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EDUARDO BANDEIRA SOUSA SILVA

O LASER DE DIODO DE 980 NM COMBINADO COM A APLICAÇÃO DE INDOCIANINA VERDE E O FLÚOR FOSFATO ACIDULADO SÃO EFICAZES NA REDUÇÃO DA PERDA MINERAL DO ESMALTE APÓS UM DESAFIO CARIOGÊNICO? - ESTUDO IN VITRO

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Orientadora: Profª Drª Marinês Nobre Dos Santos Uchôa

Este trabalho corresponde à versão final da dissertação defendida pelo aluno Eduardo Bandeira Sousa Silva, e orientado pela Profª Drª Marinês Nobre Dos Santos Uchôa

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“Sempre fui sonhador, é isso que
me mantém vivo”

Racionais Mc’s

RESUMO

Apesar de apresentarem maior coeficiente de absorção pelo esmalte e, devido a essa característica, serem mais eficazes no controle da desmineralização do esmalte, os lasers de alta potência não são amplamente empregados na prática privada nos países em desenvolvimento. O uso de lasers vermelho e infravermelho de alta potência parece ser uma alternativa promissora. Entretanto, não há relatos na literatura que tenham avaliado como o flúor na forma de fluoreto de cálcio (CaF2) atua na redução da desmineralização do esmalte dentário irradiado. O laser de diodo tem como limitação seu baixo coeficiente de absorção em dentes humanos, com isso tem sido indicado o uso de corantes como a Indocianina Verde (IV) para melhorar a absorção e, consequentemente, ação do laser na estrutura do esmalte. Dessa forma, este estudo teve como objetivo avaliar os efeitos da utilização de indocianina verde na aplicação de laser de diodo associado à aplicação de FFA na absorção de flúor pelo esmalte dentário e na redução da perda mineral do esmalte após desafio cariogênico in vitro. Para isso, foram utilizados 55 espécimes de esmalte dentário humano desmineralizado, divididos em 5 grupos: 1- Esmalte desmineralizado, 2- Esmalte desmineralizado + laser diodo, 3- Esmalte desmineralizado + FFA, 4- Esmalte desmineralizado + laser diodo + , 5- Esmalte desmineralizado + FFA + IV + laser diodo. Após os tratamentos, os espécimes foram submetidos a ciclagens de desmineralização e remineralização. A perda ou ganho de mineral do esmalte foi realizada por análise de microdureza. A normalidade dos dados foi verificada pelo teste de Kolmogorov-Smirnov. O teste de Kruskal-Wallis foi então realizado para determinar se havia alguma diferença estatisticamente significativa na perda mineral entre os grupos de tratamento. Como uma diferença significativa foi encontrada, o teste de Student-Newman-Keuls foi conduzido. Um nível de significância de 0,05 foi usado para todos os testes estatísticos. A análise dos resultados para profundidade da lesão demonstrou que a aplicação do gel FFA foi eficaz na redução da progressão da lesão em comparação com o grupo controle, bem como com a indocianina + laser, laser sozinho e a terapia combinada. Além disso, o laser de diodo e a terapia combinada não inibiram a progressão da lesão quando comparados com o grupo controle. Além disso, exceto para o grupo gel FFA não foram observadas diferenças entre os diferentes tratamentos em relação ao perfil mineral do esmalte. O ΔS demonstra que a irradiação do esmalte cariado com laser de diodo sozinho, bem como com indocianina verde + laser de diodo não foi eficaz na inibição da progressão da lesão durante o desafio cariogênico. Os resultados mostram que a aplicação de 1,123% de APF-gel reduziu significativamente a perda mineral do esmalte quando comparada ao laser sozinho, indocianina verde + laser de diodo. Concluindo assim que a irradiação com laser de diodo, a λ 980 nm, combinada com a aplicação de gel de FFA a 1,23% foi eficaz na inibição da progressão da lesão de cárie in vitro. No entanto, não foi possível detectar nenhum efeito adicional quando o esmalte cariado foi tratado com a combinação de laser de diodo e FFA em comparação com o efeito isolado da irradiação a laser ou do tratamento com gel de FFA.

Palavras-chave: Ciclagem de pH; Desmineralização do esmalte; Flúor fosfato acidulado; Laser Diodo.

