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ORIGINAL ARTICLE

Effects of antibacterial photodynamic therapy on salivary mutans streptococci in 5- to 6-year-olds with severe early childhood caries

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Abstract Antibacterial photodynamic therapy (A-PDT) has been shown to kill oral bacteria in the planktonic culture, dental plaque, and biofilm. This study sought to assess the antimicrobial effect of A-PDT with toluidine blue O (TBO) and diode laser on salivary mutans streptococci in 5–6-year-olds with severe early childhood caries (SECC). This case-control study was conducted on 56 children with SECC divided into four groups, namely 0.1 mg/mL TBO, diode laser (633 nm, 20 mW, 6 J/cm^2), combination of the two, and no intervention control group. A-PDT was performed on days 1 and 3. Salivary samples were collected before and after A-PDT on days 1 and 3, and 1 and 2 weeks after the second intervention (day 3). Samples were cultured on mitis salivarius agar, and after incubation, the colonies were counted. Data were subjected to repeated measures ANOVA, ANCOVA, and paired comparisons with least square difference and Tukey's test. Bacterial count significantly decreased on days 1 and 3, and 1 and 2 weeks after the second intervention. Bacterial count also decreased following the use of TBO and laser separately, but these reductions were not significant ($P > 0.05$). Within the limitations of this study, antimicrobial efficacy of TBO + laser was higher than that of diode laser or TBO alone. Durability of treatment increased with double-dose therapy. This modality may be used to decrease the colony count of salivary mutans streptococci in children with SECC.

Keywords Severe early childhood caries · Antibacterial photodynamic therapy · Toluidine blue O · Diode laser

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Introduction

Tooth decay is the third most common disease worldwide after cardiac disease and cancer. *Streptococcus mutans* is the main cause of caries due to its colonization and biofilm formation on tooth surfaces [1]. Severe early childhood caries (SECC) is a destructive form of dental caries prevalent among infants and pre-school children, and is among the most serious dental problems in childhood [2, 3]. The severe form of early childhood caries (ECC) is characterized by a dmfs score higher than 4 at the age of 3 years, higher than 5 at the age of 4 years, and higher than 6 at the age of 5 years [4–6]. ECC is defined as the presence of one or more decayed (cavitated or non-cavitated), restored or lost (due to carious lesions) surfaces in each deciduous tooth of a 71-month-old or younger child [5, 6]. High prevalence of ECC in developing and even some developed countries, despite the implementation of preventive measures, is of concern [4]. Clinical manifestations of ECC include pain, acute and chronic abscess, fever, and swelling of the lips or cheeks [7]. It can lead to masticatory problems, malnutrition, gastrointestinal problems, speech problems, and embarrassment [7, 8]. Growth retardation especially in terms of height and weight has also been reported [7–9].

A correlation has been shown between SECC and iron deficiency anemia, which significantly affects growth and development [10]. Consequences of ECC include increased risk of new carious lesions, increased frequency and cost of dental treatments, the need for emergency dental visits, carious lesions in permanent teeth, increased absent days from kindergarten, and development of malocclusion in some cases [7, 8, 11].

Several factors such as host susceptibility and the presence of acidogenic and aciduric bacteria affect the initiation and progression of childhood caries. *S. mutans* is the primary culprit responsible for development of caries [12]. Salivary concentration of mutans streptococci in children with ECC is higher than the rate in healthy population [8].

Antibacterial photodynamic therapy (A-PDT) also known as photo-activated disinfection is a new treatment modality incidentally discovered in the early twentieth century [13]. A-PDT is a new approach for inhibition of cariogenic bacteria and prevention of periodontal disease. Absence of genotoxic and mutagenic effects in this method guarantees its long-term safety. The advantages of this method include elimination of bacteria in a very short time (within a few seconds to a few minutes), no injury to the adjacent tissues, access to areas with complex anatomy, low risk of bacteremia in immunocompromised patients, and high reproducibility [14, 15]. Toluidine blue O (TBO) and methylene blue are among the common photosensitizers (PSs) used in the oral cavity. TBO associated with laser irradiation has been suggested for bacterial eradication and has been used for reducing the bacterial count especially mutans streptococci [16].

