Antimicrobial Effect of Diode Laser with Silver Nanoparticles against Enterococcus faecalis.

(In Vitro Study)

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Abstract

Introduction: The goal of a root canal treatment is to get rid of the bacteria and its byproducts and stop germs from coming back into the root canal system once it has been mechanically disinfected. Unfortunately, it was discovered that employing conventional procedures was insufficient for completely cleaning the dentinal tubular system and root canals, making this goal more challenging to be fulfilled. Irrigating agents can't completely kill all microorganisms in the deeper layers since they don't penetrate deep enough. Dentists have lately used diode lasers in clinics for a variety of dental applications, with positive outcomes for dentinal disinfection.

Aim:to determine the bactericidal effect of diode laser (445 nm) and silver nanoparticles on Enterococcus faecalis.

Methodology: Fifty extracted maxillary anterior human teeth with one root canal were inoculated with Enterococcus faecalis and organized into five groups; (I) Control group, (II) Sodium hypochlorite group, (III) Laser group, (IV) Silver nanoparticles group and (V) Laser with silver nanoparticles group. After the treatment protocol, the samples were analyzed by colony forming units (CFU) for analysis of the remaining bacteria. Also, they were stained with dye to detect live and dead bacteria using a confocal laser scanning microscope (CLSM). Lastly, the specimens were examined to detect the glutathione peroxidase, superoxide dismutase and ROS levels.

Results: CFU: The control group showed the highest bacterial load followed by the SNP group then the laser group then SNP-L group and finally the NaOCl group with a significant difference between them. CLSM: The SNP group showed the highest bacterial load percentage reduction followed by the NaOCl group then the laser group then SNP-L group and finally the control group with a significant difference between them. Antioxidant markers GPX and SOD showed highest levels in positive control group and

lowest levels in combination group. ROS level was significantly high in the combination group and significantly decreased in control group.
Conclusion: Silver nanoparticles had an antibacterial effect on E.feacalis which could be enhanced using low power diode laser.
Key Words: Enterococcus faecalis, NaOCl, SNP, diode laser
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Introduction:

The goal of a root canal treatment is to remove bacteria and its byproducts while also preventing re-infection of the root canal system after mechanical disinfection. [(1)] Unfortunately, it was discovered that using the conventional methods was insufficient for completely cleaning the root canal system, making its accomplishment more challenging. [(2-4)] Because irrigating solutions don't penetrate far enough, they can't completely kill all the microorganisms in the deeper layers. [(5)]Though, dentists have been using diode lasers for a variety of dental applications, with positive outcomes for teeth disinfection.[(6-8)]

Due to the presence of endogenous photosensitizing chromophores in pathogenic microbes, blue light in the 400–470 nm range of the spectrum is currently becoming more widely recognized as a phototherapy–based antimicrobial agent with significant antimicrobial activity against a wide range of bacterial and fungal pathogens. Additionally, it is less likely to give rise to resistance formation than antibiotics and less harmful to host cells than ultraviolet radiation. [(9-11)]

The widely recommended root canal irrigant is sodium hypochlorite due to its effectiveness in eradicating microorganisms and ability to dissolve tissue. [(12)] However, because it may result in tissue destruction of the tissues, direct application of sodium hypochlorite may be hazardous to the host. [(13,14)]

High bactericidal effects have been achieved in dentistry by using nanotechnology. Such as silver nanoparticles, due to their small size and higher surface area compared to conventional materials, have antibacterial qualities and compatible physical characteristics. As a result, it is frequently employed in dentistry to stop the growth of bacteria in a variety of applications. [(15-18)] Through membrane disruption, they have an antimicrobial impact on both Gram-positive and Gram-negative bacteria. [(19)]

Enterococcus faecalis whichis a normal inhabitant of the oral flora and a Gram-positive facultative anaerobic microorganism, is linked to endodontic treatment failure as well as in periradicular lesions including primary and secondary endodontic infections. E. faecalis is discovered in 40% of primary endodontic infection cases, while it is more frequently discovered in 67–77% of secondary periradicular infection cases. [(20,21)]

Therefore, Enterococcus faecalis is the most prevalent bacteria causing root canal treatment failures.[(22–24)]It is resistant to antibacterial drugs like NaOCl in various treatments. [(25,26)]Due to biofilm production and penetration into the dentinal tubules, it is tolerant to calcium hydroxide pastes and alkaline pH, which generally suppress other bacteria. [(27,28)]

Aim:

To assess the antimicrobial effect of low power diode laser 445 nm with silver nanoparticles against Enterococcus faecalis.

Materials and methods:

1. Samples selection:

A total of fifty extracted maxillary anterior human teeth with one root canal were collected. All roots were intact with mature apices.

2. Samples preparation:

The teeth were scaled with ultrasonic scaler to remove all adhering soft tissues and kept in saline solution at room temperature

until time of use. Then, they were decapitated at the level of cemento-enamel junction using high speed diamond disc to facilitate the mechanical preparation of the root canals, which the working length was established by subtracting 0.5 mm from the apical foramen and was standardized at 15mm. The canals were instrumented using ProTaper Universal rotary files.

