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Antibiofilm activity of three irrigation protocols activated by ultrasonic, diode laser or Er:YAG laser *in vitro*

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Abstract

Aim To investigate the impact of three irrigation protocols, activated by three different methods, on mature biofilms of *Enterococcus faecalis* in vitro.

Methodology Root canals in 280 single-rooted teeth were instrumented using a rotary Ni-Ti system. Biofilms of *E.faecalis* were generated based on a previously established protocol. Samples were randomly divided into 3xperimental (n=80) and one control (n=40) group based on the irrigation protocol employed: group 1 *(NaOCl + Etidronic acid)*, 1:1 mixture of 6% NaOCl and 18% etidronic acid; group 2 *(NaOCl - EDTA)*, 3% NaOCl followed by 17% EDTA; group 3 *(NaOCl - EDTA - NaOCl)*, 3% NaOCl followed by 17% EDTA and a final flush of 3% NaOCl. Saline served as the control. Samples were further divided into 4 subgroups (n=20) based on the activation method: subgroup A, no activation; subgroup B, ultrasonic activation; group C, diode laser; group D, Er:YAG laser . Confocal Laser Scanning Microscopy was used to assess bacterial viability in situ. Radicular dentin powder was obtained for analysing the colony forming units (CFU/mL). Data were analysed by appropriate statistical analyses with $P = 0.05$.

Results All experimental irrigation protocols caused complete destruction of the biofilm in the root canal lumen. Within the dentinal tubules, all groups had a significantly higher percentage of dead bacteria than the saline control ($P < 0.05$). There was no significant difference between NaOCl + etidronic acid and NaOCl-EDTA-NaOCl ($P > 0.05$), whereas both groups brought about more bacterial reduction than NaOCl-EDTA ($P < 0.05$). There was no significant difference between diode laser and Er:YAG laser in any of the groups ($P > 0.05$). Both diode and Er:YAG laser were more effective than ultrasonic activation and conventional syringe irrigation in reducing *E.fecalis* biofilms ($P < 0.05$).

Conclusions The use of NaOCl after or in combination with a chelator caused the greatest reduction of *E.faecalis*. Diode laser and Er:YAG laser activation were superior to ultrasonics in dentinal tubule disinfection.

Introduction

Reduction of intracanal bacteria is an imperative step in attaining a successful outcome of root canal treatment (Shuping *et al.* 2000, Siqueira 2001). Insufficient eradication of intraradicular bacteria could be attributed to the complex morphology of the root canal system as well as the organisation of intracanal bacteria into biofilms (Sundqvist *et al.*1998, George & Kishen 2005). Bacteria in biofilms are resistant to many of the antimicrobial agents used in endodontics (Distel *et al.* 2002). The root canal microflora in post-treatment disease shows a predominance towards gram positive bacteria, in addition to facultative and obligate anaerobes (Sundqvist *et al.*1998, Peciuliene *et al.* 2001, Rôças *et al.* 2004). In one classic study, 67% of failed cases of root canal treatment were found to harbour *Enterococcus faecalis* in contrast to its detection in 18% of primary endodontic infections (Sundqvist *et al.*1998). The argument towards this contention is that *Enterococci* are transient microbes in the oral cavity. However, any change in the microenvironment can create conditions that are favourable for infection by *E.faecalis* (Zehnder & Guggenheim 2009) leading to the development of periradicular disease.

Mechanical debridement alone can bring about a 100-1000 fold bacterial reduction, but complete eradication does not appear possible (Cvek *et al.* 1976, Wu & Wesselink 1995, Dalton *et al.* 1998,). Chemomechanical preparation of the root canal system aims at realizing the goal of killing bacteria as well as destroying intracanal biofilms. However, rendering canals bacteria-free appears to be an elusive goal. Mechanical instrumentation along with irrigation with 0.5% sodium hypochlorite (NaOCl) has been shown to be unable to attain complete eradication of bacteria (Cvek *et al.* 1976). Hence, contemporary endodontic research realizes the importance of root canal cleaning and shaping, and focuses on different antimicrobial agents as irrigants and intracanal medicaments.

