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ORIGINAL ARTICLE

Effect of using diode laser on *Enterococcus faecalis* and its lipoteichoic acid (LTA) in chronic apical periodontitis

Zhaohui Zou[1] · Junu Bhandari[1] · Baiyan Xiao[1] · Xiaoyue Liang[1] · Yu Zhang[1] · Guohui Yan[1]

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Abstract

The purpose of this study was to evaluate the effect of diode laser irradiation on *Enterococcus faecalis* (*E. faecalis*) and its lipoteichoic acid (LTA). Ninety-six freshly extracted single-rooted teeth were divided into six groups, $n = 8$ per group. Groups 1, 2, 3, and 4 as laser group (810 nm PILOT™ Diode Laser, 400 μm fiber diameter, continuous mode, 30 s time) with powers at 1.0 W, 1.5 W, 2.0 W, and 2.5 W respectively. Group 5 or positive control group (3 ml of 1% sodium hypochlorite (NaOCl) irrigation) and group 6 or negative control group (3 ml of normal saline (0.9% NaCl) irrigation). Root canal samples were collected before and after receiving laser irradiation and irrigation solution. Cultivable bacteria were determined by counting the colony (CFU/ml). Evaluation of temperature on the external root surface of teeth was done with K type thermocouple using laser at different powers. Enzyme-linked immunosorbant assay (ELISA) was performed to measure the LTA levels and the correlations between *E. faecalis* count, LTA levels, and rise in temperature were observed using Pearson's correlation test. *E. faecalis* LTA was subjected to laser irradiation and its structural damage was examined by thin layer chromatography (TLC). Compared with the control groups, all laser groups showed a decreased colony counts and decreased LTA levels with statistically significant difference ($p < 0.05$). The bactericidal effect and LTA reduction of laser was better at 2.5 W power. Laser at 2.5 W power had temperature rise of more than 7 °C which is beyond the safe thermal threshold level. No statistically significant correlation was found between *E. faecalis* count, levels of LTA, and rise in external root surface temperature ($p > 0.05$). TLC results showed a structural damage in the glycolipid moiety of *E. faecalis* LTA. Diode laser can effectively reduce the *E. faecalis* count and its LTA levels.

Keywords Diode laser · *Enterococcus faecalis* · Lipoteichoic acid · Apical periodontitis

Introduction

Enterococcus faecalis is the most prevalent species found in periradicular lesions of teeth that have been treated endodontically and is a major etiologic agent of persistent apical periodontitis [1]. They are also resistant to most disinfectants and antiseptics as they can persist under extreme conditions infecting the dentinal tubules up to a depth of 800 μm [2]. *E. faecalis* and its byproducts result in various pulpal and periradicular inflammations so their removal from the root canals is essential for successful endodontic treatment [3].

Virulence factors of *E. faecalis* are responsible for resistance against defense mechanisms of the host and production of pathological changes [4, 5]. Lipoteichoic acid (LTA) is one of the major virulence factors in the cell wall of *E. faecalis* [6] often released from bacteria during cell division and even after cell death which persists within the root canals for long period of time resulting in chronic inflammation [7]. *E. faecalis* LTA contributes to the bacterial adherence, release of several inflammatory mediators like TNF-α, interleukin 1ß (IL-1ß), interleukin 6 (IL-6) [8], and biofilm formation [9]. Accumulating reports have shown that *E. faecalis* biofilm formation inside dentinal tubules is a crucial factor in the etiology of refractory apical periodontitis [10].

Studies have shown that irrigating solutions like sodium hypochlorite can disinfect the root canals as well as they can

Zhaohui Zou and Junu Bhandari contributed equally to this work.

Zhaohui Zou and Junu Bhandari are co-first authors.

✉ Zhaohui Zou
zouzhaohui2005@sina.com

[1] Department of Endodontics, Tianjin Medical University Stomatology Hospital, Building Number 12, Qixiangtai Road, Heping District, Tianjin 300070, China

also inactivate the lipoteichoic acid of *E. faecalis* by deacylation [11–13]. In recent years, different types of lasers have been employed in root canal disinfection. Lasers have been used as an effective adjunct for successful elimination of root canal bacteria along with chemo mechanical methods [14]. Gutknecht et al. have reported the decrease of bacteria by 99.9% when high power diode laser was used on extracted human teeth experimentally infected with *E. faecalis* [15]. Lasers have now been proven as a valuable adjunct in the elimination of bacteria thereby reducing the incidence of post endodontic treatment failures [16].

