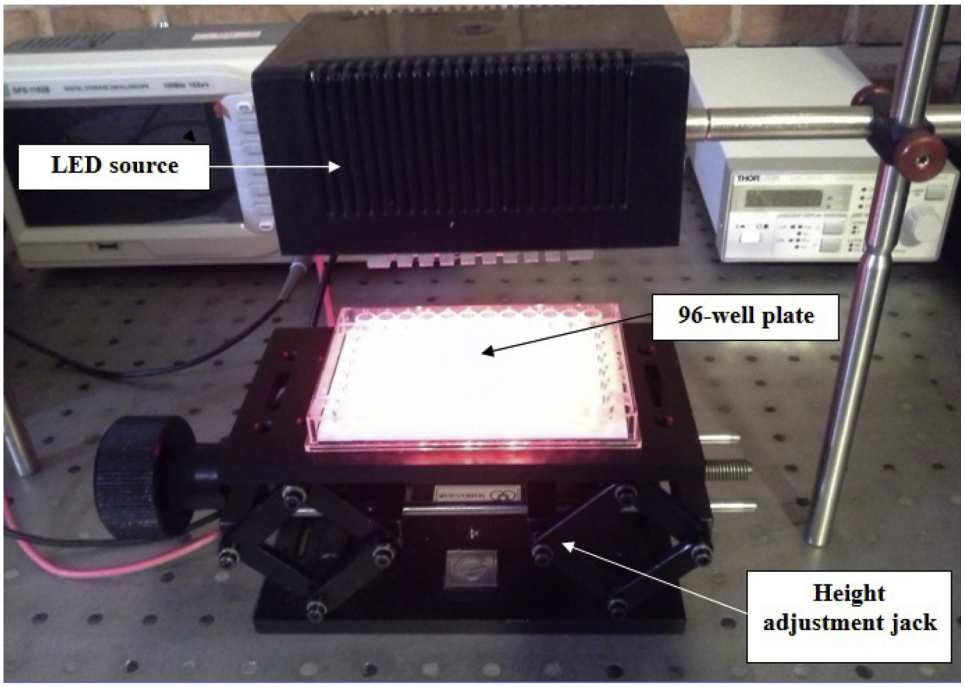
In these experiments, reference bacteria were added to the same volume of 5-ALA and then were illuminated by the LED irradiations. For this purpose, 5-ALA was mixed with equal volume of the reference bacteria and then the resulting samples were incubated at 37 °C for 4 h. At the next step, the samples were illuminated by the 30 mW/cm^2 LED system for 5 and 15 min. Finally, colonies were counted same as the main control group. All the experiments related to these four groups were repeated 3 times and then were statistically analyzed.

2.6. Laser induced fluorescence experiments

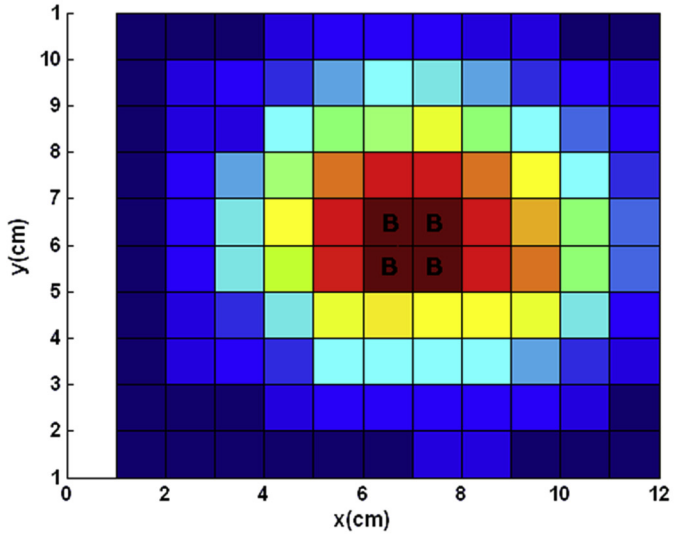
In order to demonstrate the potentials of 5-ALA for the diagnosis of cariogenic bacteria, laser induced fluorescence experiments were performed using 5-ALA in both in-vitro and Ex-vivo models.