Sodium hypochlorite (NaOCl) in varying concentrations is the most commonly used root canal irrigant, owing to its ability to kill bacteria, destroy biofilms and dissolve vital and necrotic tissue. Although there is no clear consensus on the sequence of irrigation during root canal treatment, the most commonly employed irrigation sequence involves use of NaOCl (1%-6%) and 17% ethylene diamine tetraacetic acid (EDTA). This regime has been termed soft chelation (De Deus *et al.* 2008). An alternating irrigating regimen comprising of NaOCl and EDTA, followed by a final flush of

NaOCl has also been recommended. An alternative irrigation protocol employing a mixture of a weak chelator with NaOCl has also been proposed (Lottanti *et al.* 2009), a protocol termed continuous chelation (Neelakantan *et al.* 2012).

Activation of irrigants appears to be an important method of increasing antibacterial and antibiofilm activity of root canal irrigants, not only within the root canal, but also within the anatomical complexities of the root canal system and dentinal tubules. Sonics, ultrasonics and lasers are widely researched as potential irrigant activation methods (de Gregorio *et al.* 2009, De Moor *et al.* 2009, Cachovan *et al.* 2013, Halford *et al.* 2012). The activation of irrigants by ultrasonics results in increased velocity, flow and exchange of irrigants, resulting in movement of the irrigant into the anatomical areas of the root canal system which would otherwise be inaccessible by conventional syringe irrigation (Lee *et al.* 2004). Lasers have been shown to bring about reduction in intracanal bacterial load (Meire *et al.* 2009). In addition to this, targeted photosensitiser delivery based photoactivated disinfection also appears promising (George & Kishen 2007). A more recent development is the use of pulsed erbium:yttrium-aluminum-garnet (Er:YAG) lasers for activation of root canal irrigants, a technology termed photon induced photoacoustic streaming (PIPS) (de Groot *et al.* 2009). Er:YAG laser uses a low energy laser light to generate a photoacoustic shockwave, which streams irrigants throughout the root canal system (DiVito *et al.* 2012). The efficacy of these three activation methods on the three irrigation regimens mentioned earlier is not known.

This study was undertaken to examine the antibiofilm efficacy of three irrigation protocols (mixture of NaOCl andetidronic acid, NaOCl followed by EDTA and NaOCl-EDTA-NaOCl) activated by ultrasonics, diode laser or Er:YAG laser, on mature *E. faecalis* biofilms grown on root dentine *in vitro*.. The null hypothesis was that the experimental irrigation protocols did not result in a better disinfection than the control group, and that the ultrasonic agitation, diode laser or Er:YAG laser activation did not improve the antibiofilm efficacy of the irrigation regimens**.**

Materials and methods

Sample preparation

Freshly extracted single-rooted mandibular premolars, with a closed apex (n=280) were used. These teeth had been extracted according to each patient's individual treatment plan, which was not related to the current study. The sample collection and study methodology were approved by the Institutional Review Board and Ethics Committee of the University. The teeth were stored in 0.01% NaOCl solution and maintained hydrated until use. The canals were accessed, and the length of the teeth was determined by inserting a size 10 stainless steel K- file into the canal until the file tip was just visible at the apical foramen. The cusp was reduced until each tooth measured 19 mm in length. The working length was defined as 1 mm short of the apical foramen at 18 mm.

