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Antimicrobial Efficacy of Laser Activated Irrigation and Ultrasonic Activated Irrigation Against e-faecalis – An Invitro Study

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Introduction

The goal of root canal treatment is the efficient disinfection of the root canal system and prevention of reinfection [1]. Traditionally, it is accomplished by a combination of mechanical instrumentation, irrigant disinfection and interappointment intracanal medication. Following mechanical instrumentation, large areas of the root canal system remained untouched, regardless of the system and/ or technique applied for biomechanical preparation [2,3]. Irrigants have been traditionally delivered using a syringe and needle (Haapasalo et al. 2010). The problem with this irrigation technique is inadequate replacement of the irrigant throughout the root canal system as the highest streaming velocity is present only in the lumen of the needle and around the tip of the needle [4]. Sodium hypochlorite (NaOCl) is the most commonly used root canal irrigant because it can dissolve organic tissue, kill microorganisms, and act as a lubricant. However, the high surface tension of sodium hypochlorite (NaOCl) prevents direct contact of the irrigant with the dentinal walls of the anatomical complexities [5].

Paque et al. (2009) reported that following NaOCl syringe/needle irrigation and instrumentation, 40–60% of the canal still contains cultivable bacteria.[6]. Furthermore, because of high surface tension, sodium hypochlorite penetrates only 130 µm into dentinal tubules, while

bacteria can colonize the dentinal tubules deeply up to 1100 µm from the canal lumen. [7] High temperature and agitation have been applied to increase the efficacy of sodium hypochlorite, so as to overcome the inherent shortcomings of NaOCl as an irrigant using syringe and needle. Accordingly, different agitation techniques have been proposed to improve the efficacy of irrigation solutions, including hand agitation as well as sonic, ultrasonic devices and lasers. [8,9,10]

Ultrasonic and sonic systems were introduced as a means to increase the effectiveness of chemomechanical preparation and effective cleaning of the root canals. Carver et al [11] found that significantly reduced bacterial counts compared with hand and rotary instrumentation alone. It involves the use of ultrasound with a small file or smooth wire (size 10–20) oscillating freely in the root canal to induce powerful acoustic microstreaming so that the smear layer and organic content can be removed from the root canal [12] It has been demonstrated that ultrasound may result in acoustic cavitation, so explosions and implosions generating pressure waves which create shear stress along the root canal walls, which may be sufficient to remove smear layer and biofilms. Such active root canal irrigation has been shown to facilitate the disruption of biofilms and make cell the membrane of bacteria more permeable to NaOCl.[13]

Lasers have also been proposed as an alternative to conventional approach in cleaning, disinfecting and shaping of the root canal or as an adjuvant to conventional chemo-mechanical preparation in order to enhance debridement and disinfection. It has been seen that solid-state laser systems with short pulse durations can induce pressure waves in water, including the near-infrared Nd:YAG laser and more recently the middle infrared Er:YAG and Er,Cr:YSGG lasers [14,15]. Diode lasers has been effective against E.faecalis owing to the affinity of their wavelengths for bacterial cells. (Gutknecht N 2004) .Moreover, laser irradiations are able to penetrate deep into dentinal tubules because they are not absorbed by dental hard tissues. So a 63% reduction of bacterial population can be achieved at a depth of 750 µm [16]

The major cause of endodontic failure is the survival of microorganisms in the apical portion of root filled teeth. E.faecalis can adhere to the root canal walls, accumulate, and form communities organized in biofilm, which helps it resist destruction by enabling the bacteria to become 1000 times more resistant to phagocytosis, antibodies and antimicrobials than non biofilm producing organisms. The antimicrobial resistance of biofilm bacteria has been attributed to the protective barrier provided by the extracellular polymeric matrix. Biofilms grow in a nutrient -deprived ecosystem as it concentrates trace elements and nutrients by physical trapping and electrostatic interaction. E.faecalis resists intra canal medicaments like calcium hydroxide by maintaining pH homeostasis.[17] Its prevalence in infection ranges from 24% - 77%. The organism possesses several virulence factors, and ability to survive effects of root canal treatment and persist as a pathogen in root canals. Our challenge as endodontic specialist is to implement methods to effectively eliminate this micro- organism during and after root canal treatment.

