International Journal of Medicine, 2 (2) (2014) 63-67

International Journal of Medicine

Journal home page: www.sciencepubco.com/index.php/IJM
doi: 10.14419/ijm.v2i2.3406
Research Paper

The effect of chlorine dioxide on *Enterococcus faecalis* in exponential, stationary and starvation phases in extracted human teeth - an invitro study

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Abstract

Background: *Enterococcus Faecalis* (*E faecalis*) is isolated usually from failed root canal treated teeth. It can bind to dentin and co-aggregate with other organisms. The organism is resistant to the intracanal medicaments and irrigants. It has the ability to produce bio-films and survive within the root canal. They may develop resistance at different phases of bacterial growth cycle.
Objective: To study the effect of chlorine dioxide on different phases of *E faecalis* growth cycle for 1 and 3 min duration.
Methodology: *E faecalis* ATCC strain (29212) was cultured in different growth phases on dentinal blocks of extracted human teeth. After treatment with chlorine dioxide, left out colonies were counted from dentinal shavings. Observation of the remaining biofilm of different phases was made using the scanning electron microscope and comparison between them was done.
Results: In all 3 phases, at the end of 1 and 3 min, significantly ($p<0.01$, $p<0.001$ respectively) less *E faecalis* colonies were observed when compared to initial count. When effect of chlorine dioxide on *E faecalis* colonies was compared between the three phases, at the end of 1 min, significantly ($p<0.05$) less *E faecalis* colonies were observed in exponential phase than in starvation phase. However, the *E faecalis* colony count during stationary phase was significantly ($p<0.05$) less than the colony counts in both exponential and starvation phases. At the end of 3 min, there was no significant difference in *E faecalis* colony count between exponential and starvation phases. However, the *E faecalis* colony count during stationary phase was significantly ($p<0.05$) less when compared to the colony counts in both exponential and starvation phases.
Conclusion: Our study showed that starved cells of *E faecalis* were more resistant to 13.8% chlorine dioxide irrigant when treated for 1 and 3min.

Keywords: *E faecalis, Chlorine dioxide, Dentinal blocks, Biofilms.*

1. Introduction

Microbial infection of root canal has proved to be the cause of periradicular pathosis (Moller et al. 1981). *Enterococcus faecalis* (*E faecalis*) is a predominant organism that plays a major role in the etiology of persistent periradicular lesions after root canal treatment (Rocas et al.2004). Within the root canal system, *E faecalis* has the ability to adapt to environmental changes and remain as pathogen in the root canal which makes its elimination very difficult (Haapasalo et al.2000).
Normally, bacteria are encapsulated in extracellular matrix known as biofilms where they aggregate and co-aggregate with each other (Nair 1987). Biofilm mode of growth is a survival strategy of microbes (Tronstad and Sunde 2003). Biofilm forming capacity of microorganisms depend on surface attributes of substratum and can vary according to existing environmental and nutritional conditions (Kristichet al.2004).
Disinfection of root canal is accomplished by chemo-mechanical debridement. The mechanical portion is accomplished by instrumentation whereas the chemical component is by various chemical irrigants. The irrigant must possess anti-bacterial property, should dissolve the necrotic tissue, and have low surface tension, substantivity, lubrication, harmless effect on microhardness and roughness of root canal dentin (Haapasalo and Qian 2008). It was proved that 1% sodium hypochlorite, 0.2 to 0.4% iodine potassium iodide and 0.5% chlorhexidine killed *E faecalis* within 5min. But, 0.05% concentration of iodine potassium iodide required 5min to 1hr duration for the same effect (Stuart et al. 2006). Dunavant et al. showed that 1% and 6% sodium hypochlorite were the most effective irrigant against biofilms (Dunavant et al.2006). The chelating agent like EDTA solution had no effect on *E faecalis* biofilms (Arias-Moliz et al.2009). The physiological state of bacteria plays a major role in the outcome of antimicrobial treatment. Liu et al. demonstrated that *E faecalis* in starvation phase could produce biofilms with reduced efficiency when compared to that of exponential and stationary phases (Liu etal.2010). It was also reported that starved cells treated with sodium hypochlorite have the potential to survive 1000 to 10,000times higher when compared to that of exponential and stationary phases (Portneir et al.2005). There are reports that chlorine dioxide oxidizes and kills *E faecalis* and other bacteria by altering the transport of nutrients across the cell membrane (Eddy et al. 2005).

