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Assessment of Photodynamic Therapy and Nanoparticles Effects on Caries Models

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Abstract

AIM: To assess the antibacterial competence of 650 nm diode laser, Methylene Blue (MB) and Silver Nano-Particles (Ag NPs) on *Streptococcus mutans* in biofilm-induced caries models.

MATERIAL AND METHODS: One hundred eighty specimens were prepared and equally divided into 6 groups. One group was untreated (control), and the others were subjected to either MB, laser, Ag NPs, the combination of MB and Laser or MB, laser and Ag NPs.

RESULTS: Comparison of the log10 mean Colony Forming Units per millilitre (CFU/ml) values of each of the treated 5 groups and the control group was found statistically significant (P-value < 0.05).The combination of MB, laser and Ag NPs recorded the greatest reduction (95.28%). MB alone represented the least capable (74.09%). The efficiency differences among the Ag NPs treated group; the Laser treated group and the combined MB/Laser treated group were found statistically insignificant.

CONCLUSION: The combination of MB, 650 nm diode laser and Ag NPs may be among the highly effective modern antimicrobial therapeutics in dentistry.

Introduction

Dental caries in humans is still among the most prevalent diseases [1]. Caries treatment traditional procedures do not eliminate all the microorganisms in the residual dental tissues [2] [3]. Residual organisms interfere with amalgam or veneer durability in restorative dentistry [4]. Systemic and topical antibiotics and conventional disinfectants together with mechanical cavity preparation of caries do not completely disinfect dental biofilms and caries-related lesions [4].

Experimental chemical induction of caries through enamel demineralisation and direct acid exposure has been criticised as it lacks the bacterial biofilm interactions that characterize real caries formation *in vivo* [5] [6]. The caries-like enamel lesions formed in *Streptococcus mutans (S. mutans)* biofilm models have been found to fulfil all of the principal histological features of natural caries and has been used as a pre-clinical model for evaluation of caries-preventive agents [7] [8].

Modern antimicrobial therapeutics including photodynamic therapy (PDT) and metal nanoparticles (NPs) during caries treatment reflects the need to find out protocols giving the least residual microorganisms after mechanical caries removal. Unlike conventional antimicrobial agents, PDT based on photosensitising agents' activation by a light source is non-invasive, repeatable without developing drug resistance and easily reaches deep situated areas. It may kill microorganisms in a few minutes when the proper energy density is delivered. Its precise lesion selectivity depending on photosensitizers' careful topical application and irradiation site can be further enhanced with optical fibres assistance [9] [10]. The interaction of laser light and dental hard tissues is determined by its wavelength, pulse energy, duration of exposure, and repetition rate [11]. Diode laser

irradiation can penetrate up to 1000 µm into the dentinal tubules; the penetration power of chemical disinfectants is limited to 100 µm [12].

NPs have a greater surface-to-volume ratio than non-nanoscale particles of the same material, and therefore are more reactive [13]. The antimicrobial energy of NPs has been attributed to their multi-cationic and multi-anionic large surface and its positive charge density [14]. NPs combined with polymers or coated onto surfaces show antimicrobial applications within the oral cavity [15] [16]. Silver compounds and NPs have been studied for dental applications including dental restorative material, and caries inhibitory solution [17]. Silver nanoparticles (Ag NPs) have been applied in many health care fields because of their broad-spectrum bactericidal properties [18].

This study aims to evaluate the bactericidal efficiency of the 650 nm diode laser, methylene blue photosensitizer (MB) and Ag NPs on *S. mutans* in induced caries model.

Material and Methods

One hundred and eighty (n = 180) dentin discs from crowns of sound extracted human molars and premolars were obtained using the high-speed diamond disc. Each disc measured in 4 x 5 x 6 mm. Its surfaces were ground flat and polished. All but one of each fragment surfaces was sealed with acid-resistant nail-varnish [19]. Fragments were sterilised in an autoclave for 20 minutes at 121^{o}C. Dentin specimens were pre-conditioned with sterile artificial saliva at 37^{o}C for 2 hours [20], and saliva was then gently aspirated.

The bacterial suspension was prepared from the reference strain of *S. mutans* 1815^{T} bacteria Cultured in Brain Heart Infusion (BHI) Broth, and then sub-cultured onto Mitis Salivaris Agar. The culture was grown under capnophilic conditions for 48 hours at 37^{o}C.