The biofilm tooth model was adapted from previously published work (Lin *et al.* 2013). Briefly, the root canals were prepared with Mtwo rotary nickel titanium instruments (VDW GmBH, Munich, Germany) to an apical size of 25, 0.06 taper using 3% sodium hypochlorite as the irrigant. Buccal and lingual grooves were created to split the tooth longitudinally in such a way that they could be re-approximated later. The split halves were then re-approximated using utility wax placed over the root tip and a dental stone encasing was fabricated. This simulated an *in vivo* closed apical system that provided resistance to irrigant flow by creating an apical vapor lock effect (Tay *et al.* 2010, Lin *et al.* 2013). The smear layer was removed by placing the root sections in an ultrasonic bath of 5.25% sodium hypochlorite and 17% EDTA for 4 min each. All the sections were rinsed in sterile water for 1 min and autoclave sterilized for 20 min at 121°C in brain heart infusion broth (BHI) (Hi Media Labs, Bangalore, India). The root sections were then incubated in BHI broth for 24 hours at 37°C to ensure no bacterial contamination.

Bacterial inoculation and biofilm generation

Enterococcus faecalis (ATCC 29212) was plated on BHI broth supplemented with 1.5% (wt/vol) agar (Hi Media Labs) and incubated anaerobically at 37°C for 24 hours. A single colony of *E. faecalis* from a BHI agar plate was collected and suspended in sterile BHI broth. The sterilized specimens were placed in sterile centrifuge tubes containing 3mL *E.faecalis* suspension ($1x10^8$ CFU/mL

determined spectrophotometrically). The specimens were incubated under anaerobic conditions at 37°Cfor 4 weeks. Fresh BHI broth was replaced every second day to remove dead cells and to ensure bacterial viability. After incubation, the specimens were removed from the tubes aseptically and gently rinsed with sterile phosphate-buffered saline (PBS) to remove the culture medium and non-adherent bacteria. Four root sections were randomly selected and observed by a field emission scanning electron microscope (JSM-7500F, JEOL Ltd., Tokyo, Japan) to verify the presence of *E. faecalis* biofilms on the dentine surfaces and within the dentinal tubules .

Treatment of biofilms

The sectioned teeth were reassembled and placed in the stone casing after which they were randomly divided into 3 experimental (n=80 per group) and one control group (n=40). The treatment groups were further divided as follows: group 1 *(NaOCl + Etidronic acid)*, 1:1 mixture of 6% NaOCl and 18% etidronic acid (Zschimmer & Schwarz, Mohsdorf, Germany); group 2 *(NaOCl - EDTA)*, 3% NaOCl (Parcan, Septodont, Saint-Maur-des-Fossés, France) followed by 17% EDTA (Pulpdent, Watertown, MA, USA); group 3 *(NaOCl - EDTA - NaOCl)*, 3% NaOCl followed by 17% EDTA and a final flush of 3% NaOCl. Sterile saline was used as the irrigant in the control group (group 4). The irrigant contact time was standardised to 6 min (for groups 2 and 3, contact time for sodium hypochlorite and EDTA were 4 min and 2 min respectively).

The specimens of each experimental group (n=20 per subgroup), and specimens of the control group (n=10) were randomly divided into four subgroups based on the activation protocol: subgroup A, no activation; subgroup B, ultrasonic activation; group C, diode laser; group D, Er: YAG laser. For specimens in subgroup A, canal disinfection was accomplished by placing a 30-gauge Max-i-probe needle as close to the working length as possible without binding. The quantity of all irrigants was standardized to 5mL. Ultrasonic activation was performed using ultrasonic files (IrriSafe, Satelec Acteon, Merignac, France) in an ultrasonic generator (EMS 600 ultrasonic unit). The irrigants were activated for 30 seconds following that fresh irrigant was placed into the canal and the process repeated until a total irrigation time of 6 min was reached.