ProTaper Universal NiTi rotary file system was used to prepare the canals in a crown-down fashion. The instruments were used in a 16:1 gear reduction handpiece powered by a torque-speed controlled endodontic motor electric motor at a speed of 250 - 300 rpm. The torque was adjusted for each file as recommended by the manufacturer; 3.5 Ncm for SX and S1, 1.2 Ncm for S2, 2.0 Ncm for F1and 2.6 Ncm for F2, F3, F4 and F5.

Ethylene Diamine Tetra Acetic acid (EDTA) 17 % paste was used as a lubricant during the mechanical preparation to remove smear layer. The irrigation protocol after each file was using 3ml for 1 min 5.25 % sodium hypochlorite (NaOCl) solution filled in a plastic syringe with a side-ended 27-gauge needle inserted passively as deep as possible inside the canals. Then, 3 ml of EDTA solution and a final irrigation with 3 ml distilled water.

After the completion of the shaping procedure, the apical foramina were sealed with glass ionomer cement from the external surface to prevent loss of solutions. The external roots surface was sealed with a layer of colorless varnish sealing the lateral canals ends and to avoid external microbial contamination.

3. Samples sterilization:

Each root was placed in a micro-tube containing sterile brain-heart infusion (BHI) broth and then all samples were autoclaved at temperature of 121 C for 20 minutes to remove all pre-existing bacteria.One root was randomly selected as the negative control which was placed in an incubator for 2 weeks to verify no bacterial growth.

4. Bacterial culture:

The Enterococcus faecalis (ATCC 29212) (E. faecalis) was maintained in culture on K-F Streptococcus agar plates and incubated at 37 °C for 24 h. Isolated colonies of pure cultures were scraped from agar plates by using sterile swab, then suspended in Brain Heart Infusion (BHI) Broth (oxoid, England) and dispersed by vortex under laminar air flow to adjust turbidity of the bacterial suspension. (Macferalad 0.5)

5. Root canals contamination and biofilm formation:

E. faecalis bacterial suspension was placed into all the root canals, each placed into a new micro-tube containing BHI broth and stored at 37 C for 2 weeks. During this period, the intra-canal suspension was replaced with 20 ml of a new suspension every 48 hours**(Fig. 1)**.

When the specimens' contamination period had elapsed, one root among the samples was randomly served as the positive control and processed for colony forming units (CFU) analysis to confirm bacterial biofilm formation in root canal system on Congo Red Agar (CRA) **(Fig.2)**. The agar plates were incubated at 37 C for 24 hours in anaerobic condition.

While the rest of the roots were dried from the BHI broth to receive the laser fiber optic and the antimicrobial solutions using a sterile dried paper points size 50 which were used once in each root.

Fig (1): Some of teeth samples after incubation.

Fig (2): Positive control CFU.

6. Root canals classification:

The samples were randomly divided into fivemain groups:

Group 1: Control
Group 2: Sodium Hypochlorite (NaOCl)
Group 3: Laser
Group 4: Silver nanoparticles (SNPs)
Group 5: SNPs + Laser

Sodium Hypochlorite group:

It is conventional antibacterial debridement using 5 ml of 5.25% NaOCl irrigation for 1 minute with 27-gauge side end needle plastic syringe that was introduced passively up to 1 mm from the working length. It was injected to fill the canal and left for 1 min.

Low power diode laser group:

Pioon dental diode laser device **(Fig.3)**was used with the 445 nm wavelength, output power of 0.1 W. The fiber optic tip of 200 microns diameter and 3 cm long. Its movement was in a spiral way from coronal to apical and vice versa, 10 movements / min, by the same operator, starting from 1 mm short of the working length, in a continuous mode and with exposure time for 1 min. Decontamination of the optical fiber tip was done after each root canal by a piece of gauze.

Fig (3): Pioon dental laser device.

Silver nanoparticles (SNPs) group:

The study used their particle size range between 30-40 nm and concentration of 200 ppm. SNPs **(Fig. 4)** were injected to fill the canal, left for 5 min and covered by aluminum foil in the dark.

Fig (4): Silver nanoparticles colloidal solution.

7. Samples after disinfection:

The canals were irrigated with 5ml of distilled water to wash out used solutions, dried with sterile paper point, and then were placed again in sterile micro-tubes filled with BHI broth and stored in an incubator at 37 C.

8. Microbial analysis (CFU):

To assess the remaining root canals contamination by Enterococcus Faecalis, the BHI broth inside all the canals was dried using sterile paper points and the canals were filled with sterile normal saline solution as a transfer fluid. The collection was made immediately after the treatment using sterile paper points size F5 left in the root canals for 1 minute. The paper points used were transferred to tubes with 1 ml of sterile saline solution and agitated in vortex mixer (Vortex AP 56, Phoenix, Araraquara, SP,Brazil) for 1 min and afterwards, serial decimal dilutions were made and seeded on petri dishes with CRA **(Fig.5)**. The plates were incubated under microaerophilic conditions at 37 ° C for 48 h. The results were obtained by means of counting colony forming units per milliliter (CFU mL 1)

Fig (5): Congo Red Agar with swab.