ABSTRACT

Despite having a higher absorption coefficient by enamel and, due to this characteristic, being more effective in controlling enamel demineralization, high- power lasers are not widely used in private practice in developing countries. The use of high-power red and infrared lasers appears to be a promising alternative. However, there are no reports in the literature that have evaluated how fluoride in the form of calcium fluoride (CaF2) acts in reducing the demineralization of irradiated dental enamel. The diode laser has a limitation due to its low absorption coefficient in human teeth, therefore the use of dyes such as Indocyanine Green (IV) has been indicated to improve absorption and, consequently, the action of the laser on the enamel structure. Thus, the objective of this study was to evaluate the effects of the use of Indocyanine Green in the application of diode laser (λ = 980 nm) associated with the application of acidulated phosphate fluoride (APF) on the absorption of CaF2 by dental enamel and on the reduction of mineral loss from enamel after an in vitro cariogenic challenge. For this purpose, 55 specimens of demineralized human dental enamel were used, divided into 5 groups: 1- Demineralized enamel, 2- Demineralized enamel + diode laser, 3- Demineralized enamel + APF, 4- Demineralized enamel + diode laser + , 5- Demineralized enamel + APF + IR + diode laser. After the treatments, the specimens were subjected to demineralization and remineralization cycling. The loss or gain of enamel mineral was performed by microhardness analysis. The normality of the data was verified by the Kolmogorov-Smirnov test. The Kruskal-Wallis test was then performed to determine if there was any statistically significant difference in mineral loss between the treatment groups. As a significant difference was found, the Student-Newman-Keuls test was conducted. A significant level of 0.05 was used for all statistical tests. Analysis of the results for lesion depth demonstrated that the application of APF gel was effective in reducing lesion progression compared to the control group, as well as to indocyanine + laser, laser alone, and combined therapy. Furthermore, diode laser and combined therapy did not inhibit lesion progression when compared to the control group. Furthermore, except for the APF group, no differences were observed between the different treatments regarding enamel mineral profile. The ΔS demonstrates that irradiation of carious enamel with diode laser alone, as well as with indocyanine green + diode laser was not effective in inhibiting lesion progression during cariogenic challenge. The results show that the application of 1.123% APF-gel significantly reduced enamel mineral loss when compared to laser alone, indocyanine green + diode laser. Thus, it was concluded that diode laser irradiation at λ 980 nm combined with the application of 1.23% APF gel was effective in inhibiting the progression of caries lesions in vitro. However, no additional effect could be detected when carious enamel was treated with the combination of diode laser and APF compared with the isolated effect of laser irradiation or APF gel treatment.

Keywords: Acidulated phosphate fluoride; Enamel; Diodo laser; pH Cycling;

SUMÁRIO

1 INTRODUÇÃO

Prevenir a cárie dentária é um dos principais objetivos da odontologia moderna. Outro objetivo principal inclui a remineralização da lesão de cárie em vez do convencional preparo cavitário e restauração (Cochrane et al., 2010). A remineralização é um processo de reparação que visa repor os minerais e ocorre diariamente pela presença de íons cálcio, fosfato e flúor encontrados na saliva (Farooq & Bugshan, 2020). A desmineralização acontece quando os ácidos bacterianos atacam o esmalte e o equilíbrio dinâmico entre a desmineralização e a remineralização mantém a superfície do dente saudável, enquanto a ruptura desse equilíbrio pode causar progressão da lesão e, eventualmente, levar à formação de cavitação (Hou et al., 2020).

O fluoreto desempenha um papel importante no aumento da remineralização. A aplicação tópica de flúor está entre as formas mais eficientes de deter ou prevenir a cárie dentária (Urquhart et al., 2019). Dentre as estratégias disponíveis de uso de fluoreto, a escovação com dentifrício fluoretado tem sido considerada a abordagem mais racional para o controle da cárie dentária (Cheng et al., 2022) e sua eficácia na prevenção da cárie está bem estabelecida. No entanto, para indivíduos com alto risco ao desenvolvimento de cárie, o emprego de outros métodos profissionais para a aplicação de fluoreto é altamente recomendado (Soares et al, 2021). Nesse sentido, o flúor fosfato acidulado é o composto mais utilizado na clínica odontológica e sua eficácia na prevenção da cárie em crianças e adolescentes foi cientificamente comprovada (Rhagavan et al, 2022). Porém, o efeito do fluoreto em reduzir a desmineralização e potencializar a remineralização do esmalte dental é parcial, pois depende de fatores como os hábitos de higiene do paciente e a frequência com que o mesmo vai ao dentista. Tal fato enfatiza a necessidade de se aperfeiçoar os métodos preventivos já existentes, com a introdução de técnicas inovadoras que possam agir como coadjuvantes na prevenção e controle da cárie dental. Nesse contexto, a laserterapia tem sido bastante estudada como uma alternativa promissora na prevenção da cárie (Chokhachi et al., 2018).

Diferentes tipos de lasers, tais como Díodo, Nd:YAG, Argônio, Er:YAG e CO2 têm sido estudados para uso em Odontologia (Brugnera et al, 2021.) No entanto, embora apresentem um maior coeficiente de absorção pelo esmalte e devido a esta característica sejam mais efetivos no controle da desmineralização do esmalte, os lasers de alta potência não são amplamente empregados em consultório particular em países em desenvolvimento (Sant'Anna et al., 2017). O uso de lasers vermelhos e infravermelhos parecem ser uma alternativa promissora. Dentre eles, o laser díodo destaca-se pelo baixo custo comparado com outros tipos de laser como o de CO2, além da acessibilidade e da segurança para a câmara pulpar do dente em que será feita a aplicação (Sant'Anna et al., 2017). Além disso, há um reduzido número de estudos que tenham avaliado os efeitos da aplicação do laser díodo sobre a prevenção de cárie e sua efetividade de reduzir a progressão da desmineralização do esmalte.

Existem quatro diferentes interações possíveis de lasers com um tecido alvo: reflexão, transmissão, espalhamento e absorção. Por absorção, o laser eleva a temperatura na superfície e cria efeitos fotoquímicos que variam de acordo com o teor de água dos tecidos. Dependendo da temperatura atingida, pode ocorrer ablação (vaporização da água nos tecidos), desnaturação das proteínas ou desidratação e queima do tecido (carbonização)(Carroll & Humpreys, 2006). A absorção requer uma molécula que absorva luz, conhecida como cromóforo, com afinidade por comprimentos de onda específicos de luz. Nos tecidos moles presentes na cavidade oral, os cromóforos são a melanina, a hemoglobina e a água, e nos tecidos duros de origem dentária, são a água e a hidroxiapatita. Existem diferentes coeficientes de absorção dependendo dos comprimentos de onda dos lasers. Os lasers de díodo dental têm comprimentos de onda que se estendem do visível ao infravermelho próximo, com uma faixa de 800 a 980 nm. Uma fibra óptica flexível de 200 a 600 mm é usada para fornecer um feixe de laser à área alvo com emissão contínua ou pulsada (Atieh et al., 2022).