Previous studies have shown the capability of PDT for eradication of oral bacteria in planktonic cultures [17, 18], dental plaque [19], and biofilm [20, 21]. However, studies on the efficacy of PDT for reducing the population of mutans streptococci are sparse. Considering the significance of decreasing the count of mutans streptococci in the oral cavity in patients with SECC, and the possible efficacy of PDT for this purpose, the present study sought to assess the effects of PDT on mutans streptococci using TBO and diode laser in 5–6-year-olds with SECC. The null hypothesis of the study was that the magnitude of reduction in mutans streptococci count at different time points post-intervention would be equal in the four groups of TBO, laser, TBO + laser, and control compared to the baseline value (day 1 before the intervention).

Material and methods

This case-control study was conducted on 5–6-year-old children with SECC. The required number of children in each group was calculated to be at least 13 based on a similar previous study [22] and assuming type I error = 5%, type II error = 20%, and study power = 80% [22] for estimation of the reduction in number of microorganisms by 95% using the ratio estimation equation. Written informed consent was obtained from the parents/legal guardians of children.

Eventually, four groups of 14 including three intervention groups and one control group were evaluated (Table 1). A total of 56 children aged 5–6 years were selected from Tehran kindergartens using randomized consecutive sampling. The inclusion criteria were dmfs greater than 6 and optimal patient cooperation. Children unable to spit, those with a history of antibiotic therapy in the past 2 weeks and subjects with systemic diseases were excluded from the study. After obtaining an approval from the Iranian Welfare Organization, a written informed consent was obtained from the parents for collection of children's salivary samples. The children received oral hygiene instructions in an informatory session, and the parents were informed that their children had to be excluded from the study in case of any antibiotic use during the course of study. In the first session before the initiation of treatment, salivary samples were collected from children. For collecting unstimulated salivary samples, the children (fasted for 2 h prior to saliva collection) were seated and requested to hold their saliva in their mouth for as long as possible. Next, 2 mL of saliva was collected by the spitting method and transferred to a sterile microtube. Children (n = 56) were then randomly divided into four groups namely TBO (group 1), laser (group 2), TBO + laser (group 3), and control (group 4). Group 1 children were requested to rinse 15 mL of 0.1 mg/mL TBO solution, hold it in their mouth for 5 min (interruptedly), and then spit it out (TBO was used as the PS in our study). The children were requested to hold it in their mouth for as long as possible and then spit it out and take another sip. This process was repeated, and the time periods were added up until a total of 5 min was reached. A total of 100 mg of TBO powder (Merck, Germany) was diluted with sterile distilled water according to the manufacturer's instructions in order to obtain 0.1 mg/mL concentration of TBO solution. The solution was stored in a completely dark room at 4–8 °C.

Red diode laser (InGa ALP; Mustang 2000, Russia) with 633 nm wavelength (continuous dominant), power of 20 mW, 6 J/cm^2 energy density, and KLO4 output nozzle with 1 cm^2 cross-sectional area was used in group 2 and was irradiated on the tongue mucosa and buccal and lingual tooth surfaces for 5 min (continuous wave mode). The entire mucosa of the tongue was laser irradiated from the tip to the base for 1 min. The entire palate was laser irradiated for 1 min. The

Table 1 Study groups

Groups	Interventions
Group 1	TBO
Group 2	Laser
Group 3	TBO + laser
Group 4	Control (no intervention)

entire buccal mucosa of the maxilla from one side to the other was laser irradiated for 90 s. The entire buccal mucosa of the mandible from one side to the other was laser irradiated for 90 s. The mean distance from the device tip to the target site was 5 mm [time (300 s) × power (20 mW) = 6 J/cm^2].

Group 3 children rinsed the TBO mouthwash (similar to group 1) and were then subjected to laser irradiation (similar to group 2) for photo-activation of TBO. In the aforementioned three groups, salivary samples were collected again 1 h later (for the purpose of standardization) for bacterial counting. Three days later (second session), salivary samples were collected, and the intervention was repeated in all three groups similar to what was done in the first session, and 1 h later, salivary samples were collected again for bacterial counting. One week later (third session, 2 weeks after the first session), salivary samples were collected again, but no intervention was performed. In the fourth session (2 weeks after the second intervention), salivary samples were collected again, and the children were referred to the Department of Restorative Dentistry for restorative treatments. In group 4 (control), salivary samples were collected on the first and third days and at 1 and 2 weeks after the second intervention. Salivary samples were immediately sent to the microbiology laboratory of the university.