The bacterial suspensions were prepared in BHI broth containing sucrose by transferring colonies from Mitis Salivaris Agar to reach an optical density adjusted to the standard turbidity of 0.5 McFarland units containing 1.5 x 10^{8} CFU/mL.

S. mutans biofilm generation on dentin specimens was formed by mixing the incubated *S. mutans* suspension with the pre-conditioned dentin fragments in the test tube. The culture was daily replaced with fresh new BHI Broth media solution (BHIS) for seven days under appropriate condition to allow for *S. mutans* biofilm formation [19].

After the 7-day incubation, fragments with biofilm were transferred to sterile plain lavender-top tubes and were randomly and equally distributed according to the planned anti-bacterial therapy into the following six groups:

Group I: Two ml MB 0.02 mg/ml was added to each sample and shaken.

Group II: Two ml sterile physiological saline solution was mixed with each sample, shaken and for 3 minutes at 0.5 cm exposed to 650 nm diode laser with 200 mW power.

Group III: Two ml yellow coloured liquid spherical shaped Ag NPs with 200 µg/ml concentration and 19 ± 5 nm particle-size was added to each sample and shaken.

Group IV: After 2 ml MB addition to each sample and shaking, 650 nm diode laser with power 200 mW was swept for 3 minutes at 0.5 cm distance.

Group V: 2 ml MB and 2 ml Ag NPs were added to the samples, and 650 nm diode laser with power 200 mW applied for 3 minutes at 0.5 cm distance.

Group VI: Specimens with biofilm were assigned as a negative control. The sterile physiological saline solution was added to the samples and was shaken.

Groups containing MB were kept covered with aluminium foil in a dark environment at 37°C for 5 minutes.

Serial dilution up to 10^{4} of the inoculums was carried out in sterile Eppendorf tubes. The microbial biofilms were detached from the fragment in a sterile physiological solution (0.9% NaCl), and 25 µl of the dilution was plated onto the surface of BHI agar plates. Plates were then incubated anaerobically at 37ºC for 24 hours in a dark field candle jar for protection against light and air.

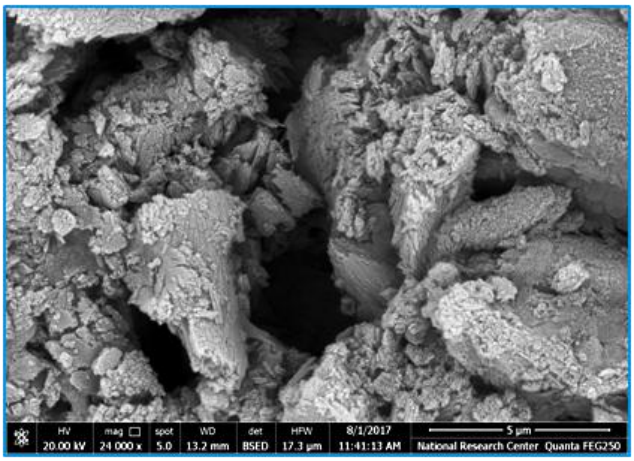

Figure 1: SEM- induced caries lesions on dentin discs

The antibacterial power of the tested therapeutic agents was assessed by:

- *Colony counting:* After incubation, the newly formed colonies were counted after 24 hours of incubation. Then the number of Colony Forming Units per millilitre (CFU/ml) was determined. Suspected colonies were confirmed to be *S. mutans* by Gram staining (crystal violet) under a light microscope.

- *Scanning electron microscope:* For evaluation of the induced caries lesions, dentin discs were rinsed with distilled water for 2 minutes to dislodge the attached biofilm. Dentin specimens were coated with a layer of gold in "S150A sputter coater" machine under vacuum. Then the outer surface was examined with a scanning electron microscope "QUANTA FEG 250" [19], (Figure 1)**.** Bacterial colonies were as well assessed under the same microscope to prove and recognise *S. mutans* bacterial shape Figure 2.

The CFU/ml results were log-transformed (log10) and analysed by the analysis of variance (ANOVA) followed by the Tukey test. A p-value < 0.05 was considered to indicate a statistically significant difference.

