COMPARATIVE EVALUATION OF THE PHYSICAL PROPERTIES AND BIOCOMPATIBILITY OF ZINC OXIDE-OZONATED EUGENOL AND CONVENTIONAL ZINC OXIDE EUGENOL AS AN ENDODONTIC SEALER – AN INVITRO STUDY

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ADHIPARASAKTHI DENTAL COLLEGE AND HOSPITAL

MELMARUVATHUR – 603319.

DEPARTMENT OF CONSERVATIVE DENTISTRY AND ENDODONTICS

CERTIFICATE

This is to certify that **DR.C.RAVI VARMAN,** Post Graduate student (2014-2017) in the Department of Conservative Dentistry and Endodontics, Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319, has done this dissertation titled **"Comparative evaluation of the physical properties and biocompatibility of zinc oxide-ozonated eugenol and conventional zinc oxide eugenol as an endodontic sealer" – An invitro study** under our direct guidance and supervision in partial fulfilment of the regulations laid down by the Tamilnadu Dr.M.G.R Medical University, Chennai – 600032 for MDS., (Branch-IV) **CONSERVATIVE DENTISTRY AND ENDODONTICS** degree examination.

Co-Guide

Dr.A.Jayasenthil MDS.,

Reader

Guide

Dr.S.Thillainayagam MDS.,

Professor & Head

Dr.S. Thillainayagam MDS.,

Principal

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Dr.C.Ravivarman

Post graduate student

DECLARATION

TITLE OF THE DISSERTATION	**"Comparative evaluation of the physical properties and biocompatibility of zinc oxide-ozonated eugenol and conventional zinc oxide eugenol as an endodontic sealer" – An invitro study**
PLACE OF THE STUDY	Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319
DURATION OF THE COURSE	3 years
NAME OF THE GUIDE	Dr.S.Thillainayagam, MDS.,
NAME OF CO-GUIDE	Dr.A.Jayasenthil, MDS.,

I hereby declare that no part of the dissertation will be utilized for gaining financial assistance or any promotion without obtaining prior permission of the Principal, Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319. In addition, I declare that no part of this work will be published either in print or in electronic media without the guides who has been actively involved in dissertation. The author has the right to reserve for publish work solely with the permission of the Principal, Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319

Co-Guide **Guide & Head of department** **Signature of candidate**

ABSTRACT

BACKGROUND: The challenge to the prognosis of endodontic therapy lies on the quality of root canal filling. The zinc oxide eugenol (ZOE) based sealers were introduced in Endodontics by **Grossman** in 1936 and they have been used in dentistry over a long period of time. It has been reported that eugenol released from ZOE is an irritant to the periapical tissues and has cytotoxic properties. To overcome these disadvantages, there is a continued research for chemical compounds with better physical properties and more biocompatibility. Ozone therapy has a long history of research and clinical application with humans because of its interesting biological properties.

AIM: The aim of the study is to evaluate and compare the physical properties and biocompatibility of zinc oxide- ozonated eugenol and conventional zinc oxide eugenol.

MATERIALS AND METHODS: Following physical properties of conventional zinc oxide eugenol and zinc oxide-ozonated eugenol were evaluated and compared. [**Group I**: Zinc oxide – eugenol; **Group II**: Zinc oxide - ozonated eugenol]. Hardness of the mixed samples was measured using Shore hardness Durometer type A.Solubility was tested as a percentage of the mass of specimen removed from the distilled water compared with the original mass of the specimens using Mettler analytical balance. pH of the fresh samples and the set samples were measured using calibrated pH meter after predetermined time intervals. Biocompatibility of the tested samples was evaluated using the MTT tetrazolium reduction assay.

RESULTS: The results of the present study have shown that the hardness and solubility of the tested samples were almost similar in both the groups. pH and biocompatibility of the samples in Group II where eugenol is ozonated was higher when compared to the samples in Group I. The proportional values obtained were statistically analysed by unpaired t-test.

