INTRODUCTION

The mechanical preparation and chemical disinfection of the root canal of the diseased tooth remain the most important procedures in endodontics. Unfortunately, it is difficult to eliminate all microorganisms and organic debris from the root canal system regardless of the irrigant used and instrumentation because of the existence of accessory canals, anastomoses, and fins. The reasons for flare-ups are numerous, but surely, one of the critical factors is viable bacteria remaining within the root canal system. Mechanical instrumentation reduces bacteria from human root canals by approximately 50%. However, auxiliary substances may be necessary to aid the removal of the microbiota in areas where instruments cannot reach. The aim of the study was to compare the vitality of Enterococcus faecalis within dentinal tubules after subjected to five root canal disinfection methods by confocal laser scanning microscopy (CLSM).

MATERIALS AND METHODS

Preparation of specimens

The method used in this study was a modification of one previously described by Haapasalo and Orstavik and Gomes et al. Sixty freshly, extracted, single-rooted, single canal mandibular premolars free of any pathology were used. The method used in this study was a modification of one previously described by Haapasalo and Orstavik and Gomes et al. Sixty freshly, extracted, single-rooted, single canal mandibular premolars free of any pathology were used.
were chosen for the study. These teeth were cleaned of hard tissues debris using ultrasonic scaler under water irrigation, and soft tissue attached to the root was cleaned by immersion in 1% sodium hypochlorite (NaOCl) for 24 h. The teeth were then washed in running water and stored in normal saline (NS) until further use. A dentin block of 10 mm prepared by decoronation and apical resection of 5 mm. The root canal was negotiated with a 10# K-file (Mani Inc., Nakaakutsu, Japan), and shaping was carried out using Gates Glidden drills (Mani Inc., Nakaakutsu, Japan) in sequence from number 1 to 3. Following this, the dentin blocks were ultrasonically activated with aqueous ethylenediaminetetraacetic acid for 4 min. The dentin blocks were washed with sterile water.

**Cultivation of bacteria**

*E. faecalis*, ATCC 29212, was grown in a Petri dish on a brain–heart infusion (BHI) agar for 24 h. For the colonization of the tooth specimens, BHI liquid media were prepared and the bacteria were then inoculated in these media. The turbidity of the prepared media was adjusted to McFarland’s 0.5 using a filter colorimeter. Five milliliters of test tubes were chosen for the colonization of tooth specimens.

**Inoculation of bacteria**

The tooth specimens and the test tubes were autoclaved at 121°C for 15 min at 15 psi pressure. The sterility of the tooth and the test tubes was confirmed by inoculation in agar media, followed by incubation for 24 h to confirm negative culture.

These tooth specimens were then individually transferred to each test tube. Three milliliters of BHI liquid medium was added to each of the test tubes. The teeth were incubated at 37°C for 4 weeks in an incubator. The BHI medium was changed twice a week.

**Preparation of samples for experimental groups**

After 28 days, the teeth were removed from the BHI media and were washed with 1010× phosphate buffered saline (PBS) on the external root surface and inside the root canal. Following this, the tooth specimens were divided into six groups as follows:

1. **Group I**, served as a control group, in which only the depth of penetration of the *E. faecalis* inside the dentinal tubules was evaluated. It was not subjected to any of the disinfection methods
2. **Group II**, 0.9% NS (Baxter, Haryana, India) irrigation
3. **Group III**, 5.25% NaOCl (Hyposept, UPS Hygienes Pvt. Ltd., India) irrigation
4. **Group IV**, 2% chlorhexidine digluconate solution (CHX, Asep-RC, Stedman Pharmaceuticals, India) irrigation
5. **Group V**, neodymium-doped yttrium aluminum garnet (Nd:YAG) laser (Fotona Fidelis III, Slovenia, Europe) with fiber size of 200 µm was used with NS at setting of 1.5 W, 15 Hz, at very short pulse for 5 s in a continuous mode
6. **Group VI**, diode laser (Sunny) with fiber size of 200 µm was used with NS at setting of 2.5 W for 10 s in a continuous mode.

For both the lasers, the fiber tip was inserted into the root canal at a distance of 1 mm from the apical foramen and moved in three consecutive cycles from apical to coronal at a constant speed of approximately 1.5 mm/s.

The tooth specimens were then washed with PBS to remove any of residual irrigating solutions. Two longitudinal grooves were prepared on the buccal and lingual surface with a diamond disc. Using a chisel and mallet, the tooth specimens were split longitudinally into two halves.

**Staining of samples for confocal laser scanning microscopy imaging**

After the tooth specimens had been split, the halves were again washed with 100 µl PBS using a micropipette to remove any debris.

For the examination of tooth specimens under the CLSM, a microscope slide was customized. The tooth specimens were then stabilized using elastomeric impression material (Express XT VPS, 3M ESPE, Germany).

After the stabilization of dentin blocks, 100 µl of prepared fluorescent stain (LIVE/DEAD BacLight stain, Invitrogen Detection Technologies, California, USA) according to the manufacturer’s instruction was applied and the blocks were incubated with the stain at room temperature for 15 min in a dark environment so that the bacteria take up the stain. After 15 min, the sample was washed with PBS to remove any residual fluorescent stain.

