Original Article

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EVALUATION OF ANTIMICROBIAL EFFICACY OF OZONATED SESAME OIL, CALCIUM HYDROXIDE AND THEIR COMBINATION AS INTRACANAL MEDICAMENT AGAINST CANDIDA ALBICANS: AN INVITRO STUDY

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ABSTRACT:

Aim: This in vitro study is to evaluate the antimicrobial effect of intracanal medicament in root canals contaminated with Candida *albicans.*

Material and Methods: Twenty four extracted human single rooted teeth were selected. Access preparations and biomechanical preparations were done. Specimens were sterilized and contaminated with Candida and incubated for 48hrs. Confirmation of Candida was done and then divided into 3 experimental and 2 control groups. Groups (n=6). A) Ozonised oil, B) Calcium hydroxide, C) Ozonised oil + Calcium hydroxide. Control- 1) Negative control (n=3), 2) Positive control (n=3). Intracanal medicament was placed into each root canal corresponding to the groups. First sampling was done after 48hrs and second or final sampling was done after one week of placement of intracanal medicament. Microbial growth was checked by counting CFU(Colony forming units).

Results: In the first sampling ozonised oil was 100% efficient, next efficient was Calcium hydroxide group, and combination of Ozonised oil + Calcium hydroxide also showed similar results. In the second or final sampling after one week ozonised oil was highly efficient when compared to other groups. Calcium hydroxide was moderately efficient whereas combination of ozonised oil + calcium hydroxide was least effective as it showed highest CFU/ml. ($p<0.0$

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

Keywords: Ozonised oil, calcium hydroxide, Candida *albicans*, root canal preparation.

INTRODUCTION:

Microorganisms may survive upto 40-70% even after Chemomechanical preparation. (Silveira *et al* .2007 ; K. Halbauer *et al*.2013).[1,2] Among microbes Candida albicans & E.*faecalis* were found to be most resistent & seen most commonly in persistent or failing root canals. As these are facultative anerobes they can survive under high ph. Such cases require interappointment dressing with an intracanal medicament.

Calcium Hydroxide has been most commonly used intracanal medicament. But even calcium hydroxide $Ca(OH)_2$ could not completely eradicate these species as it is based on high p^H and needs direct contact. Moreover its low solubility & diffusibility makes it difficult to cause an increase in p^H and gets neutralised by buffering system and acids present in deeper layers of dentin and thus decreases its bioavailability.(Sathorn *et al* .2007).[3] Moreover

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$Ca(OH)_2$ provides Ca ions necessary for growth of Candida. Hence ineffective against resistent species.(Mohammadi & Dummer 2011).[4] .

Various forms of OZONE- Gaseous, water, oil based have been used as irrigant & intracanal medicament in root canal disinfection. Ozone therapy has been found to be more efficient on anerobic bacteria which is the predominant species in the oral cavity.(K. Halbauer *et al* 2007).[2] Among these ozonated water is least cytotoxic than gaseous ozone and other irrigants like NaOCl, CHX and H_2O_2, but lacks Residual effect (half life 40min ,20ºc) and needs to be freshly prepared.(Komali *et al*. 2012,Mohammadi et *al.*2013.)[5,6]

Ozonised oil because of its viscosity remains in the root canal for prolonged periods, thus facilitating its use as an intracanal medicament. (Bocci V *et al*.2005).[7] The use of ozonated oil is still not widely used in dentistry and very few studies have been done.(Roberta Vieira Farac *et al* .2013)[8]. Sesame oil has been selected because of its cost effectiveness, antimicrobial properties , prolonged half life & healing effect. (SA Pai *et al* .2014).[9] Apart from sesame oil various plant extracts, ozonides of olive oil, sesame oil, castor oil, almond oil, carthame oil, peanut oil, jojoba oil, macadamia oil, theobroma oil, soybean oil, coconut oil, linseed oil, thistle oil, wheat germ oil, croton oil, safflower oil, avocado oil, Shea Butter, Murmuru Butter, Cupuacu Butter, Caprylic/Capric Triglyceride, Shea Butter, Rice Bran Oil, Argan Oil, Camellia Oil, Rosehip Seed Oil, Dilo Nut Oil, Pomegranate Oil, Red Raspberry Seed Oil, evening primrose oil, Moluccana Oil, palmarosa oil, rosewood oil, safflower seed oil, sunflower seed oil, pumpkin seed oil, Grapeseed oil and soybean oil have also been used in other studies. (Valter Travagli *et al* .2010. Mohamed Ali *et al*.2013).[10,11]