At present, studies focusing on the use of lasers for eliminating both the bacteria along with their virulence factors are not enough. The present study is designed to study the effect on *E. faecalis* bacteria and its lipoteichoic acid after irradiation of diode laser which may explain why laser can be used as an adjunct for elimination of *E. faecalis* in infected root canals. The efficacy of laser depends on the parameters of laser used. We compared the different powers of laser with limited rise in temperature.

Materials and method

Preparation of root samples

Ninety-six freshly extracted single-rooted teeth were collected from the department of oral and maxillofacial surgery of Stomatology Hospital of Tianjin Medical University. Teeth were cleaned and immersed in saline solution until use. Teeth free of caries, restorations, and cracks were only used for experiment. Crowns of each tooth were decoronated with a carborundum disc to standardize the length to 14 ± 0.5 mm. The patency of root canals of each tooth was checked with a number 15 K file and working length of tooth was determined using an electronic apex locator. Every tooth was prepared using hand protaper file from Sx to F3 (DENSPLY, Switzerland) with irrigation using 1% sodium hypochlorite (NaOCl). Final irrigation was done using normal saline. The apical region of each tooth was sealed with a light curing composite resin (3 M ESPE). All prepared teeth were sterilized by autoclaving at 121 °C for 20 min.

Culture and inoculum preparation

E. faecalis (ATCC 29212) strains were cultured in brain heart infusion agar under aerobic conditions and incubated at 37 °C for 24 h. The *E. faecalis* colonies were then cultured in brain heart infusion broth to obtain 0.5 McFarland standard concentration with 1.0×10^8 colony-forming units per milliliter (CFU/ml) using a spectrophotometer. The roots were inoculated with *E. faecalis* suspension using a 3.0 mL syringe with a 27-gauge needle. A sterile cotton pellet soaked in the BHI broth was used to seal the canal opening. The samples were incubated at 37 °C. During incubation, a shaker was not used. The BHI broth was changed every 24 h. After 10 days of incubation, contamination of samples was verified and divided into groups ($n = 8$ each).

Division of groups

The first, second, third, and fourth group (groups 1, 2, 3, 4) diode laser irradiation (experimental group), fifth group (group 5) 1% NaOCl irrigation (positive control group), and sixth group (group 6) normal saline irrigation (negative control group) were categorized. The laser used for this study was 810 nm, 9 W PILOT™ Diode Laser manufactured by CAO GROUP INC., 4628 W Skyhawk Dr., West Jordan, UT 84084 USA (RRID: SCR_018458). The laser fiber (diameter 400 μm) was used 1 mm short of working length in continuous up-down circumferential manner to irradiate the root canals for 30 s. Samples from root canals were collected before and after using laser with sterile paper points. The paper points were stored in sterile Eppendorf tubes with 1 ml normal saline. Similarly for the control groups, 3 ml of irrigation solution was used to irrigate the canal thoroughly for 1 min and samples were collected similarly as in laser groups.

Determination of cultivable bacterial count

The collected samples were serially diluted five times using normal saline. Ten microliters of final diluted sample was added to BHI agar plates, followed by incubation at 37 °C for 48 h. All the procedures were carried out in aseptic conditions with sterile instruments. A classic colony counting procedure was performed to determine vital *E. faecalis* bacterial counts in brain heart agar plates. After determining the colony-forming unit (CFU) in each root canal, the mean CFU/ml of each group was determined.

Evaluation of rise in external root surface temperature

Teeth were divided into four groups, 1, 2, 3, and 4, with powers of laser at 1.0 W, 1.5 W, 2.0 W, and 2.5 W respectively. Fiber tips were inserted into each root canal from the orifice to the working length and continuously moved up and down in circumferential manner for 30 s during the procedure of irradiation. The temperature measurements on the root surface were carried out using a K type thermocouple (From Institute of Biomedical Engineering Chinese Academy of Medical Sciences). During measurement, the thermocouple was in close contact to the root surface at apical region. Laser irradiation was started when the temperature at the root surface was approximately constant and similar to the room temperature. The temperatures considered (Δt) were

calculated as the difference between recorded temperatures at root surface (TRS) and recorded room temperatures (TRT): $\Delta t = \mathrm{TRS} - \mathrm{TRT}$.

