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Asian Journal of Oral Health and Allied Sciences

Original Article

Comparative evaluation of 5% sodium hypochlorite and Nd:YAG laser in reducing endotoxins level from infected root canals – An *in vivo* microbiological study

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Received : 07 April 2021
Accepted : 30 October 2021
Published : 22 November 2021

DOI
10.25259/AJOHAS_4_2021

Quick Response Code:

ABSTRACT

Objectives: Endodontic infection is one of the most challenging aspects of dental treatment which is primarily caused by Gram-negative anaerobic bacterial species. The primary objective in the management of endodontic treatment is eradication of bacteria, as well as elimination of endotoxin. The aim of this study was to compare the effectiveness of 5% sodium hypochlorite, a mixture of calcium hydroxide+2% chlorhexidine (CHX) gel and Nd:YAG lasers for decreasing the level of endotoxin in infected root canals.

Material and Methods: Forty four patients in the age group of 18–50 years were carefully chosen for the study and divided into control (Group 1, n = 11) and experimental group (Group 2, n = 33). Experimental group was further split into three subgroups according to the medicaments used. Samples were collected after the access opening (pre-operative specimens), after the biomechanical preparation (intermediate samples), and after the application of intracanal medicament for 15 days. The concentration of endotoxin in each sample was measured using quantitative chromogenic limulus amebocyte lysate assay. The data collected were statistically analyzed using SPSS for Windows (Statistical Package for the Social Sciences, Inc., Chicago, IL,) Version 15.0 Statistical Analysis Software. The level of significance was set at 0.05 for all tests.

Results: Pretreatment endotoxins level observed was 6.783EU/ml in Control group and 7.261EU/ml, 6.963EU/ml, 7.247EU/ml in experimental subgroups. After biomechanical preparation and use of medicaments endotoxins level reduced to 3.919EU/ml in Control group and 0.5222EU/ml (sodium hypochlorite) 1.164EU/ml (calcium hydroxide+chlorhexidine), 0.841EU/ml (laser).

Conclusion: This study concluded after analyzing the data that use of 5% sodium hypochlorite and Nd:YAG laser was effective in decreasing the level of endotoxins and use of intracanal medicament (2% CHX+ calcium hydroxide) for 15 days further helps to reduce the endotoxin level.

Keywords: Biomechanical preparation, Calcium hydroxide, Chromogenic limulus amebocyte lysate assay test, Endotoxin, Nd:YAG laser

INTRODUCTION

Overcoming the endodontic infection is one of the most challenging aspects of dental treatment. Endodontic microflora, namely, bacteria and their byproducts play a significant role in the development of pulpal and periapical diseases.[1] Endotoxin, which is comprised of polysaccharide, lipids, and protein, is present on the exterior cell membrane of all Gram-negative bacteria. Lipid part of endotoxin is responsible for its toxicity. Endotoxins are the virulent primary factor of Gram-negative anaerobic bacteria.[2] These endotoxins have the ability to invade the dentinal

tubules[3] from where they manage to enter the pulp chamber and stimulate the immune cells through toll-like receptor 4.[4] These stimulated cells release several cytokines which mediate the inflammatory response.[5,6] Depending on the amount of endotoxin, the inflammatory response may result in reversible or irreversible pulpitis. If the process becomes irreversible, it may lead to pulpal necrosis followed by periapical pathology[7] and bone resorption.[8] The amount of bone resorption is proportional to the amount of endotoxins produced.[8]

The main purpose of endodontic treatment of teeth with pulpal necrosis should be elimination of bacteria as well as inactivation of lipopolysaccharides.[9] The use of antibacterial dressings in the root canals help in destroying the bacteria left after the root canal preparation. Various root canal irrigants and medicaments are in use for removal of endotoxins since a very long time. Recently, laser has been introduced in this field for quick and precise treatment. Specific wavelength of laser interacts with dental tissue in a different way such as selective absorption, coagulation, ablation, sterilization, and biostimulation. All these effects make the laser a treatment of choice over conventional technique in certain situation.[10]

This study compared the effectiveness of 5% sodium hypochlorite, a mixture of calcium hydroxide + 2% chlorhexidine (CHX) gel and a role of Nd:YAG laser for decreasing the amount of endotoxins. Chromogenic limulus amebocyte lysate (LAL) assay test was employed to calculate the amount of endotoxin present in the root canals.