The diode laser had a 940 nm wavelength, 50-60Hz frequency system, which can deliver energy in pulsed or continuous wave mode, with a maximum output power of 7W, into 200-μm plain ended fibre suitable for endodontic applications (Ezlase, Biolase, San Clemente, CA, USA). The continuous wave mode was employed for this laser. The protocol for Er:YAG laser was followed exactly according to the manufacturer's instructions by a clinician proficient with the protocol. The solution was activated by a 2940-nm wavelength Er:YAG laser (Fidelis; Fotona, Ljubljana, Slovenia) at 10Hz pulse rate, 50 microseconds pulse duration and 50mJ pulse energy , fitted with a newly designed 21- mm-long, 400 microns endodontic conical fibre tip (Photon induced photoacoustic streaming - PIPS 400/14, Fotona). In the case of diode laser and Er:YAG lasers, the tips were placed into the coronal reservoir only and activated for 30 seconds. Again, additional irrigant was deposited only in cases in which the coronal reservoir was depleted, until a total irrigation time of 6 minutes was reached. For groups 2 and 3, activation was done for 30 seconds after each irrigant. After canal irrigation had been completed, the canals were irrigated with 5 mL 2M sodium thiosulfate for 30 seconds to neutralize the irrigants.

Confocal Laser Scanning Microscopic examination

Root section from each group (n=20 sections) were stained with fluorescent LIVE/DEAD BacLight Bacterial Viability stain (Molecular Probes, Eugene, OR, USA) and were viewed using a confocal laser scanning microscope (CLSM) (LSM 510, Carl Zeiss, Jena, Germany). Two to 3 random areas of the biofilm on each dentine section were scanned with a 2-mm step size by the CLSM. Simultaneous dual-channel imaging was used to display the green fluorescence (live cells) and red fluorescence (dead cells). Three dimensional reconstruction was done using the Image J software (Abramoff *et al.* 2004), and quantification of the CLSM images was done using the BioimageL software (Chávez de Paz 2009). The ratio of dead cells (red fluorescence to total fluorescence ratio) was calculated.

Dentine powder analysis

Dentine debris from the root samples (n=20) was harvested at 2 depths (200 and 400 microns) using Gates Glidden drills nos. 4 and 5 (Mani Inc., Tochigi-Ken, Japan), respectively, and collected in 1

mL of sterile BHI broth and incubated in an anaerobic environment at 37°C for 24 hours. A single run with a no.4 and 5 Gates Glidden drill along the root canal wall of the sections after separation of the fragments was considered to provide dentine powder from depths of 200 and 400 microns respectively (Krithikadatta *et al.* 2007). The content of each microcentrifuge tube was serially diluted, 100 µL of broth in 100 µL of normal saline for 5 times. Five microlitres of this diluted sample was plated on BHI agar plates and incubated for 24 hours.

Data presentation and analysis

The two main outcome measures in this study were the percentage of dead cells and the number of CFU in the powder sample as determined by plate counting. Analysis of raw data by Shapiro-Wilk test showed normal distribution and consequently parametric tests were applied for statistical analysis. Two-way ANOVA was performed to weigh the effect of the irrigation protocol and activation method as the two independent variables on the outcome (percentage of dead cells or CFU/mL). To identify the effect of activation protocols on bacterial reduction (dead cell %) in each irrigation protocol, one-way ANOVA was applied. Bonferroni's correction for multiple testing was used in one-way analysis of variance. The alpha error was set at P=0.05 for the analyses.

Results

Confocal Laser Scanning Microscopic (CLSM) analysis of biovolume and viable/dead cells in biofilm structure

The data obtained from the CLSM is tabulated (Table 1). Fig.1 shows a dense and homogenous biofilm in a pre-treatment specimen. Fig.2 (1-4) shows representative three-dimensional recontstruction of the biofilm structures obtained from different experimental groups. Since the biofilm structure was totally destroyed within the root canal lumen for all groups (except the control), only the biomass within the dentinal tubules was analyzed. Disregarding the activation method, all groups had a significantly higher percentage of dead bacteria than the saline control ($P< 0.05$). Groups 1 and 3 had a significantly higher percentage of dead bacteria than groups 2 and 4 ($P< 0.05$). There was no significant difference between group 1 and group 3 ($P> 0.05$).

Subgroups activated with diode laser or PIPS had a significantly higher percentage of dead bacteria than ultrasonic agitation or no activation in groups 1 and 3 ($P < 0.05$) while in groups 2 and 4, there was no significant difference ($P > 0.05$). There was no significant difference between diode laser and PIPS in any of the groups ($P > 0.05$).