History: Ozone was first observed by a German chemist Christian Friedrich Schonbein in 1840 when he detected an “Odorful Gas” on passing electrical discharge through water (Ozen = Odor). He is considered as father of Ozone therapy.(Shilpa Reddy A *etal.* 2013).[12]

Aim: The purpose of this study is to evaluate the antimicrobial effect of ozonated sesame oil, calcium hydroxide and their combination as intracanal medicament in root canals contaminated with Candida albicans.

MATERIALS AND METHODS:

- ✓ Materials-
- ✓ Twenty extracted human single rooted teeth.
- ✓ Sterile K-files, H-files,
- ✓ Barbed broaches, sterile Paper points.
- ✓ 3%NaOCl, 17%EDTA, 0.9%w/v NS.
- ✓ Nail varnish,
- ✓ Sterile water,
- ✓ Autoclave, Incubator.
- ✓ Candida *albicans*,
- ✓ Sabouraud’s dextrose agar medium.
- ✓ Ozonated Sesame oil(Ozone Rapid Heal,Ozorie,Mumbai)

- ✓ Calcium hydroxide powder(Deepashree products, Maharashtra,India)

Sample preparation

Twenty four freshly extracted human single rooted teeth were selected. Access cavity preparations were done and working length 1mm short of apex was determined.[Fig : 1&2]. Biomechanical preparation was done by step back technique upto size 50 kfile. During instrumentation 3ml of 3%NaOCl irrigant was used. Then subjected to 17%EDTA for 3min to remove smear layer. Finally irrigated with 5ml of 0.9%Normal Saline to wash out the residual irrigants, NaOCl & EDTA from the canal. Root apices were sealed with sticky wax and then root surfaces were coated with nail varnish with specific colour coding for group identification except the cervical openings. Then all the specimens were sterilized in an autoclave at 134ºC, 32 PSI for 5 min.

Samples were divided into 5 groups -

3.EXPERIMENTAL GROUPS : A) OZONISED OIL, B) CALCIUM HYDROXIDE,

C) OZONISED OIL + CALCIUM HYDROXIDE

2.CONTROL GROUPS: D) NEGATIVE CONTROL- No contamination and no treatment was done. E) POSITIVE CONTROL- Contamination was done but no treatment.

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

Microbial preparation

All microbial procedures were performed under aseptic conditions, in laminar flow chamber. Candida *albicans* was previously cultivated in sabouraud's dextrose agar medium. Microbial suspension was prepared to match turbidity of $1.5x10^8$ cfu/ml (equivalent to 0.5Mc.Farland standard). 10µl 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 37ºc for 48hrs.

After this period of incubation contamination confirmation of Candida was done as follows 1) direct visualization which appeared as creamy/white colored, smooth pasty colonies. 2) CFU/ml. Except negative control all the other groups showed similar counts. Negative control was zero. [Fig: 3]. 3) Germ tube test – rapid quantitative production of chlamydospore was assessed. Two different culture media (corn milk broth+5%milk) and serum milk was inoculated into it &placed in a water bath at 45ºc & results were read after 8 & 16hrs. Chlamydospores formation was observed under wet conditions when stained with LPCB (Lactophenol cotton blue stain), which was confirmatory test for Candida *albicans.*

Grouping was done & intracanal medicament was placed into 3 experimental groups (n=6).

