Photodiagnosis and Photodynamic Therapy 26 (2019) 229–234

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

Effects of photodynamic therapy with indocyanine green on *Streptococcus mutans* biofilm

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ARTICLE INFO

Keywords:
Cavity disinfection
Dentine
ICG
Laser
Ozone
Residual caries
Antimicrobial Photodynamic Therapy

ABSTRACT

Background: The current procedures for treating tooth decay are not always guaranteed to successfully remove all microorganisms which cause disease. For elimination of bacteria and prevention of their effects, different methods are recommended, such as antibacterial materials and cavity disinfectants. The aim of this study was to compare the antibacterial activity of photodynamic therapy (PDT) using diode laser with indocyanin green (ICG) on *Streptococcus (S.) mutans* biofilm with conventional methods.

Methods: Ninety human molars were divided into 9 groups: negative control, positive control, CHX, NaOCl, gaseous ozone, erbium (Er):YAG laser, diode laser, and indocyanin green/ICG, and PDT. Cavities were then cut into the teeth (2 per tooth, 20 cavities per group) and sterilized. For all of the groups, with the exception of the negative control group, 10^5 CFU/mL of the active *S. mutans* culture were inoculated into the cavities and then incubated at 37 °C for 72 h. Then, dentin chips (25 ± 5 mg) were collected from cavity walls and spread on Mueller-Hinton agar media after decimal dilution. The colonies were counted after incubation at 37 °C for 24 h.

Results: All the treatments significantly reduced the number of *S. mutans* compared with the positive control group ($p < 0.05$). The antimicrobial effectiveness of CHX, NaOCl, ozone, PDT, Er:YAG laser, and diode laser groups were similar. The lowest bacterial reduction was observed in the ICGgroup.

Conclusions: This work concludes that PDT using diode laser with ICG may be suggested on the cavity disinfection after caries excavation as an alternative to conventional methods.

1. Introduction

Dental caries is recognized as one of the most common chronic diseases in the world today, which impacts 60–90% of school children and nearly 100% of the adult population [1]. It has been described as a disease which manifests itself as progressive demineralization of dental hard tissues caused by a complicated lengthy interaction between fermentable carbohydrates and acid generating bacteria, which are laid out in bio-films, with the most common one linked to caries being *Streptococcus (S.) mutans* [2]. The integral component in the treatment of caries is to ensure that inflammation does not continue, and this is achieved through the complete eradication of the infected and necrotic tissues and microorganisms [3].

A caries lesion consists of two different layers termed as the outer layer and inner layer. The outer layer is known as infected dentin and is characterized by softened dentin with a large number of bacteria. The inner layer, known as affected dentin, is contaminated with fewer bacteria, and remineralization can occur in this layer. It is extremely difficult to make a clinical distinction between these two regions [4,5]. Thus, conventional methods usually involve the removal of both infected and affected layers before a filling is placed [6]. The rationale for this has been to prevent the development of further caries caused by remaining bacteria in the demineralized inner layer. One challenge that may arise with this approach is the possibility of nerve damage to the tooth caused by pulp exposure during the removal of tissues [7]. Following this approach, some further negative consequences can also occur, such as toothache and possibly weakening of the tooth structure [8]. More recent developments in cariology and diagnostic systems

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https://doi.org/10.1016/j.pdpdt.2019.04.005
Received 8 November 2018; Received in revised form 2 April 2019; Accepted 5 April 2019
Available online 06 April 2019

