

Original Research Article

Determination of Antimicrobial Efficacy of Calcium hydroxide, Ozonated sesame oil and their combination as intra canal medicament against *Enterococcus faecalis* – A Study in Fathima Institute of Medical Sciences, Kadapa

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Abstract

Background: Among all the microbial organisms *Enterococcus faecalis* and *Candida albicans* were the most commonly seen and most resistant organisms in persistent or failing root canals. Even after chemomechanical preparations and treatment 40 to 70% of the microorganisms survive.

Aim: This in vitro study was done to evaluate the antimicrobial efficacy of intra canal medicament in root canals charged with *Enterococcus faecalis*.

Materials and methods: Forty eight extracted human single rooted teeth were taken. Biomechanical preparations and access preparations were done. Specimens were sterilized by autoclaving and later contaminated with *Enterococcus faecalis* and incubated for 24 hours. Confirmation of *Enterococcus faecalis* was done and then divided into 3 experimental and 2 control groups. Groups (n=12) A)

Ozonized Sesame Oil, B) Calcium Hydroxide, C) Calcium Hydroxide + Ozonised Sesame Oil. Control groups Group 1:- (n=6) Negative Control, Group 2:- (n=6) Positive Control. Intra canal medicament was placed in each root canal corresponding to the groups and incubated at 37°C. First sampling was done after 24 hours and second or final sampling was done after 72 hours of placement of intra canal medicament. Microbial growth was checked by counting CFU (Colony Forming Units).

Results: In the first sampling ozonised sesame oil was 100% efficient, next efficient was Calcium Hydroxide group, and combination of Ozonised sesame oil and Calcium Hydroxide also showed almost similar results. But in the second and final sampling after 72 hours Ozonised sesame oil was highly efficient when compared to other two groups. Calcium Hydroxide was moderately efficient whereas combination of both the drugs was least effective as it showed highest CFU/ml.

Conclusion: Ozonised sesame oil was most effective for longer duration when compared to other groups and can be used as an alternative intra canal medicament.

Key words

Calcium Hydroxide, Ozonised sesame oil, *Enterococcus faecalis*, Tooth with root canal preparations.

Introduction

Among all the microbial organisms *Enterococcus faecalis* and *Candida albicans* were the most commonly seen and most resistant organisms in persistent or failing root canals. Even after chemomechanical preparations and treatment 40 to 70% of the microorganisms survive. Such cases require intermittent dressing with intra canal medicament treatment [1, 2].

The most commonly used intra canal medicament was Calcium Hydroxide. But sometimes even Calcium Hydroxide could not completely eradicate these species as it needs direct contact and high pH. Its low diffusibility and solubility makes it difficult to cause an increase in pH and gets neutralized by buffering system and acids present in deeper layers of dentin and thus decreases its bioavailability. Moreover Calcium Hydroxide provides Calcium ions necessary for growth of *Enterococcus faecalis*. Hence, ineffective against resistant species [3-7].

Different forms of Ozone – Water, Gas and Oil based products have been used as intra canal medicaments and irrigants for root canal disinfection. Among these ozonated water is least cytotoxic than the gaseous form and also other irrigants like Sodium Hypochlorite,

Chlorhexidine and Hydrogen Peroxide but lacks residual effect and needs to be freshly prepared [8-11].

Ozone therapy has been proved to be more efficient on anaerobic bacteria which is predominant bacteria in the oral cavity. Ozonated oil because of its viscosity remains in the root canal for prolonged periods, thus facilitating its use as an intra canal medicament [12]. Use of ozonated oil is still not widely used in dentistry and very few studies have been done [13, 14]. Sesame oil has been selected as a medicament because of its cost effectiveness, antimicrobial properties, prolonged half life and healing effect [15]. Apart from sesame oil various plant extracts, castor oil, almond oil, wheat germ oil, sesame oil, soyabean oil, olive oil ozonides, carthame oil, peanut oil, jojoba oil, macadamia oil, coconut oil, thistle oil, linseed oil, wheat germ oil, croton oil, safflower oil, shea butter, avacoda oil, murmur butter, caprylic triglyceride, rice bran oil, argan oil, camellia oil, roship seed oil, dilo nut oil, red raspberry seed oil, pomegranate oil, evening primrose oil, rosewood oil, grape seed oil and pumpkin seed oil have all been used in various studies [16-18].

On passing electrical discharge through water (Ozen = Oder), a German chemist named

Friedrich Schonbein detected “Odorful Gas” and named it as Ozone [19-21].

Aim

The aim of this study was to determine the antimicrobial effect of calcium hydroxide, ozonated sesame oil and their combination as intra canal medicament in root canals contaminated with *Enterococcus faecalis*.