Detection of lipoteichoic acid levels

Levels of LTA from each group were measured using Human LTA ELISA kit (Solarbio, China). Standard, control, and sample solution was added to ELISA well plate precoated with specific monoclonal antibody for LTA. The whole procedure was performed according to the instructions provided by the manufacturer.

Treatment of *E. faecalis* lipoteichoic acid with laser and sodium hypochlorite

E. faecalis LTA in powder form (Sigma-Aldrich, product number L4015; St. Louis, MO) was mixed with phosphate buffer saline to maintain a concentration of 5 mg/ml. Diode laser irradiation was done for 30 s with different powers in a 96-well plate containing 100 μl of LTA per well followed by incubation at 37 °C for 60 min. NaOCl treatment of LTA was done as described previously [11]. Briefly, nine volumes of LTA were mixed with one volume of 1% NaOCl and then incubated for 60 min at 37 degrees. The pH of the mixture was maintained around 8 using HCL. The samples were then lyophilized and stored at − 80 °C until use.

Preparation of glycolipid anchor from *E. faecalis* lipoteichoic acid and thin layer chromatography

The glycolipid anchor from *E. faecalis* LTA was prepared as described previously [13]. Acetic acid was used to cleave the phosphodiester bond of LTA. Then, 30 min ultrasonication followed by boiling for 3 h was done and samples were lyophilized. For performing TLC, a TLC silica gel plate (size 50 × 100 mm, 0.20–0.25 mm thickness) was used. The solvent form composed of 60:30:5 (v/v/v) chloroform, methanol, and water. The lyophilized samples were mixed in a solvent with 1:1:0.9 (v/v/v) chloroform, methanol, and water. After spotting the samples on the TLC plate, they were visualized under UV light first followed by staining with 5% phosphomolybdic acid and heating using a hot plate at 110 °C.

Statistical analyses

Statistical analyses were performed using SPSS software version 22.0. Student's *t* test, one way analysis of variance, and Tukey's method were performed. Pearson's correlation test was used to analyze the correlation between CFU/ml, temperature rise within the root canals, and LTA levels. All experiments were performed at least three times. The mean ± standard deviation was calculated for each group. Significance level was maintained at $p < 0.05$ (Figs. 1, 2, and 3).

Results

Colony count results

Bactericidal effect of laser (CFU/ml results)

Compared with the normal saline control group, the CFU/ml in all the laser treatment groups (groups 1, 2, 3, and 4) was decreased and the difference was statistically significant ($p < 0.05$).

In the laser group, the bactericidal effect of laser was better at 2.5 W compared with 1.0 W, 1.5 W, and 2.0 W; the difference was statistically significant ($p < 0.05$) (Tables 1, 2, 3, and 4).

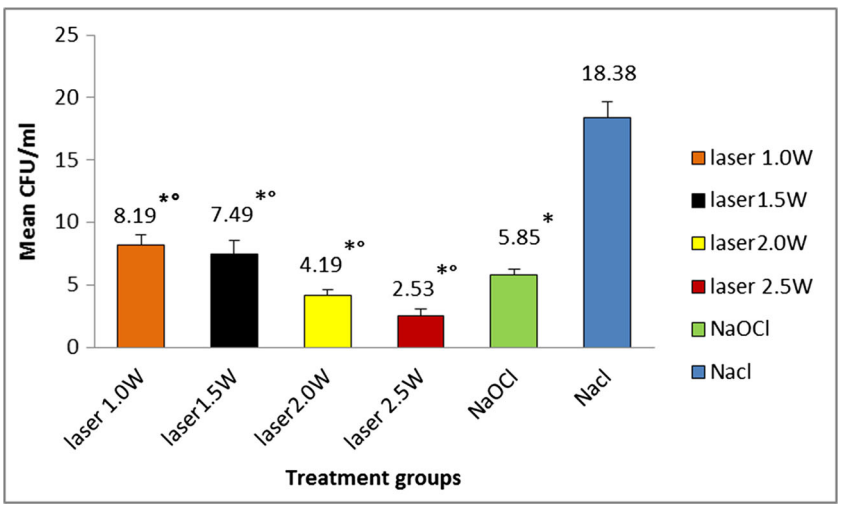

Fig. 1 CFU/ml results of each groups. Note: The asterisk indicates that compared with 0.9% normal saline group, $p < 0.05$ with statistically significant difference. The small circle means compared with the NaOCl group, the difference was statistically significant ($p < 0.05$)