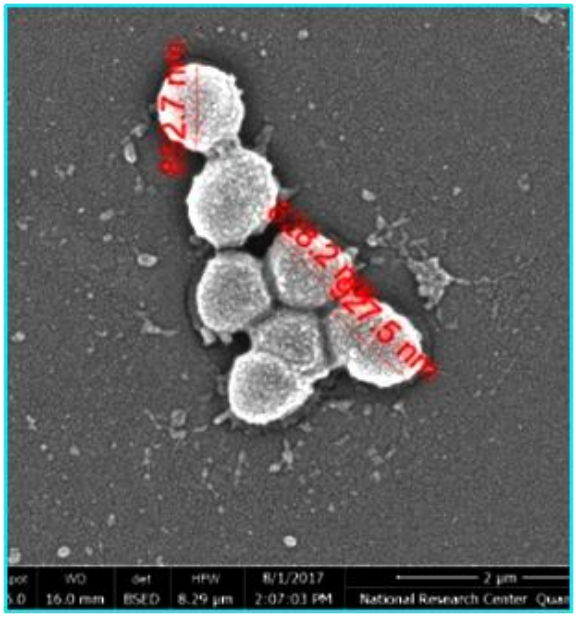

Figure 2: SEM picture of S. mutans

Results

Statistical analysis proved the high efficacy of the treatment with the combined three bactericidal agents namely Ag NPs, MB and Laser 650 nm in comparison with each of them alone. Specimens treated with Ag NPs, MB and exposed to 650 nm Laser recorded the greatest reduction percentage of CFU/ml (95.28%) compared to the control specimens. Group treated merely by 650 nm Diode Laser showed 94.27% reduction and the group treated with both 650 nm Diode Laser and MB gave 89.12% reduction. Specimens treated with MB solely recorded the lowest reduction percentage of CFU/ml (74.09%), Figure 3.

Table 1: Descriptive values of CFU/ml of the specimen's biofilms formed by exposure to S. mutans for different studied experimental conditions

	N	Minimum	Maximum	Mean	Reduction percentage of CFU/ml related to the control group
Control	30	20×10^6	140×10^6	53.23×10^6	-
Ag NPs	30	0.80×10^6	11.60×10^6	4.40×10^6	91.73%
MB	30	ND	52×10^6	13.79×10^6	74.09%
L650	30	ND	6×10^6	3.05×10^6	94.27%
MB+L650	30	0.80×10^6	20×10^6	5.79×10^6	89.12%
Ag NPs+MB+L650	30	ND	10×10^6	2.51×10^6	95.28%

ND means not detected (There were not a single CFU/ml).

There was a statistically significant reduction in the $\log_{10}$mean numbers of CFU/ml after using the combination of Ag NPs, MB and laser 650 nm irradiation in comparison to each of them separately. A significant difference was recorded by comparing the $\log_{10}$ mean CFU/ml values of the control group and the $\log_{10}$ mean CFU/ml values of each of the other groups.

Table 2: Mean values of CFU/ml ($\log_{10}$) and P-value

			P-value related to:					
Log^{10}	N	Mean ± SD	Control	Ag NPs	MB	L650	MB + L650	Ag NPs + MB+ L650
Control	30	7.65 ± 0.25	-	0.0001	0.0001	0.0001	0.0001	0.0001
Ag NPs	30	6.56 ± 0.29	0.0001	-	0.0001	0.785	0.654	0.0001
MB	27	7.07 ± 0.31	0.0001	0.0001	-	0.0001	0.0001	0.0001
L650	29	6.45 ± 0.21	0.0001	0.785	0.0001	-	0.062	0.036
MB+L650	30	6.68 ± 0.28	0.0001	0.654	0.0001	0.062	-	0.0001
Ag NPs+MB+L650	29	6.21 ± 0.44	0.0001	0.0001	0.0001	0.036	0.0001	-

All comparisons among different groups were statistically significant except the two comparisons between the Silver treated group and each of the laser treated group and the combined MB/ laser-treated group Table 1 and 2.