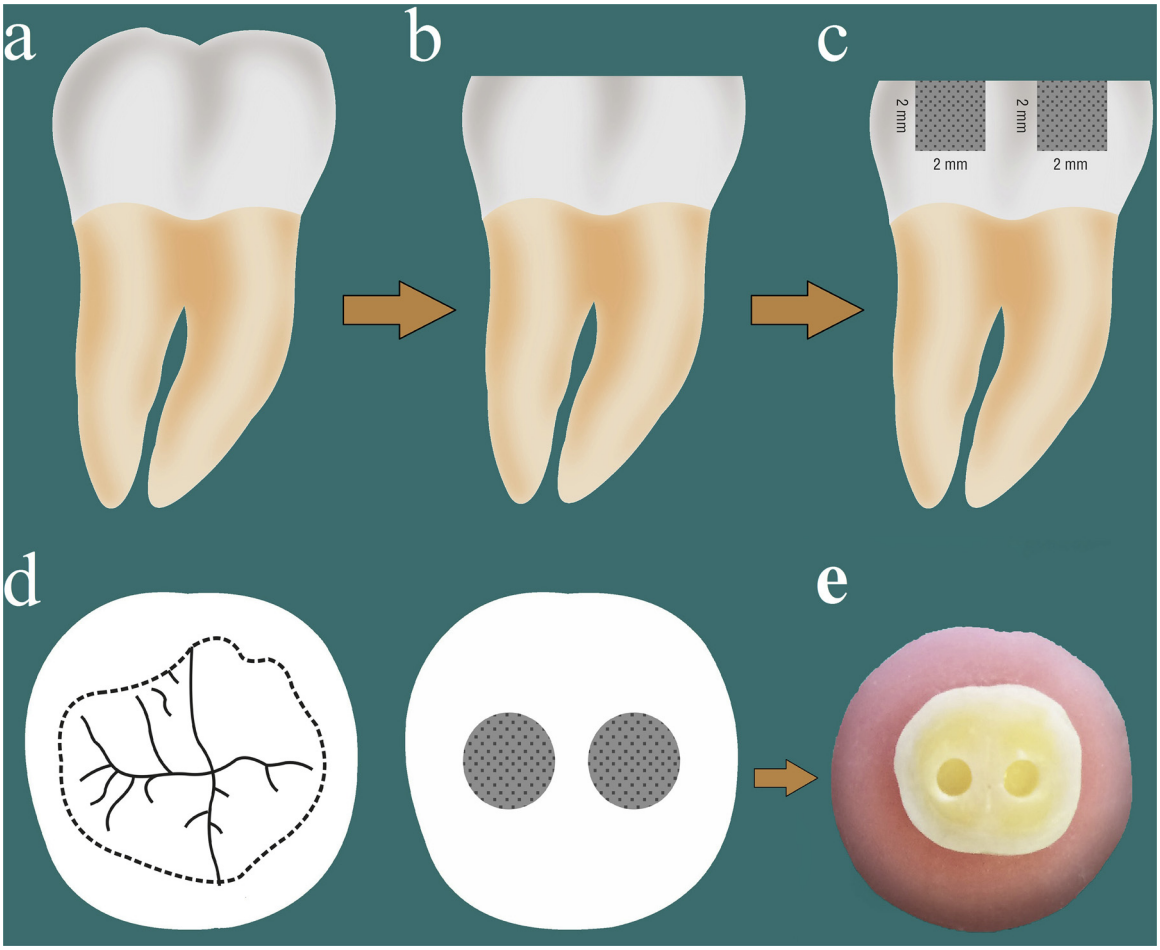

Fig. 1. Tooth cavity model.

have concentrated on a minimalist approach with the emergence of minimally invasive dentistry. Current thinking within modern restorative dentistry has considered that removing the infected layer of dentin and leaving the affected layer to protect the integrity of the tooth is a more accurate method. The current procedures for treating dental caries, whether conventional or contemporary, are not always guaranteed to successfully remove all residual tissue microorganisms, and there is no assurance that the remaining dental tissue is completely disinfected [3,9]. The bacteria left in the dentin of a cavity could maintain their activities, protect viability, and produce acid for a long time [3]. The continuity of the acidic environment promotes and increases lesion development. Thus, mineral loss and caries lesions are observed on the tooth surface under restoration [10].

The suppression of microorganisms associated with the formation of a carious lesions is an important requirement for the prevention of disease recurrence after treatment. For elimination of bacteria and prevention of their effects, different methods have been recommended, such as antibacterial adhesive systems and restorative materials, acid etching, cavity disinfectants, and use of laser and ozone [11–14]. Usually, available disinfectants containing compounds, such as chlorhexidine (CHX) digluconate, disodium ethylene diamine tetraacetic acid (EDTA) dihydrate, sodium hypochlorite (NaOCl), hydrogen peroxide, and iodine, are used to eliminate residual bacteria left in cavity preparations [15].

The use of photoactive disinfection (PAD) as a method of removing residual bacteria in the cavity prior to restoration is one of the most popular techniques used today. This method is also known as antimicrobial photodynamic therapy (PDT) or photodynamic antimicrobial chemotherapy (PACT) and offers an alternative option to eliminate oral microorganisms [16]. PDT has been characterized as the application of a photosensitizing agent (which could be any of the following: erythrosine, toluidine blue, eosin, methylene blue, rose bengal, malachite green, etc.), and light sources to areas requiring treatment [17,18].