2.6.1. Fluorescence spectroscopy

Fluorescence spectroscopy was performed to show the biological synthesis of porphyrins into the bacterial strain due to 5-ALA addition. For this purpose, 6, 10, 14, 20, 40, 80, 200, 2000 μM as well as 20 and 80 mM of 5-ALA solutions were prepared. These solutions were mixed with an equal volume as the reference bacterial solutions. Then, resulting samples were kept in a dark shaker incubator at 150 rpm and 37 °C for 4 h. After that, fluorescence spectrum of these samples was obtained using a fluorescence spectrophotometer (LS 45, Perkin Elmer, Waltham, Massachusetts, United States). The excitation and emission wavelengths were set at 380 nm and 620 nm, respectively.



(a)



(b)

Fig. 1. (a)The LED illumination system utilized in the PDI of *streptococcus sanguis* (ATCC 10,556) and (b) light intensity distribution on a surface located at 4 cm away from the LED source(dark brown cells (B) were selected for our PDI studies) [40].

2.6.2. Ex-vivo laser induced fluorescence experiments

In order to study the potential of 5-ALA to enhance the results of LIF method for the identification of decayed teeth, a series of experiments was performed. For this purpose, 12 decayed teeth were collected from the university dentistry. All the samples were kept in saline serum immediately after extraction, then were brushed completely. After that, two series of experiments were done on the teeth. First, a 10 mW continuous wave laser at 405 nm illuminated the healthy and decayed parts of the teeth. The resulting images were captured using a CCD camera. Then, the teeth were incubated by 40 mM 5-ALA solution for 4 h at 37 °C. After that, CCD imaging from the surface of healthy and decayed parts of the teeth were performed. A MATLAB code was used to analyze the quantitative changes of the obtained images (Fig. 2).

2.7. Statistical analyses

Our PDI experiments were repeated three times and the obtained results were statistically analyzed. One-way analysis of variance (ANOVA) and Tukey post hoc test were used for comparison of PDI results. Analysis was done using SigmaPlot 12.0Ink. Results with $P < 0.05$ were considered statistically significant.

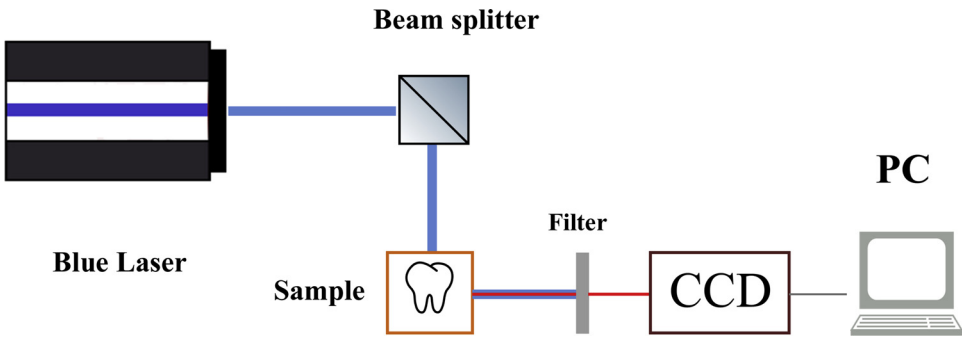


Fig. 2. Schematic arrangement of laser induced fluorescence set up for tooth decay imaging.

3. Results

3.1. Effect of the LED light on Streptococcus sanguis (ATCC 10556)

In this section, the effects of LED illumination on the viability of *Streptococcus sanguis* (ATCC 10556) are presented. Based on our results, 15 min illumination at 635 nm using the 30 mW/cm^2 LED source was led to a 0.005log10 (equal to 1.16%) viability reduction of *Streptococcus sanguis* (ATCC 10556). Therefore, according to the results of the light control group, the LED illumination (30 mW/cm^2, 15 min) had negligible effect on the survival of the bacteria.