Bacterial culture

Samples were diluted in 1:10 ratio. Mitis salivarius agar supplemented with 0.2 units/mL of bacitracin was used to culture mutans streptococci and prepared in the Microbiology Department of Shahid Beheshti University of Medical Sciences according to the standard protocol and was autoclave-sterilized at 121 °C and 15 psi pressure for 15 min before use. Using a standard titanium loop, 0.05 cm^3 of the sample was obtained close to the flame and cultured on the culture media in a checkered fashion. All cultures were done under a hood. Culture plates were then incubated at 37 °C with 10% CO2 for 24 h. Mutans streptococci colonies were of smooth type and showed strong adhesion to the medium and were dark in color. Gram-stained slides were prepared from the cultures, and the cultured bacteria were microscopically evaluated. Since a standard 0.05 mL titanium loop was used in our study, the coefficient of dilution of the loop was calculated as follows: 1 mL/0.05 = 20. A colony counter (Model 30BZG; WTW, Germany) was used for measuring the number of colonies. The obtained colony count was multiplied by the coefficient of dilution of the loop (20), and then since, the samples had been diluted 1:10, the value was multiplied by 10. The final colony count was reported in colony forming units per milliliter (CFUs/mL).

Statistical analysis

Data were analyzed using SPSS version 18 software. The mean and standard deviation (SD) of mutans streptococci count in the study groups at different time points were calculated and compared with the pre-intervention baseline value. Changes in bacterial count were recorded. Repeated measures ANOVA was used for the comparison of values obtained during six measurements between groups. The change in colony count in the TBO + laser group was statistically significant. Thus, LSD test was applied for pairwise comparisons of bacterial count at different time points. In order to compare the colony count in each treatment group and at each time point, ANCOVA was applied aiming to eliminate the effect of baseline colony count. Reductions in number of colonies at different sampling time points in different groups were statistically analyzed using one-way ANOVA. In case of significant results, pairwise comparisons were carried out using Tukey's test. Level of significance was set at $P = 0.05$ and P values ≤ 0.05 were considered statistically significant.

Results

Table 2 shows the mutans streptococci count in TBO, laser, TBO + laser, and control groups at different time points. Considering the repetition of measurements at six different time points, repeated measures ANOVA was applied for comparison of bacterial count in different groups (between-subject factor) and at six measurement time points in each group (repeated factor). The results of repeated measures ANOVA showed that the interaction effect of the mentioned two factors was statistically significant ($P < 0.05$). Figure 1 shows the mutans streptococci count in salivary samples of children in different groups and at different time points.

Based on the obtained results, mutans streptococci count significantly decreased in the TBO + laser group at all time points after the intervention. Thus, pairwise comparison of bacterial count at different time points was carried out in this group using LSD test (Table 3).

In the TBO + laser group, reduction in mutans streptococci colony count at all measurement time points was statistically significant (compared to baseline, $P < 0.05$) except for day 3 prior to the second intervention ($P > 0.05$).

In order to compare the efficacy of different interventions, the bacterial count at the time of measurement was subtracted from the baseline value at day 1, and ANCOVA was applied to eliminate the effect of baseline bacterial count (dependent variable was the subtraction of bacterial count at the time of measurement from the baseline value, and the independent variable was the type of intervention). ANCOVA revealed significant differences in bacterial count between different

Table 2 Mutans streptococci colony count in the groups at different time points (n = 14)

Group	Time point	Mean	Std. deviation	Minimum	Maximum
Control	Day 1	26,357	29,510	4000	100,000
Control	Day 3	26,142	25,434	5000	80,000
Control	1 week	24,500	21,625	3000	70,000
Control	2 weeks	24,142	24,778	3000	80,000
Laser	Before intervention day 1	29,212	22,925	3000	80,000
Laser	After intervention day 1	19,142	21,873	3000	80,000
Laser	Before intervention day 3	18,214	19,224	4000	70,000
Laser	After intervention day 3	14,428	14,410	3000	50,000
Laser	1 week	17,214	18,208	5000	70,000
Laser	2 weeks	29,857	20,709	4000	70,000
TBO	Before intervention day 1	16,285	19,628	3000	80,000
TBO	After intervention day 1	9500	17,796	1000	70,000
TBO	Before intervention day 3	12,714	9546	1000	40,000
TBO	After intervention day 3	4500	2503	1000	10,000
TBO	1 week	7500	2377	3000	12,000
TBO	2 weeks	11,214	4526	8000	25,000
TBO + laser	Before intervention day 1	32,000	28,457	7000	100,000
TBO + laser	After intervention day 1	7857	8328	0	20,000
TBO + laser	Before intervention day 3	19,500	15,026	4000	50,000
TBO + laser	After intervention day 3	4928	7819	0	30,000
TBO + laser	1 week	12,714	14,588	2000	80,000
TBO + laser	2 weeks	16,428	19,527	4000	80,000