9. Confocal Laser Scanning Microscope (CLSM):

The samples of CLSM were sectioned for detecting viable and dead bacteria on root canals walls. The sectioning was done precisely into two halves by IsoMet device **(Fig.6).** Then, each half was stained by acridine orange (AO) and propidium iodide (PI) dyes respectively just before scanning **(Fig 7).**

CLSM was used to emit 40x objectives for scanning. Multiple random areas all over the root canals were scanned. The images taken for

the median intensity of green (live with intact cell membrane) and red (dead with damaged cell membrane) bacteria and was calculated by a specific software.

Fig (6): IsoMet device.

Fig (7): Confocal Laser Scanning Microscope (CLSM)

10. Assessment of Glutathione peroxidase and Superoxide dismutase by colormetry:

Bacterial cells were harvested by centrifugation for 15 min. The bacterial lysate was used to determine the level of Glutathione peroxidase (GPx) and Superoxide dismutase (SOD)**.** The techniques were done according to the manufacturer's instructions (Biodiagnostic, CAT NO :GR25 24- Biodiagnostic CAT NO: 25 21 respectively). The enzymes activity was measured by the inhibition of nitrobluetetrazolium reduction by O_2-generated by the xanthine/xanthine oxidase system**(Fig 8)**.

11. Assessment of ROS by ELISA:

The level of ROS was measured in the Bacterial lysate using ROS elisa kit amsbio (AMS.E01R0021). The spectrophotometric absorbance was assessed at 450 nm in accordance with the manufacturer's instructions. The results were expressed as IU/mL**(Fig 8)**.

Fig (8):GPx, SOD and ROS assessments.

12. Statistical Analyses:

Data were presented as mean and standard deviation. Between group comparisons were conducted using ANOVA test followed by Tamahne post hoc test for pairwise comparisons. Significance level for statistical tests was set at 0.05.Statistical analysis was performed using SPSS software (IBM Corp.

Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.)

Results

1. CFU:

The control group showed the highest bacterial load followed by the SNP group, then the laser group then SNP-L group and finally the NaOCl group. ANOVA test showed a significant difference between the five groups (p<0.001).**(Table 1)(Fig 9 & 10)**.

Pairwise comparisons:

Tamahne post hoc test showed significant differences in bacterial loads between all pairs of groups.

Table (1): mean, standard deviation (SD) and the results of ANOVA test and Tamahne post-hoc test for comparison of bacterial loads in CFUs/ml between the five groups:

	SNP-L group	SNP group	Laser group	NaOCl group	Control group	*p* - value
Mean	309[d]	1240[b]	529[c]	39[e]	4510[a]	<0.001*
SD	97.8	222.1	41.8	12.9	604.5	