3.2. Effect of 5-ALA on Streptococcus sanguis (ATCC 10556)

In this phase, dark toxicity of 5-ALA on the viability of *Streptococcus sanguis* (ATCC 10556) is studied. Fig. 3 shows the viability of the bacteria versus the concentration of 5-ALA in the absence of light. Based on our results, 10, 20, 40, 100 μM and 1, 10 and 40 mM concentrations of 5-ALA led to the reduction of the bacterial viability equal to 0.056 log10, 1.38 log10, 1.52 log10, 1.72 log10, 2.10 log10, 2.82 log10, and 3.22log10, respectively. According to this figure, 5-ALA was toxic for concentrations more than 20 μM. In addition, based on the results of the drug control group, 5-ALA showed considerable antibacterial effects on *Streptococcus sanguis* (ATCC 10556) at concentration more than 1 mM.

3.3. Effect of 5-ALA PDI on Streptococcus sanguis (ATCC 10556)

Fig. 4 shows the bacterial viability in the presence and absence of light for different 5-ALA concentrations. For the bacterial solutions containing 10μM of 5-ALA, *Streptococcus sanguis* (ATCC 10556) encountered a 0.74 log10and 1.69 log10 reduction of viability after 5 and 15 minillumination, respectively. Therefore, based on our observations, the synergistic effects of 30 mW/cm^2 and non-toxic concentration of 5-ALA (10 μM) led to a considerable reduction of viability for both exposure times. On the other hand, for toxic concentrations of 5-ALA, the results were more remarkable. As seen in Fig. 4a, 5 min illumination of *Streptococcus sanguis* (ATCC 10556) containing 100 μM 5-ALA led to a rise of viability reduction from 1.72 log10 to 3.3log10.

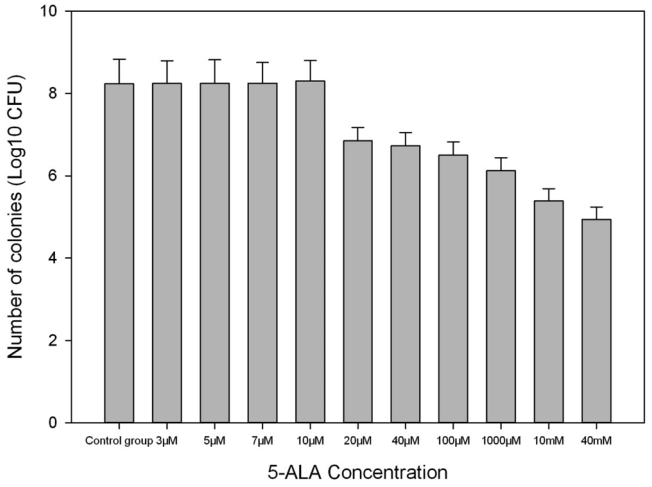


Fig. 3. The viability of *Streptococcus sanguis* (ATCC 10556) versus concentration of 5-ALA for drug control group.