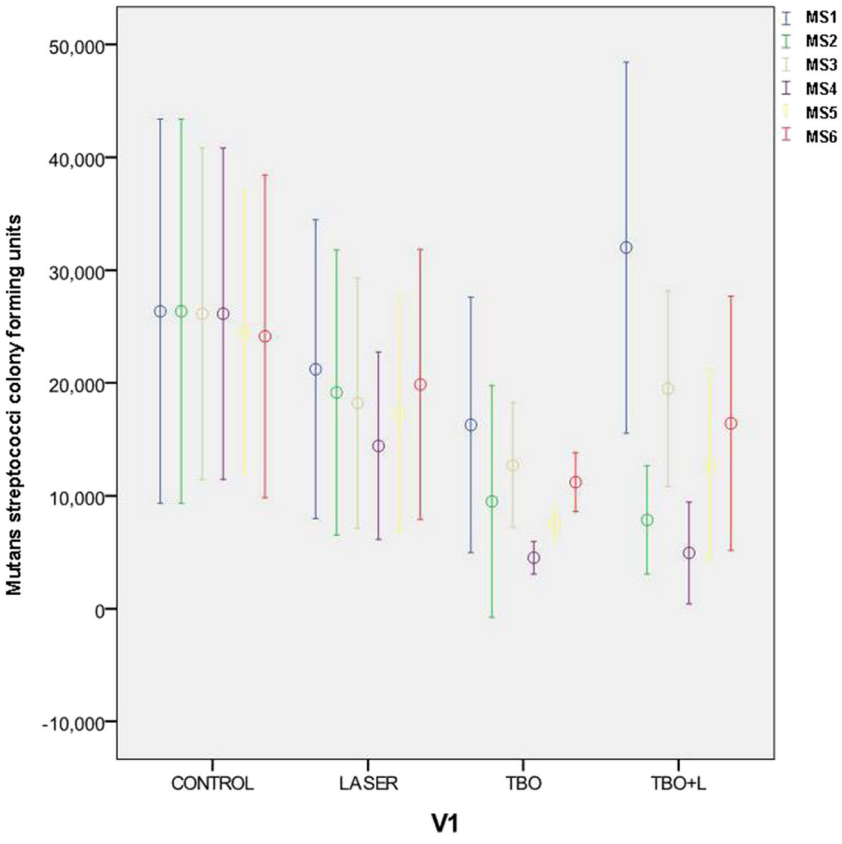

Fig. 1 Error bar of the mean count of mutans streptococci with 95% confidence interval of the mean in the study groups at different sampling time points

Table 3 Pairwise comparison of mutans streptococci colony count in the TBO + laser group (LSD test)

First time point	Second time point	*P* value
Before intervention day 1	After intervention day 1	0.001
Before intervention day 1	Before intervention day 3	0.07
Before intervention day 1	After intervention day 3	0.002
Before intervention day 1	1 week later	0.005
Before intervention day 1	2 weeks later	0.009
After intervention day 1	Before intervention day 3	0.0001
After intervention day 1	After intervention day 3	0.12
After intervention day 1	1 week later	0.14
After intervention day 1	2 weeks later	0.07
Before intervention day 3	After intervention day 3	0.0001
Before intervention day 3	1 week later	0.03
Before intervention day 3	2 weeks later	0.47
After intervention day 3	1 week later	0.01
After intervention day 3	2 weeks later	0.03
1 week later	2 weeks later	0.13

groups at day 1 post-intervention, at day 3 post-intervention, at 1 week after the second intervention, and 2 weeks after the second intervention ($P < 0.05$). However, no significant difference was found in bacterial count in different groups at day 3 before the intervention (after eliminating the effect of baseline bacterial count, $P = 0.1$). Considering the lack of a significant difference in the results of ANCOVA, no other comparisons were made in this respect.

Table 4 shows the results of pairwise comparison of groups at the second time point (day 1 post-intervention), fourth time point (third day after the second intervention), fifth time point (1 week after the second intervention), and sixth time-point (2 weeks after the second intervention).