Colony-forming unit (CFU) is a rough estimate of the number of viable bacteria or fungal cells in a sample. Viable is defined as the ability to multiply via binary fission under the controlled conditions. In contrast in a microscopic evaluation, all cells, dead and living are counted. The visual appearance of a colony in a cell culture requires significant growth - when counting colonies it is uncertain if the colony arose from one cell or 1,000 cells. Therefore results are reported as CFU/mL

(colony-forming units per milliliter) for liquids, and CFU/g (colony-forming units per gram) for solids to reflect this uncertainty (rather than cells/mL or cells/g). An advantage to this method is that different microbial species may give rise to colonies that are clearly different from each other, both microscopically and macroscopically. The colony morphology can be of great use in the identification of the microorganism present.[18]

The aim of this study was to evaluate and compare the effectiveness of two modes of irrigation: ultrasonic activation and laser activation of NaOCl using diode laser in eradicating *E. faecalis* that had been innoculated in the root canals of extracted single rooted teeth.

Materials and Methods

The Test Organism

The Enterococcus faecalis (ATCC 29212) (HiMedia Lab Pvt.Ltd. India) was procured, the organism was maintained on nutrient agar, and the purity of the cultures were periodically checked by gram staining.

Preparation of Teeth

Teeth which were extracted for orthodontic or periodontal reasons used in this study were obtained. 92 single rooted premolar with completed apices were selected. They were stored in 0.02% thymol solution at 25 ºC until ready for use. The teeth were closely examined for caries and resorptive lesions and teeth that had extensive root caries. All remnants of soft tissue and calculus was removed mechanically with an ultrasonic scaler (Acteon Satelec Suprasson P5 Booster-Dental Ultrasonic Scaler Aceteon Group,India) prior to root canal preparation.

A conventional access cavity was prepared using Endoaccess bur (Dentsply/ Maillefer, Switzerland) in each case. #10 K-type file (Dentsply /Maillefer, Switzerland) was used to confirm patency and to establish a clinical working length 1 mm short of the apical foramen. Root canals were prepared using the crown-down technique with ProTaper rotary instruments (Dentsply, Maillefer, Switzerland) to an apical size equal to #30 (F3 ProTaper, Dentsply/Maillefer ,Switzerland) using reduction gear handpiece(Xsmart Dentsply, Maillefer, Switzerland). Between instrumentations, 2 ml of 3 % NaOCl was used for irrigation. The smear layer was removed using 17% EDTA , followed by another 4-min rinse with 5.25% NaOCl .

Irrigation was performed with a 3.0 ml syringe with a 27-gauge needle (Dispo Van,Hindustan Syringes &Medical Devices Ltd.). Sterile paper points (Absorbent Paper points, Meta Biomed Co Ltd. Korea) were used to dry the canals, and the external surfaces of the teeth were then allowed to air dry The apical foramen was sealed using light-cured restorative glass ionomer cement (GC Fuji II,GC America Inc.).The other surfaces of the roots were covered with two layers of nail varnish (Lakme,true wear nail color, Hindustan Unilever Ltd.India). This was done so as to prevent microleakage. Each sample was transferred to a plastic cryo-tube containing sterile brain heart infusion (BHI) broth (HiMedia Lab Pvt.Ltd.) and autoclaved under a pressure of 15 psi at 121ºC for 30 min. Samples were incubated in their sealed tubes for 48 hrs at 37ºC. Daily inspection was done to reveal any signs of turbidity. Fourteen teeth were selected randomly to serve as negative control to ensure the absence of any organism and confirm the sterility of the samples.

Formation of Biofilm

An overnight pure culture of E. faecalis (ATCC 29212) grown in Brain Heart Infusion broth (HiMedia Lab Pvt.Ltd. India) at a concentration of 1.5 × 10 8 CFU/ml was used for inoculation. The bacterial suspension was adjusted to match the turbidity of a McFarland 0.5 scale.