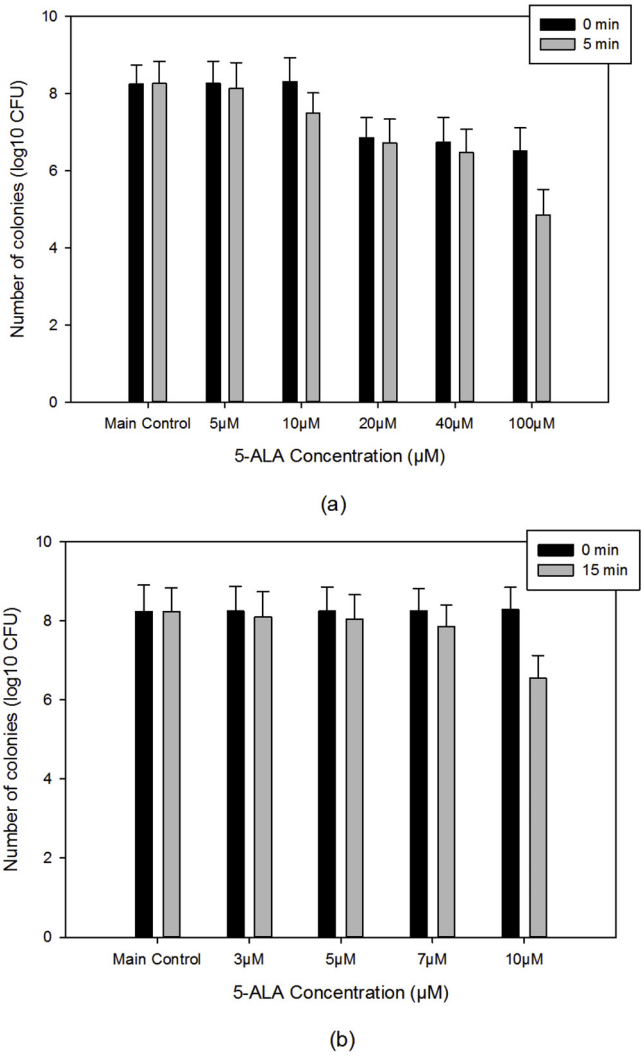


Fig. 4. Comparative representation of the bacterial viability at different concentrations of 5-ALA for (a) 5 and (b) 15 min exposure times.

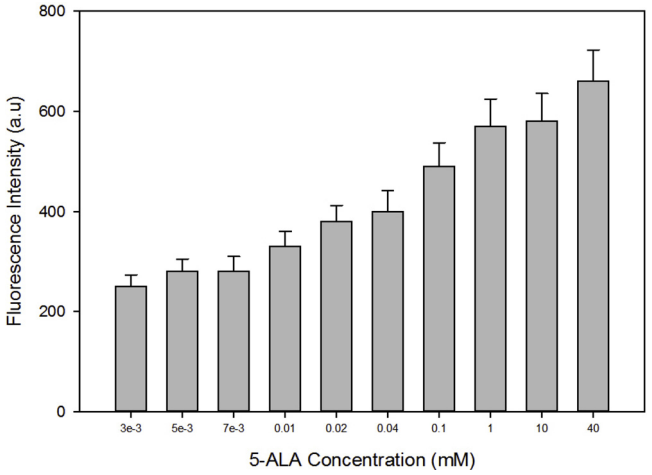


Fig. 5. The results of the fluorescence spectrophotometer. Most phorphyrin is produced at a concentration of 40 mM 5-ALA.

3.4. Fluorescence spectroscopy of Streptococcus sanguis (ATCC 10556)

The fluorescence spectroscopy of *Streptococcus sanguis* (ATCC 10556) in the presence or absence of 5-ALA are summarized in this section. For this aim, the bacteria were exposed to the excited wavelength of 380 nm and its fluorescence emission at 620 nm was recorded by the fluorescence spectrophotometer. Fig. 5 represents autofluorescence intensity of *Streptococcus sanguis* (ATCC 10556) versus 5-ALA concentration. Obviously, the height of the fluorescence peak at 620 nm increased by an increase of5-ALA concentration in the bacterial solution.

3.5. Results of ex-vivo LIF experiments

In this part, in order to identify dental caries, the positive effects of 5-ALA on intensity of LIF are investigated. Fig. 6 represents a comparison between the obtained results for two typical samples. Based on our observations, before 5-ALA incubation, the reflection of laser beam on the healthy parts of every tooth does not contain any red fluorescence light. On the other hand, in the case of decayed parts and before 5-ALA addition, a red autofluorescence was observed in all caries. For deep dental caries, as shown in Fig. 6b, the bacterial autofluorescence was more obvious and shiny. In contrast, before 5-ALA addition, the fluorescence intensity for small tooth decays was recorded weaker.

As can be seen in Fig. 6c and d, after 2 and 4 h of 5-ALA incubation, while there was still no fluorescence in healthy parts, the intensity of red fluorescence significantly increased for all dental caries. In order to get a quantitative analysis, we used a MATLAB code. The code gave us a gray scale histogram for each image. In fact, the obtained histogram revealed numerical distribution of the pixels in a 256 gray scale. According to Fig. 7, a clear difference between the histograms obtained from teeth before and after 5-ALA incubation was observed. For all dental carries, the number of pixels related to high gray numbers (especially 256) increased significantly after 5-ALA addition. For example, the arbitrary number of pixels related to gray number 256 for typically deep caries were 5×10^{-3}, 1.1×10^{-2} and 2.4×10^{-2} for 5-ALA incubation times 0, 2 and 4 h, respectively. Therefore, growth of the autofluorescence intensity was observed for both 2 and 4 h incubation times with a preference for 4 h. In other words, autofluorescence of dental caries showed an upward trend with time after 5-ALA addition. In addition, the pixels of lower gray numbers (related to lower fluorescence intensity) were clearly reduced for all the deeply decayed teeth.