Para que se obtenha o efeito desejado quando do emprego do laser, que é a prevenção da cárie por meio da modificação química e morfológica dos tecidos dentários duros, a energia do laser deve ser fortemente absorvida e eficientemente convertida em calor (Manara et al, 2020). No entanto, os efeitos do laser devem ficar restritos aos tecidos mais superficiais, sem causar danos aos tecidos subjacentes ou circundantes. No caso de comprimentos de onda do laser que não são fortemente absorvidos pelo esmalte, como é o caso dos lasers díodo, o emprego de um corante apropriado poderia absorver a luz, gerar calor e, em seguida, transferir esse calor para o tecido alvo (De Sant'anna et al, 2016). A aplicação de agentes foto absorvedores como a indocianina verde, um corante anfifílico de iodeto de tricarbocianina (massa =

751,4 Da) tem sido sugerido para aplicação antes da irradiação do esmalte dental com os lasers de alta potência com o objetivo de aumentar a absorção de energia do laser infravermelho pelo esmalte, em um esforço para induzir mudanças fotoquímicas e térmicas que o tornem mais resistente ao desafio cariogênico (de Sant'anna et al., 2009a). Esse corante é reconstituído em solução aquosa de pH 6,5 que é geralmente excitada entre 750 e 800 nm, cuja fluorescência é visualizada em torno do pico máximo de 832 nm (Reinhart et al., 2016) e exibe uma forte absorção centrada a 800 nm, e o laser de díodo neste comprimento de onda é preferencialmente absorvido pela combinação corante-esmalte. Nesse contexto, o estudo realizado por de Sant'anna et al. (2009a) demonstrou que a intensidade e área espectral integrada de carbonato tipo B (BCO32–) relacionado ao pico para 960cm–1 (PO43–pico) diminuiu significativamente apenas após o tratamento combinou o creme foto absorvente e o laser díodo. Da mesma forma, esses autores observaram uma redução do conteúdo orgânico do esmalte. Assim, tem sido sugerido que a interação entre a energia do laser, os cromóforos e o esmalte pode causar desnaturação de proteínas o que resultaria no bloqueio da via de difusão no esmalte (de Sant'anna et al., 2009b).

Além da absorção do corante no comprimento de onda de 980 nm, (de Sant'Anna, et al., 2009b) sua cinética de circulação rápida e toxicidade mínima, bem como a facilidade de sua remoção da superfície, levaram a investigações sobre a utilidade de corantes verdes de indocianina como fotossensibilizadores quando combinados com a aplicação do laser díodo com o objetivo de prevenir a cárie dentária. O estudo realizado por de Sant'anna et al., (2009b) demonstrou que no grupo irradiado com laser díodo combinado com o corante, o esmalte apresentou aumentos estatisticamente significativos na porcentagem de cálcio e fósforo em comparação com o grupo controle. Além disso, os autores observaram que houve redução do conteúdo orgânico quando o esmalte foi irradiado com o laser díodo associado ao corante. No entanto, observa-se dessa forma que não foram realizados estudos que tenham investigado o potencial do laser díodo quando combinado com o flúor fosfato acidulado em favorecer a formação de fluoreto de cálcio e como consequência, reduzir a desmineralização do esmalte dental após um desafio cariogênico.

Outro composto largamente empregado na Odontologia para aqueles indivíduos que apresentam alto risco ao desenvolvimento de cárie é o fluoreto, que atua aumentando a remineralização e reduzindo a desmineralização do esmalte dental e dessa forma, contribuindo para a redução da prevalência de cárie (Marinho et al., 2015) como já citado anteriormente . No entanto, não se tem conhecimento de pesquisas que tenham investigado o potencial do laser díodo de potencializar o efeito do fluoreto em reduzir a desmineralização do esmalte de dentes permanentes após um desafio cariogênico bem como não foram realizados estudos que tenham investigado o potencial do laser díodo quando combinado com o flúor fosfato acidulado em favorecer a formação de fluoreto de cálcio e como consequência, reduzir a desmineralização do esmalte dental após um desafio cariogênico.

Diante do exposto, este estudo teve como objetivos avaliar os efeitos do uso da indocianina verde na aplicação do laser díodo associado à aplicação de FFA sobre a absorção de fluoreto pelo esmalte dental e sobre a redução da perda mineral do esmalte após um desafio cariogênico in vitro. A hipótese do presente estudo foi que a irradiação do esmalte dentário com o laser díodo pode potencializar os efeitos do flúor fosfato acidulado em formar CaF_2 e reduzir a desmineralização do esmalte dentário.

2 ARTIGO:

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Are 980 nm diode laser and acidulated phosphate fluoride effective in reducing the enamel mineral loss after a cariogenic challenge? – In vitro study

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Short title: In vitro effect of Diode laser and fluoride on the caries progression

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Abstract

Aim: This study aimed to evaluate the effects of using indocyanine green in the application of diode laser associated with the application of FFA on the absorption of fluoride by dental enamel and on the reduction of mineral loss from enamel after an in vitro cariogenic challenge. The hypothesis of the present study was that irradiation of dental enamel with diode laser can enhance the effects of acidulated fluoride phosphate in forming CaF2 and reduce the demineralization of dental enamel.

