

Quantification of Lipoteichoic Acid of Gram Positive Bacteria after Irrigation with Sodium Hypochlorite in Root Canal-An in vitro Study

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Abstract

Bacteria play an important role in root canal infection. Lipoteichoic acid (LTA) is the one of virulence factor released by Gram positive bacteria.

The aim of this study was to quantify the LTA levels after irrigation with sodium hypochlorite.

Seventy two single rooted Mandibular premolar tooth was taken for the study. Gram positive and negative bacteria were grown in these teeth. Teeth were then irrigated with sodium hypochlorite. The dentinal shavings were collected for LTA quantification.

Sodium hypochlorite irrigated in tooth with Gram positive bacteria showed significant increase in LTA levels ($P < 0.05$)

Gram positive bacteria alone showed increase in LTA levels than that of combination of Gram positive, negative bacteria, after sodium hypochlorite disinfection.

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Introduction

Bacteria play an important role in root canal infection.¹ Biofilms represent bacterial colonies enveloped within an extracellular matrix composed of polysaccharides, proteins, and nucleic acids.² The extracellular matrix of biofilms is composed of proteins and carbohydrates. Biofilms are formed by the bacteria on any moist surface rich in nutritional components. Microbes in the root canals can grow not only as planktonic cells, in aggregates or co-aggregates, but they can also form biofilms consisting of a complex network of different microorganisms. Hence, root canal treatment aims to eliminate bacteria from the infected canal system and to prevent re-infection. Clinical studies have demonstrated that chemo-mechanical preparation and use of antimicrobial medicaments are effective in

reducing the bacterial load in root canal systems.³

Enterococcus faecium (*E. faecalis*) is a multidrug-resistant (MDR) nosocomial pathogen causing significant morbidity in debilitated patients.⁴ *E. faecalis* possesses certain virulence factors including lytic enzymes, cytolysin, aggregation substance, pheromones, and lipoteichoic acid. Endodontic pathogens differ in virulence.

E. faecalis can cause co-aggregation with other organisms and survive within root canal. *E. faecalis* produces lipoteichoic acid (LTA) within the root canal.

Streptococcus mutans play an important role in growth and physiological defects, however exact role of its LTA is not known. *Streptococcus mutans* protect against cationic antimicrobial peptides can be provided by change in main structure of LTA.^{5,6} Structurally exhibits overlap and redundancy in the processes of adhesion, colonization and innate immune resistance.

Actinomyces species belong to the primary colonizers of clean tooth surfaces and are relatively frequent isolates in endodontic infections.⁷

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The Enterococcus faecalis, Streptococcus mutans and Actinomyces Israelii bacteria are commonly seen in failed cases of root canal.⁷

Lipoteichoic acid (LTA) is a unique component of Gram-positive bacterial cellular walls.^{8, 9} It is a glycerol phosphate polymer containing sugar and two acyl groups which confer the property of anchoring in the cellular membrane. A non-acetylated form of LTA is the teichoic acid which is covalently linked to polyglycan of the cellular wall of Gram positive bacteriae.

LTA is synthesized in great amount when cariogenic bacteria find saccharose as available carbonated source.

It is well known that macrophages have a significant role in phagocytosis against bacteria and also play a pivotal role in innate immunity.¹⁰ Local inflammatory responses are developed through the production of proinflammatory cytokines and chemokines.^{11, 12}

LTA is an amphiphilic molecule. It has glycolipid anchor linked with polyglycerolphosphate or polyribitolphosphate skeleton. Polyglycerolphosphate type LTA is found in most of the Gram positive bacteria. However polyribitolphosphate type LTA is expressed in a few Gram positive bacteria like streptococcus pneumoniae. LTA triggers (TLR) Toll-like receptors 2. LTA is a ligand against TLR2 and partially dependent on TLR1, TLR6 and CD14. Various proinflammatory chemokines and cytokines produced by the activation of Toll like receptor 2, which in turn is activated by LTA.^{13,14,15}