**Significant at p<0.05*

***Different lower-case letters indicate statistical significance between each group.*

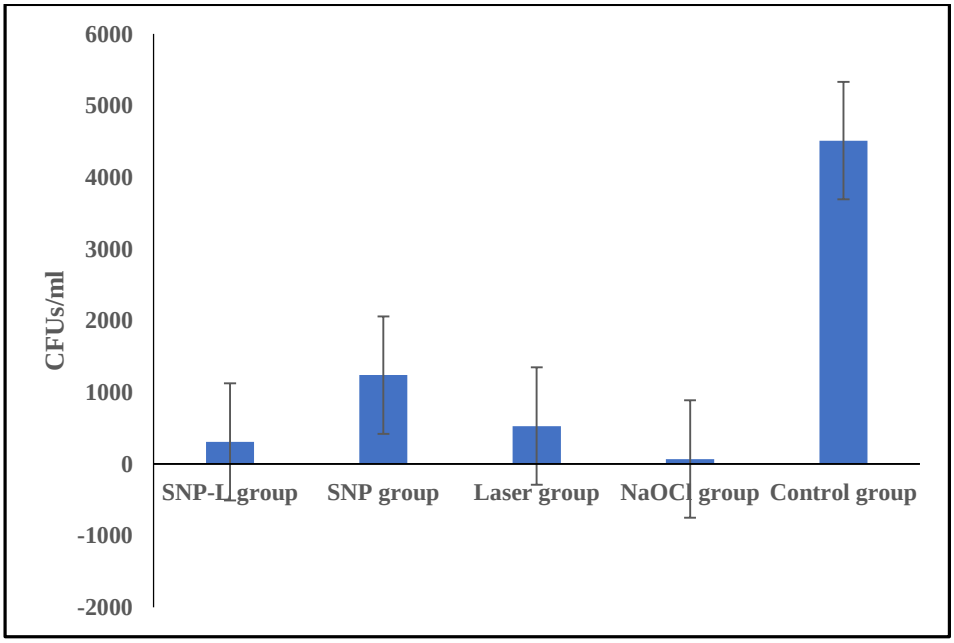
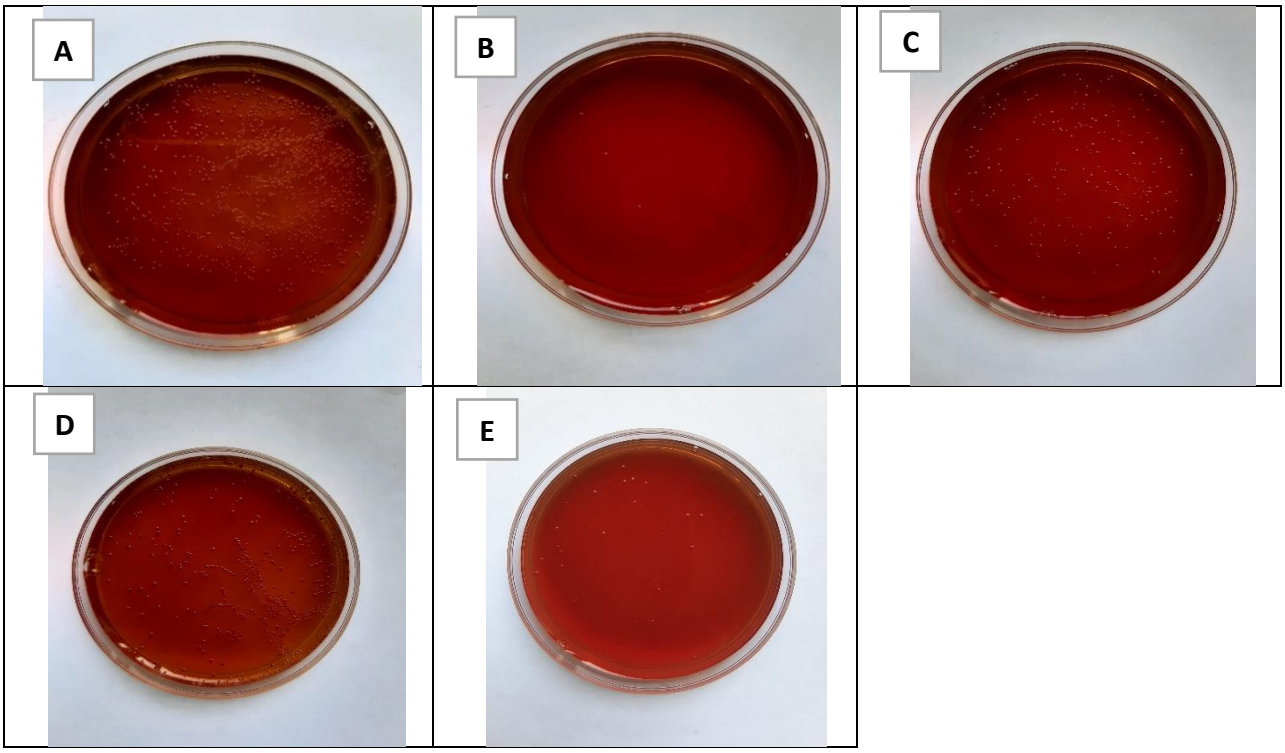


Fig (9): Bar chart representing the mean bacterial loads in the 5 groups.



Fig(10): Colony forming units agar plates showing E.faecalis. (A) Control group. (B) NaOCl group. (C) Laser group. (D) SNP group. (E) SNP-L group.

2. CLSM measuring bacterial percentage reduction:

The SNP group showed the highest bacterial load percentage reduction followed by the NaOCl group then the laser group then SNP-L group and finally the control group. ANOVA test showed a significant difference between the five groups (p<0.001).**(Table 2)(Fig 11 & 12)**.

Pairwise comparisons:

Tamahne post hoc test showed a significant difference in bacterial load percentage reduction between the SNP group and the SNP-L group. There were no significant differences between SNP group, laser group, and NaOCl group. There were no significant differences between SNP-L group, laser group, and NaOCl group. The control group showed significantly lower bacterial percentage reduction than all other groups.

Table (2): mean, standard deviation (SD) and the results of ANOVA test and Tamahne post-hoc test for comparison of bacterial percentage reduction between the five groups:

	SNP-L group	SNP group	Laser group	NaOCl group	Control group	*p* - value
Mean	43.6%[b]	49.6%[a]	47.3%[ab]	47.7%[ab]	33.9%[c]	<0.001*
SD	7.0%	3.8%	5.0%	8.9%	4.0%	