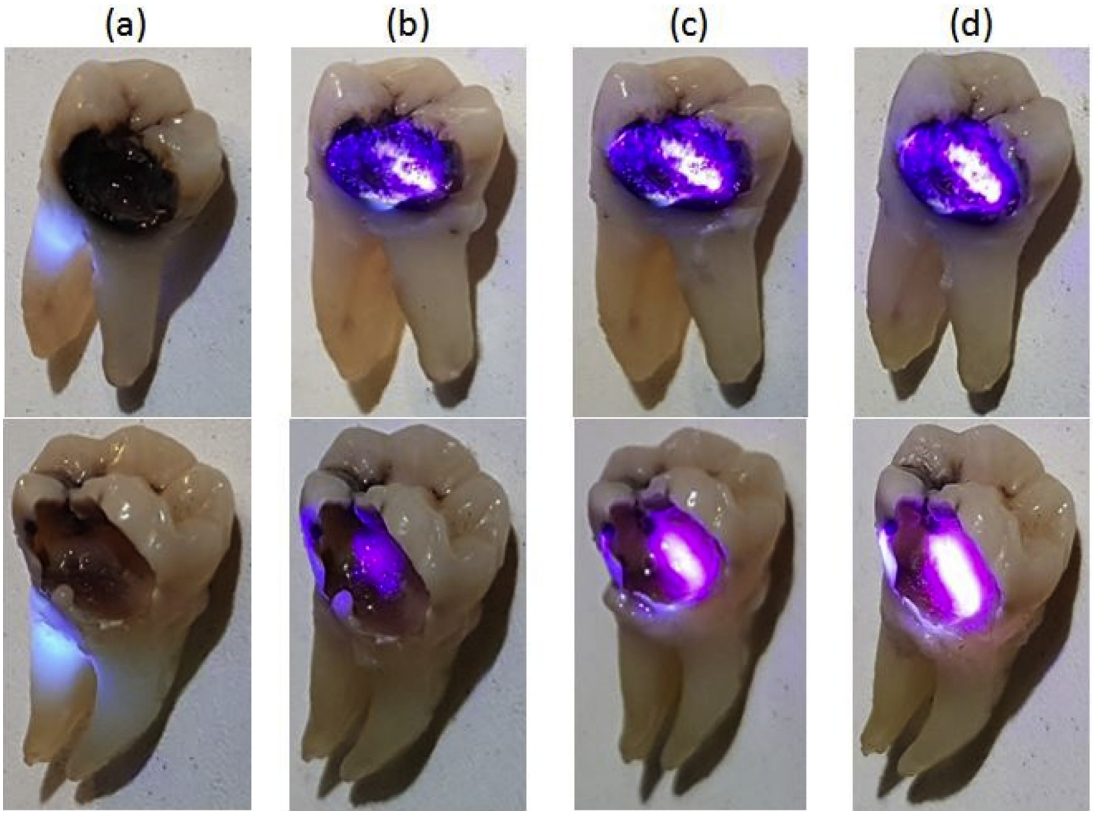


Fig. 6. LIF results of teeth. (a) Illuminating the healthy part of tooth. (b) Illuminating the decayed part of tooth. Illuminating the decayed part of tooth that incubated with 5-ALA for (c) 2 h and (d) 4 h.