MATERIAL AND METHODS

Patient selection

Forty-four patients in the age group of 18–50 years were selected from the outpatient department of conservative dentistry and endodontics, for endodontic treatment. Exclusion criteria included patient undergoing antibiotic treatment for more than 3 months, prior endodontic treatment, systemic disease, and teeth having more than 4 mm of periodontal probing depths. Inclusion criteria for both control and experimental groups included infected carious tooth which was hyper responsive to vitality test and radiographically no radiolucency present.

Institutional Ethical Committee (No. 7813/Ethics/R.cell-15) of University accepted the protocol for describing the sample collection for this study. All the patients who participated in the research sign up a written informed consent.

Medicament used for the study

5% sodium hypochlorite, calcium hydroxide + 2%CHX gel, Nd:YAG laser.

Sampling procedure

Selected patients were allocated into control (Group 1) and experimental groups (Group 2). The control group consisted of 11 patients and their root canals were irrigated by pyrogen-free normal saline. The experimental group consisted of 33 patients which were further subdivided into three subgroups according to the irrigating solution used, namely, Group 2a, Group 2b, and Group 2c: G2a irrigation by hypochlorite (n = 11), G2b: Irrigation by pyrogen-free normal saline (n = 11), and G2c disinfection by laser (n = 11). After irrigation and drying of the canals, all the samples of experimental groups received intracanal medicament of 2% CHX and calcium hydroxide for 15 days. Control group did not receive any medicament.

Sample collection

Autoclaved instruments were used for the study. Before initiating the access cavity preparation, patients were asked to rinse with CHX mouthwash. The particular tooth was isolated with a rubber dam. Cotton gauze soaked in 30% hydrogen peroxide followed by 2.5% sodium hypochlorite was used to disinfect the individual tooth. Access cavity was prepared using sterilized airotor handpiece and round bur along with manual irrigation using pyrogen-free normal saline. A preoperative sample (S1) of the canal was collected with sterile paper point (number 15 or 25, Sure-endo, Korea) by inserting it into the full length of the canal for 1 min. Paper point after removal from the canal was immediately suspended in 1 mL LAL solution for quantification of endotoxins. Working length was checked by inserting K-file (number 10) and an intraoral periapical radiograph of that tooth was taken.

Root canals of all the samples were prepared at a speed of 300 rpm with M-two rotary files. M-two instruments (10/0.04, 15/0.05,20/0.06, 25/0.06) were introduced in the canal in a single-length technique and advanced gradually up to the apex in a gentle in-and-out movement. Root canals were irrigated with 5 mL pyrogen-free saline before the commencement of next instruments. According to the groups, canals were irrigated with 5 mL of pyrogen-free saline (control group), 5 mL of 5% sodium hypochlorite (Group 2a), pyrogen-free normal saline (Group 2b), and Group 2c was irradiated with Nd:YAG laser with an energy level of 1.5 W, with 15 Hz repetition rate, 150 J/cm^2 for 15 s in contact mode. An intermediate sample (S2) of the canal was taken with sterile paper point (number 15) by inserting it into the canal for 1 min. The intracanal dressing mixture of calcium hydroxide plus 2% CHX gel (in a paste-like consistency at 1:1 ratio) was placed inside the canal by use of lentulospiral for 15 days duration. Zinc oxide eugenol paste was used to seal the coronal access.

After the 15 days, canals were reopened under a rubber dam aseptically and irrigated by 5 mL pyrogen-free saline and

agitated with master file and irrigated with 5 mL normal saline to remove calcium hydroxide + 2% CHX. The post-operative samples (S3) of the canal were taken with sterile paper points (number 15) by inserting them into the canal for 1 min.