**Significant at p<0.05*

***different lower-case letters indicate statistical significance between each group.*

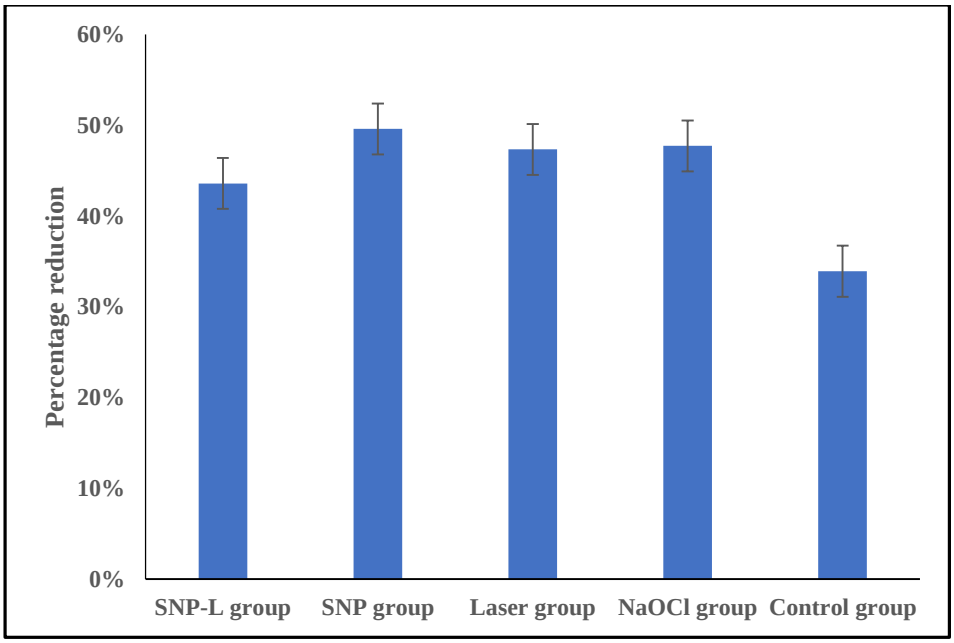
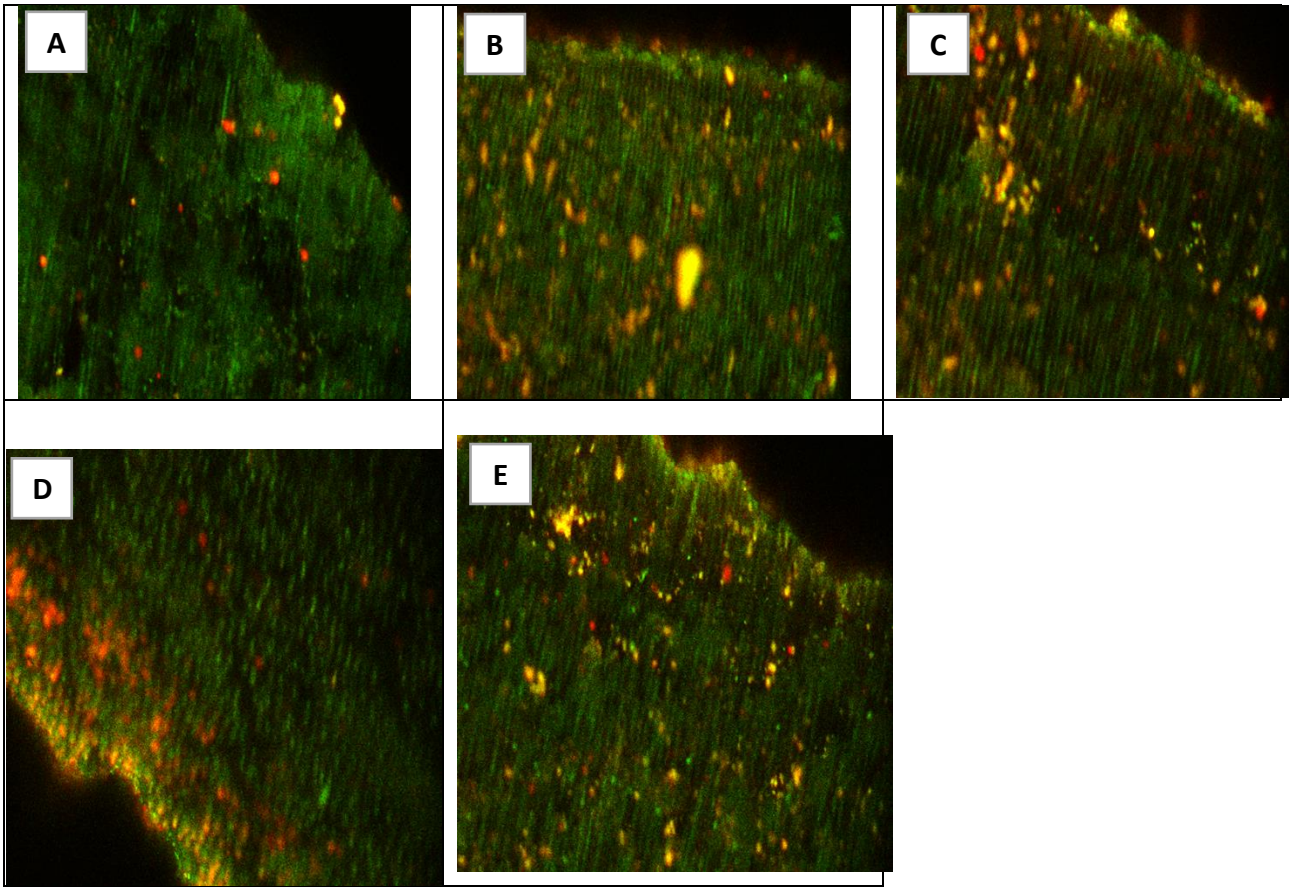


Fig (11): bar chart representing the mean bacterial percentage reduction in the 5 groups.