Design: Sixty-six previously demineralized enamel specimens (DES) were randomly divided into 5 groups (n=11/group): 1- No treatment (negative control), 2- APF gel, 3- Diode laser, 4- Diode laser + APF gel, 5- Green indocyanine + Diode laser diode + APF gel. After treatments, specimens were submitted to a pH cycling. Microhardness and analysis were carried out to quantitatively assess the enamel mineral loss. Data normality was verified by the Kolmogorov-Smirnov test. Results were analyzed using the Kruskal-Wallis test and the Student-Newman-Keuls test with a 0.05 significance level.

Results: Only 1.23% APG-gel inhibited lesion progression. The 1.23% APF-gel significantly reduced the enamel mineral loss when compared with no treatment, laser alone and Indocyanine green + Diode laser ($p<0.001$, $p<0.014$, and $p<0.001$ respectively). No difference was noted between the Indocyanine green + Diode laser + 1.23% APF-gel group and 1.23% APF-gel group.

Conclusion: The combination therapy did not provide any further improvement in the inhibition of enamel mineral loss as compared to Indocyanine green + Diode laser + 1.23% APF-gel group or 1.23% APF-gel application.

KEYWORDS: dental caries, lasers, sodium fluoride.

1 INTRODUCTION

Preventing dental caries is one of the main goals of modern dentistry. Another main goal includes remineralization of the carious lesion instead of conventional cavity preparation and restoration[1]. Remineralization is a repair process that aims to replace minerals and occurs daily due to the presence of calcium, phosphate, and fluoride ions found in saliva[2]. Demineralization occurs when bacterial acids attack the enamel and the dynamic balance between demineralization and remineralization maintains a healthy tooth surface, while disruption of this balance can cause lesion progression and eventually lead to cavitation formation[3].

Fluoride plays an important role in increasing remineralization. Topical application of fluoride is among the most efficient ways to stop or prevent dental caries[4]. Among the available strategies for using fluoride, brushing with fluoridated toothpaste has been considered the most rational approach to controlling dental caries[5] and its effectiveness in preventing caries is well established. However, for individuals at high risk of developing caries, the use of other professional methods for applying fluoride is highly recommended[6]. In this sense, acidulated phosphate fluoride is the most widely used compound in the dental clinic and its effectiveness in preventing caries in children and adolescents has been scientifically proven[7.] However, the effect of fluoride in reducing demineralization and enhancing remineralization of dental enamel is partial, as it depends on factors such as the patient's hygiene habits and how often they go to the dentist. This fact emphasizes the need to improve existing preventive methods, with the introduction of innovative techniques that can act as adjuvants in the prevention and control of dental caries. In this context, laser therapy has been widely studied as a promising alternative in the prevention of caries[8].

Different types of lasers, such as Diode, Nd:YAG, Argon, Er:YAG and CO2, have been studied for use in Dentistry[9]. However, although they have a higher absorption coefficient by enamel and, due to this characteristic, are more effective in controlling enamel demineralization, high-power lasers are not widely used in private practices in developing countries[10]. The use of red and infrared lasers appears to be a promising alternative. Among them, the diode laser stands out for its low cost compared to other types of lasers such as CO2, in addition to its accessibility and safety for the pulp chamber of the tooth in which the application will be made[10]. Furthermore, there are a small number of studies that have evaluated the effects of diode laser application on caries prevention and its effectiveness in reducing the progression of enamel demineralization.

There are four different possible interactions of lasers with a target tissue: reflection, transmission, scattering and absorption. By absorption, the laser raises the temperature on the surface and creates photochemical effects that vary according to the water content of the tissues. Depending on the temperature reached, ablation (vaporization of water in the tissues), denaturation of proteins or dehydration and burning of the tissue (carbonization) may occur[11]. Absorption requires a molecule that absorbs light, known as a chromophore, with affinity for specific wavelengths of light. In the soft tissues present in the oral cavity, the chromophores are melanin, hemoglobin and water, and in the hard tissues of dental origin, they are water and hydroxyapatite. There are different absorption coefficients depending on the wavelengths of the lasers. Dental diode lasers have wavelengths that extend from the visible to the near-infrared, with a range of 800 to 980 nm. A flexible optical fiber of 200 to 600 mm is used to deliver a laser beam to the target area with continuous or pulsed emission[12].

In order to achieve the desired effect when using a laser, which is the prevention of caries through the chemical and morphological modification of hard dental tissues, the laser energy must be strongly absorbed and efficiently converted into heat[13]. However, the effects of the laser must be restricted to the most superficial tissues, without causing damage to the underlying or surrounding tissues. In the case of laser wavelengths that are not strongly absorbed by enamel, as is the case with diode lasers, the use of an appropriate dye could absorb the light, generate heat and then transfer this heat to the target tissue[10]. The application of photoabsorbing agents such as indocyanine green, an amphiphilic tricarbocyanine iodide dye (mass = 751.4 Da) has been suggested for application prior to irradiation of dental enamel with high-power lasers with the aim of increasing the absorption of infrared laser energy by the enamel, in an effort to induce photochemical and thermal changes that make it more resistant to cariogenic challenge[14]. This dye is reconstituted in an aqueous solution of pH 6.5 that is generally excited between 750 and 800 nm, whose fluorescence is visualized around the maximum peak of 832 nm[15] and exhibits a strong absorption centered at 800 nm, and the diode laser at this wavelength is preferentially absorbed by the dye-enamel combination. In this context, the study carried out by de Sant'anna et al. (2009)[14] demonstrated that the intensity and integrated spectral area of type B carbonate (BCO32–) related to the peak at 960 cm–1 (PO43– peak) decreased significantly only after the combined treatment of the photoabsorbing cream and the diode laser. Likewise, these authors observed a reduction in the organic content of the enamel. Thus, it has been suggested that the interaction between the laser energy, the chromophores and the enamel may cause protein denaturation which would result in the blocking of the diffusion pathway in the enamel[16].