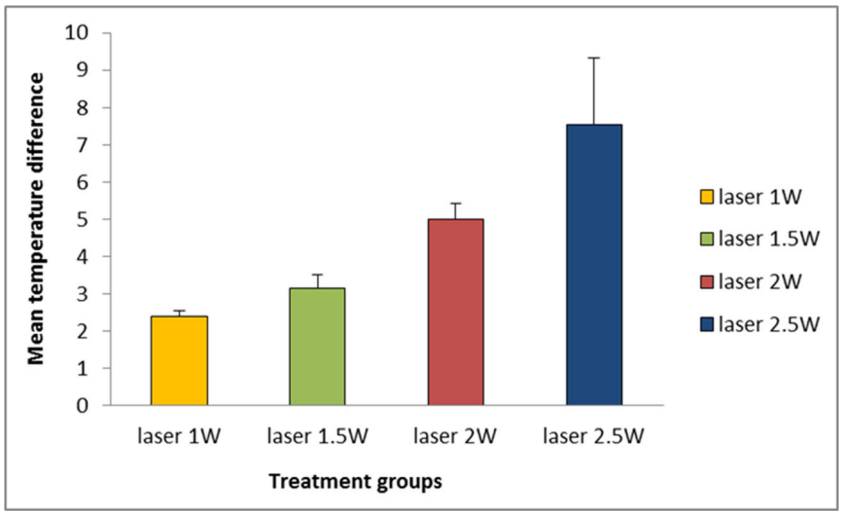

Fig. 2 Mean temperature difference results of each group

Results of temperature evaluation

Results of rise in external root surface temperature after using laser

The mean temperature difference (Δt) was less than 7 °C when the power of diode laser was set at 1.0 W, 1.5 W, and 2.0 W. The difference was not statistically significant ($p > 0.05$).

When the power of the laser was 2.5 W, the mean temperature difference (Δt) was 7.8 °C, which is higher than the safe thermal threshold level of 7 °C. The difference was not statistically significant ($p > 0.05$).

LTA level results

Reduced LTA levels after using laser

Compared with the normal saline control group, the LTA levels in all the laser treatment groups (groups 1, 2, 3, and 4) were decreased and the difference was statistically significant ($p < 0.05$).

In the laser group, the reduction in the levels of LTA was better at 2.5 W compared with 1.0 W, 1.5 W, and 2.0 W; the difference was statistically significant ($p < 0.05$).

Correlation analysis between CFU/ml, LTA (ng/ml), and rise in external root surface temperature (Δt)

Pearson's correlation analysis results

When laser was used at power of 1.0 W, there was a positive but not significant correlation ($p > 0.05$) between CFU/ml and LTA ($r = 0.435$), between CFU/ml and rise in temperature ($r = 0.368$) and between LTA and rise in temperature ($r = 0.451$).

When laser was used at power of 1.5 W, there was a positive correlation between CFU/ml and LTA ($r = 0.695$). A negative correlation was observed between CFU/ml and rise in temperature ($r = -0.513$) and between LTA and rise in