However, there are no reports revealing the effect of chlorine dioxide against *E faecalis* in exponential, stationary and starvation phases of its life cycle. Hence, aim of our study was to check the efficacy of 13.8% chlorine dioxide against *E faecalis* in exponential, stationary and starvation phases.

2. Materials and methods

2.1. Dentinal block preparation

Eighty extracted human mandibular premolars were collected from the dental clinics of Manipal College of Dental Sciences. Teeth were ultrasonically cleaned and were then decoronated at cemento-enamel junction with the help of diamond disc. Two longitudinal grooves were made on opposite sides of the roots of the teeth and split opened. Coronal portion of the roots were then prepared into blocks each of 4x4x1mm size without scraping. The blocks were then dipped in sodium hypochlorite for one minute and washed with saline. Following this, blocks were irrigated with ethylene diamine tetra acetic acid (EDTA) for 1min to remove the smear layer produced during the procedure. The blocks were autoclaved at 121ºC for 15min. Total of eighty blocks were randomly selected for the study. Twenty blocks were used for the study under each of exponential, stationary and starvation phases.

2.2. Contamination with *Enterococcus faecalis*

2.2.1. Exponential phase

Twenty blocks (10 blocks each under 1 and 3 minutes) were used for the study under exponential phase using nutrient rich brain heart infusion (BHI) broth as medium. The blocks were kept in the orbital shaker for 4hrs to induce exponential phase and were further incubated at 37º C for 96hrs (11). ATCC (American type cell culture) strain of *E faecalis* (29112) was cultured. Each dentinal block was kept in one ml BHI broth suspended in Eppendorf tube of 2cm size. *E faecalis* was innoculated into the Eppendorf tube containing the blocks. It was adjusted to contain $1x10^8$ CFU/ml corresponding to 0.5 Mcferland's tube. This suspension was incubated at 37^0 c at air orbital shaker at 100 rotations /min (MTS 2, IKA, Staufen, Germany).

2.2.2. Stationary phase

Another twenty blocks (10 blocks each under 1 and 3 minutes) in BHI broth were kept in the shaker for 12hrs to induce stationary phase and incubated at 37ºC for 96hrs. *E faecalis* was innoculated into the Eppendorf tube containing the blocks. It was adjusted to contain $1x10^8$ CFU/ml corresponding to 0.5 Mcferland's tube. This suspension was incubated at 37^0 c at air orbital shaker at 100 rotations /min (MTS 2, IKA, Staufen, Germany).

2.2.3. Starvation phase

Other twenty blocks (10 blocks each under 1 and 3 minutes) were then kept in anaerobic condition for 96hrs to induce starvation phase (Fig 1). *E faecalis* was innoculated into the Eppendorf tube containing the blocks. It was adjusted to contain $1x10^8$ CFU/ml corresponding to 0.5 Mcferland's tube. This suspension was incubated at 37^0 c at air orbital shaker at 100 rotations /min (MTS 2, IKA, Staufen, Germany).

2.2.4. Control group

Remaining 20 blocks were kept as control in BHI medium containing *E faecalis*. They were treated with normal saline (10 blocks for 1minutes and 10 blocks for 3 minutes).

2.3. Treatment with chlorine dioxide

Following the contamination with *E faecalis*, twenty blocks for each of the exponential, stationary and starvation groups were taken and subjected to treatment of chlorine dioxide for 1 (10 blocks) and 3 (10 blocks) minutes (Chlorine dioxide 13.8%, Frontier Pharmaceuticals, U.S.A). After the time duration, the blocks were washed with phosphate buffer saline and dentinal shavings were collected from the canal portion of teeth. Four blocks from each group (2 each from 1 and 3 minutes subgroups) were prepared for scanning electron microscopic observation.