In recent years, indocyanine green (ICG) has been among the materials investigated as photosensitizing agents. It is often used in cancer treatment and ICG angiography in the medical field [19]. In dentistry, it is preferred as a supportive treatment in intra-oral cancer therapies and periodontal therapy, and successful outcomes have been obtained [20]. However, there are few published studies on the antibacterial effect of ICG on the cavity disinfection after excavation of caries.

The aim of this research was to evaluate the antibacterial activity of PDT using a diode laser with ICG on *S. mutans* biofilm against conventional methods with the null hypothesis being that there will be no difference in antibacterial activity between the conventional methods and the PDT method made with ICG.

2. Materials and methods

2.1. Ethical issues

The study protocol was reviewed and approved by the ethics committee of the Tokat Gaziosmanpasa University, Turkey; the protocol number is 16-KAEK-008.

2.2. Study design

Ninety freshly extracted human non-carious third molar teeth were used in this study. Teeth with enamel hypoplasia, fractures, or

restoration were excluded. All calculus and other remnants were removed completely from the teeth surfaces, and the teeth were cleaned with pumice for 15 s. To prepare the specimens, the enamel was removed from the occlusal part of the teeth. All teeth were mounted in self-curing acrylic resin blocks with their occlusal surfaces exposed, and then flat dentinal surfaces were achieved using a slow-speed (< 5000 r.p.m.) diamond bur. On the now-flat occlusal surface of each tooth, two cylindrical cavities were prepared (with dimensions 2 mm diameter, 2 mm depth) without causing pulp exposure (Fig. 1).To remove the smear layer after the preparation, 2.5 ml of 17% EDTA solution (MD-Cleanser, Meta Biomed, Korea) was applied to each cavity, and then the cavities were rinsed with 5 ml of distilled water. The teeth were sterilized by autoclaving for 15 min at 121 °C. After sterilization, 10 teeth were disinfected to confirm the accuracy of the sterilization and were evaluated as a negative control group.

2.3. Inoculation of the cavities

S. mutans activated overnight in Mueller Hinton Broth with 1.5% sucrose was reactivated for 18 h in the same media. 10^5 CFU/mL of the active *S. mutans* cultures were inoculated into dried cavities and incubated at 37 °C for 72 h. Ten of the inoculated teeth were allocated in 8 operation groups.

Teeth were randomly divided into 9 groups of 10 teeth each (20 cavities) together with the negative control. The groups tested were:

1 *Group (negative control group);* after the teeth were sterilized, specimens were not infected by *S. mutans* and any procedures were made.
2 *Group (positive control group)*; after the teeth sterilized, each cavity was infected by *S. mutans* (10^5 CFU/mL), but no disinfection techniques were applied.
3 *Group:* After inoculation of the cavities with *S. mutans* (10^5 CFU/mL), 0.5 ml of 2% CHX (ceraxidin-C, Imicryl, Konya, Turkey LOT 13224) was applied to each cavity, and the solution was left in the cavity for 60 s.
4 *Group:* After inoculation of the cavities with *S. mutans* (10^5 CFU/mL), 0.5 ml of 2.5% NaOCl (Wizard, Rehber Chemistry, Istanbul, Turkey LOT VM00001) was applied to each cavity, and the solution was left in the cavity for 60 s.
5 *Group:* After inoculation of the cavities with *S. mutans* (10^5 CFU/mL), 60 µg of gaseous ozone (OzonyTron XP, MIO International Ozonytron, Germany) was applied to each cavity for 60 s.
6 *Group:* After inoculation of the cavities with *S. mutans* (10^5 CFU/mL), a 2,940-nm wavelength Er:YAG laser (Kavo Key 3+, KaVo, Biberach, Germany) was applied to each cavity in 1 W, 100mj, and 10 Hz parameters by using periodontics tips for 60 s.
7 *Group:* After inoculation of the cavities with *S. mutans* (10^5 CFU/mL), a 940-nm wavelength diode laser (Epic, Biolase Tech., CA, USA) was applied to each cavity in 1 W power and continuous modes using E4 tips for 60 s.
8 *Group:* After inoculation of the cavities with *S. mutans* (10^5 CFU/mL), 25 mg of sterile ICG (Pulsion Medical Systems SE, Feldkirchen, Germany LOT 25DE03101) was mixed with 5 ml of sterile distilled water in a sterile bottle, and 0.5 ml of ICG solution was applied to each cavity and the solution was left in the cavity for 60 s.
9 *Group*: After inoculation of the cavities with *S. mutans* (10^5 CFU/mL), 25 mg of sterile ICG (Pulsion Medical Systems SE, Feldkirchen, Germany LOT 25DE03101) was mixed with 5 ml of sterile distilled water in a sterile bottle. ICG solution (0.5 ml) was applied to each cavity, and then cavities were irradiated for 60 s with a 940-nm wavelength diode laser in 1 W power and continuous mode using E4 tips.