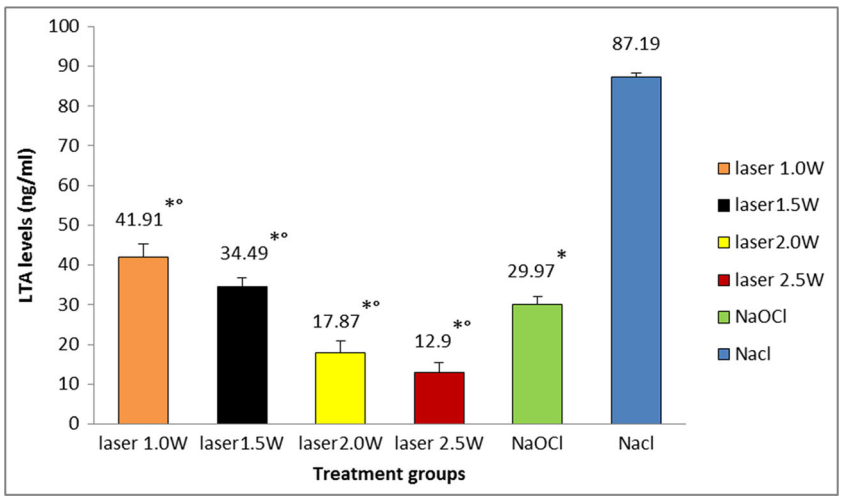

Fig. 3 Mean LTA levels (ng/ml) of each group. Note: The asterisk indicates means compared with 0.9% normal saline group, $p < 0.05$ with statistically significant difference. The small circle means compared with the NaOCl group, the difference was statistically significant ($p < 0.05$)

Table 1 *E. faecalis* count (CFU/ml) results after diode laser irradiation

S. n.	Groups	*N*	Min	Max	CFU/ml ($\bar{x} \pm SD$)
1	Diode 1.0 W	8	6.93	9.04	$8.19 \pm 0.85^{\times\circ}$
2	Diode 1.5 W	8	5.39	8.70	$7.49 \pm 1.02^{\times\circ}$
3	Diode 2.0 W	8	3.75	5.03	$4.19 \pm 0.44^{\times\circ}$
4	Diode 2.5 W	8	2.10	3.45	$2.53 \pm 0.52^{\times\circ}$
5	NaOCl	8	5.07	6.45	$5.85 \pm 0.39^{\times}$
6	0.9% normal saline	8	16.46	19.84	18.38 ± 1.30

×, denotes the statistically significant values when compared to normal saline control group; °, denotes the statistically significant values when compared to NaOCl control group

temperature ($r = -0.499$). The correlations were not statistically significant ($p > 0.05$).

When laser was used at power of 2.0 W, there was a negative correlation between CFU/ml and LTA ($r = -0.091$). A positive correlation was observed between CFU/ml and rise in temperature ($r = 0.379$) and between LTA and rise in temperature ($r = 0.319$). The correlations were not statistically significant ($p > 0.05$).

When laser was used at power of 2.5 W, there was a positive but not significant correlation ($p > 0.05$) between CFU/ml and LTA ($r = 0.214$), between CFU/ml and rise in temperature ($r = 0.654$) and between LTA and rise in temperature ($r = 0.381$).

In NaOCl (positive control) group, there was no statistically significant correlation between CFU/ml and LTA levels ($r = 0.018$, $p > 0.05$).

In NaCl (negative control) group, there was no statistically significant correlation between CFU/ml and LTA levels ($r = 0.350$, $p > 0.05$).

Thin layer chromatography results

Figure 4 shows the identification of free fatty acids generated from the glycolipids after laser treatment. Glycolipids from laser- and NaOCl-treated *E. faecalis* LTA were prepared and TLC was performed.

Table 2 Rise in external root surface temperature after diode laser irradiation

S. n.	Groups	*N*	Min	Max	(Δt) ($\bar{x} \pm SD$)
1	Diode 1.0 W	8	2.31	2.57	2.45 ± 0.14
2	Diode 1.5 W	8	2.72	3.40	3.15 ± 0.35
3	Diode 2.0 W	8	4.77	5.35	5.15 ± 0.49
4	Diode 2.5 W	8	6.84	9.30	7.80 ± 1.06

Note: In all the four laser groups, the difference in temperature rise (Δt) was not statistically significant ($p > 0.05$)

Table 3 Results of mean LTA levels (ng/ml) after diode laser irradiation

S. n.	Groups	*N*	Min	Max	LTA (ng/ml) ($\bar{x} \pm SD$)
1	Diode 1.0 W	8	37.31	46.39	$41.91 \pm 3.34^{\times\circ}$
2	Diode 1.5 W	8	29.85	36.63	$34.49 \pm 2.23^{\times\circ}$
3	Diode 2.0 W	8	12.07	21.86	$17.87 \pm 3.06^{\times\circ}$
4	Diode 2.5 W	8	9.47	16.93	$12.90 \pm 2.50^{\times\circ}$
5	NaOCl	8	27.53	33.64	$29.97 \pm 2.12^{\times}$
6	0.9% normal saline	8	85.18	88.59	87.19 ± 1.15

×, denotes the statistically significant values when compared to normal saline control group; °, denotes the statistically significant values when compared to NaOCl control group

Lane 1: Glycolipid from *E. faecalis* LTA. Lane 2: Glycolipid from diode laser–treated LTA. Lane 3: Glycolipid from NaOCl-treated LTA.

