Original paper

Viability of bacteria associated with root caries after Nd:YAG laser application in combination with various antimicrobial agents: An in vitro study

Janina Golob Deeb[1,A–F], Nitya Reddy[1,A–C,F], Todd Kitten[2,A–D,F], Caroline Kelly Carrico[3,C,E,F], Kinga Grzech-Leśniak[1,4,A,C–F]

[1] Department of Periodontics, School of Dentistry, Virginia Commonwealth University, Richmond, USA
[2] Department of Oral and Craniofacial Molecular Biology, Philips Institute for Oral Health Research, School of Dentistry, Virginia Commonwealth University, Richmond, USA
[3] Department of Dental Public Health and Policy, School of Dentistry, Virginia Commonwealth University, Richmond, USA
[4] Laser Laboratory, Department of Oral Surgery, Wroclaw Medical University, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

Dental and Medical Problems, ISSN 1644-387X (print), ISSN 2300-9020 (online) *Dent Med Probl*. 2023;60(4):649–655

Address for correspondence
Kinga Grzech-Leśniak
E-mail: kinga.grzech-lesniak@umw.edu.pl

Funding sources
None declared

Conflict of interest
None declared

Acknowledgements
None declared

Received on July 16, 2023
Reviewed on August 15, 2023
Accepted on August 30, 2023

Published online on November 20, 2023

Cite as
Golob Deeb J, Reddy N, Kitten T, Carrico CK, Grzech-Leśniak K. Viability of bacteria associated with root caries after Nd:YAG laser application in combination with various antimicrobial agents: An in vitro study. *Dent Med Probl.* 2023;60(4):649–655. doi:10.17219/dmp/171690

DOI
10.17219/dmp/171690

Abstract

Background. The neodymium-doped yttrium aluminum garnet (Nd:YAG) laser has various therapeutic applications in dentistry, including the treatment of dentin hypersensitivity and the bacterial reduction therapy in periodontology. The addition of antimicrobial agents may enhance the impact of the laser on bacterial viability.

Objectives. This in vitro study aimed to assess the effect of Nd:YAG laser application in combination with various chemical antimicrobial agents, including hydrogen peroxide (H_2O_2), sodium hypochlorite (NaOCl), chlorhexidine (CHX), and sodium fluoride (NaF), on the viability of bacteria implicated in the etiology of root caries.

Material and methods. Three oral bacterial species were examined: *Streptococcus mutans* (*S. mutans*); *Streptococcus sanguinis* (*S. sanguinis*); and *Enterococcus faecalis* (*E. faecalis*). The bacteria were grown in broth at 37°C, and then treated with the chemical agents and/or irradiated with an Nd:YAG laser for 30 s. Each treatment modality was repeated 3 times: group 1 – no treatment; group 2 – 0.5% H_2O_2; group 3 – 0.5% NaOCl; group 4 – 0.12% CHX; group 5 – 2% NaF; group 6 – Nd:YAG laser irradiation; group 7 – laser and 0.5% H_2O_2; group 8 – laser and 0.5% NaOCl; group 9 – laser and 0.12% CHX; and group 10 – laser and 2% NaF. The viability of the bacteria was determined by plating them, counting viable colonies, converting the data into colony-forming units (CFUs)/mL, and transforming them into the log form. Statistical analysis was performed using the two-tailed paired *t* test.

Results. Irradiation with an Nd:YAG laser alone did not show a statistically significant effect against any of the bacterial species. The only effective antimicrobial used alone was CHX for *S. mutans*. Chlorhexidine with Nd:YAG resulted in a greater reduction in *S. mutans* and *E. faecalis* than either treatment alone. Meanwhile, H_2O_2 with Nd:YAG also showed an enhanced *S. mutans* reduction. Treatment with 0.5% NaOCl in conjunction with Nd:YAG brought the most significant reduction in viability for all bacteria in comparison with other treatment modalities.

Conclusions. The Nd:YAG laser combined with 0.5% NaOCl resulted in the most substantial reduction in bacterial survival as compared to the antimicrobials or the Nd:YAG laser used alone.