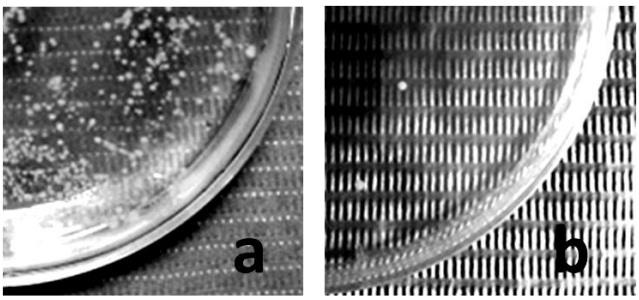

Figure 3: Bacterial colonies counted in different groups; a: control; b: Ag NPs+MB+L650

Discussion

Traditional mechanical cavity preparation of caries lesions does not guarantee complete eradication of bacteria. The principal objective of caries removal is to eliminate infected, necrotic hard tissues and microorganisms that may cause persistent

inflammation and treatment failure. Removal of the infected dentin has a direct influence on the clinical success of the restoration [4]. The caries treatment procedures widely practised presently does not eliminate all the microorganisms in the residual dental tissues [2] [3]. Cavity disinfection is an adjunctive approach to reduce the bacteria in the residual dental tissues left after cavity preparation. Considering the importance of efficient disinfection and elimination of microorganisms from the cavity, this study aimed to assess the efficacy of five different disinfection methods, which could be used as an adjunct to mechanical debridement.

S. mutans was used in this study because it is generally believed that this bacteria is known as the principal cariogenic pathogen [21]. Dental caries involves the adherence of bacteria and development of biofilms on a tooth surface [22]. Biofilm is an aggregate of microorganisms in which cells adhere to each other and to a surface [22]. Formed biofilm confers resistance against antibodies and antimicrobial agents to this microorganism [23]. Different in vitro biofilm models have been described [24] because of their easy handling and simplicity; monospecies batch cultures are preferred in in-vitro secondary caries models. For investigating antimicrobial agents, the cariogenic challenge is induced by the addition of sucrose. Steiner-Oliveira et al., [25] used a *S. mutans* monospecies biofilm model with initial specimen immersion in artificial saliva, growth medium change every 24 h and periodical exposures to sucrose, the same was used in our experiment. Hetrodt et al., 2018 [26] used dentin-enamel discs subjected to a *S. mutans* monospecies biofilm model with 0.5% sucrose to a McFarland turbidity standard of 0.5.

The mechanism of disinfection by PDT is via the irradiation of a photosensitizer. PDT mechanism of bactericidal action may include bacterial cell wall damage; cytoplasm membrane augmented permeability and nucleic acid strand breakage [27]. It is a host-friendly technique for the elimination of microorganisms whereas other methods such as the direct application of antibiotic therapy can be potentially harmful to the host [28]. MB as a photosensitizer combined with the laser irradiation kills the bacterium [29]. Azizi et al., 2016 [30] found that combination of MB and laser irradiation decreases the final number of *S. mutans* colonies more than MB without laser irradiation, which is in agreement with our results. PDT can effectively decrease the number of bacteria present in the biofilm. PDT and Ag NPs have become a trustworthy alternative antibacterial therapy for plaque-related diseases such as dental caries [9] [10]. In the current study, the addition of Ag NPs to the PDT (650 nm Diode Laser and MB) significantly reinforced the bactericidal potential of the applied therapy. In their study, Afkhami et al., [31] used conventional PDT with indocyanine green ICG (1 mg/ml)/810 nm diode laser (200 mW, 30 seconds), modified PDT Ag NPs/ICG/810 nm diode laser (200 mW, 30 seconds) against *Enterococcus faecalis*, diode laser alone 810 nm (1 W, 4 times for 10 seconds), Ag NPs alone (100 ppm). Matching with our results, they found the greatest reduction in colony-forming units with the modified PDT and the lowest with the conventional PDT. Pagonis et al., [32] also used poly (lactic-co-glycolic acid) nanoparticles with MB and red light at 665 nm supportive of combining NPs with PDT.

In 2016, Gomez et al., [33] claimed that *S. mutans* contain endogenous photosensitizer porphyrins and that just 5-minute exposure to 380-440nm wavelength violet-blue light killed *S. mutans* biofilm without any photosensitizer. This may explain the laser 650nm bactericidal effect when used alone in our current work. In the current experiment, the red diode laser was used with sucrose-biofilm, according to the advice given by Gomez et al., [33]. Our results showed laser bactericidal effect (6.45 *log10* and 94.27% CFU/ml) more than laser/MB (6.68 *log10* and 89.12%) with an insignificant difference. This may be due to the presence of endogenous photosensitizer in *S. mutans*. The used MB (0.02 mg/ml) with incubation period (5 minutes) might be insufficient. It has been used with higher concentrations [34] and longer incubation periods [32] for allowing adequate penetration in S. mutans. Afkahami et al., in 2016 [31] reported results showing 97% bacterial (Enterococcus faecalis) reduction with laser (810 nm, 1 W, 4 times for 10 seconds) alone versus 68% with laser/photosensitizer (Indocyanine green (1 mg/mL)/810 nm DL (200 mW, 30 seconds).