Colony-forming units (CFU) of viable cells were determined on sabouraud dextrose agar plates in triplicates of 1:10 dilution (phosphate buffer saline). Sabouraud dextrose agar plates were incubated in 5% CO_2 at 37ºC in carbon dioxide incubator and the number of colony-forming units was counted at 24hrs. Plates containing 30 to 300 *E faecalis* colonies were used preferentially for data analysis.

2.4. Optical density (OD) measurement

Optical density is the optical thickness which measures the total light blocking power of a certain medium with certain thickness. The effect of drugs against bacterial multiplication can be measured by this method. Increased optical density value indicates that the drug is not effective. Optical density of chlorine dioxide against *E faecalis* at exponential, stationary and starvation phases was measured at 405nm using photospectrometer (Bio-Rad laboratories India).

2.5. Tooth preparation for scanning electron microscope (SEM)

Dentinal blocks were washed with phosphate buffer saline. They were then fixed with 4% glutaraldehyde for 8hrs at 4 to 6ºC. Following this, super fixation was done with 1% osmium tetroxide for 4hrs at 5ºC. Blocks were then dehydrated with ascending concentrations of ethanol (30%, 50%, 70%, 90%, and 100%). Critical point drying was done by keeping the preparations in dryer. Preparations were processed for gold sputtering. Blocks were examined in SEM (Cambridge, England) at 20Kv. Different images of the entire dentinal blocks were captured and recorded. Quantitative analysis and comparison of areas of biofilm formed by different groups were done.

3. Statistical analysis

Statistical analyses were carried out using statistical software Graph Pad Prism version 3.00 Graph Pad Software, Inc. San Diego CA. One-way ANOVA was applied to analyze the data and Bonferoni's post hoc test was used to see the significance between different groups.

The study was approved by Institutional Ethical Committee of Manipal University.

4. Results

4.1. Optical density (OD) measurement results

Optical density reading (OD405nm) of *E faecalis* grown in distilled water (DW) medium is low and it remained almost the same in log and stationary phases and started decline phase. This indicates that distilled water is not favoring the bacterial growth. When peptone water is used as a medium, there is a moderate increase in optical density reading. However, brain heart infusion broth medium showed high optical density in all four phases indicating faster and higher bacterial growth. (Figure1).

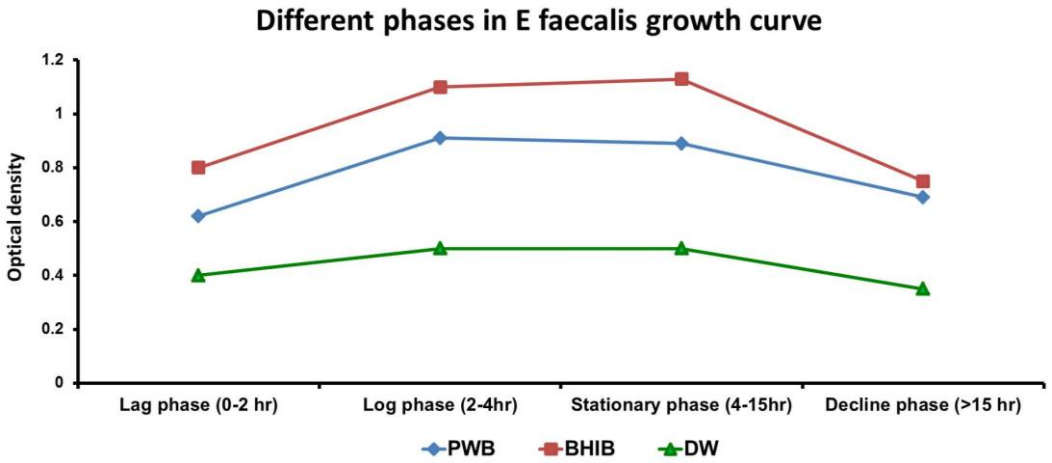

Fig. 1: Line chart showing different phases in growth curve of *E faecalis*. It can be observed that optical density (OD) reading in distilled water (DW) medium is low and it remains almost the same in all four phases indicating that DW is not favoring the bacterial growth. When peptone water (PWB) is used as a medium, there is a moderate increase in OD Reading. However, brain heart infusion broth (BHIB) medium showed high OD in all four phases indicating faster and higher bacterial growth.