Keywords: antimicrobials, root caries, neodymium laser

Introduction

Periodontitis is a multifactorial chronic inflammatory disease in a susceptible host, initiated by bacteria and driven by the interaction between the biofilm and the host immune response, resulting in tissue destruction and the development of periodontal pockets.[1] The progression and treatment of periodontal disease cause attachment loss, gingival recession and root exposure, which, apart from being esthetically unpleasing, may lead to dentin hypersensitivity and root caries.[2] Root exposure can occur independently as a consequence of aggressive tooth brushing, and the presence of thin alveolar housing or gingival phenotype. Additionally, recession is associated with aberrant frenal attachments, mucogingival deficiencies, the orthodontic therapy, the positional characteristics of the teeth,[3] and natural aging.[4]

The roots may become susceptible to developing caries, which presents as progressive lesions on the root surfaces exposed to the oral environment due to some degree of periodontal attachment loss.[5] The demineralization of the root surface is twice as rapid as in the case of enamel.[6] The pooled prevalence of root caries is reported at 41.5%, and is growing due to the increasing human life span and dentition longevity.[7] Furthermore, isolation, access, the adhesive properties of root surfaces, and the lack of retention in preparations, associated with the root form and anatomy, present a challenge in the treatment of root caries lesions.

The microflora associated with root caries is different from that found in dentinal caries,[8] with *Streptococcus mutans* (*S. mutans*), *Streptococcus sanguinis* (*S. sanguinis*) and *Enterococcus faecalis* (*E. faecalis)* being 3 of the bacterial species implicated in root caries etiology.[8] The onset and progression of caries on the root surface occur due to the bacteria metabolizing fermentable carbohydrates into acids, which initiate the demineralization of the root surface by removing calcium (Ca) and phosphate ions from the surface apatite.[9] While this process starts in enamel at pH of 5.5, pH of only 6.4 is enough for demineralization to begin on less mineralized cementum and dentin on the surface of the exposed root. This lower degree of mineralization makes the initiation and progression of root caries considerably faster.[10]

Lasers have gained significant popularity in dentistry since the 1990s, and are used for various kinds of treatment.[11,12] The neodymium-doped yttrium aluminum garnet (Nd:YAG) laser has a wavelength of 1,064 nm and can penetrate deeper into the tissue, targeting dark pigments, such as melanin and hemoglobin, as its chromophores. The Nd:YAG laser, through exerting a photothermal effect, is capable of killing bacteria by evaporation, destruction or denaturation, which results in their devitalization or inactivation.[13,14] It can be achieved with a quartz fiber-optic tip, 200–320 μm in diameter, placed into the periodontal pocket[15] up to 5 mm[16] to target pigments, with a little effect on the non-pigmented tissues. Due to its ability to target the pigmented and inflamed gingival tissues, the Nd:YAG laser can effectively treat periodontal disease, leading to periodontal regeneration, with new cementum, periodontal ligament and alveolar bone observed during a histological analysis.[17]

Antimicrobial agents, such as chlorhexidine (CHX), have broad antimicrobial activity, as do the irrigants hydrogen peroxide (H_2O_2) and sodium hypochlorite (NaOCl), and are commonly used in dentistry to control supragingival plaque. Chlorhexidine is a potent allopathic reagent that has been used as a wide-spectrum antiseptic agent since 1950 to target Gram-positive and Gram-negative bacteria, fungi, and some viruses, and has the ability to inhibit the formation and development of bacterial plaque for several hours.[18,19] Hydrogen peroxide has been used in dentistry for more than 70 years[20]; it shows a wide-spectrum antimicrobial activity against bacteria, yeasts, fungi, viruses, and spores.[21] Sodium hypochlorite is also known for its antimicrobial effect, as well as fast bactericidal action and non-toxicity at a proper concentration.[22] When combined with curettage, NaOCl effectively reduces soft tissue inflammation in periodontics,[23] and using 0.1% NaOCl during periodontal surgeries could potentially improve the healing and regeneration of the connective tissue.[24] The American Dental Association (ADA) Council on Dental Therapeutics proposed using diluted NaOCl (0.1–0.5%) as an antiseptic mouth rinse for its rapid bactericidal action, relative non-toxicity, the lack of color and staining, a low cost, and having no known contraindications.[25] Applying sodium fluoride (NaF) to tooth surfaces is a well-established and commonly used method for preventing caries, as it promotes the remineralization of enamel and inhibits the production of bacterial acids.[26] Furthermore, in vitro studies have demonstrated the inhibition of demineralization by NaF combined with a carbon dioxide (CO_2) laser.[27]