Each cavity of the teeth in all groups was rinsed with 2.5 ml of sterile distilled water for 30 s. The cavities were sealed with a sterile cotton sponge and temporary filler (Cavit™ G, 3 M ESPE, Deutschland LOT 542332). Then, the specimens were incubated at 37 °C for 72 h to establish an infected cavity.

2.4. Antibacterial activity determination

Following incubation, the temporary fillings were removed, and the standardized amounts of dentin chips (25 ± 5 mg) were collected from cavity walls using new and sterile carbide round burs, which were in a freezer (−25 °C) with a low-speed handpiece. Collected dentin chips were placed into sterile Eppendorf tubes containing 1 ml of sterile distilled water and dispatched to the Microbiology Laboratory for analysis.

After operation, the remaining *S.mutans* on dentin dust samples were spread on Mueller-Hinton agar media after decimal dilution. The colonies were counted after incubation at 37 °C for 24 h.

2.5. Statistical analysis

Quantitative data were obtained regarding the arithmetic mean and standard deviation. The one way analysis of variance (ANOVA) test was used to compare the CFU/ml among groups. For post-hoc comparisons between the pair-wise groups, the Tukey HSD test was used. A p-value < 0.05 was considered significant. Analyses were performed using SPSS 19 (IBM SPSS Statistics 19, SPSS,Inc., an IBM Co., Somers, NY).

3. Results

According to the results, all the treatments significantly reduced the number of *S. mutans* compared with the positive control group ($p < 0.05$) (Fig. 2). The antimicrobial effectiveness of CHX, NaOCl, ozone, PDT, erbium (Er):YAG laser, and diode laser groups were similar. The lowest bacterial reduction was observed in the ICG group, and there was a significant difference between the ICG group and the other groups. Nevertheless, there was significant difference between the number of the microorganisms of the positive control group and those of the ICG group ($p < 0.05$) (Table 1). It was found that the amount of the growth in the PDT group was less than the Er:YAG laser and diode laser groups, although this was not statistically different. The antimicrobial effectiveness to all tested methods can be ranked from the strongest to the weakest as follows: CHX, NaOCl, ozone, PDT, Er:YAG laser, diode laser, ICG.

4. Discussion

The results of this study supported the hypothesis that antibacterial activity of the PDT method made by ICG will not differ from other methods. It was discovered as part of this research that *S. mutans* were substantially decreased when using the PDT technique in combination with ICG. However, with regard to the quantity of the microorganisms grown, there were no significant variations among the groups except in the positive control and ICG group.

It was recommended that the possible dangers caused by bacterial activity could be eradicated if, after the cavity was prepared, an antibacterial cavity cleanser was used [21] and the use of NaOCl as an effective antimicrobial agent was proposed [22]. This solution can deproteinize the collagen fibrils. Furthermore, it was noted that NaOCl breaks down into its constituent components, namely sodium chloride and oxygen, which may result in some oxidation in the dentin matrix [23] and causes a reduction in the elastic modulus and flexural strength of the dentin [24]. It could also interfere in restoration performance and affect the resin penetration into the dentin structure and/or the polymerization of monomers in the demineralized dentin, allowing microleakage, while some studies showed that deproteinization did not affect the bond strength of the adhesive systems [24–26]. In addition, NaOCl