Laser can structurally damage the glycolipid moiety of LTA from *E. faecalis*: TLC analysis was performed to examine the release of free fatty acids from the lipid moiety in *E. faecalis* LTA after treating with laser. TLC result shows identical patterns of *E. faecalis* LTA treated with diode laser and *E. faecalis* LTA treated with NaOCl confirming its deacylation. But, the intact *E. faecalis* LTA has different Rf. values originating due to difference in length and saturation degrees of its fatty acid chains.

Discussion

The main goal of endodontic treatment is to make the tooth free of infection and eliminate the pulpal and periapical diseases. In order to achieve this goal, it is important to reduce the microbial content inside the root canals along with their toxic byproducts. Studies have pointed out that presence of *E. faecalis* is strongly associated with chronic apical periodontitis and failure of endodontic treatments [17]. The conventional root canal therapy with chemo mechanical preparation can effectively reduce the bacterial colonization inside the root canal system but it cannot completely eradicate the toxic byproducts named as virulence factors [18].

In the present study, we found that intra canal irradiation of diode laser at 2.5 W can effectively reduce the *E. faecalis* content inside root canals and its sterilization effect was better than NaOCl. The reduction of bacteria using diode laser is mainly due to the reduction of dentin permeability and smear layer removal [19]. Diode laser can penetrate to a dentinal depth of about 500 μm to reduce the bacterial contamination [20]. According to a study by Beer et al., diode laser of 810 nm wavelength could eliminate 98.8% of root canal and laser was described as "modern state-of-the-art instrument for endodontics" [21]. These studies suggest that using laser during root

Table 4 Correlation analysis between CFU/ml, LTA (ng/ml), and temperature

CFU/ml		CFU/ml Pearson correlation	Sig. (2-tailed)	LTA levels Pearson correlation	Sig. (2-tailed)	Temperature Pearson correlation	Sig. (2-tailed)
1.	Diode 1.0 W	1	–	0.435	0.282	0.368	0.369
2.	Diode 1.5 W	1	–	0.695	0.055	− 0.513	0.194
3.	Diode 2.0 W	1	–	− 0.091	0.830	0.379	0.355
4.	Diode 2.5 W	1	–	0.214	0.611	0.654	0.078
5.	NaOCl	1	–	0.018	0.966	–	–
6.	NaCl	1	–	0.350	0.395	–	–
LTA levels							
1.	Diode 1.0 W	0.435	0.282	1	–	0.451	0.262
2.	Diode 1.5 W	0.695	0.055	1	–	− 0.499	0.208
3.	Diode 2.0 W	− 0.091	0.830	1	–	0.319	0.441
4.	Diode 2.5 W	0.214	0.611	1	–	0.381	0.352
5.	NaOCl	0.018	0.966	1	–	–	–
6.	NaCl	0.350	0.395	1	–	–	–
Temperature							
1.	Diode 1.0 W	0.368	0.369	0.451	0.262	1	–
2.	Diode 1.5 W	− 0.513	0.194	− 0.499	0.208	1	–
3.	Diode 2.0 W	0.379	0.355	0.319	0.441	1	–
4.	Diode 2.5 W	0.654	0.078	0.381	0.352	1	–
5.	NaOCl	–	–	–	–	–	–
6.	NaCl	–	–	–	–	–	–

canal treatment could be an alternative to traditional irrigants and medicaments used for successful elimination of bacterial contamination during root canal therapy.

Analysis of temperature rise due to intra canal laser application is necessary since it can impair adjacent periodontal tissues. In our present study, we found the powers below 2.0 W to be within the safe range for root canal treatment. Previous studies have reported a thermal threshold level of 7 °C for impairing periodontal tissues and 10 °C for alveolar bone [22, 23]. When we used the diode laser at 2.5 W power, the rise in temperature was greater than 7 °C which can impair the periodontal tissues. So using laser at a power with increased rise of temperature is avoided to preserve the adjacent periodontal tissues and alveolar bone.