A 0.01-ml aliquot of the suspension was inoculated into each canal using a sterile insulin

Syringe (Dispovan, Hindustan Syringes &Medical

Devices Ltd.). Then the samples were incubated for 4 weeks under aerobic conditions at 37ºC. The inoculum inside the canal was replaced with fresh bacterial suspension every other day. Every seventh day, random sampling was done for gram staining to confirm the purity and viability of the E. faecalis cultures. The entire inoculation procedure was carried out in a UV Chamber. After the incubation period, fourteen inoculated teeth were chosen to serve as positive controls.

Experimental Groups

Group 1 (n=32) - Ultrasonic activated irrigation using a stainless steel noncutting wire (#20) (Irrisafe, Satelec Acteon Group,India) was used driven by an ultrasonic device (Acteon Satelec Suprasson P5 Booster-Dental Ultrasonic Scaler) at power setting of 5-7 W (frequency 30 KHz, displacement amplitude about 30 mm according to the manufacturer) for 20 seconds with 3 times activation of the irrigant. The tip of the Irrisafe was kept 1-2 mm from the apical stop with gentle withdrawing motion. Irrisafe was vibrating freely in the canal. The irrigant was flushed out and renewed after each activation cycle.

Group 2 (n=32) – Laser activated irrigation using NaOCl was activated by diode laser 980nm (Zolar Photon Plus, Zolar Technology & MFG,India) irradiation using an endodontic fiber with a diameter of 200 µm and, with pulse energy of 75 mJ at 25 Hz & the panel settings of 2.5 W/25 Hz were used. The fiber was kept 5 mm away from the most apical preparation and then kept stationary for 5 seconds. A mark was put on the fiber with a black marker at 13 mm in order to position it in the root canal at this depth. This protocol was repeated four times, without removing the tip from the root canal. At the end of this procedure, the canal was flushed with 2 mL 2.5% NaOCl using a syringe with an endodontic needle

Group 3- Positive control(n=14) –To confirm that the innoculated teeth have developed E.faecalis biofilms. The positive control served to determine the total bacterial number and was used to determine the percentage survival of bacteria by the various experimental protocol

Group 4-Negative control (n=14) –Teeth which have not been inoculated with E.faecalis are taken as negative control. This ensures the absence of any other infection & also confirms the sterility of the experimental protocol

Estimation of Bacterial Colonies

The canals were dried with sterile paper points (Absorbent Paper points, Meta Biomed Co Ltd. Korea). Sterile BHI broth, according to the volume of each canal (average 10 µl), was then added into the canals, followed by transfer of each tooth to another sterile cryo-tube and incubation at 37ºC for 24 hrs. Twenty four hours later, the BHI broth was dried out of the root canals, and the canals were refilled with normal saline as a transfer fluid. Sampling from inside the canals was done using a sterile #25 H-file (H-Type Files, Mani Inc, Japan), and circumferential filing was performed for 20 s to collect dentin chips, mostly from the coronal and midparts of the canal.

A sterile #35 K-file (Mani Inc.,Japan) was used for sampling from the apical part by reaming for 20 s. Sterile paper points were used to collect the transfer fluid and dentin chips. Sterile paper points and sampling H- and K-files had been placed into a test tube containing 10 ml of sterile saline and vortexed for 20 s. Fifty microliters of the vortexed saline was applied to Bile Esculin agar culture plates (HiMedia Lab Pvt.Ltd.,India) and incubated at 37ºC for 48 h. All procedures were carried out under sterile and aseptic conditions. Random samples were processed for gram staining and examined under a light microscope to confirm the presence of E. faecalis.

The CFU/ml for each plate was calculated using a bacterial colony counter (Digital Colony Counter, Labtronics, Panchkula, India). The mean and standard

deviation of CFU values were calculated for the samples. Degrees of disinfection in the experimental subgroups were calculated in relation to the positive controls. The CFU counts from the untreated positive control served as the baseline for comparisons throughout the study. Survival rates were calculated by the ratio of the CFUs of each treatment group to the CFU count of the positive control. Statistical analysis was done using one way ANOVA for comparison between the groups, followed by Post hoc Tukey's test to confirm the results.