In addition to the absorption of the dye at a wavelength of 980 nm[16] its rapid circulation kinetics and minimal toxicity, as well as the ease of its removal from the surface, led to investigations into the usefulness of indocyanine green dyes as photosensitizers when combined with the application of diode laser with the aim of preventing dental caries. The study carried out by de Sant'anna et al., (2009)[16] demonstrated that in the group irradiated with diode laser combined with the dye, the enamel presented statistically significant increases in the percentage of calcium and phosphorus compared to the control group. In addition, the authors observed that there was a reduction in the organic content when the enamel was irradiated with the diode laser associated with the dye. However, it is thus observed that no studies have been carried out that have investigated the potential of the diode laser when combined with acidulated phosphate fluoride to favor the formation of calcium fluoride and, as a consequence, reduce the demineralization of dental enamel after a cariogenic challenge.

Another compound widely used in Dentistry for those individuals who are at high risk of developing caries is fluoride, which acts by increasing remineralization and reducing demineralization of dental enamel and thus contributing to the reduction of the prevalence of caries[17] as previously mentioned. However, there is no knowledge of research that has investigated the potential of the diode laser to enhance the effect of fluoride in reducing the demineralization of permanent tooth enamel after a cariogenic challenge, nor have there been studies that have investigated the potential of the diode laser when combined with acidulated phosphate fluoride to favor the formation of calcium fluoride and, as a consequence, reduce the demineralization of dental enamel after a cariogenic challenge.

In view of the above, this study aimed to evaluate the effects of using indocyanine green in the application of diode laser associated with the application of FFA on the absorption of fluoride by dental enamel and on the reduction of mineral loss from enamel after an in vitro cariogenic challenge. The hypothesis of the present study was that irradiation of dental enamel with diode laser can enhance the effects of acidulated fluoride phosphate in forming CaF2 and reduce the demineralization of dental enamel.

2 MATERIAL AND METHODS

2.1 Ethical concerns

As this study included experiments with human teeth, it was carried out after approval by the local Ethics Committee (CAAE: 76697023.2.0000.5418).

2.2 Sample size calculation

The sample size was calculated based on the hypothesis of superiority of one combined treatment (Indocyanine green + diode laser) over another (Indocianine green + diode laser + APF gel), according to the following formula[18]:

$$n_1 = \frac{(z_\alpha + z_\beta)^2 \left[r\theta_1(1-\theta_1) + \theta_2(1-\theta_2) \right]}{(\theta_1 - \theta_2 - \delta)^2}$$

Where, α represents the type I error (equal to 0.05), β the type II error (equal to 0.20), r the allocation rate of 1/1 (equal to 1), δ the value of clinical importance to attest superiority (equal to 0.10) and θ1 and θ2 represented the mineral loss rate of each treatment assuming a % of mineral loss after cariogenic challenge of 9.3 for Indocyanin green + diode laser and 3.98 for Indocyanin green + diode laser + APF gel. The calculation resulted in an n of 11 specimens per group. The calculation was performed in the G* Power program.

2.3 Experimental design

For this in vitro study, we used fifty-five previously demineralized enamel specimens (DES). Lesion formation was confirmed using polarized light microscopy analysis. Thus, 55 specimens were randomly divided into 5 groups. Each group consisted of 11 buccal or lingual demineralized dental enamel specimens as follows:

Group 1 - No treatment (Control).

Group 2 - 1.23% APF-gel.

Group 3 - Diode laser.

Group 4 - Indocyanine green + Diode laser.

Group 5 - Indocyanine green + 1.23% APF-gel + Diode laser.

After treatments, specimens were submitted to a pH cycling. The enamel mineral loss or gain was evaluated using microhardness analysis. Data normality was verified using the Kolmogorov-Smirnov test. Results were analyzed using the Kruskal-Wallis test and the Student-Newman-Keuls test and the 0.05 significance level was adopted.

2.4 Teeth selection and enamel specimen preparation

To carry out this in vitro investigation, we gathered thirty-six sound-impacted human third molars. The visual inspection evidenced that the teeth were found to have more than two-thirds of the root formed. After extraction, the teeth were cleaned and stored in 0.1% thymol solution in the refrigerator. A magnifying colony counting lens (Phoenix CP 600 plus, Phoenix, Ararquara, São Paulo, Brasil) was used to check for the presence of extrinsic and/or intrinsic changes in the enamel surface and to verify if they were free of apparent caries, macroscopic cracks, and abrasions, as well as staining. Subsequently, 0.1% (w/v) thymol-distilled water solution was used to sterilize the teeth, which were individually immersed in 20 mL volumes for five days[18]. Afterword's, the teeth were sectioned mesiodistally with a water-cooled diamond saw and a cutting machine to obtain the enamel blocks with 2-mm² (Isomet, Buehler, Lake Bluff, IL, USA). Each enamel specimen was polished for 30 s using a 5-µm alumina/water suspension micropolish (Instrumental, Jabaquara, Sao Paulo, Brazil) to allow fresh enamel to be exposed washed with purified water, and twicely sonicated for 5 minutes. An acid-resistant varnish was applied to each the specimen's surface, leaving a 1-mm^2 window of exposed enamel in the middle third of buccal surface to be submitted to treatments, pH cycling, and analyses.