Dentin powder analysis for the quantitative assessment of the viable biofilm bacteria (Table 1)

Data from this analysis revealed a significant reduction of viable bacteria in all groups compared to the control ($P<0.05$). At 200 microns depth, all groups except the control had a 7 log reduction of bacteria (no growth). At a depth of 400 microns, specimens of group 1 had a 5 log reduction of bacteria when diode laser or PIPS were used for activation. This was significantly less than the CFU/mL of group 3 using the same activation methods ($P<0.05$).

Discussion

The relatively complex anatomy of the root canal system hampers its complete debridement (Chow 1983). When the pulp becomes completely necrotic and infected, the pulp space and the root canal walls contain numerous bacteria, and these bacteria may also penetrate the dentinal tubules to reach the external surface of the root (Love & Jenkinson 2002). This results in a periradicular inflammatory lesion, and may also be considered an important cause for failure of root canal treatment (Trope & Bergenholtz 2002). Hence, some authors recommend placing an intracanal medicament such as calcium hydroxide to achieve adequate microbial control (Byström *et al.* 1985, Shuping *et al.* 2000). In contrast, some argue that placing an intracanal medicament does not significantly improve the success of root canal treatment (Peters *et al.* 2002, Peters & Wesselink 2006). Hence, in the absence of any conclusive evidence on the role of intracanal medicaments, the irrigation protocol should be relied upon to bring about adequate microbial control.

This study aimed at comparing the bacterial reduction brought about by three common irrigation protocols in root canal treatment. Root canal irrigation protocols commonly employ the proteolytic agent sodium hypochlorite and a chelating agent such as EDTA. While the primary actions of sodium hypochlorite pertain to tissue dissolution and bacterial reduction, the application of EDTA

aims to remove the smear layer that is formed during mechanical instrumentation (Zehnder 2006). An important aspect of this study was that, with the exception of the control group, the biofilm in the canal lumen was destroyed in all the groups. Hence the bacterial viability within the biomass inside the dentinal tubules was analysed. The results of the present study showed that the maximum bacterial reduction was brought about by group 1 (NaOCl + Etidronic acid) and group 3 (NaOCl-EDTA-NaOCl). With regard to the irrigation protocol, the use of a disinfecting agent such as NaOCl after the use of EDTA, and the use of continuous chelation brought about significantly higher bacterial reduction. This means that chelation allowed better penetration of NaOCl into the dentinal tubules facilitating bacterial reduction (Zehnder 2006). The process of continuous chelation prevents the formation of a smear layer. Hence, it is possible that NaOCl was able to penetrate the dentinal tubules better in this situation (Lottanti *et al.* 2009). It may also be speculated that the continuous chelation protocol was able to penetrate better into the bacterial biofilm matrix and disrupt it, thereby allowing better contact of NaOCl with the bacteria.

The use of NaOCl-EDTA-NaOCl has been shown to completely remove the smear layer and bring about erosion of the root canal surfaces (Baumgartner & Mader 1987). Such a process of accelerated dentine erosion may predispose to weakening of the root structure (Niu *et al*. 2002). It is also possible that the slightly longer contact time of NaOCl and the chelating agent combination in group 1 (6min) compared to the contact time with NaOCl (4 min) and EDTA (2min) in group 3 could have resulted in better results in group 1.

Several techniques have been proposed to improve the efficacy of irrigants. These include changes in concentration, temperature, addition of surfactants, and activation (Stojicic *et al.* 2010). This study revealed that diode lasers and Er:YAG laser were superior to conventional syringe irrigation and ultrasonic activation, in groups 1 and 3 in reducing the overall biomass within the dentinal tubules. Hence, the null hypothesis is partially rejected. While the maximum percentage of dead bacteria was found when NaOCl+Etidronic acid was activated by PIPS, this was not significantly less than diode laser activation. Similarly, these results were not significantly different from diode and PIPS activation of NaOCl-EDTA-NaOCl. This result on the effectiveness of PIPS is in accordance with a previous report (Peters *et al.* 2011). PIPS, which works via a Er:YAG laser,