Results

The CFU counts from the untreated positive control served as the baseline for comparisons throughout the study. Survival rates were calculated by the ratio of the CFUs of each treatment group to the CFU count of the positive control. Statistical analysis was done using One Way ANOVA **(Table1),** which showed statistical significant difference between the groups. This was followed by Post hoc Tukey's test **(Table 2)**, which showed that there was no statistically significant difference between the ultrasonic activated & laser activated groups.

There was a Statistical significant difference between Laser activated and Positive control groups. There was a Statistical significant difference between Ultrasonic and Positive control groups. The results showed that both treatment protocols significantly affected the survival rate of E. faecalis compared with the positive control. There was a significant difference between all groups except between the laser activated and ultrasonic activated. However, there is no significant difference in the survival rate of Enterococcus faecalis after treatment with laser and ultrasonics.

Graph 1 shows the survival rate of E.faecalis after both the treatment protocols. Laser activated irrigation showed lower survival rate of E.faecalis as compared to ultrasonic activated irrigation but this is not statistically significant.

Discussion

This study compared the effectiveness of ultrasonic and laser activation of sodium hypochlorite, in the eradication of an *Enterococcus faecalis* biofilm. The use of these two instruments permitted examination and comparison of the effectiveness of laser energy induced cavitation against the acoustic streaming achieved by the ultrasonic activation of the irrigant. The results of this study illustrate clearly that the use of both Passive Ultrasonic Irrigation (PUI) and Laser activated irrigation are effective in removing pulp tissue and bacteria from the root canal. These results are in line with a study done by Lee et al [9] where he compared the ability of syringe irrigation and ultrasonic irrigation to remove artificially placed dentin debris from simulated canal irregularities.

Ultrasonic irrigation was more effective than hand irrigation in removing vital pulp tissue or dentine debris from the root canal. Another study by Gutarts and Nusstein [19] suggested that 1 min of ultrasonic irrigation after hand/rotary instrumentation resulted in significantly cleaner canals and isthmuses. Acoustic streaming and cavitation induce a powerful streaming of the NaOCl in the root canal during PUI and are an explanation for the efficacy of ultrasonic irrigation

Studies that investigated the mechanism of laser energy mediated cavitation effects that have shown that the phenomenon is brought about by the rapid implosion of a vapour bubble. However, most of these studies have visualised these mechanisms at work in plastic containers or glass capillary tubes that simulate a root canal [20,21]system. It is thus assumed that the pattern of vapour cavity generation and implosion would be duplicated when the irrigant is activated in a similar manner within the root canal. In this study, the apices of the root canals were blocked with GIC and nail varnish prior to irrigation

and activation of the irrigant. This creates an apical air lock, which has been shown to reduce the efficacy of hand irrigation. The occlusion of the apices also limited the forward expansion of the vapour bubble generated by the laser, and prevented the expulsion of the irrigant out of the root canal, especially as these canals had been prepared with a size #40 (0.06 taper) instrument to 1 mm beyond the apex.

It is evident that the higher level of energy imparted by the Er,Cr:YSGG Laser is able to permit greater penetration of the sodium hypochlorite into the dentinal tubules to cause more effective eradication of the *E. faecalis* bacterial cells. There are a number of studies that have shown the effectiveness of various antimicrobial agents against bacteria. However, much of the work has been conducted with the use of planktonic cultures [22] which do not accurately reflect the manner in which bacteria grow within the root canal system. Nair (1987) had shown, with the aid of light and electron microscopy, that bacteria grow as a biofilm within the radicular dentinal walls, Since then, *in–vitro* studies have attempted to replicate *in-vivo* conditions by cultivating bacteria as a biofilm (Gutknecht *et al*., 1996; Spratt *etal*., 2001). However, the "biofilm" had been developed over periods ranging between 24 to 48 hours. Our study has cultivated biofilms for longer period of time i.e up to 4 weeks.