For the development of novel antimicrobials, a suitable target is LTAs enzyme. This is supported by an altered cell morphology, cell division and its growth in case of mutation of LTA gene of *S. aureus*, *Listeria monocytogenes*, *Bacillus subtilis* and *Bacillus anthracis*.¹⁶

Sodium hypochlorite is the most commonly used irrigant in Endodontics, because of its efficacy against wide range of micro-organisms. Biofilms have been shown to be resistant to sodium hypochlorite.¹⁷ It has also been proven to disrupt the structure of biofilm and eliminate bacteria from root canal.¹⁷

Primary root canal flora consists of Gram negative microorganisms. It is important to quantify the liberation of LTA from the coaggregation of Gram positive and Gram negative flora.

The aim of this study is to quantify the release of LTA of *E faecalis*, *Streptococcus mutans*, *Actinomyces Israelii*, and these bacteria with *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Tanerella forthysia* after irrigation with sodium hypochlorite.

Materials and methods

The study was approved by ethical committee of manipal university (IEC/925/2016). Seventy two single rooted mandibular premolar teeth were taken for the study. Crown was decoronated with diamond mandrel. Working length was determined and biomechanical preparation was done. The canals were enlarged with protaper file upto F2 size (Dentsply,mailfer, USA). During shaping and cleaning EDTA and 5.25% sodium hypochlorite (manipal pharmacy) were used as irrigants. The teeth were then subjected to autoclave.

Teeth were divided into two groups

Group A: Teeth inoculated in eppendorf tube with combination of following bacteria, *E. faecalis* + *Streptococcus mutans* + *Actinomyces Israelii*.

Group B: Teeth inoculated in eppendorf tube with Gram positive and Gram negative bacteria (*E faecalis*+ *Streptococcus mutans* + *Actinomyces israelii*+ *Porphyromonas gingivalis*+ *Prevotella intermedia*+*Fusobacterium nucleatum*+ *Tanerella forthysia*)

Group A is further divided into three groups (n=12)

Group A1: Teeth subjected to bacteria and not exposed to any irrigants (Control)

Group A2 : Teeth subjected to saline irrigation

Group A3: Teeth contaminated with bacteria were irrigated with 5.25% sodium hypochlorite

Group II is divided into (n=12)

Group B1: Teeth subjected to both groups of bacteria(control)

Group B2: Teeth subjected to saline irrigation

Group B3: Teeth irrigated with sodium hypochlorite

Pure cultures of the test strains, *E. faecalis* ATCC strain 19433, *Streptococcus mutans* ATCC 25175 strain, *Actinomyces israelii* ATCC 12103 strain, *Porphyromonas gingivalis* ATCC 33277 strain, *Prevotella intermedia*,

ATCC15032 strain ATCC Fusobacterium nucleatum 25586 and Tanerella forthysia ATCC 43037 were prepared in sterile BHI broth (Becton, Dickinson and Co.).

These bacterial cells were grown as a suspension and incubated at 37°C for 24 hours. One group of teeth were submerged in the BHI Broth containing *E. faecalis*, *Streptococcus mutans* and *Actinomyces israelii*. The other group of teeth were suspended in BHI Broth of *E. faecalis*, *Streptococcus mutans*, *Actinomyces israelii*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Tanerella forthysia* and were incubated at 37°C for 21 days to allow biofilm formation. BHI (Brain Heart Infusion) broth was added every 2 days for the growth of microorganisms. After 21 days of contamination, bacterial colony counts were confirmed with colony forming unit (CFU) per millilitre (9.2×10^6 CFU/mL).

Following this, the groups were irrigated with 5.25% sodium hypochlorite and saline with 27gauge needle. The needle was kept 1mm short of Working length and irrigated.

Then the teeth were washed with phosphate buffered saline. Dentinal shavings from tooth were then taken with the help of H-File (Dentsply, mallifer, USA). The shavings were then processed for LTA extraction by ELISA method (My Biosource, sandiego, CA).