Limited research has been published on the effect of chemical antimicrobial agents combined with an Nd:YAG laser on the viability of bacterial strains associated with root caries. Therefore, this in vitro study aimed to evaluate the effectiveness of H_2O_2, NaOCl, CHX, and NaF as adjuncts to Nd:YAG laser irradiation on the viability of *S. mutans*, *S. sanguinis* and *E. faecalis*, with the ultimate goal of assessing the efficacy of their application in the prevention of root caries. The proposed hypothesis is that combining a chemical agent with an Nd:YAG laser will lead to a more substantial reduction in viable *S. mutans*, *S. sanguinis* and *E. faecalis* colonies than using the chemical agent or the laser alone.

Material and methods

Bacterial cultures

Three oral bacterial species associated with root caries were used as representatives in the study, including *S. mutans* (UA159), *S. sanguinis* (SK36) and *E. faecalis*. The bacteria were grown individually and treated in parallel. The bacterial species were obtained from the freezer stocks kept at –80°C, with 5 µL of a single-use aliquot inoculated into 5 mL of the Brain Heart Infusion (BHI) broth (Becton Dickinson, Franklin Lanes, USA), and then incubated overnight in an aerobic environment at 37°C. A Genesys™ 150 spectrophotometer (Thermo Fisher Scientific, Emeryville, USA) measured the optical density (OD) of the cultures at 660 nm (OD_{660}), which were then normalized to an OD of 0.5. Ten 150-microliter aliquots of each bacterial strain were transferred to a 96-well plate, to non-adjacent wells to provide 10 treatment groups for each experiment. The stock solutions of the chemical antimicrobial agents (3% H_2O_2, 5.25% NaOCl, 2% CHX, and 75% NaF) were diluted in sterile distilled water to 4 times the desired final concentration. Then, 50 µL of each diluted agent was added to the 150 µL of bacteria already present in each well, resulting in the concentrations listed below for each study group.

Laser irradiation was performed on the designated study groups in a sterile biological safety cabinet, with the Nd:YAG and/or chemical agent treatment done individually to ensure that each bacterial culture received contact with the chemical agent and/or the laser for 30 s. Following Nd:YAG laser irradiation, the treated samples were diluted 50-fold into fresh BHI broth, and then further diluted before being spread onto BHI plates with the use of an Eddy Jet 2 spiral plater (Neutec Group Inc., Farmingdale, USA). The plates were incubated in anaerobic conditions at 37°C for 24–48 h. Each plate was examined, with viable colonies counted and converted into colony-forming units (CFUs)/mL, which were log-transformed for statistical analysis.

Table 1. Model results for the average colony count after treatment

Model	*F*-value (ANOVA)	*p*-value
Bacteria	12.91	**<0.0001**
Irrigant	170.92	**<0.0001**
Laser (Y/N)	134.98	**<0.0001**
Bacteria*Irrigant	7.71	**<0.0001**
Irrigant*Laser(Y/N)	32.13	**<0.0001**
Bacteria*Laser(Y/N)	0.91	0.4055
Bacteria*Irrigant*Laser (Y/N)	3.34	**0.0015**

Y – yes; N – no; values in bold indicate statistical significance ($p < 0.05$).