Following this, an antifade mountant (Dakocytomation, Glostrup, Denmark) was applied over the sample. A coverslip was placed and the samples were then subjected to CLSM imaging.

**Confocal laser scanning microscopy imaging**

The dentin segments were examined under CLSM (LSM 510. Carl Zeiss). The respective absorption and emission wavelengths were 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide. The mounted specimens
were observed using a 10× oil lens and a 63× oil lens with an additional zoom of 3×.

RESULTS

Group I showed bacteria present in dentinal tubules to a depth in a range of 965.45–1175.78 µm [Figure 1a]. For the experimental groups, the zone of dead bacteria (ZDB) was measured for each sample. It is observed from Figure 1b-f and Table 1; Group II did not show any dead bacteria for all the samples. Group III had a partial ZDB that measured in a range of 88.45–110.43 µm. Group IV also had a partial ZDB that measured in a range of 109.89–194.14 µm. Group V did not show any viable bacterium in dentinal tubules. In fact, the ZDB was equal to the entire thickness of dentin that measured in a range of 897.89–1145.10 µm. Similarly, Group VI had ZDB which measured in a range of 760.93–1110.12 µm. The results were subjected to statistical analysis using the one-way ANOVA test using Bonferroni corrections with value of \( P < 0.05 \) considered as statistically significant. Viable bacteria inside dentinal tubules are significantly higher with NS when compared to all other groups. The ZDB is not significant when NaOCl is compared to CHX. There are viable bacteria present with NaOCl and CHX when compared to lasers. There are no viable bacteria present with Nd:YAG and diode lasers. The ZDB is not significant when Nd:YAG is compared to diode laser [Figure 2].

DISCUSSION

*E. faecalis* was chosen because it has been shown to be associated with failed endodontic cases[8,9] and has the ability to invade whole length of dentinal tubules to 1100 µm or close to the cementum.[10-17] It is also important to validate the bactericidal action of different disinfection methods using a resistant microorganism such as *E. faecalis*.[3,6,13-17-20]

CLSM can be used as an alternative to other method of evaluation, for evaluating the penetration depth inside dentinal tubules.[21] The fluorescent stain used in the present study utilizes a mixture of SYTO 9 and propidium iodide. An appropriate mixture of the SYTO 9 and propidium iodide stains bacteria with intact cell membranes (live bacteria) as fluorescent green whereas bacteria with damaged membranes (dead bacteria) as fluorescent red. Bacteria are capable of invading the periluminal dentin to a depth of 1100 µm.[12] The present study has also proved the same with bacteria invading the dentinal tubules nearing to a depth of 1200 µm.

Total elimination of bacteria from dentinal tubules cannot be achieved by irrigants alone.[22] It has also been demonstrated that the penetration of irrigants inside dentinal tubules is not more than 160 µm.[6,23-25] NS did

Table 1: Measurement of zone of dead bacteria for the respective disinfection methods

| Groups | Methods  | ZDB (µm) |
|--------|----------|----------|
| II     | NS       | 0        |
| III    | NaOCl    | 88.45-110.43 |
| IV     | CHX      | 109.89-194.14 |
| V      | Nd: YAG  | 897.89-1145.10 |
| VI     | Diode    | 760.93-1110.12 |

NS: Normal saline, NaOCl: Sodium hypochlorite, CHX: Chlorhexidine digluconate, Nd: YAG: Neodymium-doped yttrium aluminum garnet, ZDB: Zone of dead bacteria

Figure 1: Representative confocal images (a) *Enterococcus faecalis* colonized inside dentinal tubules. (b) No zone of dead bacteria seen with normal saline. (c and d) Partial zone of dead bacteria observed with sodium hypochlorite and chlorhexidine digluconate respectively. (e and f) Total zone of dead bacteria observed with neodymium-doped yttrium aluminum garnet and diode laser
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Figure 2: Average values of zone of dead bacteria for the disinfection groups

not show any dead bacterium. Previous studies have also shown that NaOCl and CHX were more effective than NS in killing bacteria.[26-28]

NaOCl and CHX showed limited ZDB. The reason for the limited ZDB by NaOCl is its inactivation caused by dentin.[29] The limited ZDB by CHX is because of its inability to dissolve necrotic tissue remnants and it is less effectiveness on Gram-negative than on Gram-positive bacteria.[30-32]

The Nd: YAG as well as diode laser showed no viable bacteria, and its effectiveness reaches to a depth of more than 1000 µm inside dentinal tubules. These findings are accordance with previous studies.[33-41] A possible explanation for the greater antibacterial action of lasers is that the light emitted by the laser creates a “light fog” in the dentin and does not have the characteristics of a concentrated beam anymore. The enamel prisms and dentin tubules act as optical fibers that propagate this laser light to the dentinal periphery of the root.[42-44]

**CONCLUSION**

Conventional root canal irrigants have a limited action inside dentinal tubules beyond which viable bacteria are present. This can be a reason for failure postendodontic treatment. In such cases, lasers proved to be a valuable adjunct in elimination of bacteria,[45] and can help reduce the incidence of postendodontic treatment failures.

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**Conflicts of interest**

There are no conflicts of interest.

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