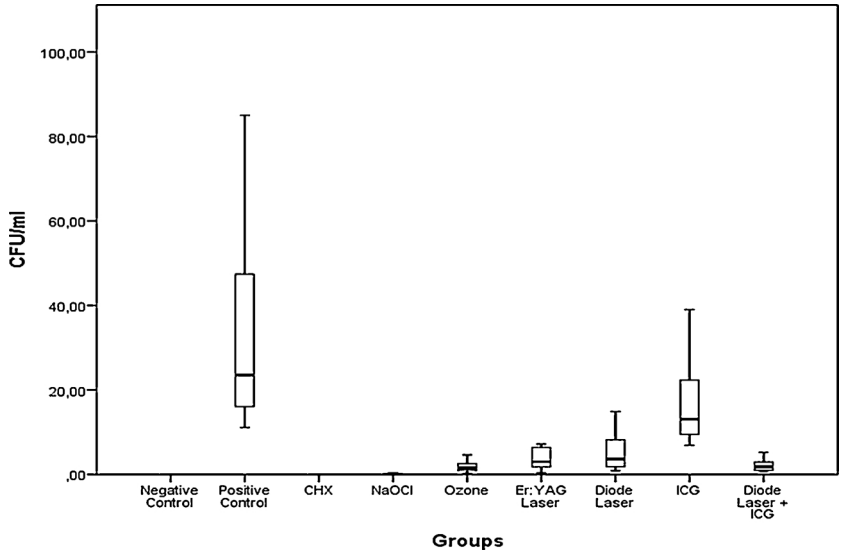

Fig. 2. Distribution of CFU/ml according to groups.

Table 1
Logarithmic values and standard deviation of the means in CFU/ml of the number of microorganisms isolated from the cavities of groups.

Groups	CFU/ml
Negative Control	0 ± 0 (A)
Positive Control	32.65 ± 22.68 (B)
CHX	0 ± 0 (A)
NaOCI	0.14 ± 0.23 (A)
Ozone	1.74 ± 1.25 (A)
Er:YAG Laser	3.69 ± 2.49 (A)
Diode Laser	5.9 ± 5.67 (A)
ICG	18.22 ± 14.18 (C)
ICG + Diode Laser (PDT)	2.11 ± 1.26 (A)

Different uppercases indicate significant statistical difference.

may present cytotoxic and genotoxic effects [27,28]. In the current study, 2.5% NaOCl was used, and it showed considerably high antibacterial effectiveness on *S. mutans*. Considering the cytotoxic and genotoxic effects, as well as the effects on the performance of the restoration and dentin structure, NaOCl may not be suitable for cavity disinfection after caries excavation.

It has been noted that CHX is effective against both gram-positive and gram-negative microbes and is popular for eliminating bacterial contaminants [29]. Due to these antibacterial properties, its use in the cavity is advised before placement of the restoration [30]. The effectiveness of CHX at the antimicrobial level is linked to the changes in the bacterial osmotic equilibrium brought about by the cationic molecule binding to negatively charged bacterial cell walls [31], and the collagen matrix structural integrity could be controlled and managed by CHX [32]. In the present study, there was a correlation with the Kapdan et al. [30] study in that the most significant decrease in bacterial activity was observed in the CHX group. They reported that the most effective antibacterial treatment for *S. mutans* was the chemical cavity disinfectant (2% chlorhexidine solution). However, it remains controversial whether the application of a chlorhexidine solution in a cavity preparation influences the effectiveness of a self-etching adhesive and ultimately the marginal microleakage of resin composite restorations [33]. Also, its long-term usage can leave undesirable side effects, such as dysgeusia, metallic taste, and tooth and restoration discoloration, which may lead to the patient's dissatisfaction [34]. Therefore, attainment of a disinfection method without the undesirable side effects with appropriate efficacy against *S. mutans*, which is the main etiology of dental caries, is of the utmost importance. As a result, to provide an alternative method, the antibacterial activity effects of PDT made by ICG combined with diode laser was evaluated in the study.

The application of ozone gas has been advocated for use in dentistry for the sterilization of cavities, root canals, periodontal pockets, and herpetic lesions [35]. Ozone is a strong, fast oxidizer of cell walls and cytoplasmatic membranes of bacteria and is considered one of the best bactericidal, antiviral, and antifungal agents [36]. Kapdan et al. [30] noted that the ozone exposure could be also an efficient disinfectant when it is used in appropriate concentrations and periods. Anand et al. [37], reported that ozonated water has the best antibacterial properties in *S. mutans* and *E. faecalis* among the study groups ($Ca(OH)_2$, chlorhexidine, and wine). However, it was noted by Hauser-Gerspach et al. [38] that, for open occlusal carious lesions, the gaseous ozone application as well as 1% chlorhexidine gel application for 30 s was not effective in reducing microorganisms. The present study revealed that 60 µg and 60 s of ozone treatment for disinfecting cavities was very efficient in eliminating *S. mutans,*leading to the conclusion that in case of determining the appropriate indications, the use of ozone for cavity disinfection is an effective technique. However, several issues have been raised with regard to the use of ozone, in particular that inhalation of ozone gas for long periods can result in damage to the lungs and other organs [37].