Laser irradiation parameters

A LightWalker Nd:YAG laser (Fotona, Ljubljana, Slovenia) with a wavelength of 1,064 nm and a 300-micrometer fiber tip was used for the irradiation of the bacterial cultures in direct contact at 150 mJ, 20 Hz and 3 W for 30 s in the micro-short pulse (MSP) mode (pulse duration of 100 µs). A disinfected aluminum foil barrier was applied to isolate the treated wells during laser irradiation and prevent the contamination of other wells by spatter. Each experiment was repeated 3 times.

Study groups

The groups were formed as follows:
– group 1: bacteria (*S. mutans*, *S. sanguinis* or *E. faecalis*);
– group 2: bacteria (*S. mutans*, *S. sanguinis* or *E. faecalis*) + H_2O_2 (0.5%);
– group 3: bacteria (*S. mutans*, *S. sanguinis* or *E. faecalis*) + NaOCl (0.5%);
– group 4: bacteria (*S. mutans*, *S. sanguinis* or *E. faecalis*) + CHX (0.12%);
– group 5: bacteria (*S. mutans*, *S. sanguinis* or *E. faecalis*) + NaF (2%);
– group 6: bacteria (*S. mutans*, *S. sanguinis* or *E. faecalis*) + Nd:YAG;
– group 7: bacteria (*S. mutans*, *S. sanguinis* or *E. faecalis*) + Nd:YAG + H_2O_2 (0.5%);
– group 8: bacteria (*S. mutans*, *S. sanguinis* or *E. faecalis*) + Nd:YAG + NaOCl (0.5%);
– group 9: bacteria (*S. mutans*, *S. sanguinis* or *E. faecalis*) + Nd:YAG + CHX (0.12%); and
– group 10: bacteria (*S. mutans*, *S. sanguinis* or *E. faecalis*) + Nd:YAG + NaF (2%).

Statistical analysis

The combined effect of the irrigants and the laser on the log CFU bacterial count was assessed using the analysis of variance (ANOVA) model. Post hoc pairwise comparisons were adjusted using Tukey's adjustment. The significance level was set at 0.05.

Results

Significant differences were observed in bacterial recovery with regard to the irrigant (the chemical antimicrobial agent) used, the Nd:YAG laser and the bacterial species for all combinations. Table 1 summarizes the models, while Table 2 presents pairwise comparisons for the effect of the irrigants with and without the laser (irrigant + laser vs. irrigant alone), and Table 3 shows pairwise comparisons for the effect of the laser with and without the irrigants (laser + irrigant vs. laser alone).

Table 2. Pairwise comparisons of the effect of the irrigants with and without the laser on the average colony count [log CFU/mL])

Comparison	Bacterial species	Irrigant	Estimated average change	*SE*	Adjusted *p*-value (Tukey's test)
Nd:YAG laser vs. no laser	*E. faecalis*	CHX	–5.23	0.81	<0.0001*
		H_2O_2	–2.06	0.81	0.7396
		NaF	–0.03	0.81	>0.9990
		NaOCl	–5.74	0.81	<0.0001*
		none	0.08	0.81	>0.9990
	S. mutans	CHX	–0.26	0.81	>0.9990
		H_2O_2	–3.27	0.81	0.0240*
		NaF	0.06	0.81	>0.9990
		NaOCl	–6.63	0.81	<0.0001*
		none	–0.03	0.81	>0.9990
	S. sanguinis	CHX	–5.24	0.81	<0.0001*
		H_2O_2	–1.52	0.81	0.9885
		NaF	0.05	0.81	>0.9990
		NaOCl	–6.41	0.81	<0.0001*
		none	–0.08	0.81	>0.9990

SE – standard error; Nd:YAG – neodymium-doped yttrium aluminum garnet; *E. faecalis* – *Enterococcus faecalis*; *S. mutans* – *Streptococcus mutans*; *S. sanguinis* – *Streptococcus sanguinis*; CHX – chlorhexidine; H_2O_2 – hydrogen peroxide; NaF – sodium fluoride; NaOCl – sodium hypochlorite; CFU – colony-forming unit; * statistically significant.