Fig(12): Colony forming units agar plates showing E.faecalis. (A) Control group. (B) NaOCl group. (C) Laser group. (D) SNP group. (E) SNP-L group.

3. Assessment of Glutathione peroxidase and Superoxide dismutase by colormetry:

a- Glutathione peroxidase:

The control group showed the highest level followed by the NaOCl group then the laser group then SNP group and finally the SNP-L group. ANOVA test showed that there was a significant difference between the five groups (p<0.001).**(Table 3) (Fig 13)**

Pairwise comparisons:

Tamahne post hoc test showed significant differences in glutathione level between all pairs of groups.

Table (3): mean, standard deviation (SD) and the results of ANOVA test and Tamahne post-hoc test for comparison of levels in nmol/ml between the five groups:

	SNP-L group	SNP group	Laser group	NaOCl group	Control group	*p* - value
Mean	24.8[e]	34.4[d]	56.7[c]	64.2[b]	77.2[a]	<0.001*
SD	1.7	2.5	3.5	2.7	4.3	

**Significant at p<0.05*

***Different lower-case letters indicate statistical significance between each group.*

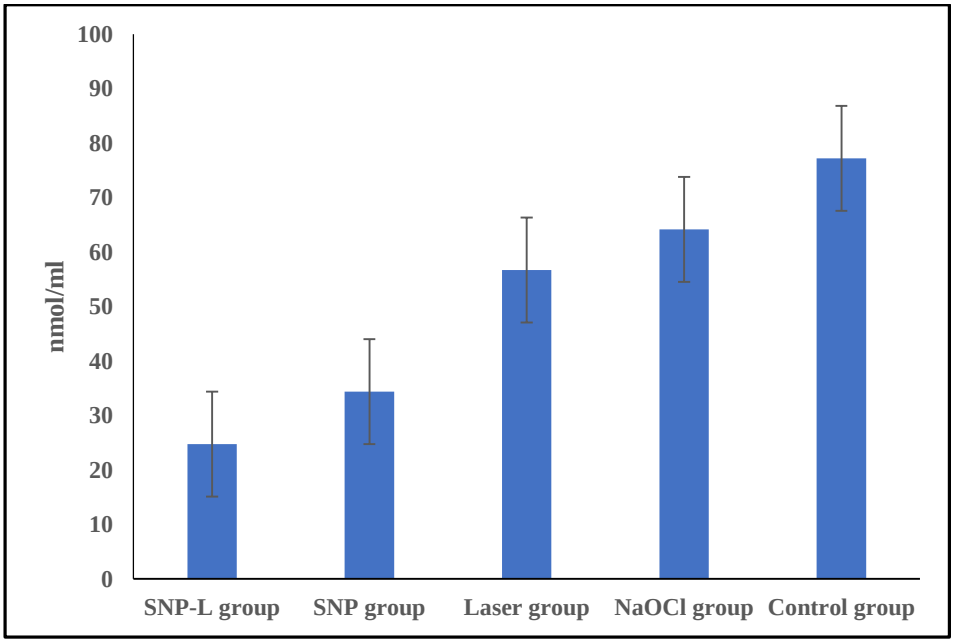


Fig (13): Bar chart representing the mean GPx level in the 5 groups.

b- Superoxide dismutase:

The control group showed the highest level followed by the NaOCl group then the laser group then SNP group and finally the SNP-L group. ANOVA test showed that there was a significant difference between the five groups (p<0.001). **(Table 4) (Fig 14)**

Pairwise comparisons:

Tamahne post hoc test showed no significant difference in superoxide levels between the control group and the NaOCl group. There were significant differences between all the other pairs of groups.

Table (4): mean, standard deviation (SD) and the results of ANOVA test and Tamahne post-hoc test for comparison of levels in u/ml between the five groups:

	SNP-L group	SNP group	Laser group	NaOCl group	Control group	*p* - value
Mean	1.5^{d}	2.5^{c}	3.5^{b}	4.9^{a}	5.1^{a}	<0.001*
SD	0.3	0.2	0.3	0.6	0.4	