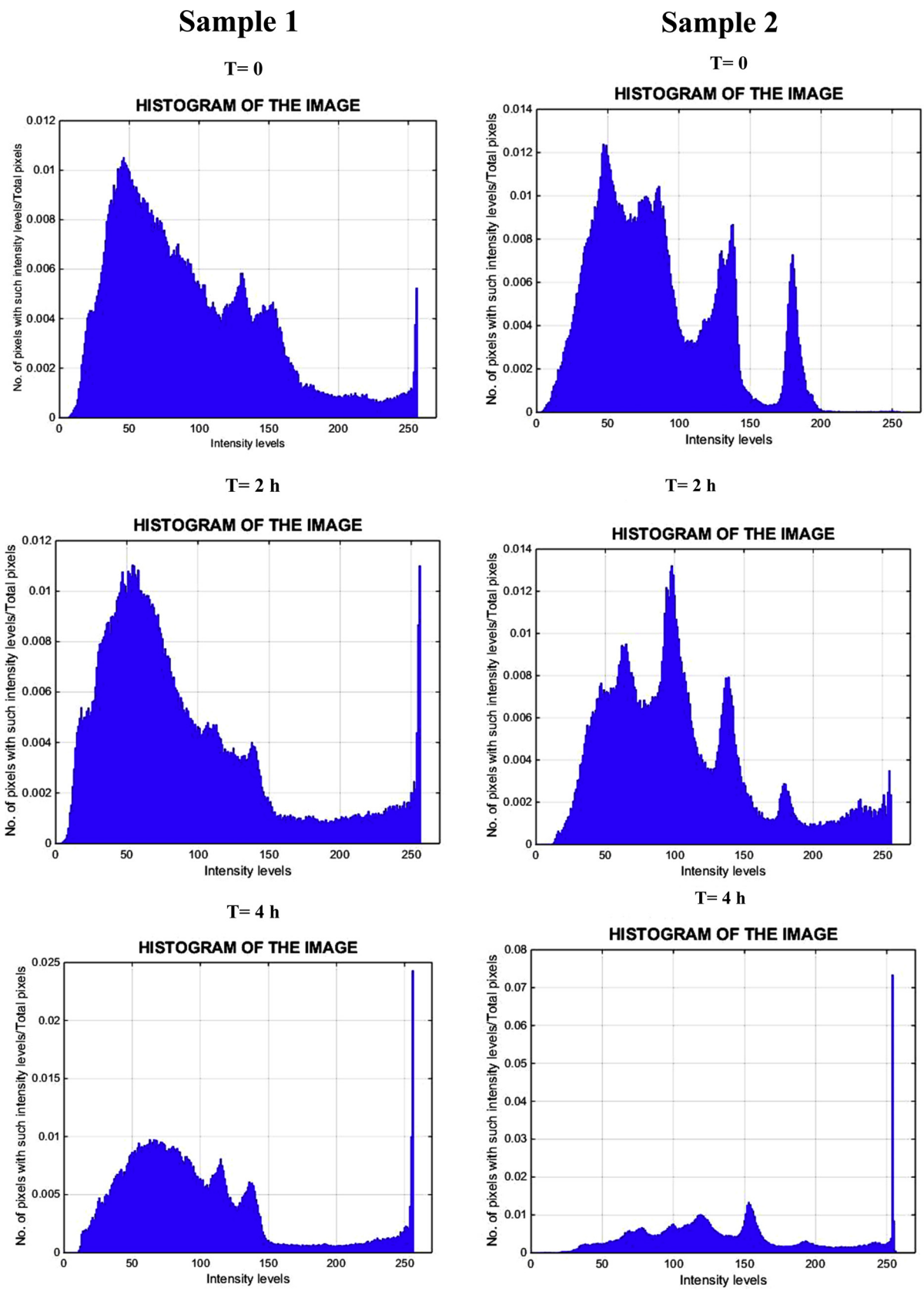


Fig. 7. Histogram of two samples without (T = 0) and with (different incubation times T = 2 and T = 4 h) 5-ALA incubation.

4. Discussion

According to our experimental results, the addition of 5-ALA into the bacterial solution showed a considerable toxicity in concentrations over 20 μM. The obtained results are in accordance with some reports that investigated the toxicity of porphyrins [41–44]. For example Wilson et al. showed that adding hematoporphyrin led to ~90% reduction of *S.sanguis* viability [43]. According to Ito et al. 5-ALA at mM concentration levels induces oxidative stress through the production of reactive oxygen species (ROS). These observations can be explained by the initial attack on the cell membrane by ROS produced in the medium outside the cell and also provide insight into possible uses of 5-ALA in cancer chemotherapy [45]. Stojiljkovic et al. also studied antimicrobial and antiviral properties of several porphyrins based on their ability to catalyze peroxidase and oxidase reactions, absorb photons and generate reactive oxygen species (ROS) and partition into lipids of bacterial membranes [44].