Thus, samples were collected after the access opening (pre-operative specimens), after the biomechanical preparation (intermediate samples), and after the application of intracanal medicament for 15 days. In this way, three samples were collected at three intervals from each group and subgroups. The pre-operative (S1), intermediate (S2), and post-operative root canals endotoxin samples (S3) were placed in 1.5 mL microcentrifugation tubes containing 1 mL pyrogen-free water. Then, each microcentrifugation tube containing endotoxin was stored in a chilled ice box and transferred to the microbiology laboratory for endotoxin evaluation.

Preparation of sample for endotoxin detection

All the samples obtained were stored at −200°C, for extracting endotoxin from the paper point. Before extracting endotoxins, all the samples were placed at room temperature for 45 min and then microcentrifugation tubes were agitated in the vortex for 60 s. A 50 µl solution of microcentrifugation was used as an endotoxin sample.

Determination of endotoxins concentration

A quantitative endpoint assay test was used to measure the endotoxin concentrations by Thermo Scientific Pierce LAL Assay Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, USA). Preparation of Endotoxin Standard Stock Solutions done as follows –

One milliliter endotoxin-free water was added to 15 EU *Escherichia coli* endotoxin standard vial to make endotoxin standard stock of 15 EU/mL concentration, and the solution was vigorously shaken on a vortex mixer for at least 15 min before use. Final concentrations (i.e., 0.1, 0.25, 0.5, and 1 EU/mL) were prepared using the manufacturer's instructions.

The LAL reagent was prepared immediately before use by adding 1.4 mL of endotoxin-free water into LAL powder containing vial and swirl gently to dissolve the powder. Foaming should be avoided and the solution should not be vortex. Chromogenic substrate was prepared by adding 6.5 mL of endotoxin-free water into the vial to yield a final concentration of 2 mL.

Statistical analysis

The data collected for each group were tabulated into the spreadsheet and statistically analyzed using SPSS for Windows (Statistical Package for the Social Sciences, Inc., Chicago, IL) Version 15.0 Statistical Analysis Software. One-way analysis of variance was used to compare the mean within the group variances followed by Turkey *post hoc* test. The level of significance was set at 0.05 for all tests.

RESULTS

Turbidimetric chromogenic LAL assay was used to evaluate endotoxin level. The endotoxin level at different time intervals (before irrigation, after irrigation, and after removal of medicaments) was collected. Data thus obtained were subjected to statistical analysis.

Table 1 shows the mean value of endotoxin levels of all groups. The level of endotoxin was higher in all pre-treatment samples as compared to intermediate and post-treatment. The pre-treatment endotoxins level was 6.783 EU/mL in control group, 7.261 EU/mL in Group 2a (sodium hypochlorite), 6.963 EU/mL in Group 2b (calcium hydroxide and CHX), and 7.247 EU/mL in 2c (laser group). After the biomechanical preparation and irrigation with sodium hypochlorite, Nd:YAG laser, and pyrogen-free water, the intermediate endotoxin level was decreased; and the highest reduction was observed with sodium hypochlorite, that is, 0.522 EU/mL. After 15 days of intracanal dressing, the mean value of endotoxin level was lowest with sodium hypochlorite subgroups followed by Nd:YAG laser, calcium hydroxide + CHX, and control groups.

Table 2 shows the percentage change in the endotoxin values at different levels of the treatment procedure. The percentage change in the endotoxins level between pre and intermediate samples was highest with sodium hypochlorite and lowest with saline solution. The percentage changes in endotoxin levels between pre-operative and intermediate samples show the poorest response with normal saline as compared to other irrigating solutions. The percentage change in endotoxin level between pre-operative and post-operative endotoxin samples is higher for sodium hypochlorite as compared to the laser subgroup.

DISCUSSION

The primary objective of biomechanical preparation and disinfection of infected canals should be to eliminate not only microorganism but also their byproducts, that is, endotoxins.[11] The microorganisms responsible for primary endodontic infection consist of Gram-positive, Gram-negative, and mainly anaerobic microorganisms.[1] However, secondary infection is the leading cause of root canal treatment failure, and it results from existing Gram-positive and Gram-negative bacteria. Endotoxins are present on the outer cell wall of anaerobic bacteria. It consists of polysaccharides, lipids, and proteins. The toxic effect is mainly due to lipids.[12] Endotoxins are released during reproduction of bacterial death which causes sequence of biological reaction leading to an inflammatory and periapical

Table 1: Mean value of endotoxins level in control and experimental groups at different time intervals.