Materials and methods

Materials

- Forty Eight extracted human single rooted teeth,
- Sterile Paper Points,
- Sterile K Files, H Files,
- 3% NaOCl, 17% EDTA, 0.9% Normal Saline (w/v),
- Sterile Water,
- Nail Varnish,
- *Enterococcus faecalis*
- Autoclave, Incubator
- Brain Heart Infusion Agar Medium
- Eppendorf tubes
- Ozonated Sesame Oil (Ozone Rapid Heal, Ozorie – Mumbai)
- Calcium Hydroxide Powder (Commercially available)

Sample preparation

Forty eight freshly extracted human single rooted teeth were selected and studied in April 2013 in Fathima Institute of Medical Sciences, Kadapa. Access cavity preparations were done and working length 1mm short of apex was determined. By step back technique Biomechanical preparation was done up to size 50 K – File. During instrumentation 3 ml of 3% Sodium Hypochlorite irrigant was used. Then subjected to 17% EDTA for 3 minutes to remove the smear layer. After that final irrigation with 5 ml of 0.9% Normal Saline to wash out the residual irrigants, NaOCl and EDTA from the canal. Sealing of the root apices with sticky wax and then root surfaces were coated with nail varnish with specific colour coding for the group

identification except the cervical openings. Then all the specimens were sterilized in an autoclave at 121⁰C, 15 to 20 lb pressure for 10 minutes.

Samples were divided into 5 groups

Three Experimental Groups:

- A) Ozonised Oil,
- B) Calcium Hydroxide,
- C) Ozonised Oil + Calcium Hydroxide

Two Control Groups:

- D) Negative Control - No contamination with *Enterococcus faecalis* and No treatment with medicament,
- E) Positive Control – Contamination done with *Enterococcus faecalis* but no treatment with medicament.

Samples of corresponding groups with specific colour coding were suspended in eppendorf tubes.

Preparation of Microbes

Under aseptic precautions all microbial procedures were performed. *Enterococcus faecalis* was previously cultivated on Brain Heart Infusion Agar medium. Microbial suspension was prepared to match turbidity of 1.5 X 10⁸ CFU/ml (equivalent to 0.5 Mc. Farland Standard). 10 µl of microbial suspension was inoculated into each root canal with automated micropipette and cervical openings were sealed with temporary cement. Microplates containing specimens were incubated at 37⁰C for 24 hours. Confirmation of *Enterococcus faecalis* was done after the incubation period as follows:

- Colony appearance on Brain Heart Infusion Agar,
- Colony appearance also on Sheep Blood agar,
- Catalase test,
- Oxidase test,
- Growth in 6.5% NaCl,
- Esculin Hydrolyzation and lastly
- PYR test positive.

Tooth were grouped and intra canal medicament was placed into 3 experimental groups (n=12).

Preparation of intra canal medicament and placement

Ozonized Sesame Oil: With the help of an automated micropipette Ozonated oil is deposited in canals in 1:2 ratio of microbial suspension that is 10 µl of *Enterococcus faecalis*: 20 µl of Ozonated oil.

Calcium Hydroxide Solution: A powder form of 48 gm% of Calcium Hydroxide is available commercially. 1 ml of Calcium Hydroxide solution with sterile water is prepared. From this 20 µl is taken and inoculated into each canal with micropipette. The prepared Calcium Hydroxide is carried into canals with automated micropipette in 1:2 ratio of microbial suspension that is 10 µl of *Enterococcus faecalis*: 20 µl of Calcium Hydroxide as intra canal medicament.

Ozonated Oil and Calcium Hydroxide Combination: 10 µl *Enterococcus faecalis* suspension + 10 µl of Ozonated oil + 10 µl of Calcium Hydroxide solution of intra canal medicament.

- Negative Control – No treatment and contamination was done.
- Positive Control – No treatment but contamination was done.

Into each root canal of corresponding group intra canal medicament was placed and sealed with temporary cement.

Sampling

First Sampling

After 24 hours of placement of intra canal medicament first sampling was done. Using K – files, H – files and paper point's dentinal scrapings were collected from all root canals and transferred into eppendorf tubes containing 1 ml of peptone water which is the nutrient medium for growth of microbes. Then the tubes were agitated on cyclomixer for 1 minute. Aliquots of 0.1 ml were seeded into petridishes containing Brain Heart Infusion agar medium and incubated at 37⁰C for 24 hours. After this period microbial growth was measured by CFU/ml. After

sampling the root canals were sealed with temporary cement.

Second Sampling

After 72 hours of placement of intra canal medicament second sampling was done. Samples were collected from root canals similar to first sampling. Results were submitted to logarithmic transformation. Kruskal – Wallis and Dunn's tests were used for comparison among the groups. Friedman's test was used for comparison among the samples within each group. The significant level was set at 5% for all analyses.