Table 3. Pairwise comparisons of the effect of the laser with and without the irrigants on the average colony count [log CFU/mL]

Comparison	Bacterial species	Laser	Irrigant	Estimated average change	*SE*	Adjusted *p*-value (Tukey's test)
Irrigant vs. no irrigant	*E. faecalis*	Nd:YAG	CHX	–6.81	0.99	<0.0001*
			H_2O_2	–3.11	0.99	0.2959
			NaF	–0.06	0.99	1.0000
			NaOCl	–9.28	0.99	<0.0001*
	S. mutans	Nd:YAG	CHX	–8.89	0.99	<0.0001*
			H_2O_2	–4.24	0.99	0.0101*
			NaF	0.08	0.99	1.0000
			NaOCl	–8.89	0.99	<0.0001*
	S. sanguinis	Nd:YAG	CHX	–8.57	0.99	<0.0001*
			H_2O_2	–3.86	0.99	0.0388*
			NaF	0.14	0.99	1.0000
			NaOCl	–8.57	0.99	<0.0001*

* statistically significant.

Figure 1 shows that the Nd:YAG laser demonstrated a synergistic effect with NaOCl, reducing significantly more *E. faecalis* than NaOCl alone (–5.74 log CFU/mL; adjusted $p < 0.0001$) or the laser alone (–9.28 log CFU/mL; adjusted $p < 0.0001$), and reducing substantially more *S. mutans* than NaOCl alone (–6.63 log CFU/mL; adjusted $p < 0.0001$) or the laser alone (–8.89 log CFU/mL; adjusted $p < 0.0001$). Furthermore, combining the Nd:YAG laser with NaOCl reduced more *S. sanguinis* than NaOCl alone (–6.41 log CFU/mL; adjusted $p < 0.0001$) or the laser alone (–8.57 log CFU/mL; adjusted $p < 0.0001$).

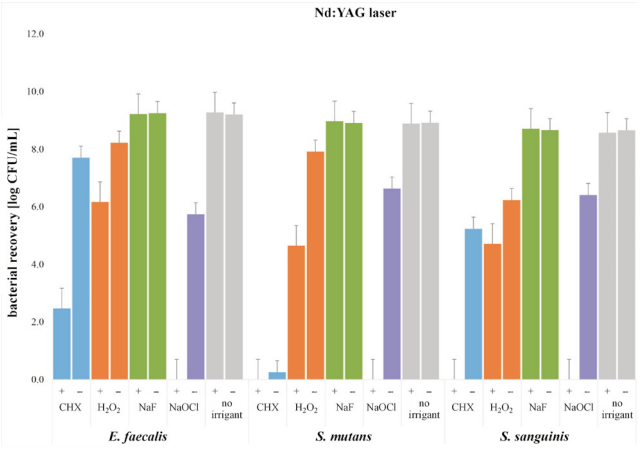

Fig. 1. Mean bacterial recovery [log CFU/mL] with a standard error (*SE*) with regard to various treatment modalities

+ Nd:YAG laser used; – no laser used.
The significance of pairwise comparisons is reported in Tables 2 and 3.

The Nd:YAG laser had a synergistic effect with H_2O_2, reducing significantly more *S. mutans* than H_2O_2 alone (–3.27 log CFU/mL; adjusted $p = 0.0240$) and the laser alone (–4.24 log CFU/mL; adjusted $p = 0.0101$). However, the combination of Nd:YAG and NaOCl was more effective than Nd:YAG and H_2O_2 for *S. mutans* (–4.6 log CFU/mL; adjusted $p = 0.0020$ (data not presented)).

The Nd:YAG laser acted synergistically with CHX, killing significantly more *S. sanguinis* than CHX alone (–5.24 log CFU/mL; adjusted $p < 0.0001$) or the laser alone (–8.57 log CFU/mL; adjusted $p < 0.0001$). The same was true for the Nd:YAG laser combined with CHX, which killed significantly more *E. faecalis* than CHX alone (–5.23 log CFU/mL; adjusted $p < 0.0001$) or the laser alone (–6.81 log CFU/mL; adjusted $p < 0.0001$). For *S. mutans*, CHX was effective on its own, with no additional benefit from the Nd:YAG laser (adjusted $p = 0.300$).