As observed in Table 4, bacterial count in TBO + laser group was significantly different than that in other groups at all measurement time points ($P < 0.05$).

In the TBO and laser groups, reduction in colony count was greater than that in the control group, but the three intervention groups were not significantly different in this respect ($P > 0.05$).

Discussion

This study evaluated the efficacy of laser-activated antimicrobial agents against mutans streptococci in salivary samples of patients with SECC as a new therapeutic approach.

Different lasers have been used in relevant studies, but diode laser is the most commonly used laser in clinical trials including our study [23]. We used diode laser with 20 mW power, 633 nm wavelength, and 6 J/cm^2 energy density in two irradiation cycles. The second irradiation was done at day 3. Diode lasers are more cost-effective and user-friendly and easily transferable compared to other laser types. They are also battery operable and can be used in body cavities [24]. PDT lasers with lower exposure dose have greater usage than those with higher exposure doses [23].

In our study, the selected PS was TBO, which is the most commonly used PS in studies for dental purposes. TBO has a wide absorption band at the red light wavelength (620–660 nm) with a maximum absorbance at 630 nm [22, 25]. Diode laser used in our study had maximum absorbance very close to that of TBO. TBO is a blue-violet, hydrophilic solution that is toxic even in the dark and has destructive effects on the cell membrane.

Our study showed that TBO combined with laser irradiation can decrease the number of mutans streptococci. The highest antibacterial efficacy in our study was observed in the TBO + laser group followed by the TBO and laser irradiation groups. However, reduction in mutans streptococci count was only significant in the TBO + laser group, which can be explained by the mechanism of action of PDT. Several studies have shown the effectiveness of PDT with PS in reducing oral bacterial count. Williams et al. in their study in 2003 evaluated the susceptibility of *S. mutans* to photo-activated disinfection when the microorganism was placed in an environment simulating a carious lesion and showed that antibacterial treatment by photo-activation with laser (633 nm) and TBO could cause significant reduction of oral bacteria especially *S. mutans*. These results are in agreement with our findings [26]. Zou et al. in an in vitro study in 2008 studied the effects of PDT with hematoporphyrin monomethyl ether (HMME) on the

Table 4 Pairwise comparison of treatment groups in terms of changes in mutans streptococci colony count at different time points

Group 1	Group 2	*P* value 2nd time point	*P* value 4th time point	*P* value 5th time point	*P* value 6th time point
TBO	Laser	0.69	0.88	0.88	0.89
TBO	Laser + TBO	0.001	0.02	0.036	0.02
TBO	Control	0.39	0.32	0.7	0.95
Laser	Laser + TBO	0.0001	0.02	0.04	0.03
Laser	Control	0.96	0.76	0.99	0.99
Laser + TBO	Control	0.0001	0.001	0.04	0.04

viability of *S. mutans* strains in biofilm. They demonstrated that PDT with HMME was capable of destroying *S. mutans* strains in the biofilm [1]. Lima et al. in 2009 reported that PDT with diode laser (two different energy powers of 47 and 94 J/cm^2) and TBO was effective for eradication of microorganisms present in dentin caries in the clinical setting and that it can be used for elimination of bacteria before tooth restorations [27]. The results of the clinical trial by Lima et al. were in accord with our findings. Rolime et al. in 2012 reported that use of TBO (in comparison with other PSs) eliminated 99.9% of mutans streptococci [28]. They reported the highest efficacy of PDT in use of TBO compared to other studies and their findings were in agreement with our study results.

Numerous variables can affect A-PDT protocol such as the photon sources with different physical parameters, PSs with different concentrations and method of exposure [29]. The maximum absorbance of PS should be close to the wavelength of the radiated light in order to ensure the formation of reactive oxygen species responsible for elimination of bacteria [30–32]. Photosensitizers used in studies mostly include methylene blue and TBO which are both phenothiazine dyes with maximum absorbance at 656 and 635 nm, respectively [33].

Wainwright in 1998 indicated that photodynamic inactivation of microorganisms depends on the chemical structure of the PS and duration of incubation of bacterial cells with the medication [34]. Bacterial cell wall damage, increased permeability of cytoplasmic membrane, and breakage of nucleic acid chains following A-PDT are also probable [34].