Intracanal medicament Preparation and Placement-

A) Ozonated oil - ozonated oil is carried into canals with automated micropipette in 1:2 ratio of Microbial suspension i.e, 10µl of candida : 20µl intracanal medicament ozonated oil. [Fig:4]

B) Calcium hydroxide- $Ca(OH)_2$ solution preparation

48gm % of $Ca(OH)_2$ is present in commercially available $Ca(OH)_2$ powder. 1ml of $CaOH_2$ solution with sterile water is prepared. From this 20µl is taken & inoculated into each canal with micropipette. [Fig : 5]. The prepared $Ca(OH)_2$ solution is carried into canals with automated micropipette in 1:2 ratio of Microbial suspension i.e, 10µl of Candida: 20µl $Ca(OH)_2$ intracanal medicament.

C)Ozonated oil + Calciumhyroxide-10µl Candida Microbial suspension : 10µl of Ozonated oil + 10µl of $Ca(OH)_2$ solution of intracanal medicament. [Fig :6]

D) Negative control-no contamination and no treatment was done.[Fig:7]

E) Positive control-contamination was done but no treatment.[Fig:8]

Intracanal medicament was placed into each root canal of corresponding group and sealed with temporary cement (ZnO).[Fig: 9,10,11,12,13]

Sampling

Table 1.[Fig 3] .

First sampling was taken after 48hrs of placement of intracanal medicament .

Dentinal scrapings were collected from all root canals using k-files, H-files and paper points & transferred into eppendorf tubes containing 1ml of peptone water which is the nutrient medium for growth of microbes.[Fig: 14]. Then the tubes were subjected to agitation for 1min . Aliquots of 0.1ml were seeded into petridishes containing sabouraud's dextrose agar medium & incubated at 37ºC for 48hrs. After this period microbial growth was measured by CFU/ml. After sampling the root canals were sealed with temporary cement.

Second sampling was taken after1week of placement of intracanal medicament –

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.

RESUTS:

Microbial colony count (10^5) in initial, post medication (after 48hrs) & final sample (after 1week).

After inoculation of candida into root canals. Sample (n=6).

Groups	1	2	3	4	5	6	mean	Std dev
A	610	615	613	608	606	611	3663	3.271085
B	620	612	624	610	621	618	3705	5.43139
C	625	621	623	624	625	620	3738	2.097618
D	0	0	0	0	0	0	0	0
E	630	628	626	629	625	623	3761	2.639444

[A=Ozonised oil group, B=Calcium hydroxide, C=combination of Ozonised oil + calcium hydroxide, D=Negative control,E=positive control].

First sampling after 48hrs of placement of intracanal medicament Table 2,[Fig:15]

	1	2	3	4	5	6	mean	Std dev
A	0	0	0	0	0	0	0	0
B	0	1	0	2	0	0	3	0.83666
C	1	0	3	0	1	0	5	1.169045
D	0	0	1	2	0	0	3	0.83666
E	632	628	628	629	626	622	3765	3.331666

Second, final sampling after 1 week of placement of intracanal medicament. Table 3[Fig:16,17,18]

	1	2	3	4	5	6	mean	Std dev
A	2	21	11	1	0	2	37	8.280499
B	47	0	62	0	42	23	174	25.69047
C	122	74	91	108	33	39	467	36.2404
D	0	3	4	5	1	2	15	1.870829
E	632	630	629	633	624	623	3771	4.135215

Table 4: AFTER INOCULATION POST MEDICATION FINAL SAMPLE

GROUPS	INITIAL	AFTER 48hrs	AFTER 1WEEK
A) Ozonated oil	3663 ± 3.2	0 ± 0	37 ± 8.2
B)Calcium hydroxide	3705 ± 5.4	3 ± 0.83	174 ± 25.6
C) Ozonated oil + calcium hydroxide	3738 ± 2.0	5 ± 1.16	467 ± 36.2
D) i) Negative control	0 ± 0	3 ± 0.8	15 ± 1.87
E) ii) Positive control	3761 ± 2.63	3765 ± 3.33	3771 ± 4.13

The data was statistically analyzed with Kruskal-Wallis test & Dunn's Post-Hoc test to assess the differences in antimicrobial efficacy between groups. Friedman's test was used for comparison among the samples 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 48hrs in post medication samples Ozonised oil showed lowest CFU/ml. $Ca(OH)_2$ and Ozonised oil+Ca(OH)2 combination also showed similar results.