It has been documented in thestudies that effective elimination of the debris and smear layer can be achieved using Er:YAG and Er,Cr:YSGG laser irradiation [39,40].The antibacterial effects of a laser are mainly governed by what the laser light does to the target cell, tissue, or organism. These can be photochemical (caused by the generation of free radicals and other reactive species), photothermal, photoablative (caused by the disruption of chemical bonds) or photomechanical (caused by the shock waves generated by the dispersion of a plasma) [41]. Türkün et al. [42] were able to show that using an Er,Cr:YSGG laser with different energy outputs had the same effect on the antibacterial activity on *S. mutans* as that of the tested chlorhexidine gluconate-based cavity disinfectant.In the current study, theEr:YAG laser has produced satisfactory results, though it may not be as efficient as CHX, NaOCl, and ozone treatment. We believe that since the use of Erbium lasers in cavity preparation is a modern technique, the use of the Er:YAG laser as a means of cavity disinfection during cavity preparation would be beneficial.

Lasers with a wavelength in the range of infrared waves, such as diode lasers, have been effective in the obturation of dentinal tubules and antibacterial activity [43]. Diode lasers have a wavelength of 800–1064 nm. Hemoglobin and pigmented tissues and materials are

most affected by the 810–830-nm wavelength of this laser [44]. Gutknecht et al. [45]reported that chemical agents can only penetrate up to a depth of 100 µm, whereas a diode laser can destroy bacteria up to a depth of 500 µm in dentin at a wavelength of 980 nm. Similarly, Lee et al. [46] proved the superiority of laser treatment when they showed that they could remove 97.7% of *S. mutans* species by exposing dentin disks measuring 500 µm with laser irradiation.Their study also revealed that the antibacterial effect of diode lasers at 7 W was reduced to 50.9% and 20.1% when the dentin thickness was increased to 1000 and 2000 mm, respectively [46]. When the data of the current study were evaluated, the 940 nm wavelength diode laser demonstrated better results than the ICG group, they were not as good as those of the Er:YAG laser. It is evident that the performance and effectiveness of the diode laser may be dependent on a number of factors, such as wavelength, repetition rate, pulse energy of laser irradiation, duration of exposure, optical properties of the tissue, and the type of dentin chips used.

The use of PDT through the application of nontoxic dyes as photo sensitizer agents and visible light with the appropriate wavelengths is a proven technique for eliminating dangerous cells. The walls of the cells are destroyed by the reactive oxygen generated when light is absorbed by photo sensitizer agents [47,48]. It is also cited as an optional treatment for dentine disinfection and dental caries [49]. Boehm and Ciancio [50] reported that ICG combined with diode laser may be useful as a photodynamic adjunct for reduction of bacterial load in periodontal pockets. Beytollahi et al. [51] indicated that ICG-mediated photo-elimination exhibited significantly stronger inhibitory effects on biofilm formation and, consequently, the CFU/mL in *S. mutans*. Foschi et al. [52] suggested that PDT causes oxygen depletion during irradiation and that the limited oxygen supply in dentinal tubules leads to a greater rate of oxygen consumption than reperfusion in the photochemical reaction. In the present study, it was intended to eliminate the biofilm bacteria, *S. mutans*, without triggering any detrimental effects on the dentin surface and surrounding tissues through PDT using ICG combined with a 940 nm diode laser. Although this disinfection strategy is not higher than the conventional methods, it showed better results in terms of the amount of the grown microorganisms than the other methods (control group and ICG alone). It seems promising to give similar results to conventional methods. Further research on this method under a variety of conditions, for example changing the application and irradiation time, the irradiation wavelength, the cariogenic bacteria and the concentration, is recommended.

5. Conclusion

Based on the results of this study, the use of PDT with a diode laser and ICG is proposed as an alternative to standard techniques for cavity disinfection after caries excavation. Disinfection of residual caries dentin with this strategy requires further investigation with in vivo and in vitro studies.

Acknowledgments

The authors declare no conflicts of interest related to the materials tested in the present study. The study protocol was reviewed and approved by the Ethics Committee of the University of Tokat Gaziosmanpasa, Turkey; the protocol number is 16-KAEK-008. This work was supported by the Tokat Gaziosmanpasa University Scientific Research Projects Commission (Project No: 2016/17).

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