CONCLUSION: Within the limitations of the study, it could be concluded that ozone with interesting biological properties seems to be a better treatment modality in improving the properties of eugenol. However further clinical trials and randomized control studies are required in this field to use ozonated eugenol in combination with zinc oxide powder to use it as an endodontic sealer invivo.

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LIST OF ABBREVIATIONS

ADA	:	American dental association
Al	:	aluminium
ANSI	:	American national standards institute
ATP	:	adenosine triphosphate
COX	:	cyclooxygenase
DNA	:	deoxyribose nucleic acid
DUWLs	:	dental unit water lines
IC	:	inhibitory concentration
IL-β	:	Interleukin-1β
IRM	:	intermediate restorative material
LDH	:	lactic dehydrogenase
LPS	:	Lipopolysaccharide
MTT	:	(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)
NaOCl	:	sodium hypochlorite
NF	:	nuclear factor
O_3	:	ozone
OSHA	:	occupational safety and health administration
ZOE	:	zinc oxide eugenol
PDGF	:	platelet derived growth factor
PDT	:	photodynamic therapy
PRCL	:	primary root carious lesions
pH	:	potential of hydrogen
ppm	:	parts per million
RNA	:	ribose nucleic acid
TNF	:	tumour necrosis factor
TGF-β	:	transforming growth factor
VEGF	:	vascular endothelial growth factor

INTRODUCTION

Success in Endodontic therapy is based on diagnosis, treatment planning, knowledge of tooth anatomy and the traditional concepts of debridement and obturation. **Schilder** describes the final objective of endodontic procedure as “the complete obturation of the root canal space”. It is the sealing of complex root canal system from the periodontal bone that ensures the health of attachment apparatus against the breakdown of endodontic origin[1].

Endodontic sealers are used in the obturation of root canal system to achieve a fluid tight seal throughout the canal including the canal irregularities. An ideal root canal sealer should provide excellent seal when set, should have dimensional stability, biocompatibility and help to resolve periapical lesions[2].

The Zinc Oxide Eugenol (ZOE) based sealers were introduced in Endodontics by **Grossman** in 1936. ZOE sealers have a history of successful use over extended period of time and has both anaesthetic and antimicrobial properties[3]. However the eugenol released from ZOE is reported to be an irritant to periapical tissues and said to have cytotoxic properties[4]. Every attempt made to overcome these disadvantages and to replace ZOE completely has failed, so there is continued research for chemical compounds with better physical properties and biocompatibility.

In search of other therapeutic modalities, ozone therapy has a long history of research and clinical applications with humans because of its interesting biological properties. The word ozone was first introduced by **Schonbein** in 1840 when he subjected oxygen to electric discharge and noted the odour of electric matter[5]. Ozone is said to have antimicrobial, immune-modulatory, immune-stimulating, anti-inflammatory, analgesic and haemostatic property. Ozonation is an energetic process that results in an oxygen molecule being split into singlet oxygen, the singlet oxygen then combines with diatomic oxygen to form ozone. Three systems of generating ozone are: 1.Ultraviolet system 2.Cold plasma system 3.Corona discharge system. The modes of applications of ozone can be in gaseous form, aqueous solution or in the form of ozonated oils.

Antimicrobial action: Being a superoxide, ozone is 5 times more effective than chlorine. It affects only the unhealthy cells such as cancer cells, organisms like bacteria, viruses, fungi, parasites which have lost their protective mechanism and those devoid of anti-oxidants and free radical scavengers.

Anti-inflammatory and Analgesic action: Ozone helps in the synthesis of biologically active substance such as interleukins, leukotriene and prostaglandins thereby reducing the inflammation and pain.

Anti-cariogenic action: The strongest naturally occurring acid produced by acidogenic bacteria is pyruvic acid. Ozone has the ability

to decarboxylate pyruvic acid to acetic acid and said to enhance the remineralisation of teeth.