	Group 1 Control group	Group 2 Experimental group		
	(disinfection by normal saline)	Subgroup 2a (disinfection by hypochlorite) followed by calcium hydroxide+ chlorhexidine	Subgroup 2b disinfection by normal saline followed by calcium hydroxide+ chlorhexidine	Subgroup 2c (disinfection by laser) followed by calcium hydroxide+chlorhexidine
S1 (pre-operative)	6.783	7.261	6.963	7.247
S2 (interoperative)	3.429	1.695	3.532	2.324
S3 (post-operative)	3.919	0.522	1.164	0.841
P-value	0.246	0.0000053	0.000067	0.00000037

Table 2: Mean percentage change in endotoxin level in control and experimental groups between different time intervals.

	Group 1 Control group	Group 2 Experimental group		
	(disinfection by normal saline)	Subgroup 2a (disinfection by hypochlorite) followed by calcium hydroxide+ chlorhexidine	Subgroup 2b disinfection by normal saline followed by calcium hydroxide+ chlorhexidine	Subgroup 2c (disinfection by laser) followed by calcium hydroxide+chlorhexidine
Pre-operative + intermediate	48.72	76.36	47.711	68.75
Intermediate + post-operative	-19.828	68.79	66.33	61.58
Pre-operative + post-operative	41.715	92.91	82.40	88.83

bone resorption.[12] Schein *et al*.[13] were the first to report endotoxin inside the root canal. Schonfeld *et al*.[14] have proven that endotoxins highly correlated with the presence of inflammation in the tissue. Since then, many researchers and clinicians are actively involved in studies related to the toxicity of endotoxins and their removal.

In this present study, assessment of levels of endotoxins in the infected canal was done employing chromogenic limulus amebocyte lysate assay test. Both the groups showed the highest amount of endotoxins in the pre-treatment samples. There was a fall in the mean value of endotoxins level from 6.783 EU/mL to 3.532 EU/mL [Table 1] after biomechanical preparation and irrigation with pyrogen-free normal saline in the control group. A possible reason for the initial decrease in the endotoxin level can be attributed to the action of biomechanical preparation and particular debris removal with irrigation action which leads to removal of certain endotoxins levels as well. This observation was consistent with the findings of Valera *et al*.[15] The root canal acts as the receptacle of LPS, can seep into periapical tissues, and plays a part in the inflammatory process.[16] Thus; it becomes necessary to do biomechanical preparation along with irrigants to remove the remaining toxins.

Sodium hypochlorite which is a gold standard in endodontic irrigation due to its potent antimicrobial activity and effective dissolution efficacy was incorporated in this study. The other reason being that sodium hypochlorite dissolves organic and fat degrading fatty acids resulting in the formation of fatty acid salts (soap) and glycerol (alcohol) which ultimately leads to decrease in the surface tension of the remaining solution.[16] Sodium hypochlorite reduces the effect of amino acids by forming water and salt. The release of hydroxyl ions contributes to bringing down the pH of the solution. Sodium hypochlorite acts as tissue solvent due to the presence of hypochlorous acid and releases chlorine. Chlorine in combination with the protein amino group forms chloramines. Hypochlorous acid (HOCl-) and hypochlorite ions (OCl-) proceed further for degradation and hydrolysis of amino acid. Chloramines interfere with the metabolism of the cell which is formed during chloramination reaction between chlorine and the amino (NH) group.[17] Chlorine (strong oxidant) due to its antimicrobial action inhibits bacterial enzymes resulting in irreversible oxidation of SH groups (sulfhydryl group).