4.2. Colony forming units

In the present study, roots of teeth in all the groups were inoculated with about 8±1.4 lakhs colonies of *E faecalis* colonies initially and this value is taken as initial count of *E faecalis*. This initial count was compared with colony formation after the time interval of 1 and 3minutes in exponential, starvation and stationary phases.

In exponential phase, at the end of 1min, 4.5±1 lakh *E faecalis* colonies were observed which was significantly ($p<0.01$) less when compared to initial number of 8 lakh *E faecalis* colonies. At the end of 3min, 3.9±0.85 lakh colonies were observed which was significantly ($p<0.001$) less when compared to initial count of 8 lakh *E faecalis* colonies. (Figure 2)

In starvation phase, at the end of 1 min 5.1±1.2 lakh *E faecalis* colonies were observed which was significantly ($p<0.01$) less when compared to initial number of 8 lakh *E faecalis* colonies. At the end of 3min, 4.33±0.74 lakh colonies were observed which was significantly ($p<0.001$) less when compared to initial count of 8 lakh *E faecalis* colonies. (Figure 2)

In Stationary phase, at the of 1 min 3.63±0.8 lakh *E faecalis* colonies were observed which was significantly ($p<0.01$) less when compared to initial number of 8 lakh colonies. At the end of 3min, 2.43±0.7 lakh *E faecalis* colonies were observed which was significantly ($p<0.001$) less when compared to initial count of 8 lakh *E faecalis* colonies. (Figure 2)

Above results clearly indicate that more number of *E faecalis* colonies were killed when treated with chlorine dioxide for relatively longer duration (3min) in all three phases.

When effect of chlorine dioxide on *E faecalis* colonies was compared between the three phases, at the end of 1 min, 4.5±1 lakh E faecalis colonies were observed in exponential phase which was significantly ($p<0.05$) less when compared to *E faecalis* colony count (5.1+1.2 lakhs) in starvation phase. However, the *E faecalis* colony count during stationary phase was 3.63±0.8 lakh which is significantly ($p<0.05$) less when compared to the colony counts in both exponential and starvation phases. (Figure 2)

When effect of chlorine dioxide on *E faecalis* colonies was compared between the three phases, at the end of 3 min, there was no significant difference in *E faecalis* colony count between exponential phase (3.9±0.85 lakh) and starvation phase (4.33±0.74 lakhs). However, the *E faecalis* colony count during stationary phase was 2.43±0.7 lakh which is significantly ($p<0.05$) less when compared to the colony counts in both exponential and starvation phases. (Figure2).