2.5 Caries-like lesion production

To produce the caries-like lesion, each enamel specimen was kept individually in 12,5 mL (6.25 mL solution/ mm^2 of exposed enamel) of 0.05 M acetate buffer solution 50% saturated with respect to hydroxyapatite, pH 5.0, for 48 hours at 37 ºC[19]. Before the caries-like lesion production, this solution was submitted to fluoride, calcium, and phosphate analysis to assess its subsaturation degree. Eleven specimens of demineralized enamel served as the in vitro caries baseline and was not submitted to pH cycling. Thus, fifty-five DES were randomly allocated to five groups.

2.6 Enamel treatment with acidulated phosphate fluoride

The specimens of groups 2 and 5 were individually submitted to the a single 1.23% APF-gel application pH 3.5 (Allplan, Aparecida, São Paulo, Brazil), with a cotton swab, for four minutes. Then, the fluoride excess was removed with a sterile gauze.

2.7 Enamel irradiation with Diode laser

The specimens of groups 3, 4 and 5 were irradiated with a 980 nm Diode laser GaAlAs (CHEESE GIGAA, China), after the application of Indocyanine green. Before laser irradiation, the specimens of group4 and 5 were submitted to a one minute application of Indocyanine green powder (Sigma-Aldrich, St. Louis, MO, USA; Lot: BCCK8901; neutral pH) dissolved in a 2% Carbopol (2 mg/mL)[20]. Indocyanine green gel was applied to the exposed area of the samples with a microbrush. We performed laser irradiation on the exposed enamel area of each specimen by scanning the area for 40 seconds. This was done by manually moving the laser fiber tip while maintaining a 5 mm distance from the handpiece to the enamel specimen. The infrared diode laser irradiation was carried out using a 300 µm optic fiber conductor with a pulse diode laser at 980 nm wave length, a 0.8 W output power and an energy density of 5.33 J/mm2. The optic fiber oriented perpendicularly to the enamel surface with its tip held in contact mode for 40 s 400 µm fiber tip diameter in a continuous mode laser source (CW) and sweeping motion[21]. The same distance was kept for all samples by positioning the laser handpiece in a pH meter holder.

2.8 Demineralization and remineralization pH cycling

The pH cycling model used in this study was based on that described by Featherstone et al., (1988)[22], with modifications as suggested by Steiner-Oliveira et al., (2008)[23]. Samples from groups 1 to 5 were submitted to the pH cycling regime. Each specimen was individually kept in demineralizing solution (6.25 mL/mm^2 exposed enamel) containing 2.0 mmol/L calcium, 2.0 mmol/L phosphate in acetate buffer 75 mmol/L, and pH 4.6, for 3 h. After this period, the specimens were placed in a remineralizing solution (3.12 mL/mm^2 of exposed enamel) containing 1.5 mmol/L of calcium, 0.9 mmol/L of phosphate and 150 mmol/L of KCl in 20 mmol/L of cacodylic buffer, pH 7.0, for an average of 21 h. The demineralization and remineralization solutions were changed in the fourth day of cycling, which was repeated for eight days. Between the demineralizing and remineralizing phases, and at the end of pH cycling, the specimens were washed with purified water for 10 seconds and dried with absorbent paper. To prevent microbial growth, both solutions contained thymol. After pH cycling, the specimens were kept in a closed, humid and refrigerated environment until they were submitted to microhardness analysis.

2.9 Cross- sectional microhardness testing

To verify the effects of various treatments on the inhibition of caries-like lesion progression, after pH-cycling, the specimens were carefully sectioned longitudinally through the border of the exposed enamel, ensuring that the cut passes through the area of interest. Each section was then embedded in acrylic resin to stabilize the sample for further analysis. Following embedding, the specimens were serially flattened and polished to create a smooth surface suitable for detailed examination, using longitudinal microhardness testing using a Future Tech FM-ARS microhardness tester (Future-Tech Corp., Tokyo, Japan) with a Knoop indentor, under a 25 g load for 5 s. Thirty-six indentations were made on each specimen, with the long axis of the diamond penetrator aligned parallel to the external enamel surface. These indentations were taken at different points but maintained standardized distances to ensure consistency. The indentations were arranged in three rows, spaced 10 µm apart, with each row containing 12 indentations. The first indentation was performed at 10 µm from the enamel surface and extended to 180 µm depth. The first 6 indentations in each row were made at 10-µm intervals between 10 and 60 µm, whilst the remaining impressions were performed at 20-µm intervals from 60 µm to 180 µm. This method allowed for a detailed systematic evaluation across the lesion and into the underlying sound enamel following pH-cycling[22].

2.10 Determination of the enamel mineral profile

The mean hardness values from the three rows at each distance from the enamel surface were averaged and expressed as the Knoop hardness number (KHN). This provided a single representative hardness value for each depth from the enamel surface. Subsequently, the area of demineralized enamel was calculated by plotting the microhardness values against the lesion depth and performing numerical integration using the trapezoidal rule. The integration allowed for an estimation of the total area under the curve, representing the extent of demineralization. The ΔS (change in mineral content) was then calculated by finding the difference between the area corresponding to sound enamel (indicating no demineralization) and the area of the demineralized enamel. This parameter reflects the extension of the enamel mineral loss after pH cycling[23].