The present study showed a reduction in the level of endotoxins after 15 days from the sample treated with calcium hydroxide and CHX. These results are consistent with Oliveira *et al*.[18] who found no endotoxins after medication with calcium hydroxide and CHX. It may be due to the combined use of calcium hydroxide and CHX. The calcium hydroxide possesses antibacterial activity, biocompatibility, the capability to reduce periapical exudate, and the skill to dissolve necrotic tissue remnant after biomechanical preparation.[12,19] CHX, on the other hand, is a positively charged lipophilic molecule and the most common oral preparation, that is, CHX gluconate

is water soluble and at physiological pH, which is helpful in the destruction of microorganisms.[20] The positively charged molecule of CHX enters the bacteria cell membrane through active or passive transport mechanism after interacting with lipopolysaccharides and phospholipids present on the outer cell membrane of bacteria.[21] The effectiveness of CHX is when the positive charge of the molecule interacts with negatively charged phosphate groups on the microbial cell walls. Thus, the association of CHX and calcium hydroxide boosts up the antimicrobial activity of calcium hydroxide resulting in improved efficacy against resistant microorganisms without altering their biological properties.[22] Safavi and Nicholas and other researchers have concluded in their studies that calcium hydroxide transforms lipid A into fatty acid and amino sugars which are a toxic components. Signoretti *et al*. have found that the increased detoxifying effect of combined use of CHX and Ca(OH)2 is due to the spawning of excessive reactive oxygen species. Thus, calcium hydroxide helps in detoxifying the root canal.[12] The combination of calcium hydroxide plus 2% CHX was placed in the canal for 15 days for endotoxins reduction because maximum pH (12.12) and maximum calcium ion (140.18 mg/L) are releases after 15 days.[22]

Besides causing an inflammatory reaction, these bacterial endotoxins adhere to the mineralized tissue, thereby stimulating resorption of bones, synthesis, and release of osteoclast stimulating factors and osteoclastogenesis.[23] Thus, it becomes necessary to eliminate lipopolysaccharide from the root canal. Otherwise, it may lead to periapical pathosis. Rooney *et al*.[24] were the first to use the laser for root canal sterilization. The antibacterial effect depends on the temperature interactions of the microorganism with biofilm. Subsequent heating of the substrate causes an increase in temperature which is high enough to kill attached microorganisms.[25] Lasers have various purposes in endodontics, but there are limited studies regarding endotoxin removal by lasers.

In the samples treated with Nd:YAG laser, the endotoxin content was high in pre-treatment samples with a mean value of 9.705 EU/mL. After biomechanical preparation and disinfection with Nd:YAG laser (1.5 W, 15 Hz, 150 J/cm^2 for 15 s), a significant reduction in endotoxin content was noticed with a mean value of 2.836 EU/mL. The lowest concentration of endotoxins was reported after 15 days of intracanal dressing (mean value 1.087 EU/mL) [Table 1]. This initial reduction in the level of endotoxins following laser irradiation may be because of the high temperature achieved by laser endotoxin moiety leading to its disintegration. After 15 days, it was seen that endotoxins level reduced further. Cell which is in synchronous with the results obtained in previous groups wherein the synergistic action of $Ca(OH)_2$ and CHX has augmented antimicrobial effect which prevents the growth of bacteria for 15 days and does not allow any new endotoxins to form.

In the present study, a significant reduction of endotoxin content was reported with the use of a laser; this finding is consistent with Rooney *et al*.[24] The result of this study is not consistent with Yasuda *et al*.[26] where they found that the bactericidal effect of Nd:YAG laser decreased in curved canals due to hindrance in the spiral motion of optical fiber.

CONCLUSION

From our study, we can conclude that intracanal irradiation with laser does not intend to substitute the conventional method of disinfection but it can be used as an adjunct to sodium hypochlorite. The additional application of 2% CHX + calcium hydroxide as an intracanal medicament for 15 days helps to decrease the endotoxin levels significantly. This study by no means is a final one. Further study should be undertaken with more sample size to assess the role of NdYAG laser in reduction of endotoxins in infected root canals. On the other hand to find out alternative modalities for root canal sterlization which may further improves the efficacy of endotoxins removal in a more precise and faster manner.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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How to cite this article: Tikku AP, Verma P, Bharti R, Gupta P, Singh B. Comparative evaluation of 5% sodium hypochlorite and Nd:YAG laser in reducing endotoxins level from infected root canals – An *in vivo* microbiological study. Asian J Oral Health Allied Sci 2021;11:7.