Chemical antimicrobial agents

Sodium fluoride did not reduce the viability of *S. mutans*, *S. sanguinis* or *E. faecalis* when used alone or in conjunction with the Nd:YAG laser. The only statistically significant reduction in bacterial growth by H_2O_2 was observed for *S. mutans* when it was used with the Nd:YAG laser, though it did not eliminate all *S. mutans* bacteria (–3.27 log CFU/mL; adjusted $p = 0.0240$). Chlorhexidine proved to be an effective monotherapy for *S. mutans*, as it reduced the bacterial count to undetectable levels whether or not laser irradiation was used. Additionally, CHX was more effective on *S. sanguinis* (–5.24 log CFU/mL; adjusted $p < 0.0001$) and *E. faecalis* (–5.23 log CFU/mL; adjusted $p < 0.0001$) when used alongside the Nd:YAG laser.

The most extensive effect of NaOCl occurred in combination with the Nd:YAG laser; the synergistic effect was observed for all 3 bacterial species.

Bacterial strains

Enterococcus faecalis was reduced to undetectable levels with the NaOCl and Nd:YAG laser combined treatment. When used separately, neither NaOCl nor the Nd:YAG laser was able to achieve the same level of *E. faecalis* reduction. The combination of NaOCl and the Nd:YAG laser had a synergistic antimicrobial effect and was the most effective treatment for *E. faecalis*.

For *S. mutans*, the most effective treatment was CHX as a monotherapy or in conjunction with the Nd:YAG laser, with both resulting in undetectable amounts of bacterial recovery after treatment. While the synergistic effect of NaOCl and the Nd:YAG laser was observed, *S. mutans* reduction with CHX was not enhanced by the addition of the Nd:YAG laser.

Streptococcus sanguinis achieved the highest reduction when CHX or NaOCl were used in conjunction with the Nd:YAG laser. Combining either CHX or NaOCl with the Nd:YAG laser resulted in undetectable bacterial levels, and was the most effective synergistic antimicrobial treatment for *S. sanguinis*.

This in vitro experiment evaluated only 3 bacterial species, providing a small-sample representation with regard to abundant bacteria engaged in the complex interactions present in the clinical environment.

Discussion

In this investigation, we showed the effect of the synergistic application of an Nd:YAG laser and chemical antimicrobial agents on reducing the viability of *S. mutans*, *S. sanguinis* and *E. faecalis* in vitro. Based on our data, NaOCl combined with the Nd:YAG laser resulted in the greatest growth reduction to undetectable levels for all 3 bacterial species. However, the laser as individual treatment was unable to reduce counts for any of the tested bacteria. A previous study confirmed the bactericidal and synergistic effect of the Nd:YAG laser when combined with CHX, H_2O_2 or NaOCl in reducing periodontal pathogens, specifically *Porphyromonas gingivalis* (*P. gingivalis*) and *Fusobacterium nucleatum* (*F. nucleatum*).[28] In both investigations, the laser parameters were selected to reflect the clinical settings used for periodontitis treatment, so that the protocols could be clinically translated and implemented as part of a supportive periodontal therapy (SPT) in patients with attachment loss, identified as being at high risk of caries.

Chlorhexidine is an effective antimicrobial agent capable of reducing a bacterial load by 97% when used as a preoperative rinse. However, it is not indicated for continued long-term use due to its side effects, such as altered taste and the staining of the teeth.[29,30] In the present study, CHX effectively reduced the *S. mutans* count to undetectable levels as a monotherapy, and also worked in combination with the Nd:YAG laser.