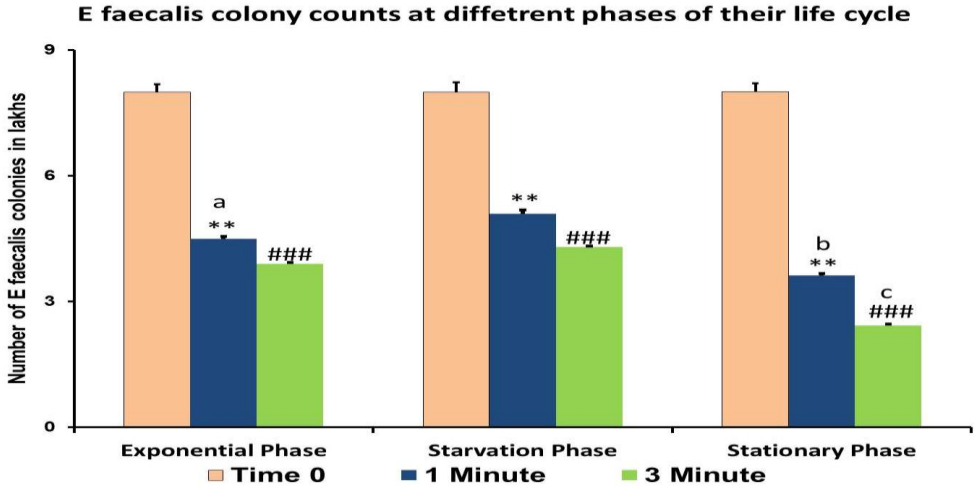

Fig. 2: Graph showing the initial *E faecalis* count and after treatment with chlorine dioxide at different phases of its life cycle. In exponential phase, ** initial count Vs 1min $P<0.01$ and ### initial count Vs 3min $P<0.001$, In starvation phase, ** initial Count Vs 1min $P<0.01$ and ### initial count Vs 3min $P<0.001$, In stationary phase, ** initial count Vs 1min $P<0.01$ and ### initial count Vs 3min $P<0.001$. When compared across the phases, at the end of 1 minute, a. exponential phase Vs starvation phase $P<0.05$, b. exponential and starvation phases Vs stationary phase $P<0.05$. At the end of 3 Minutes, c. exponential and starvation phases Vs stationary phase $P<0.05$.

4.3. Scanning electron microscope (SCM) observation (figure 3)

Scanning electron microscopic observation showed that biofilm was not eliminated completely in all the three phases. Pictures "a" and "b" show biofilm that were not eradicated by chlorine dioxide at time duration of 3min in exponential phase. Photographs, c and d- show biofilm remaining after 1min exposure to irrigant in exponential phase. Here dentinal surfaces are mostly covered by biofilm. Photographs e and f reveal biofilms at starvation phase exposed to irrigant at 1min and 3min. Though the formation of biofilm is less here, the existing biofilm might be more resistant. Figures g and h show the remaining biofilm after the treatment in stationary phase and biofilm appear to be scanty indicating more effectiveness of the drug. Arrow indicates areas of biofilm eradication. Photographs I and j show co-aggregation of bacteria which is a step of biofilm formation after 24hrs of incubation. (Figure3).
Figure 3 a - j: Scanning electron microscopic photographs:

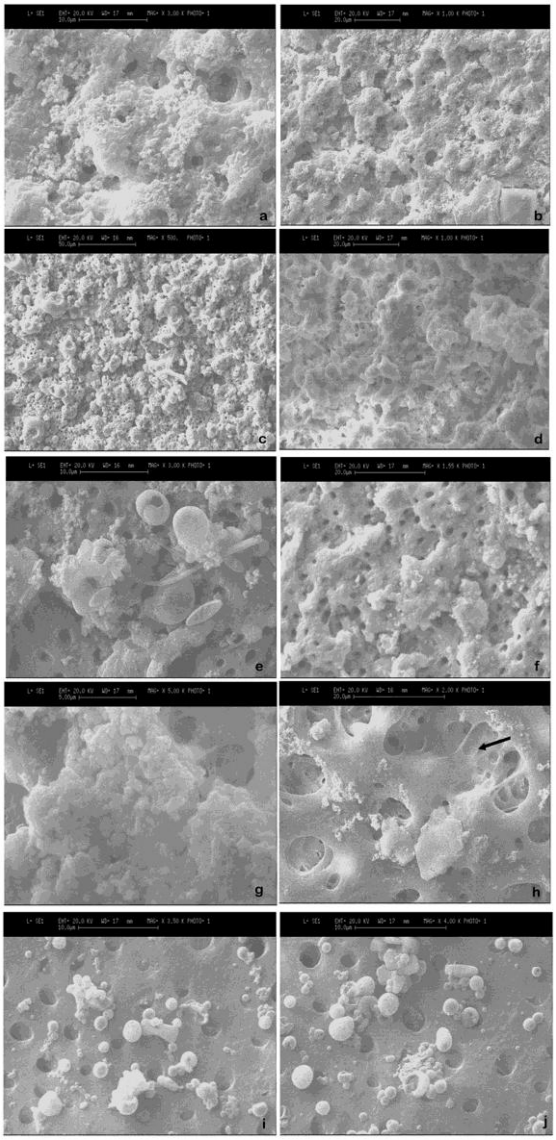

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Fig. 3: A and B show biofilms that were not eradicated by chlorine dioxide at time duration of 3min in exponential phase, C and D show biofilm remaining after 1min exposure to irrigant in exponential phase, E and F show biofilms at starvation phase exposed to irrigant at 1min and 3ming and H shows eradicated areas in stationary phase after 1min and 3min Exposure. Arrow indicates areas of biofilm eradication. I and J show blocks contaminated with *E faecalis* observed after 24hrs of incubation showed co-aggregation between cells.

5. Discussion

Bacteria persisting in biofilms within the root canal system, their byproducts and metabolites are responsible for chronic periapical pathosis (Sundquist 1994). In our study, single bacterial isolate was used in different physiological conditions to check the efficacy of the irrigant. Biofilm formation may be influenced by the nature of the substratum (Siegrist et al. 1991). Coronal portion of the roots were used because of increased density of detinal tubules and the ability of *E faecalis* to penetrate deep inside the tubules (Stuart et al. 2006). Stabilized chlorine dioxide was proved to be less effective than NaOCl against other micro-organisms (Lundstrom et al. 2010). Factors such as collagen may be responsible for bacterial invasion (Kayaoglu et al. 2009). Chlorine dioxide eliminated majority of cells of *E faecalis* within the duration of 30minutes (Eddy et al. 2005). An irrigant cannot be kept in continuous contact with the dentin for longer duration in clinical conditions. So in this study efficacy of the irrigant was checked in time durations of one and three minutes.

The colony counts obtained after the treatment with chlorine dioxide on starved cells showed higher values compared to that of exponential and stationary phases. This is in agreement with the previous studies and may be attributed to release of catabolite repression in starvation phase (Liu et al.2010, Mohamed et al.2006) It may also be due to a glucose dependent transcriptional regulator responsible for change of fsr and down steam proteases (Mohamed et al. 2006). Biofilms in exponential phase were more resistant to chlorine dioxide than in stationary phase, which may be attributed to increase in density of biofilms in exponential phase. This could also be due to increase in levels of calcium in nutrient rich conditions (Rosanova et al.1991).

Reason for increased number of survival colonies in starvation phase when compared to exponential phase may be due to their acclimatization to the anaerobic conditions. Under scanning electronic microscopic observation, density of biofilm in starvation phase was decreased which may be due to lack of multiplication of surviving cells. But, the existing cells developed resistance to the chemicals. Within the root canal system starved cells utilize serum as nutritional source through periodontal ligament and alveolar bone (Figdor et al. 2003). In our study, after prolonged starvation, the bacteria might not have been more virulent due to deprived serum. Biofilm in stationary phase was least resistant and S.E.M observations revealed fewer areas with biofilm than in exponential phase. This could be due to factors such as lack of calcification of biofilm and less dense biofilm matrix which could have enhanced penetration of irrigant effectively through the biofilm leading to significant decrease in the *E faecalis* count. But, the biofilm observed under SEM in stationary phase was denser than that of starvation phase, may be due to higher production of biofilm during stationary phase than that of starvation phase. In our study, treatment with chlorine dioxide failed to eliminate the biofilms completely in all three phases of *E faecalis* life cycle. Hence, further woks need to be carried out to establish whether using other irrigants along with chlorine dioxide can help in total eradication of *E faecalis* in root canals.

6. Conclusion

Our study showed that starved cells of *E faecalis* were more resistant to 13.8% chlorine dioxide irrigant when treated for 1 and 3min. Study also revealed that these microbes in exponential phase were more resistant than in stationary phase. Chlorine dioxide was effective in reducing the *E faecalis* bacterial counts, but failed to eliminate them completely.

Acknowledgements

We acknowledge Prof Verma, Professor of Material Research Centre, Indian Institute of Science, Bangalore for permitting us to utilize Scanning Electron Microscope.

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