Results

Microbial colony count (10⁸) has done after initial, post medication (after 24 hours) and final sample (after 72 hours) and tabulated.

Analysis of the data was done statistically with Kruskal – Wallis test and Dunn's Post – Hoc test to assess the differences in antimicrobial efficacy between groups. For comparison of samples Friedman's test was used within each group (P < 0.05). The initial sample revealed similar CFU/ml for all groups except negative control. The highest microbial count was observed in positive control. After 24 hours in post medication samples Ozonised oil showed lowest CFU/ml. Calcium Hydroxide and Ozonised oil + Calcium Hydroxide combination also showed similar results.

In final samples after 72 hours ozonised oil revealed lowest counts, Calcium Hydroxide showed moderate counts, combination of Ozonised oil + Calcium Hydroxide showed highest counts. Of all combination of Ozonised oil + Calcium Hydroxide was proven least effective as it showed highest colony count whereas Calcium Hydroxide alone is the second best and Ozonised oil alone is proven to be most efficient intracranial medicament (**Table – 1 to Table – 4**).

Table - 1: Colonies after Initial inoculation.

Groups	1	2	3	4	5	6	Mean	Std Dev
A	314	312	315	310	302	317	1870	3.26
B	318	304	320	306	314	306	1868	4.81
C	318	322	311	316	309	314	1890	2.19
D	0	0	0	0	0	0	0	0
E	320	324	318	312	315	310	1899	2.72

(A = Ozonised oil group, B = Calcium Hydroxide, C = Combination of Ozonised Oil and Calcium Hydroxide, D = Negative Control, E = Positive Control).

Table - 2: First sampling after 24 hours of placement of intra canal medicament.

Groups	1	2	3	4	5	6	Mean	Std Dev
A	0	0	0	0	0	0	0	0
B	0	1	0	1	0	0	2	0.724
C	1	0	2	0	1	0	4	1.032
D	0	0	1	1	0	0	2	0.724
E	328	330	326	318	321	316	1939	3.215

Table - 3: Second sampling after 72 hours of placement of intra canal medicament.

Groups	1	2	3	4	5	6	Mean	Std Dev
A	2	18	9	2	0	1	32	7.326
B	28	0	30	0	26	15	99	18.20
C	58	36	45	52	18	17	226	25.38
D	0	2	4	3	1	2	12	1.453
E	340	328	330	342	330	318	1988	3.828

Table - 4: After Inoculation Post Medication Final Sample.

Groups	Initial	After 24 hours	After 72 hours
A) Ozonized Oil	1870 ± 3.26	0 ± 0	32 ± 7.326
B) Calcium Hydroxide	1868 ± 4.81	2 ± 0.724	99 ± 18.20
C) Ozonated Oil + Calcium Hydroxide	1890 ± 2.19	4 ± 1.032	226 ± 25.38
D) Negative Control	0 ± 0	2 ± 0.724	12 ± 1.453
E) Positive Control	1899 ± 2.72	1939 ± 3.215	1988 ± 3.828

Discussion

In secondary or persistent infections *Enterococcus faecalis* can be found in root canals. This species has the ability to colonize and invade the dentin and seems to be resistant to Calcium Hydroxide treatment [22-27].

With Sodium Hypochlorite and Chlorhexidine solution irrigations a wide spectrum of microorganisms can be killed in endodontic infections. However, during the treatment they act for a short time and often cannot penetrate inside some parts of the root canal system. Therefore the use of intracanal dressings is

necessary to allow a longer duration of action against microorganisms in root canal and prevent the proliferation of microorganisms, acting as a mechanical barrier to reinfection [28].

For teeth with apical lesions Calcium Hydroxide as intracanal medicament is most appropriate. Healing rates will improve about 10% and approach the success rate for endodontic treatment of teeth with vital pulps. Combination of Calcium Hydroxide with CMCP has previously been shown to be more capable of inhibiting the growth of bacteria than Chlorhexidine and Calcium Hydroxide combined with sterile saline. However CMCP was found to be cytotoxic to the target periodontal ligament cells by inhibiting cell viability and proliferation. In our present study Calcium Hydroxide was found to be moderately efficient against *Enterococcus faecalis*. This is in agreement with other studies.