After one week in final samples ozonised oil revealed lowest counts, Calcium hydroxide showed moderate CFU/ml, combination of ozonised oil+calcium hydroxide showed highest CFU/ml. Ozonised oil was proven most efficient intracanal medicament. Calcium hydroxide second best, whereas combination of ozonised oil + calcium hydroxide was proven least effective as it showed highest colonies.

DISCUSSION:

Candida *albicans* a dimorphic fungi can be found in secondary or persistent infections in root canals. (Siqueira *et al*.2002). This species has the ability to colonize and invade the dentin and seems to be resistant to calcium hydroxide dressing.(Sen BH *et al*. 1997).[13]

Irrigating solutions such as NaOCl and CHX have a wide spectrum of action on the microorganisms present 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.(Siqueira *et al*.1998).[14]

$Ca(OH)_2$ intracanal medicament is most appropriate for teeth with apical lesions and healing rates will improve about 10% and approach the success rate for endodontic treatment of teeth with vital pulps. Histological periapical repair after obturation of infected root canals in dogs revealed better healing with $Ca(OH)_2$ in 2 appointments than 1 appointment.(Trope *et al.* 99).[15]

Calcium hydroxide alone is less effective against C. *albicans*(Mohammadi *et al*.2012).[16]

Hasselgren *et al*.(1988) found $Ca(OH)_2$ to improve debridement efficacy of NaOCl when root canals were pretreated with $Ca(OH)_2$. It enhances the tissue-dissolving capability of sodium hypochlorite, and this may confer an advantage to multiple-visit root canal treatment where NaOCl would be used following a period of $Ca(OH)_2$ medication.(Hasselgren *et al*.1988).[17]

Waltimo *et al.(*1999) and Mohammadi *et al*.(2012) stated that Calcium hydroxide has been found to be ineffective against C. *albicans* (Mohammadi *et al*.2012).[16] He demonstrated that *C. albicans* is highly resistant to $Ca(OH)_2$. Because *C. albicans* survive at a wide range of pH values, the alkalinity of saturated $Ca(OH)_2$ solution

may not have any effect on *C. albicans*. In addition, $Ca(OH)_2$ pastes may provide the Ca ions necessary for the growth and morphogenesis of *Candida*. These mechanisms may explain why $Ca(OH)_2$ has been found to be ineffective against *C. albicans*.(Waltimo *et al.*1999). [18]

Combination of $Ca(OH)_2$ with CMCP has previously been shown to be more capable of inhibiting the growth of bacteria than CHX and $Ca(OH)_2$ combined with sterile saline.

(Penesis *et al.*2008).[19] However CMCP was found to be cytotoxic to the target periodontal ligament cells by inhibiting cell viability and proliferation.(Chang *et al.* 1999).[20] $Ca(OH)_2$ affects the set of eugenol sealers. It accelerates the set and those using eugenol sealers should be aware of this effect. There is a concern that, the interaction between calcium hydroxide and zinc oxide eugenol sealers, at the time of root-canal filling, the retention of calcium hydroxide on the canal wall might effect the quality of the seal and influence the prognosis of treatment.(Margelos *et al.* 1997).[21], Kim & Kim *et al*.2002). [22]

In our present study Ca(OH)2 was found to be moderately efficient against Candida.albicans. This is in agreement with other studies(. Silveira *et al* (2007), Roberta Vieira Farac et al. (2013).