The selection of *E. faecalis* as the bacteria for the present study was due to its frequent association with persistent endodontic infections (Molander *et al*, 1998; Sundqvist *et al*., 1998). In many of these studies, it was found that not only was *E. faecalis* the most commonly recovered microorganism from root canals of teeth with persistent endodontic disease, it was also often present within the canal as a mono-infection. The results of these studies clearly demonstrate the dominance of *E. faecalis* in retreatment cases. The significant proportion of root canals of teeth exhibiting signs of persistent infection from which *E. faecalis* is recovered as a pure culture justifies the use of this microorganism for this study.

The choice of sodium hypochlorite for the present study was due to its widespread use in endodontic practice, its broad spectrum of antimicrobial action and its ability to hydrolyse necrotic pulp tissue. Due to short period of time for irrigation (1 min) and the small volume of irrigant employed (6 ml) in this study, it was deemed that a concentration of sodium hypochlorite that was sufficiently high to exert its antimicrobial effect was necessary. Moreover, no instrumentation was performed in this study, so the eradication of the *E. faecalis* biofilm was dependent solely on the antimicrobial effect of sodium hypochlorite. Even with the use of an antimicrobial irrigant in the form of 3% sodium hypochlorite, the canals could not be rendered bacteria-free, and this is most likely because the bacteria were exposed to sodium hypochlorite for only 1 min and only a small volume of 6 ml was used.

Most studies that had examined the use of the diode laser in the eradication of *E. faecalis* employed the laser without an irrigant present within the canal and directly irradiated the root canal (Gordon *et al* 2007; Schoop *et al*, 2007; Wang *et al*, 2007). With the exception of the Wang *et al*. (2007) study, most of these investigations had cultivated *E. faecalis* for relatively short periods, ranging from 4 hours to 7 days (Schoop *et al*., 2007; Gordon *et al*., 2007). Based on a previous investigation, cultivation of the *E. faecalis* biofilm for a period of 4 weeks yielded a dense, multi-layered aggregation of cells within an amorphous extracellular matrix (Plutzer, 2009). It is evident that in our model that a 4 week period permitted the growth of a dense, mature biofilm, with cells that clearly had penetrated the dentinal tubules.

The teeth used in this study usually has complex anatomy i.e mandibular premolars. The presence of isthmuses and

fins will test the ability of laser activated irrigation to clean these normally inaccessible areas, and may provide greater insight into the effectiveness of such an irrigation protocol. The method with which the teeth were sampled i.e paper point method was easy and convenient in recovering most, if not all, of the bacteria within the root canal, including those that were residing within the dentinal tubules .Also, this technique of sampling is non-destructive, and can be applied in *in vivo* studies. Care was also taken to ensure that the bacteria sampled would be from within the root canal, as the bacteria biofilm that may have developed on the surface of the root would have been eliminated by soaking the entire tooth in 4% sodium hypochlorite for 1 hour.

Conclusion

Within the limitations of the study it was thus concluded that;

Ultrasonic and laser activated irrigation, in conjunction with the use of an antimicrobial irrigant, resulted in reduction of *E. faecalis* cells from within the root canal. This superior ability to disinfect the root canal system predictably may potentially remove the need for the placement of an inter-appointment intra-canal medicament

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Legends Table

Table 1: Comparison of The Four Groups Using One Way **Anova**

	Number of values	**Mean**	**Std. Deviation**	**Std. Error**	**Number of groups**	**P value**
Ultrasonic activated irrigation	92	0.5455	0.2528	0.02775	4	< 0.0001
laser activated irrigation	92	0.4986	0.2426	0.02663		

positive control	92	100	0	0		
negative control	92	0	0	0		

Table 2: Posthoc Analysis

Tukey's Multiple Comparison Test	**Mean Diff.**	**Q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Ultrasonic activated irrigation vs laser activated Irrigation	0.04699	2.444	No	Ns	-0.02380 to 0.1178
Ultrasonic activated irrigation vs positive control	-99.45	5172	Yes	***	-99.53 to -99.38
Ultrasonic activated irrigation vs negative control	0.5455	28.37	Yes	***	0.4748 to 0.6163
laser activated irrigation vs positive control	-99.5	5174	Yes	***	-99.57 to -99.43
laser activated irrigation vs negative control	0.4986	25.93	Yes	***	0.4278 to 0.5693
positive control vs negative control	100	5200	Yes	***	99.93 to 100.1