CONFOCAL SCANNING LASER MICROSCOPY (CSLM)

Two teeth were randomly selected for confocal laser scanning microscopy (Carl Ziess) observation before irrigation to confirm the growth of biofilms. Both teeth were grooved with help of mandrel. They were dehydrated with ascending concentrations of alcohol. Later it was fixed with 2%glutraldehyde. Each tooth was split open and was stained with propidium iodide for observation under microscope.

Bacterial preparation

The dentinal shavings of each group were collected separately in a Broth. To the bacterial suspension in 300ml of distilled water, 300ml of 90%phenol (hot) was added and vortexed for 15 minutes and Centrifuged at 10000rpm for 20 minutes . Then the solution was kept at 65 to 70° C in water bath for 15 minutes, followed by chill suspension in ice, Centrifuged at 8500 rpm for 15 minutes. Supernatant was transferred to sterile eppendorf tube. 300 µl of 90%phenol was added

and centrifuged. The resultant supernatant was collected in other tube to which 50µl of sodium acetate and 0.5ml of 10 volumes of 90% ethanol (1ml supernatant and 10ml ethanol) was added. Samples were at -20°C till centrifugation. Then they were centrifuged at 4000rpm at 4° C for 10minutes. The supernatant was discarded. To the bacterial sediment 100ul of D/W (distilled water) was added and transferred to eppendrotube. Then the sample suspension were made in 5ml of 0.2% NHCl and heated to 95°C for 10min. Then cells were washed six times with ice cold distilled water and resuspended in 200ml of distilled water. To this suspension 200µl of 90%phenol was added and was centrifuged at 10000rpm for 30min. This phase contained LTA (lipoteichoic acid).

ELISA (Enzyme linked immune absorbent essay)

ELISA kit of human LTA cells (My Biosource, San diego, CAUSA) was used for detection of LTA.

Standard, control, sample solution were added to an ELISA plate which had been precoated with specific monoclonal antibody for LTA, supplied by manufacturer. Anti LTA antibodies labeled with biotin were added to unite with streptavidin conjugated with horse radish peroxidase forming an immune complex. The plate was incubated for 60 minutes at 37° C and washed for removal of unbound enzymes. The reaction was observed after addition of a substrate solution containing hydrogen peroxide and chromogen.

Statistical Analysis

Statistical analysis was done with the help of ONE –WAY ANNOVA and Post-hoc Tuckey's test by SPSS software version 20.

Results

In group A gram positive bacteria was used alone.

In group B gram positive and negative was used together.

On intra group comparision, there was no significant difference between LTA released from a)Gram positive, Gram negative and b)Gram positive bacterial groups, when irrigated with saline ($P > 0.05$).

Similarly no differences were seen between both the groups of teeth that were not irrigated ($P > 0.05$).

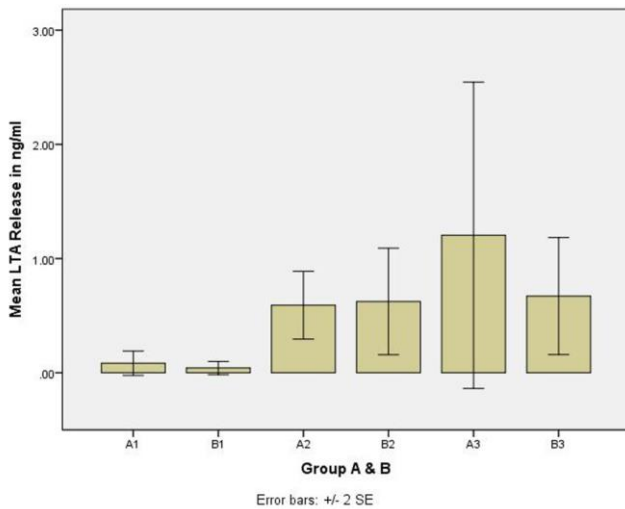


Figure 1. Mean LTA values in ng/ml.