Ozone is chemical compound consisting of 3 oxygen atoms (triatomic oxygen). It is one of the most important gases in 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 O_3 is created when oxygen molecule receives an electrical discharge breaking into two O_2 atoms. The individual atoms combine with other O_2 molecule to form O_3. (Carlos Goes Nogales *et al* .2008).[23] Medical grade ozone is a mixture of pure oxygen and pure ozone in the ratio of 0.05% to 5% of O_3 with 95% to 99.95% of O_2 .(Ahmed *et al.*2013)[24]

There is evidence of use of Ozone as a disinfectant from 1881. During World War 1, Ozone was used medically to treat wounds and other infection. O_3 has extensive use in medicine and dentistry. There is evidence in literature since 1990 of use of O_3 in dentistry. Ozone is used in dentistry as gaseous form, ozonated water and as ozonated oils. Artificially there are 3 different systems of generating O_3 gas-1) Ultra violet system-produces low concentrations of O_3. 2)Corona discharge system-produces high concentrations of O_3. 3) Cold plasma system. (Komali G *et al* 2012).

(Nagayoshi *et al.*2004) have shown that O_3 water has almost the same antimicrobial activity as 2.5% NaOCl, especially in combination with ultrasonic canal treatment, with low cellular toxicity. Ozone water can be considered to be a potential root canal disinfectant and is less cytotoxic than NaOCl which can cause necrosis while ozone water is exceptionally biocompatible.(Nagayoshi

*et al.*2004)[25] .O_3 can be used as an irrigant and intracanal medicament in root canals.

In a study conducted by (Chandra *et al*.2013), Ozonated oil with ZnO combination demonstrated good clinical & radiographic success at 12months follow up & so can be considered an alternative obturating material in infected primary teeth. [26]

Since O_3 was unstable in gas form, O_3 oil was used in this study. (Ahmed et al.2013). The O_3 present in oily vehicle could have advantages over gaseous or aqueous media.(Oizumi *etal*.1998;Noetzel *etal*.2009).[27,28] Since the oil remains in contact with the surface of root canal for prolonged period of time, exercising its functions for a longer period. (Guinesi *et al.*2011).[29]

Preparation of Ozonated oil-

Bubbling of O_3 gas 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-3fatty acid (Leinolenic acid), 2double bonds in omega 6 fatty caid(Leinoleic acid),1 double bond in omega -9 fatty acid(Oleic acid). They react with O_3 – to form ozonoids – aldehydes, ketones, peroxides i.e Reactive Oxygen Species(ROS), Lipid Oxidation Products (LOP) (Bocci V *et al*.2005).[30]

O_3 is strong oxidising agent which is responsible for antimicrobial property. 1mole of O_3-2moles of aldehydes &1mole of Hydrogen peroxide-(ROS,LOP)

Mecchanism of action- Ozonoids can induce disruption of microbial cell wall (Lipopolysaccharide moiety) & 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(Shapiro *et al*.1996).[31] Therefore, the antimicrobial activity of the ozonized 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 antibacterial effects of ozonized oil on anaerobic bacterial species commonly found in endodontic infections (Sequiera *et al*.2000).[32]

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.

1) Peroxide value (PV) represents the quantity of peroxide expressing in milliequivalents of active O_2 contained in 1000 g of the sample.

2) Acid Value. The acid value (AV) is an index that expresses, in mg, the quantity

of potassium hydroxide required to neutralise the free acids presents in 1 g of the substance.

3) Iodine Value. The iodine value (IV) represents the quantity of iodine (in grams) that will react with the double bonds in 100 grams of sample.

4) 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.

5) Colour- adequately ozonised oil is colourless. (Valacchi G. *et al*.2010).[33]

EFFICIENCY GRADING:

Strong- >3000 meq , medium – 1500 to 1700meq , light<1000meq.

Middle concentration PV – 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 sesame oil used in the present study has PV-1500-700meq (Ozorie ,Ozone Forum of India). Ozonated oils can augment the wound healing process and is being used in various fields of medicine.(S.A Pai *et al.*2014) .

In a study conducted by (Silveria *et al*.2007), the efficiency of O_3 oil was proven equivalent to CMCP & 2.5% NaOCl. (Silveria *et al*.2007).