2.11 Statistical analysis

The primary variable analyzed was the mineral loss of dental enamel (ΔS), with different enamel treatments serving as the sources of variation. Data normality and homogeneity of variance were verified using the Shapiro-Wilk test and the Levene test respectively. The Kruskal-Wallis test was then performed to determine whether there were any statistically significant difference in mineral loss among the treatment groups. Since a significant difference was found, the Student-Newman-Keuls test was subsequently conducted to identify which specific group differed from each other. A significant level of 0.05 was used for all statistical tests. Tests were performed on the G* Power program.

3 RESULTS

3.1 Lesion depth and Enamel mineral loss

Analysis of the results for lesion depth shown in Table 1, demonstrated that the APF-gel application was effective in reducing lesion progression as compared with the control group ($p < 0.001$) as well as with the Indocyanine + Laser, Laser alone, and the combined therapy. In addition, the Diode laser and the combined therapy did not inhibit lesion progression when compared with control group ($p < 0.2589$ and $p > 0.2646$ respectively). Moreover, except for the APF-gel group, no differences were observed among treatments.

Table 1. Medians ± Interquartile ranges of lesion area of each treatment according to each group (n=11).

Treatments	Lesion area (Kg_f x mm^2)			
Control	63364.43	±	13949.86	B
1.23% APF- gel	71633.40	±	4853.40	A
Diode laser	58370.10	±	10124.95	B
Indocyanine green + Diode laser	60766.33	±	10714.65	B
Indocyanine green + Diode laser + 1.23% APF-gel	58792.70	±	19213.32	B

*Distinct capital letters evidence statistically significant difference among groups.

Regarding the enamel mineral profile (ΔS), results from table 2 demonstrate that irradiation of carious enamel with Diode laser alone as well as with Indocyanine green + Diode laser was not effective in inhibiting lesion progression during the cariogenic challenge. This table also shows that the 1.23% APF-gel application significantly reduced the enamel mineral loss when compared with laser alone, Indocyanine green + Diode laser ($p < 0.001$, $p < 0.014$, and $p < 0.001$ respectively). Results also evidence that the combination therapy did not provide any further improvement in the inhibition of enamel mineral loss as compared to 1.23% APF-gel application **(**$p < 0.001$).

Table 2. Medians and interquartile ranges of the enamel mineral loss (vol% x μm) according to each treatment (n=11).

Treatments	Enamel mineral loss(ΔS) (vol% x μm)			
Control	1541.34	±	2150.84	B
1.23% APF gel	3608.82	±	1032.15	A
Diode laser	919.30	±	1378.78	B
Indocyanine green + Diode Laser	681.00	±	674.13	B
Indocyanine + Diode laser + 1.23% APF gel	2395,17	±	1791,33	A

*Distint capital letters evidence statistically significant difference among groups.

4 DISCUSSION

With the experimental conditions of this vitro study with previously demineralized enamel and simulating a high cariogenic challenge, we demonstrated that laser irradiation combined with 1.23% APF-gel application did not provide additional effect in hindering early caries lesion progression. This finding is in line with results provided by Amer et al. (2023)[24], who performed Energy Dispersive X-Ray analysis and concluded that the combination therapy of a 940 nm Diode laser and 1.23% APF-gel application was not effective in enhancing the Ca/P ratio. These authors also noted that this treatment significantly reduced this enamel property. However, no cariogenic challenge was used in their study. On the other hand, different findings were obtained in the study of Barbosa et al. (2013)[25], who claimed that the lowest percentage of surface microhardness loss was provided by the 980 nm Diode laser irradiation and 2% sodium fluoride application after a pH cycling. However, it is known that during the caries process, the highest enamel mineral loss occurs in the lesion body which is in the enamel subsurface[24].

In the current study, results showing that the combination treatment did not statistically differ from the laser alone as well as from the 1.23% APF gel alone, demonstrate that in regard to the enamel mineral loss, no synergism could be detected. A reasonable explanation as why we did not find a synergistic effect because of the laser plus fluoride therapy, could be that the 980-nm wavelength has a low absorption coefficient by enamel – 1.0 cm^{-1} [24]. In this way, surface heat and consequently surface changes such as melting and fusion should not be the mechanism operating in the inhibition of lesion progression. In line with this assumption, results from Amer et al. (2023)[24] study evidenced under scanning electron microscopy analysis, that enamel submitted to APF application and irradiated with a 940 nm wavelength produced broad areas of obliterated enamel rod endings, with narrow areas of recrystallized enamel elevated above the surface and some cracks. However, no calcium fluoride-like crystal was note on the enamel surface. Thus, it seems that reduction in the carbonate content and in the organic matrix may play a role in this mechanism[9].

Results of the current study indicated that Indocyanine green did not enhance the 980 nm Diode laser effect in reducing the lesion progression of carious enamel after a cariogenic challenge in vitro. These results disagree with the conclusion of de Sant'Anna's study[14] demonstrating that the combination of IG and Diode laser, favored a significant increase in the calcium and phosphorus concentrations in the enamel surface. However, these authors quantified the acid resistance of the enamel surface determining the ratio (experimental/control) of the of calcium and phosphate percentage evaluated using elemental analysis, while in the current investigation, we quantified the mineral content in the entire subsurface lesion using microhardness analysis which expresses the direct enamel mineral content. In addition, in their study, authors used human primary enamel, and it is known to have a higher type B carbonate than enamel of permanent teeth, which turns this substrate more susceptible to dental caries[26]. Carbonate fits less inadequately in the lattice, producing derangements in the hydroxyapatite structure, reproducing a less stable and more acid-soluble apatite phase[26].