Pereia et al., [35] used 0.1 mg/ml MB and 660nm laser, alone and conjugated. They reported that photodynamic inactivation with MB and laser showed a great reduction in CFU/ ml of the *S. mutans* biofilm while laser alone and MB alone did not yield a noticeable bactericidal effect. MB, when used alone in our experiment, showed antibacterial activity. Using broth dilution assay, Soria-Lozano et al., [36] needed 0.025 mg/ml MB and metal halide lamp emitting 420-700 nm to reach 99.9% *S. mutans* inhibition. Araujo et al., [37] showed *S. mutans* bacterial reduction of 73% for MB when these photosensitizers were used at 25 mg/L, and a reduction of 48% was observed for MB at 5 mg/L using a red laser for one minute. Our results were comparable and showed 89.12% bacterial reduction using MB 0.02 mg/ml and 650 nm laser for 3 minutes. Neves et al., [38] assessed the clinical effect of PDT against *Lactobacillus spp.* And *S. mutans in* deciduous molars. They used 0.01% MB dye followed by irradiation with an In GaAlP diode laser (λ-660 nm; 40 mW; 120 J/cm2; 120 seconds). They did not find any significant difference in the number of colony-forming units (CFU) for any of the microorganisms. In our study, MB 0.02 mg/ml and 650 nm laser for 3 minutes 200 mW showed 89.12% bacterial reduction. In our study, 3 minutes, 200 mW were made. In their study, the specimens were only irradiated with a laser

for 120 seconds, 40 mW. The highest bactericidal power depends definitely on the energy density. With the used parameters in our experiment, the score 95.29% was achieved when Ag NPs, MB and 650 nm diode laser co-worked.

Ag NPs can lyse the cells and prevent their proliferation via several mechanisms [39] [40]. Ag NPs show multiple antibacterial mechanisms such as adherence and accumulation on the bacterial surface. Ag NPs damage cell membranes are leading to structural changes, which render bacteria more permeable [41]. Ag NPs prevent DNA duplication and the expression of ribosomes and other cellular proteins. It also interferes with energy transfer cycles of the bacteria [42]. We used Ag NPs with 200µg/ml concentration and 19 ± 5 nm particle-size, and results showed 91.73% decrease in the bacterial count. Cristóbal et al., 2009 [43] detected the minimum inhibitory concentration of AgNPs against *S. mutans* in with sucrose addition as 101.98 ± 72.03µg/ml for 8.4 nm, 145.64 ± 104.88 µg/ml for 16.1nm and 320.63 ± 172.83 µg/ml for 98 nm. Sucrose addition enhances the cariogenic power of *S. mutans* and gives more realistic results because it's almost always present in our diet [43]. The smaller the nanoparticle, the more it releases Ag+ ions, and their antibacterial effect can be better [43]. Holla et al., in 2012 [20] set 40 µg/ml as the minimum inhibitory concentration and minimum bactericidal concentration needed for nano-silver base inorganic anti-microbial agent against *S. mutans in vitro* using broth dilution assay.

In vitro biofilm caries models have been widely used to study the carious process under laboratory controlled conditions, in an attempt to simulate the clinical development of carious lesions [7] [44]. Dental caries results from interactions among different cariogenic microorganisms. In our study *S. mutans,* monospecies biofilm model was used. Differences in experimental conditions may explain discrepancies between in vitro under controlled conditions and in vivo studies. Whereas monospecies biofilm models based only on the cultivation of *S. mutans* [45] [46] do not mimic the metabolic interactions that occur among the diverse microbiota of a clinical dental biofilm, the outcomes obtained by high-complexity microbial models based on microcosm cultivation are directly dependent on inoculum source [47]. Even though a model cannot capture all of the details involved with caries formation, it can give us a means of performing reproducible experiments under controlled conditions [48].

In conclusion, this in vitro study recognises that the addition of Ag NPs to diode laser and MB enhance their antibacterial efficiency against *S. mutans* in caries models. This modern therapeutic combination has a high potential for use in operative dentistry for *S. mutans* eradication.

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