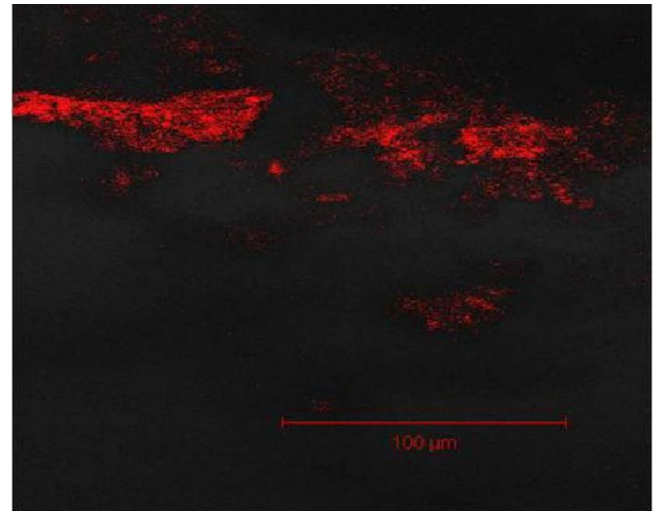


Figure 4. Biofilm formation after 21 days of incubation

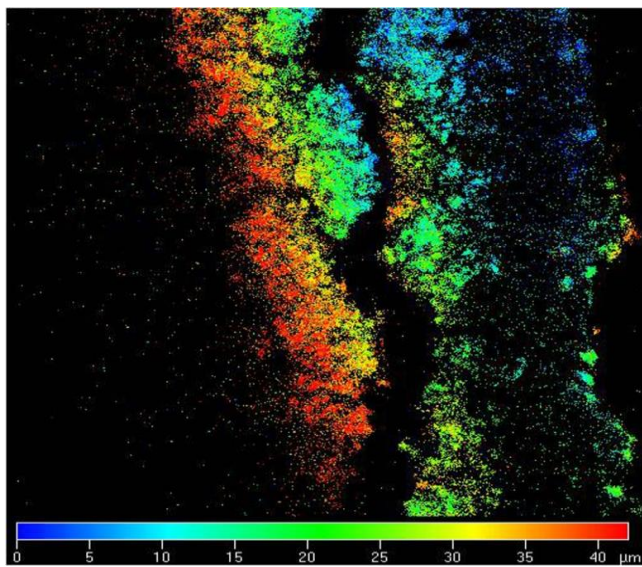


Figure 2. Biofilm formation after 21 days of incubation.

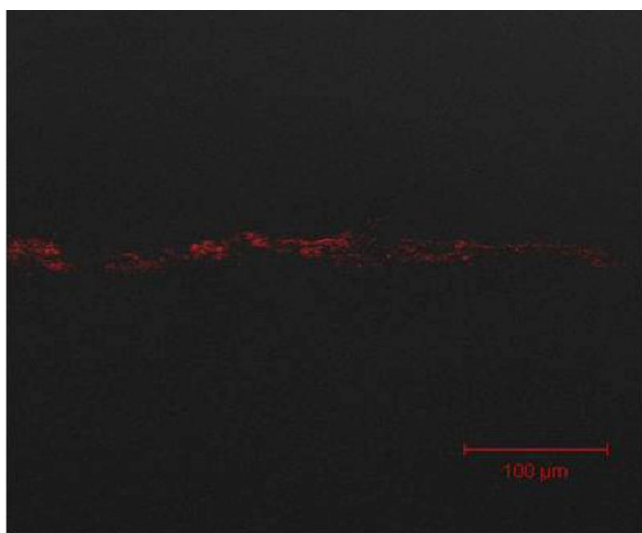


Figure 3. Biofilm formation after 21 days of incubation

Significant differences were observed in LTA levels of Gram positive bacteria irrigated with sodium hypochlorite than that of combination group irrigated with sodium hypochlorite. LTA levels increased in the Gram positive bacteria ($P < 0.05$).

On inter group comparison, there was no significant difference between the groups irrigated with saline and sodium hypochlorite in Gram positive and Gram positive, negative bacteria ($P > 0.05$).