functions by a direct shock wave mechanism, producing a photomechanical effect when the laser is pulsed in liquid (Doukas & Flotte 1996, de Moor *et al.* 2010). The working mechanism of erbium laser activated irrigation is increased fluid dynamics and movement due to expanding and imploding vapour bubbles at the fibre tip. Shock waves may also contribute to the cleaning action, but this has not been demonstrated in the root canal. This is important considering that the results varied with the activation method, despite smear layer removal prior to bacterial inoculation. Er:YAG laser is also effective in removal of the smear layer from the root canal walls (Takeda *et al.* 1998). Furthermore, explanation for the results achieved by the PIPS group lies probably in the better irrigant dynamics, and also in the increased reaction rate (Macedo *et al*. 2010). This explains why PIPS activation of groups 1 and 3 was associated with superior bacterial reduction.

This study revealed that all experimental irrigation protocols were associated with zero bacteria at 200 microns depth. The results at a depth of 400 microns showed a 5 log reduction of bacteria when diode laser or PIPS were used to activate NaOCl+etidronic acid or NaOCl-EDTA-NaOCl. Furthermore, the reduction in bacteria at 400 microns was significantly less when the continuous chelation protocol was employed. Previous studies on the efficacy of PIPS in detruction of bacterial biofilms as well as dentinal tubule disinfection, suggested that the effects are a non-thermal, photoacoustic subablative process (Pedulla *et al.* 2012), resulting in an increased reaction kinetics of NaOCl (Stojicic *et al.* 2010, Macedo *et al.* 2012). The findings of the present study support this hypothesis. As discussed earlier, it can be hypothesised that more effective removal of the smear layer by group 1 and further increase in the reaction kinetics of NaOCl by the lasers could be the reason for the results obtained in this study. Furthermore, the results of this study also show that diode lasers possibly bring about an increase in the kinetics of the irrigants similar to PIPS. While this may be true for pulsed diode lasers (Hmud *et al*. 2010), such a correlation has thus far not been drawn for diode lasers in continuous mode. Continuous diode laser irradiation has not been evaluated thus far for irrigant activation. It is possible that the results for the diode laser in this study could be attributed to the increase in temperature of the irrigant by the laser.

There are no clear recommendation in the literature on irrigation or activation times. While some papers recommend only a 20 seconds activation (de Moor *et al.* 2010, Peters *et al.* 2011), a 1

min exposure time has also been recommended to increase the kinetics of NaOCl activity (Macedo *et al.* 2010). However, several factors can influence the activity: amount of sclerotic dentine, preparation sizes and most importantly, the microbial load within the root canal (Svensater & Bergenholtz 2004, Paque *et al.* 2006, Aydin *et al.* 2007). The present study used a 4 min activation for all activation methods. Although this time frame is in excess of previous reports for PIPS, this helped achieve standardised exposure times. However, none of the samples were associated with negative cultures at the 400 microns depth. This could be because of the mature biofilm model that was used. Future studies should look into the effects of lower activation times on mature biofilm models. Furthermore, it is important to understand that root canal anatomy is a complex entity and direct extrapolation of these results should be performed with caution. Future work should also focus on the role of these irrigation protocols and activation methods on disinfection of complex root canal systems.

Conclusions

The intracanal biofilm was entirely destroyed in all experimental groups. NaOCl + etidronic acid or NaOCl-EDTA-NaOCl resulted in better dentinal tubule disinfection than NaOCl - EDTA. Also, activation of these irrigants with diode laser or PIPS brought about superior dentinal tubule disinfection compared to conventional and ultrasonically activated irrigation

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Legends to Figures:

Figure 1 Three dimensional CLSM image of root canal wall the prior to treatment, showing dense biofilm

Figure 2 Representative three dimensional CLSM images of the dentinal tubules showing apparently live (green) and apparently dead (red) bacteria of the groups activated by PIPS. 1: NaOCl + etidronic acid; 2: NaOCl - EDTA; 3: NaOCl - EDTA - NaOCl; 4: Saline

Table 1. Outcome data gathered in the current study and their statistical analysis; percent of apparently dead bacterial cells in the biofilm within the dentinal tubules, assessed by confocal laser microscopy after different treatment regimes, as well colony-forming units at different dentin depths (200 and 400 microns).