CONCLUSION:

Ozonised Oil was proven effective & can be used as an alternative intracanal medicament because of its prolonged activity, antimicrobial & 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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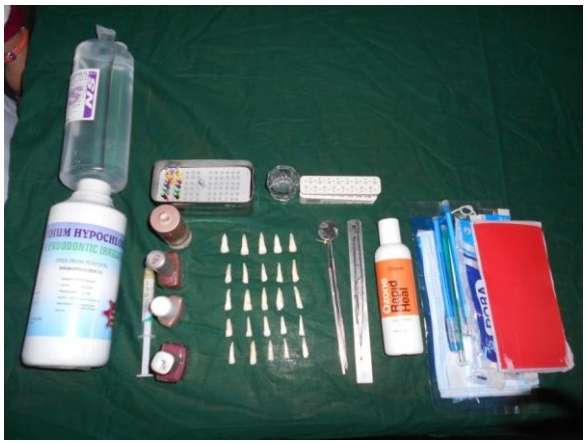
FIGURES:

[Fig 1] Materials

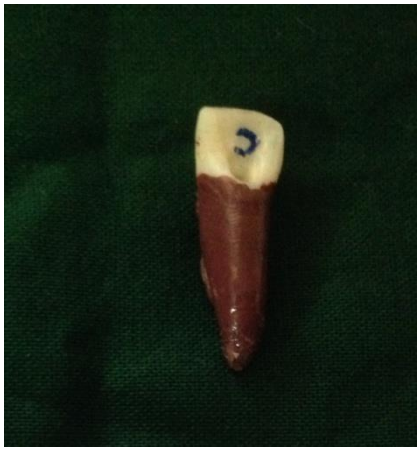


[Fig 2] Samples

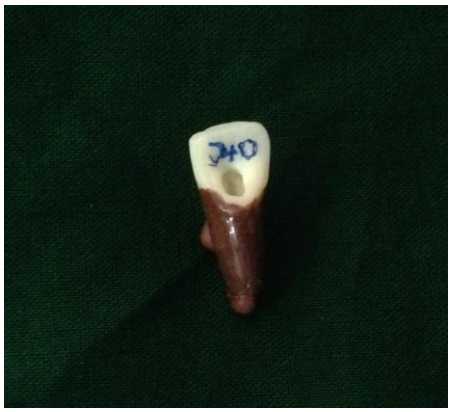
[Fig 3]Confirmation of candida albicans growth by CFU

[Fig 4] Ozonised oil group

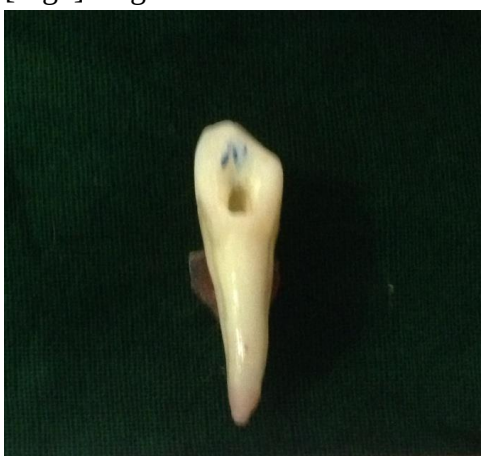
[Fig 5] Calcium hydroxide group



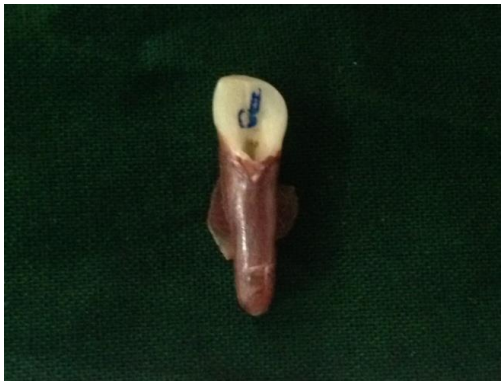
[Fig 6] Ozonised oil + Calcium hydroxide group