Although individual effects of the LED light had negligible effect on the viability, the synergistic effects of light and 5-ALA on the survival of the bacteria was obviously considerable. This result is justified due to the rapid growth of reactive oxygen species during light illumination and consequently photo oxidation of neighboring bacterial substances. As a result, these photochemical processes led to the death of the bacteria. These results are in accordance with some studies on PDI of bacteria using 5-ALA [46–48]. For example, Ming Hsieh et al. studied antibacterial photodynamic inactivation of *S. aureus* and *P. aeruginosa* using 5-ALA. They employed 1, 2.5, 5 and 10 mM concentrations of 5-ALA and a system of red LEDs with the energy density of 216 J/cm^2 [49]. According to their results, survival of both *S. aureus* and *P. aeruginosa* were decreased with a growth of 5-ALA concentration. Also Dutta et al. investigated PDI of *Leishmania amazonensis mutants* considering 5-ALA incubation. In fact, these bacteria had been engineered to accumulate uroporphyrin when treated with 5-ALA [50].

On the other hand, in our in-vitro fluorescence spectroscopic experiments, a difference between the emission intensity of the main control group and drug control groups was observed. This difference establishes formation of porphyrin after 5-ALA incubation into the bacteria. This claim is approved based on previous reports about the biological synthesis of porphyrin derivatives in cells and microorganism [51–54].

Finally, we investigated the positive effects of adding 5-ALA into LIF results on the teeth. According to our results, although no fluorescence was seen in the healthy parts of teeth before and after 5-ALA addition, there was an intrinsic auto fluorescence in the decayed parts of the teeth. Several groups have reported these observations already. According to their studies, fluorescence from dental caries was observed when the tooth was exposed to the suitable laser beam. Based on those reports, the observed fluorescence arises from natural porphyrins presented in the decayed parts of tooth.

Furthermore, 5-ALA addition led to a significant growth in the fluorescence intensity of decayed parts of these teeth. This result relies on the fact that the microorganisms in dental caries, especially bacteria, transform 5-ALA to porphyrin derivatives. This claim was previously validated by several reports [55,56]. For example, Fotinos et al. have shown the growth of porphyrin concentration after 5-ALA incubation of *Escherichia coli*, *E. coli* Ti05, *Pseudomonas aeruginosa* and *Staphylococcus aureus* [57]. According to their report, the type and concentration of the produced porphyrins depends on the type of bacteria. Therefore, 5-ALA incubation of dental caries leads to an increase of fluorescent porphyrins in caries and that in turn leads to a growth in LIF intensity. This significant growth in LIF intensity will be valuable especially for the identification of early stages of caries formation. Hence, based on our experimental results, fluorescence spectroscopy method is proposed in order to confirm the existence of dental plaque bacteria utilizing efficient concentrations of 5-ALA.

Although our experimental results seem promising but vast experiments are crucially required to determine the 5-ALA theranostic potentials for dental purposes. For example, several studies are needed to decrease the porphyrin formation time in the bacteria through introduction of the suitable catalyst to accelerate the reaction. Finding the appropriate time for 5-ALA to porphyrin transformation may be considered as a basis for future clinical LIF diagnosis and PDI treatment studies. In addition, future researches will be focused on introducing other fluorescent agents in order to exclusively increase LIF intensity originated from dental bacteria. As the main conclusion, the present paper is a prelude for next researches about the theranostic potentials of 5-ALA in dentistry. Similar experiments for other cariogenic bacteria especially *S. mutans* as the leading cause of dental caries are proposed for future works.

5. Conclusion

The aim of this paper is to study thetheranostic potentials of 5-ALA in dentistry. According to our experimental results, 5-ALA plays a dual modality role for diagnostic and inactivation purposes. Based on our observations, in spite of the intrinsic fluorescence observed in dental carries, the 5-ALA incubation leads to a significant growth in the LIF intensity. In addition, 5-ALA shows desirable results in PDI of *Streptococcus sanguis* (ATCC 10556). This dual modality of 5-ALA makes it a potent theranostic agent for dentistry applications.

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