The mechanism of action of A-PDT is based on a reaction that involves a non-toxic PS (such as methylene blue, TBO, aluminum disulphonatephthalocyanin, or carbon derivatives) and visible light. The combination of these two factors in the presence of oxygen initiates a cascade of biological events by generation of reactive oxygen species and consequent apoptosis and cell death [15]. The stimulated PS is combined with molecular oxygen and produces reactive oxygen species that cause cell damage and cell death [15]. The aforementioned three factors are harmless and inactive when alone, but in combination with oxygen, they are converted to toxic agents causing the death of tumor cells and microorganisms. Due to this mechanism, diseased tissues have greater odds of being targeted, because only the cells that are exposed to the PS, light, and oxygen simultaneously undergo cytotoxic changes [35]. Since Gram-positive bacteria have a porous cytoplasmic membrane, they allow greater penetration of PS into their cytoplasm compared to Gram-negative bacteria [36]. Thus, TBO is a suitable PS for Gram-positive mutans streptococci. On the other hand, Gram-positive bacteria are more easily killed by A-PDT than Gram-negative bacteria [37]. Gram-positive bacteria are more susceptible to A-PDT; this difference is attributed to the structural differences in their cell wall, because Gram-negative bacteria have a complex lipid bilayer while Gram-positive bacteria have only one lipid layer and a relatively permeable outer layer [38].

Inactivation of mutans streptococci is mainly done through membrane injury following lipid peroxidation; [39] which is the possible mechanism of action of TBO in A-PDT. Difference in intracellular concentrations or location of different PSs can explain the difference in antibacterial efficacy [40]. On the other hand, it should be noted that TBO can easily react with the bacterial cell membrane, and due to maximum solubility in hydrophobic parts of the membrane and its minimum molecular weight (305.83 g/mol), it easily diffuses and exerts its antimicrobial effect; thus, it is widely used in studies related to A-PDT [36, 41].

Our study showed significant differences in colony count at all time points compared to baseline in TBO + laser group. However, colony count in the study groups at day 3 before the second intervention (regardless of the effect of baseline colony count) was not significantly different. This finding may indicate that one-time intervention may not be sufficient for reduction of microorganisms. Combined use of TBO and laser in our study significantly decreased mutans streptococci count even after 2 weeks post-intervention. This finding may be due to the conduction of intervention in two cycles.

Some studies showed that use of laser alone was not effective for elimination of microorganisms [24, 42, 43]. In our study, reduction in bacterial count in the laser group was not significantly different from the control group, which confirms the results of previous studies.

Reducing the number of *S. mutans* colonies as the main culprit responsible for development of tooth caries leads to preservation of tooth structure and decreases the risk of tooth loss at early adulthood and subsequently reduces the costs related to prosthetic treatments. This method is non-invasive and can be easily tolerated by young children. It can be applied along with fluoride therapy and helps improve children's cooperation. Furthermore, A-PDT with application of TBO and diode laser can be performed after caries removal to disinfect the area before restoring the tooth and prevent caries recurrence. Also, due to its simplicity and reproducibility, this method can be periodically used to reduce oral microbial pathogens. A-PDT can be especially effective for dental caries, because decay is a localized lesion and PS can be injected into these lesions by a syringe [44].

One limitation of A-PDT is that it cannot be used in inaccessible areas like the tonsils and the pharynx, which can be good sources for regrowth of bacteria and recurrence of infections. Researchers should come up with a resolution for application of A-PDT in inaccessible areas. Diode laser used in our study had maximum absorbance

very close to that of TBO, which was among the strengths of our study. Another strength of our study was assessment of samples obtained from children in the clinical setting and the comparison of results of different treatment sessions, which, to the best of our knowledge, has not been done before.

In conclusion, A-PDT may be used to decrease the salivary count of mutans streptococci in children with SECC. Future studies with different light sources, optical parameters, and PS concentrations are recommended. Use of newer radiation methods such as fiber diffusers and ball-shaped tips (that are more compatible with the oral cavity) is also suggested. Hopefully, in near future, this method will be used as a home care technique using low-cost laser devices.

Further studies are also required to evaluate the effect of repetition of interventions in longer treatment courses with longer follow-ups of patients in order to suggest a preventive protocol for patients with SECC.

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Compliance with ethical standards The study design was approved by the Ethics Committee of Shahid Beheshti University (acceptance number: 703).

Conflict of interest The authors declare that they have no conflict of interest.

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