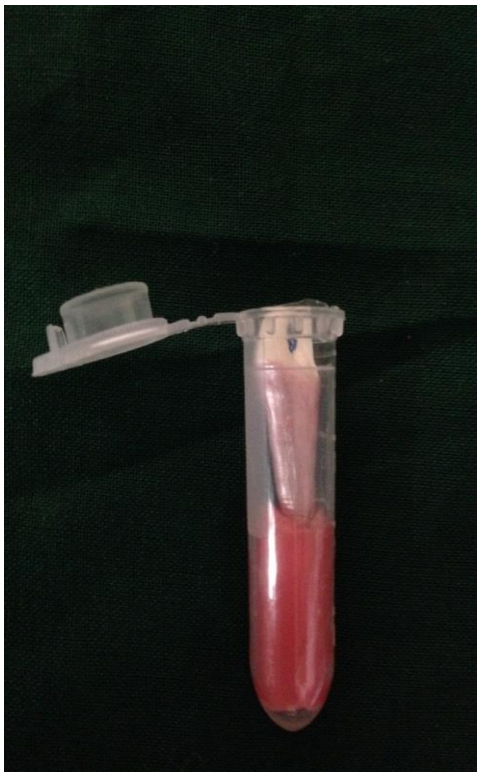
[Fig7] Negative Control



[Fig 8] Positive control group

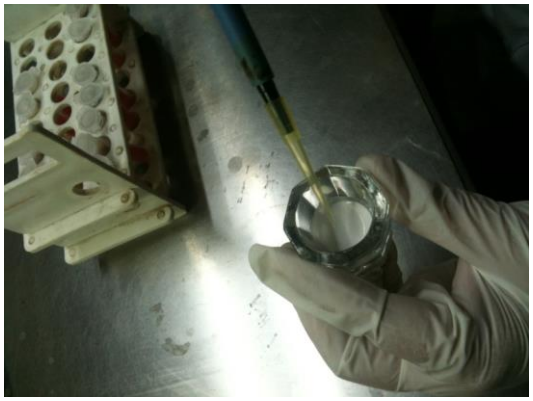


[Fig 9]Tooth suspended in eppendorf tube



[Fig 10] Intracanal medicament carried into specimen using automated micropipette

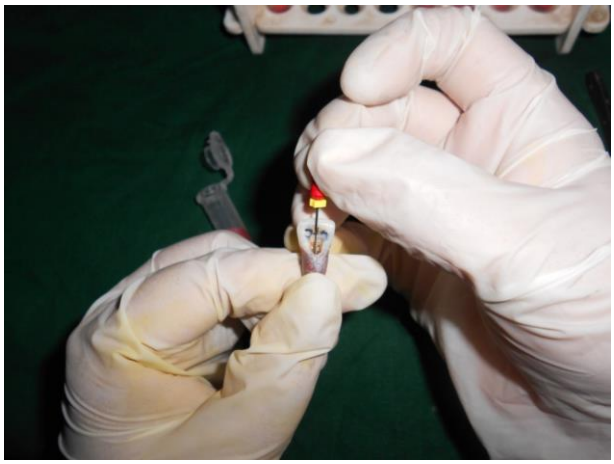
[Fig11] Preparation of calcium hydroxide solution