**Significant at p<0.05*

***Different lower-case letters indicate statistical significance between each group.*

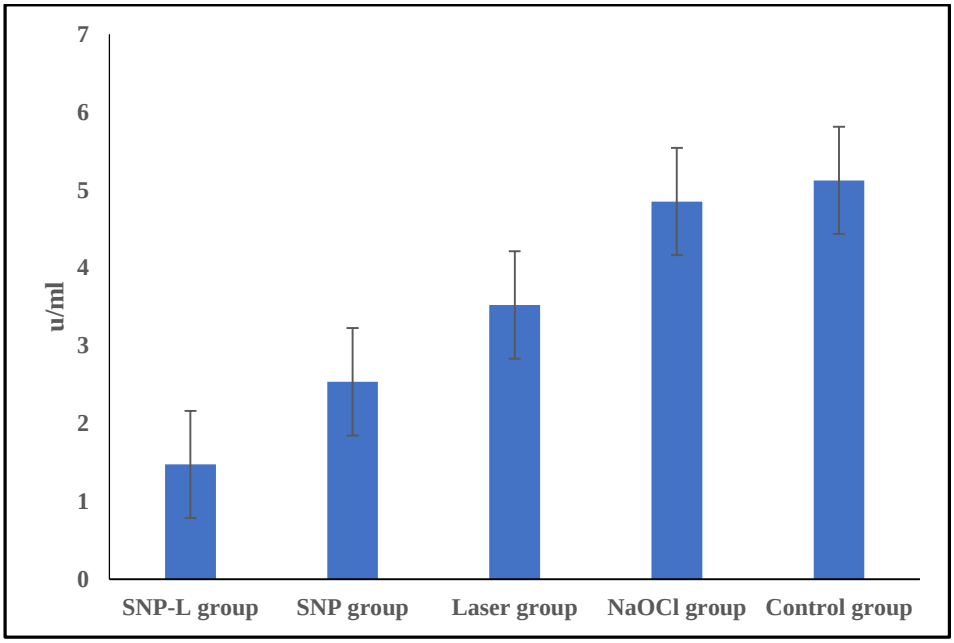


Fig (14): Bar chart representing the mean SOD level in the 5 groups.

c- ROS:

The SNP-L showed the highest ROS level followed by the SNP group then both the laser and NaOCl groups and finally the control group. ANOVA test showed that there was a significant difference between the five groups (p<0.001). **(Table 5) (Fig 15)**

Pairwise comparisons:

Tamahne post hoc test showed no significant difference in ROS level between the laser group and the NaOCl group. There were significant differences between all the other pairs of groups.

Table (5): mean, standard deviation (SD) and the results of ANOVA test and Tamahne post-hoc test for comparison of ROS level in IU/ml between the five groups:

	SNP-L group	SNP group	Laser group	NaOCl group	Control group	*p* – value
Mean	2.9^{a}	2.3^{b}	1.5^{c}	1.5^{c}	1.1^{d}	<0.001*
SD	0.2	0.1	0.1	0.3	0.2	

**Significant at p<0.05*

***Different lower-case letters indicate statistical significance between each group.*

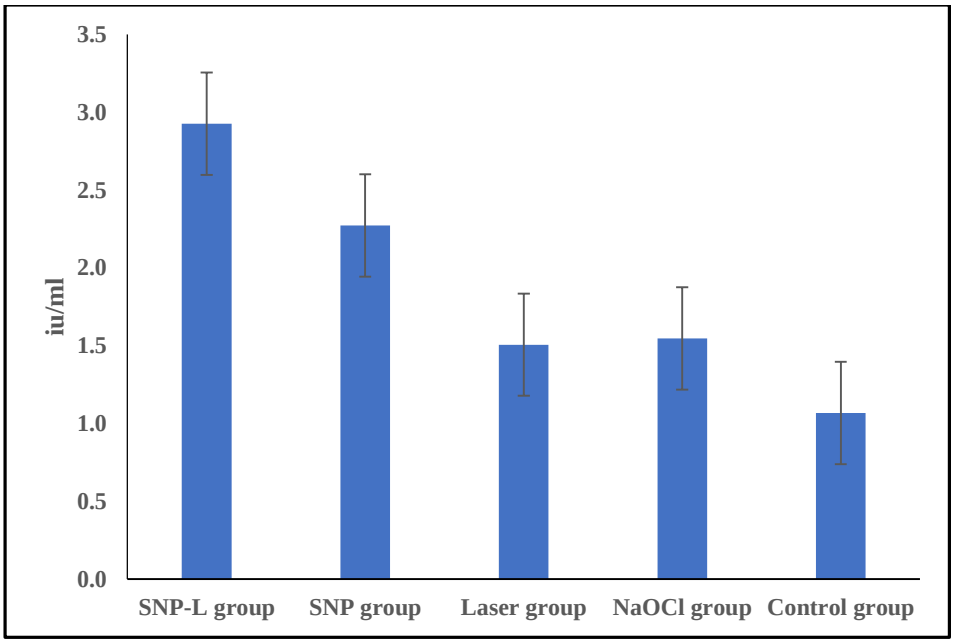


Fig (15): Bar chart representing the mean ROS level in the 5 groups.

Discussion:

The purpose of this study was to test the antimicrobial effect of low power diode laser 445 nm with silver nanoparticles against Enterococcus faecalis.That is the oral cavity's most resilient speciesleading to root canal treatment failure. [29]It possesses virulence-enhancing substances such enzymes, cytolysin and lipoteichoic acid. It was chosen for this investigation because it has a history of exhibiting antimicrobial resistance to a wide range of medications and has been linked to resistant apical periodontitis. [30]

Sodium hypochlorite was chosen for the main reference since it is the universal optimum effective irrigant for root canal therapy. It can dissolve organic debris, get rid of biofilm and the remnants of necrotic tissue. [31]Hypochloric acid is thought to be the cause of the sodium hypochlorite's action on inorganic material because it combines with insoluble proteins to produce soluble products that make it possible to remove the superficial smear layer. [32]Nevertheless, sodium hypochlorite harms the periapical tissues and decreases the elastic modulus and flexural strength of dentine.