Ozone is a chemical compound consisting of three oxygen atoms. It is one of the most important gases in the stratosphere due to its ability to filter UV rays which is critical for the maintenance of biological balance in the biosphere. Ozone is produced naturally by the following natural methods, from electrical discharges following thunderstorms. Ozone is created when oxygen molecule receives an electrical discharge breaking into two oxygen atoms. The individual atoms combine with Oxygen molecule to form Ozone. Medical grade ozone is a mixture of pure oxygen and pure ozone in the ratio of 0.05% to 5% of Ozone with 95% to 99.5% of Oxygen. There is evidence of use of Ozone as disinfectant from 1881. Ozone was used medically to treat wounds and other infections. Ozone has extensive use in medicine and dentistry. There is evidence in literature since 1990 of use of ozone in dentistry. Ozone is used in dentistry as gaseous form, ozonated water and as ozonated oils. Artificially there are 3 different systems of generating Ozone gas a) Corona discharge system – producing high concentrations of ozone. b) Ultraviolet system –

producing low concentrations of ozone c) Cold plasma system.

Ozone water form has almost the same antimicrobial activity as 2.5% NaOCl especially in combination with ultrasonic canal treatment, with low cellular toxicity shown by Nagayoshi, et al. 2004. Ozone water can be considered to be a potential root canal disinfectant and compatible biologically [29]. Ozone can be used as an irrigant and intracanal medicament in root canals.

Ozonated oil with Zinc oxide combination demonstrated good clinical and radiographical success at 12 month follow up and so considered an alternative material in infected primary teeth. Since ozone was unstable in gaseous form, ozone oil was used in this study. The ozone present in oily vehicle could have advantages over gaseous or aqueous media. Since the oil remains in contact with the surface of root canal for prolonged period of time, exercising its functions for a longer period [30, 31].

Preparation of Ozonated oil

Ozone gas is bubbled through plant, vegetable extracts which are rich in omega – 3,6,9 unsaturated fatty acids. They contain double bonds between C atoms. They contain 3 double bonds in omega – 3 fatty acids (linolenic acid), 2 double bonds in omega 6 fatty acids (linolenic acid), 1 double bond in omega – 9 fatty acid (Oleic acid). They react with ozone – to forms ozonoids – aldehydes, ketones, peroxides that is Reactive Oxygen species (ROS), Lipid Oxidation products (LOP). Ozone is strong oxidizing agent which is responsible for antimicrobial property. 1 mole of ozone – 2 moles of aldehydes and 1 mole of Hydrogen peroxide.

Mechanism of Action

Action of ozonoids is to induce disruption of microbial cell wall and cell membrane, unsaturated fatty acids in the oil may also have antimicrobial effects, which can be due to their

incorporation in the cytoplasmic membrane, inducing lethal structural perturbations, disruption of the membrane integrity, and release of intracellular constituents. Therefore the antimicrobial activity of the ozonised oil may be result of action of aldehydes, unsaturated fatty acids and hydrogen peroxide. Indeed, the oxidant effects of hydrogen peroxide may help to explain the excellent antimicrobial effects of ozonised oil on anaerobic bacterial species commonly found in endodontic infections.

The word ozonated is expressed as the amount of peroxides existing in the ozonated derivative used. Quantitative evaluation of the therapeutic effect of topically applied ozonated sesame oil has been developed.

- Acid Value – The acid value is an index that expresses in mg, the quantity of potassium hydroxide required to neutralize the free acids present in 1 gm of the substance.
- Peroxide Value – represents the quantity of peroxide expressing in milliequivalents of active Oxygen contained in 1000 gm of the sample.
- Iodine Value – The iodine value represents the quantity of iodine (in grams) that will react with the double bonds in 100 grams of sample.
- Color – adequately ozonised oil is colourless.
- Viscosity Measurement – Viscosity evaluation is a useful technique because it is fast, giving an estimation of the double bonds present in the sample. In fact, the greater the ozonation time the higher the product viscosity because of the disappearance of the double bonds.

Grading of the Efficiency

Light < 1000 meq, Medium – 1500 to 1700 meq and Strong > 3000 meq. Ozonated sesame oil used in the present study has peroxide value 1500 to 1700 meq. (Ozorie, ozone Forum of India). Middle concentration peroxide value 1500 to 1700 meq has the most beneficial effect

in accelerating the wound closure ratio. The doses used were given by Ozone forum of India providers of ozonated sesame oil. Ozonated oils can augment the wound healing process and is being used in various fields of medicine. In one study, the efficiency of ozonated oil was proven equivalent to CMCP and 2.5% Sodium Hypochlorite [31].

Conclusion

Of the Calcium Hydroxide, Ozonated oil and their combination Ozonated oil was proven effective and can be used as an alternative intracanal medicament for *Enterococcus faecalis* infection in root canal because of its prolonged activity, antimicrobial and wound healing properties. It also saves time of the clinician as it can be used alone rather than using calcium hydroxide in combination with other medicament.

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