Lipoteichoic acid (LTA) of *E. faecalis* is responsible for the induction of inflammatory mediators resulting in pain, periapical pathology, and bone resorption in involved tooth [24]. From the results of ELISA, we also observed a decrease in the levels of *E. faecalis* LTA after using laser. Previous studies shows that sodium hypochlorite could also effectively reduce the levels of *E. faecalis* LTA when used during root canal preparation but regarding the effect of laser on the LTA of gram-positive bacteria like *E. faecalis* is not known till date [16, 17].

Thin layer chromatography performed in our study also showed that treating *E. faecalis* LTA with diode laser results in deacylation of the LTA glycolipid moiety structure which is similar to NaOCl-treated *E. faecalis* LTA. Laser was used at 2.0 W since at 2.5 W the rise in temperature was beyond the safe thermal threshold of 7 °C so this power was not considered safe for the periodontal tissues even though it had better bactericidal effect. Disrupting the structure of LTA after using laser could make it inactive [18]. Accumulating studies also suggest that *E. faecalis* LTA could be inactivated by deacylating its fatty acid chains which results in impairment of immune stimulating activity of LTA [25]. The rationale for performing this in vitro experiment is to provide a valuable information to the clinicians about the antibacterial effects of diode laser along with its effects on LTA.

Laser used during root canal therapy has many advantages over conventional therapeutic methods [26]. From our present study also, it is found that using laser at a safe thermal threshold level, it can effectively reduce bacterial contamination along with its virulence factors responsible for the production of inflammatory mediators in chronic apical periodontitis. However, there was no statistically significant correlation between bacterial colony counts, levels of LTA, and temperature rise. The use of NaOCl for root canal treatment is mainly due

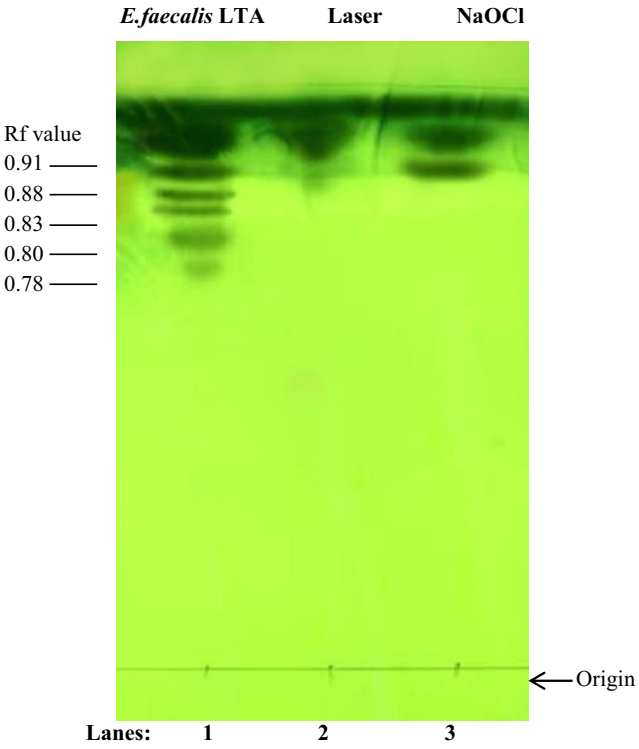

Fig. 4 TLC plate after staining with 5% Phosphomolybdic acid

to its low cost and ability to remove residual microorganisms but increased concentrations of it are cytotoxic and can reduce the elastic and flexural strength of dentin [27]. Laser on the other hand is an easy means of root canal disinfection with no harmful effects to the dentin and surrounding apical tissues if used according to the standard protocol.

The reduction of *E. faecalis* contamination and inactivation of lipoteichoic acid shown in our study may justify the high rate of success in repairing periapical lesions when diode laser is used in clinics for endodontic treatments [28].

Conclusion

Diode laser irradiation during root canal treatment can eliminate both *E. faecalis* bacteria and its LTA in cases of chronic apical periodontitis. The laser can also damage the structure of LTA and make it inactive.

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Compliance with ethical standards

Conflict of interest There authors declare that there are no conflicts of interest.

Ethical considerations The ethical committee of Tianjin Medical University, Stomatology Hospital, Tianjin approved this study (TMUhMEC2019035).

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