GROUP	PERCENTAGE OF DEAD BACTERIA	200 MICRONS	400 MICRONS
1: NaOCl + Etidronic acid			
A: No activation	$65.7 \pm 1.48^{a,A}$	No growth ^{a,A}	$4.8 \times 10^{3} \pm 0.55 \times 10^{2a,A}$
B: Ultrasonic activation	$66.8 \pm 1.61^{a,A}$	No growth ^{a,A}	$3.8 \times 10^{3} \pm 0.67 \times 10^{2a,A}$
C: Diode laser	$91.2 \pm 1.54^{b,A}$	No growth ^{a,A}	$410 \pm 77^{2b,A}$
D: PIPS	$93.6 \pm 2.03^{b,A}$	No growth ^{a,A}	$300 \pm 63^{2b,A}$
* *NaOCl - EDTA*			
A: No activation	$51.1 \pm 1.21^{a,B}$	No growth ^{a,A}	$2.7 \times 10^{5} \pm 0.32 \times 10^{5a,B}$
B: Ultrasonic activation	$54.3 \pm 0.69^{a,B}$	No growth ^{a,A}	$1.9 \times 10^{5} \pm 0.15 \times 10^{5a,B}$
C: Diode laser	$62.9 \pm 0.78^{a,B}$	No growth ^{a,A}	$2.4 \times 10^{5} \pm 0.19 \times 10^{5a,B}$
D: PIPS	$58.9 \pm 0.82^{a,B}$	No growth ^{a,A}	$2.3 \times 10^{5} \pm 0.25 \times 10^{5a,B}$
3. NaOCl - EDTA - NaOCl			
A: No activation	$70.3 \pm 1.01^{a,A}$	No growth ^{a,A}	$2.1 \times 10^{3} \pm 0.18 \times 10^{4a,A}$
B: Ultrasonic activation	$69.3 \pm 1.46^{a,A}$	No growth ^{a,A}	$1.8 \times 10^{3} \pm 0.25 \times 10^{4a,A}$
C: Diode laser	$87.5 \pm 1.69^{b,A}$	No growth ^{a,A}	$1020 \pm 14^{4a,C}$
D: PIPS	$89.92 \pm 1.57^{b,A}$	No growth ^{a,A}	$850 \pm 12^{4a,C}$
4. Saline (control)			
A: No activation	$1.91 \pm 0.42^{a,C}$	$2.2 \times 10^{7} \pm 0.70 \times 10^{7a,B}$	$3.1 \times 10^{7} \pm 0.49 \times 10^{7a,C}$
B: Ultrasonic activation	$1.92 \pm 0.46^{a,C}$	$2.1 \times 10^{7} \pm 0.67 \times 10^{7a,B}$	$3.2 \times 10^{7} \pm 0.28 \times 10^{7a,C}$
C: Diode laser	$2.83 \pm 0.32^{a,C}$	$1.9 \times 10^{7} \pm 0.27 \times 10^{7a,B}$	$2.9 \times 10^{7} \pm 0.18 \times 10^{7a,D}$
D: PIPS	$2.97 \pm 0.36^{a,C}$	$2 \times 10^{7} \pm 0.30 \times 10^{7a,B}$	$3 \times 10^{7} \pm 0.20 \times 10^{7a,D}$

Within each group, values with identical lower case superscript alphabet indicates no significant difference ($P > 0.05$); Between groups, for the same subgroup, values with identical upper case superscript alphabet indicates no significant difference ($P > 0.05$)