Sodium hypochlorite can be replaced with silver nanoparticles in intracanal irrigation for endodontic treatment. It was discovered that dentine's mechanical qualities were not considerably impacted by root canal irrigation with a silver nanoparticle solution.[33] Another study found that sodium hypochlorite can be replaced with silver nanoparticles in intracanal irrigation for endodontic treatment. As it had superior tissue biocompatibility and was as potent as sodium hypochlorite in the elimination of both Enterococcus faecalis and Staphylococcus aureus.[34]

Irrigating solutions have insufficient penetration depth, thus cannot destroy the microorganisms in the deeper areas inside the dentinal tubules. Diode laser has been used with promising results for dentinal disinfection. Thus, in the present study, 445 nm diode laser has been used against E. faecalis.Blue light has attracted increasing attention because of its intrinsic antimicrobial effect which does not require the introduction of exogenous photosensitizers as in the photodynamic therapy (PDT) and the less harmful to the cells than ultraviolet irradiation. [35]As well as the use of fine diameters of optic fibers (200-320 µm)

facilitate the lasereffective delivery into the root canal aiding the reduction of bacterial contamination. [36]

The most popular method used in vitro for evaluatingirrigants antimicrobial efficacy along with laser irradiation is the Agar diffusion test. The results of microbiological cultures demonstrate a positive bactericidal effect of SNP solution as an endodontic irrigation on E.faecalis, but was less significant than that occurred with NaOCl at 5.25 % concentration. That agreed with Hendi et al. [53]As well as Rodrigues et al [15]study, which demonstrated that SNP solution used was not able to eradicate bacteria present in dentinal tubules at all time intervals tested and thus was significantly less antimicrobial action compared with sodium hypochlorite in cervical and middle regions and in superficial and deep areas. While, found that they had similar results using other concentrations and durations. [19, 37, 38]

The potent antibacterial efficacy of cationic silver nanoparticles might be due to electrostatically interaction between positively charged nanoparticles with the negatively charged bacterial cells leading to loss of cell wall, increasing permeability, leakage of intracellular components and killing of bacteria. [39-41]In addition to, damage to enzymatic system, plasmids and DNA simultaneously providing the bacteria least capacity to develop resistance leading to cell death. [42-45]AgNPs have a high surface area to volume ratio that allows the optimal possible interaction with bacterial surfaces leading to a higher antimicrobial activity. [46]

Possible explanations for these observations of its lower antimicrobial effect would be the limited interaction between positively charged AgNPs and negatively charged bacterial cells during the shorttime period of root canal irrigation and the resistance of the biofilm matrix resulting in limited bacterial killing. Despite the antibacterial effectiveness of AgNPs in dentistry, cells cytotoxicity and dentin staining are possible adverse effects may exist, thus made it a controversial agent for in vivo application. [47]Although previous studies have assured that AgNPs cytoxicity is concentration dependent, further studies are required to optimize the use of AgNPs for root canal disinfection. [48]

Also, this study showed that SNP-L group had a higher antibacterial activity than laser or silver nanoparticles groups. This coincides with Safan et al[36] that used diode 980 nm laser irradiation with SNP against Lactobacillus acidophilus bacteria which had more bactericidal effect than SNP alone. While this study revealed that the laser group had a statistically significant lower mean bacterial count than the silver group. That coincides with Sadony et al.[49] but does not coincide with results founded in Hendi et al study. [53]The commonly accepted hypothesis for the 445 nm blue laser is the elimination of highly cytotoxic reactive oxygen species in a similar way to photodynamic therapy. [50]

ROS are formed as intermediate products in a variety of physiological conditions. Different detoxification enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), as well as other antioxidants, such as flavonoids, ascorbic acids, vitamin E, and glutathione, strongly regulate the concentration of these substances in cellular organelles (GSH). Nanoparticle-induced free radical generation causes GSH to become oxidized, which triggers the onset of oxidative stress. [51]Our results showed that increased ROS level and decreased level of GPX and SOD especially in SNP groups. this is can be explained by that Ag nanoparticles enter the cell and break down inside, generating Ag+ ions that damage the mitochondria. As byproducts of the electron transport chain, ROS build up and damage and degrade the operation of mitochondria, depolarize the mitochondrial membrane, harm mtDNA (mitochondrial DNA), and peroxide lipids and protein components, ultimately resulting in apoptosis.[52]

Conclusion:

Silver nanoparticles had an antibacterial effect on E.feacalis which could be enhanced using low power diode laser 445 nm. This is considered as an adjunctive endodontic disinfection to using the traditional sodium hypochlorite irrigation.

Declarations:

Consent for Publication: I certify that the work has been approved by all the authors for submission.

Competing interests: none

Funding: Authors received no fund

Conflicts of Interest: No conflicts of interest as regard publication of this paper were declared by the authors.

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