Hydrogen peroxide has been used as a mouth rinse for plaque control and the treatment of oral infections. A recent review reported that H_2O_2 had no side effects, but was not superior to CHX in antiplaque efficacy, and in reducing gingival inflammation and the oral bacteria count.[18] The concentration of H_2O_2 used in most studies is 1.5%, which is lower as compared to the present study. However, higher concentrations, such as 3%, did not cause mucosal irritation in an animal model at a maximum contact time of 7 min, and it is the concentration that is most commonly available over the counter.[19] Further examination of the hydroxyl radicals generated during the photolysis of H_2O_2 showed that they were a powerful oxidizing agent capable of inducing oxidative damage to oral bacteria.[19] The results of the present study align with these findings, as the Nd:YAG laser combined with H_2O_2 had an increased bactericidal effect on *S. mutans* as compared to either treatment alone.

While we do not fully understand the mechanisms underlying the synergistic effect found when combining laser treatment with NaOCl, there is evidence that thermal energy can potentiate the effect of NaOCl. Indeed, the intracanal heating of NaOCl in endodontic therapy has been shown to increase bacterial reduction as compared to the ultrasonic and non-heated agitation techniques.[31] Therefore, we speculate that the thermal effects from the Nd:YAG laser contributed to the enhanced bactericidal effect of NaOCl.

Sodium fluoride did not cause bacterial reduction when used alone or in combination with the Nd:YAG laser, which is in agreement with the studies reporting that various concentrations of fluorides did not significantly decrease the growth of *P. gingivalis* or *S. mutans* on titanium disks,[22] and even found a slight increase in bacterial growth with a 1% gel concentration.[23] Sodium fluoride, when applied to a tooth surface, reduces the demineralization and promotes the remineralization of enamel. Fluoride-treated teeth exhibit higher pH values, as fluoride inhibits the production of bacterial acids, which proves its antimicrobial rather than direct bactericidal effect.[26] The observations of the present study align with these reports, as we did not find any bacteria-reducing effect with NaF alone or when used alongside the Nd:YAG laser.

The prevention of root caries is important for periodontists, as 2/3 of periodontally treated patients may develop root caries during the first 4 years of periodontal maintenance. Furthermore, the incidence of new root caries persists longitudinally at 4, 8, 12, and 14 years of periodontal maintenance.[32–36] Moreover, a cross-sectional study reported a high prevalence of root caries and high caries risk rates in 20% of patients referred for periodontal treatment.[37] The current study showed that using an Nd:YAG laser with low concentrations of NaOCl may constitute an effective method that is easy to implement during SPT when treating patients at high risk of caries.

The application of the Nd:YAG laser with the settings used herein is already established for periodontitis treatment, and similar findings as in this study have been reported for the reduction of bacteria associated with periodontitis when the Nd:YAG laser was used in conjunction with chemical agents (H_2O_2 and NaOCl).[28,38–41] Such chemical agents could be applied along the gingival margin, over the exposed root surfaces before using the laser in the maintenance therapy.

Limitations

Limitations to the present study include its in vitro nature. Thus, determining the clinical significance of the observed effects of the Nd:YAG laser and chemical irrigants in the periodontal therapy remains unclear. Furthermore, evaluating only 3 bacterial species and the small sample size means the findings cannot be clinically extrapolated.

Conclusions

Treatment with an Nd:YAG laser and low concentrations of chemical antimicrobial agents provided synergistic effects, reducing the viability of bacterial species associated with root caries. In comparison with the chemical antimicrobial agents or the Nd:YAG laser used alone, the greatest reduction in bacterial viability was achieved when using the Nd:YAG laser with 0.5% NaOCl.

Ethics approval and consent to participate

Not applicable.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

ORCID iDs

Janina Golob Deeb https://orcid.org/0000-0002-3189-0136
Nitya Reddy https://orcid.org/0000-0002-4206-2707
Todd Kitten https://orcid.org/0000-0001-6097-7583
Caroline Kelly Carrico https://orcid.org/0000-0001-9521-9854
Kinga Grzech-Leśniak https://orcid.org/0000-0002-5700-4577

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