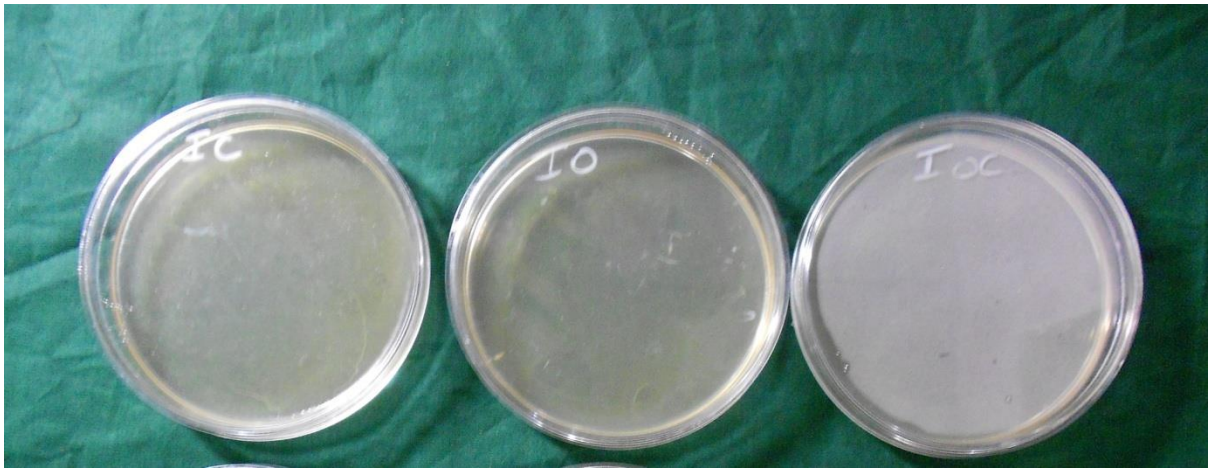
[Fig 12] Intracanal medicament Ozonised oil

[Fig 13]Intracanal medicament $Ca(OH)_2$ powder

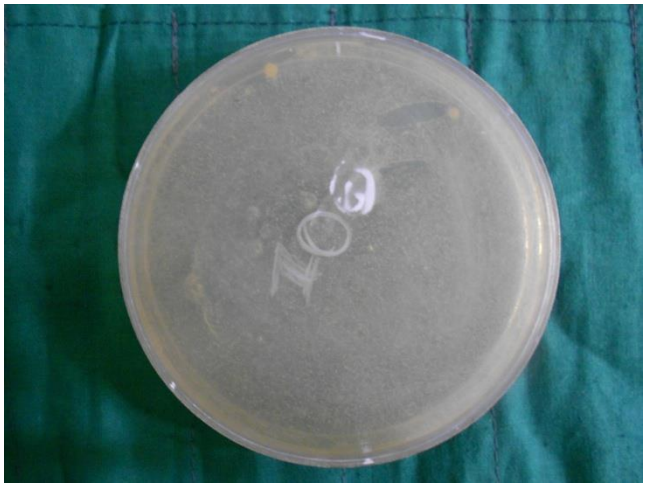
Fig14]Dentinal scrapings collected from root canals using k files



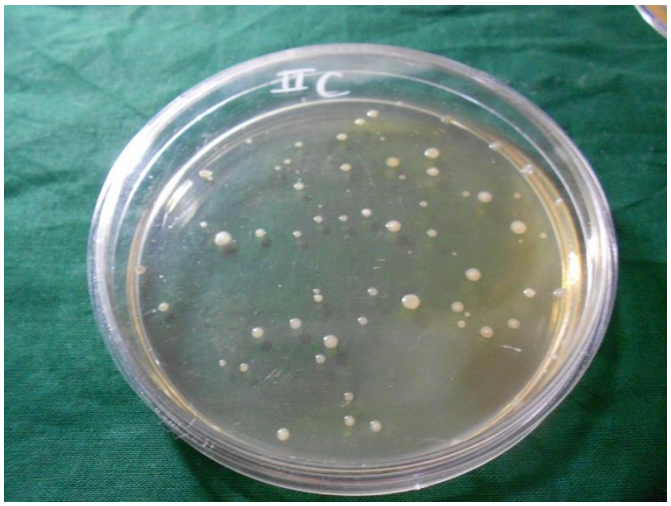
[Fig 15]First sampling result-Calcium hydroxide,Ozonised oil, Combination of Ozonised oil+ Calcium hydroxide



[Fig16]Second sampling result-Ozonised oil



[Fig17] Second sampling result –Calcium hydroxide



[Fig18] Second sampling result –Combination of Ozonised oil+Calcium hydroxide