As expected, the APF treatment demonstrated to be effective in inhibiting lesion progression of carious enamel produced by the cariogenic challenge. This discovery is in accordance with several early investigations demonstrating its caries prevention effectiveness[6]. The APF- gel application was performed in the current study considering that 1.23% APF-gel is the most used product in dental practice. In addition, it is well known that 1.23% APF- gel application results in the formation of spherical or globular precipitates on the enamel surface, which resemble calcium-fluoride-like deposits. These precipitates, being loosely bound, can act as a fluoride reservoir[27]. Thus, during a cariogenic challenge such as the pH cycling used in our study, fluoride from these deposits can be released to humper the enamel mineral loss, contributing to a protective effect against dental caries[28,29]. In line with this assumption, other study[30] concluded that enamel irradiation with a 940 nm Diode laser activated the APF and significantly favored fluoride uptake by the enamel surface as clearly noticed using scanning electron microscopy analysis.

Regarding the lesion depth, our results showed that only the 1.23% APF- gel treatment was effective in significantly decreasing lesion extension as compared to control group. This finding may partially be explained considering that when enamel sample is treated with APF-gel application and submitted to a pH cycling, the loosely fluoride (CaF_2) increases the ions saturation of the pH cycling solutions[22]. However, the same mechanism did operate when enamel was treated by the combination therapy. This result is in line with conclusion of a recent systematic literature review[31]. It is important to note that this in vitro study had limitations, such as not involving biofilm formation and not fully simulating the complexity of the oral environment. However, it simulated a condition of high cariogenic challenge and demonstrated and evaluated the effects of using indocyanine green in the application of diode laser associated with the application of FFA on the absorption of fluoride by dental enamel and on the reduction of mineral loss from enamel after an in vitro cariogenic challenge, being a pioneer in testing this treatment combination in this study model. We emphasize that further studies are still needed to evaluate the effects of the application of combined diode laser in reducing demineralization, given the need to seek alternative treatments that can help prevent caries disease.

In conclusion, the results of the present study confirmed that Diode laser irradiation, at λ 980 nm, combined with 1.23% APF- gel application was effective in inhibiting caries lesion progression in vitro. However, we were not able to detect any additional effect when the carious enamel was treated with the combination of Diode laser and APF as compared with the isolated effect of laser irradiation or APF gel treatment.

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3 CONCLUSÃO

A aplicação de indocianina verde associada à aplicação de laser de díodo não foi capaz de melhorar os efeitos do flúor fosfato acidulado na desmineralização do esmalte após desafio cariogênico.

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ANEXOS

Anexo 1 – Certificado de submissão do manuscrito

---------- Forwarded message ---------
De: **International Journal of Paediatric Dentistry** <no-reply@atyponrex.com>
Date: qua., 4 de dez. de 2024 às 21:10
Subject: Manuscript submitted to International Journal of Paediatric Dentistry
To: Marines Nobre dos Santos <mnobre@unicamp.br>

Dear Marines Nobre dos Santos,

Your manuscript "Are acidulated phosphate fluoride and 980 nm Diode laser effective in reducing the enamel mineral loss after a cariogenic challenge? – In vitro study" has been successfully submitted and is being delivered to the Editorial Office of *International Journal of Paediatric Dentistry* for consideration.

You will receive a follow-up email with further instructions from the journal editorial office, typically within one business day. That message will confirm that the editorial office has received your submission and will provide your manuscript ID.

Thank you for submitting your manuscript to *International Journal of Paediatric Dentistry*.

Sincerely,
The Editorial Staff at International Journal of Paediatric Dentistry

By submitting a manuscript to or reviewing for this publication, your name, email address, and affiliation, and other contact details the publication might require, will be used for the regular operations of the publication, including, when necessary, sharing with the publisher (Wiley) and partners for production and publication. The publication and the publisher recognize the importance of protecting the personal information collected from users in the operation of these services and have practices in place to ensure that steps are taken to maintain the security, integrity, and privacy of the personal data collected and processed. You can learn more by reading our data protection policy. In case you don't want to be contacted by this publication again, please send an email to IJPDedoffice@wiley.com.

Anexo 2 – Parecer do CEP

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

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Informações	PB_INFORMAÇÕES_BÁSICAS_DO_P	21/02/2024		Aceito

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Bairro: Areião **CEP:** 13.414-903
UF: SP **Município:** PIRACICABA
Telefone: (19)2106-5349 **Fax:** (19)2106-5349 **E-mail:** cep@fop.unicamp.br

Básicas do Projeto	ETO_2268533.pdf	19:12:44		Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	modelo_regulamento_biorrepositorioeduardo.pdf	21/02/2024 19:11:13	EDUARDO BANDEIRA SOUSA SILVA	Aceito
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Folha de Rosto	FOLHADEROSTO.pdf	22/12/2023 09:48:05	EDUARDO BANDEIRA SOUSA SILVA	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PIRACICABA, 21 de Fevereiro de 2024

Assinado por:
jacks jorge junior
(Coordenador(a))

Endereço: Av.Limeira 901 Caixa Postal 52, Prédio Administrativo, Segundo Piso, Setor de Secretarias de Ensino
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