Significant differences were observed between tooth not irrigated and tooth irrigated with saline, sodium hypochlorite groups ($P < 0.05$).

Similarly, comparing groups of Gram positive bacteria, there was significant difference between all the groups.

Sodium hypochlorite showed increase in levels of LTA than that of saline and tooth not treated with irrigant ($P < 0.05$).

Figure 1 showing mean LTA values in ng/ml.

Confocal laser scanning microscope Images showing biofilm formation after 21 days of incubation (Fig 2,3,4).

Discussion

Virulence factors released by bacteria are the major cause of failure of root canal treatment. LTA released within the root canal system can activate inflammatory mediators. The irrigant used to eradicate bacteria should neutralize the liberation of LTA. In this study sodium hypochlorite was used because it is the most commonly used and proven to eradicate the

biofilm. LTA is released within the root canal after sodium hypochlorite irrigation. The mixed microflora was selected because bacteria have been shown to co-aggregate with each other building an environment that is adherent to the surface.¹⁸

In this study there were no significant differences between the two irrigants, When treated on tooth contaminated with Gram positive bacteria. Sodium hypochlorite irrigated tooth released more LTA which may due to more damage to bacteria. Three different Gram positive bacteria were chosen to see liberation of LTA when they co aggregated with each other. LTA presence within the root canal system makes the defense mechanism innate.

LTA can begin series of reactions by binding to specific receptors. The combination of Gram positive and negative was chosen in another group to quantify liberation of LTA after forming biofilm. It has been shown that *E faecalis* co aggregates with other Gram negative bacteria.

Persistent infection can be contributed from an inflammatory response evoked by release of bacterial products that diffuse through dentinal tubules towards the pocket. Such bacteria can invade radicular dentinal tubules from periodontal pocket.¹⁹

Thus quantification of the levels of LTA after interaction with Gram negative bacteria is of clinical significance, which may hinder the cell death of Gram positive bacteria.¹⁹

Cvitkovitch and coworkers discovered the quorum-sensing CSP signaling system in *Streptococcus mutans* and showed that this system is essential for genetic competence in *S. mutans*. It is involved in biofilm formation.²⁰

Coaggregation interactions between *E. faecalis* and *F. nucleatum* have shown to produce infection in periapical region gaining protection against medicament. Coaggregation results in exchange of nutrients and survival of bacteria.¹⁸ The slow metabolic rate of microorganisms in biofilms as well as the extracellular matrix of the biofilm works to impede the effectiveness of many antimicrobials.

Saline was used in this study because it is commonly used solution and was used to compare release of LTA with sodium hypochlorite. In Group B there was no significant difference in LTA level between tooth irrigated with sodium hypochlorite and saline. Additional contributing factors for their use may be safety. Saline

probably might not have killed the bacteria, as a result the LTA liberation was decreased.

Sodium hypochlorite, an antimicrobial agent is used regularly to reduce microbes in root canal. The ideal property of an irrigant should be effective against most of microorganisms.²¹ Sodium hypochlorite in Group B was ineffective probably due to inactivation of LTA by Gram negative bacteria. Further studies should be conducted to determine coaggregation and LTA release. Sodium hypochlorite, because of its less cost and ability to remove residual microorganisms is used commonly in dentistry. Sodium hypochlorite treated LTA impaired toll like receptor activation and induction of inflammatory mediators. Due to this the LTA action is inactivated.²²

LTA is mainly liberated after cell death of bacteria in the system.²³ Sodium hypochlorite resulted in more lysis of Gram positive bacteria in group I, as a result there was more release of LTA.

The mixed microorganisms in biofilm may inhibit the activity of one another.²⁴ The limitation of this study was that LTA activity was not determined.

Conclusions

Sodium hypochlorite, when irrigated to tooth that is infected with Gram positive and negative organisms in combination showed less liberation of LTA, than when irrigated to tooth infected with Gram positive bacteria alone. Further studies are required to see the correlation between LTA release and bacterial coaggregation in mixed flora.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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