Antihypoxic action: ozone brings about the rise of partial pressure of oxygen and acts as super oxygenator by bringing oxygen to tissues, assisting in its natural healing process[6].

In dentistry, ozonated oil applied on herpes labialis infection and osteomyelitis has demonstrated faster healing rate. It has been also used in the treatment of avascular osteonecrosis of the jaw. Ozone has strong bactericidal activity against plaque biofilm and can be used to disinfect denture bases from Candida albicans. Zinc oxide-ozonated sesame oil when used as a root filling material has demonstrated good success rate(93.3%) when compared to ZOE(63.3%)[7]. In restorative dentistry, gaseous ozone has been used in the treatment of incipient lesions since it causes reduction in the number of microorganisms[8]. In Endodontics, ozonated water had nearly the same antimicrobial activity as 2.5% NaOCl with fewer side effects when used as an irrigant[9].

Ozonator D-400 uses a highly efficient corona discharge system rated at 400mg/hr. of constant ozone output. It can be used to ozonate water and oil. Due to the highly unstable nature of ozone in aqueous form, we opted use ozone in the form of oils. Eugenol is a member of phenyl propanoids, a yellow oily liquid extracted especially from clove oil (80-85%), which can be ozonated to overcome its disadvantages like irritating action on periapical tissues and cytotoxic properties.

Hence, the purpose of the study is to evaluate and compare the physical properties and biocompatibility of ozonated ZOE and conventional ZOE for being used as an endodontic sealer.

AIM AND OBJECTIVES

Aim of the study:

To evaluate and compare the physical properties and biocompatibility of zinc oxide-ozonated eugenol and that of conventional zinc oxide eugenol.

Objectives of the study:

1. To evaluate the physical properties such as hardness, solubility and pH of zinc oxide- ozonated eugenol and conventional zinc oxide eugenol.
2. To evaluate the biocompatibility of zinc oxide-ozonated eugenol and conventional zinc oxide eugenol.
3. To compare and correlate the physical properties and biocompatibility of zinc oxide- ozonated eugenol and conventional zinc oxide eugenol.

REVIEW OF LITERATURE

Root canal therapy depends on the integrally related treatment phases: microbial control, cleaning and shaping, and effective sealing of the root canal system. The success of treatment depends on the execution of the final phase i.e. sealing of the root canal. Endodontic filling materials may be considered like true implants as they touch and are based in vital tissues of the body. The main components of a root filling are: a solid core material and a sealer. ZOE sealers have a history of successful use over extended period of time. It has both anaesthetic and antimicrobial properties[10].

Torabinejad M (1993)[11] stated that sealers help to prevent leakage, reduce the possibility of residual bacteria from the canal to invade the periapical tissues and to resolve periapical lesions.

McMichen FR et al (2003)[12] stated that the handling properties of root canal sealers and its clinical behaviour may be characterised by lab tests on their physical properties. These include flow, solubility, working time and setting time. He also stated that these materials should possess adequate working time for clinical handling and treatment procedures.

ISO standards: ISO 6876: 2001[13]

- Flow of endodontic sealer should have a diameter not less than 20mm.
- Working time of the sealer to be less than 30 min

- Setting time of the sealer should be greater than 30 min and upto72hrs.
- Film thickness should not be more than 50µm
- Dimensional change should not to exceed 1.0 % in shrinkage or 0.1% in expansion.
- Solubility of the sealer shall not exceed 3% of mass fraction.
- Radio-opacity should be equivalent to or not less than 3mm of aluminium.

Grossman stated that Zinc oxide eugenol sealer have a history of successful use over an extended period of time. An ideal root canal sealer should possess following properties [14].

1. It should be tacky when mixed, to be able to provide adhesion/sealing between the canal walls and the core obturating material.
2. Flowability in its non-set state to promote luting.
3. It should be non-irritating to the periapical tissues.
4. It should possess a hermetic sealing ability.
5. It should be radiopaque.
6. It should possess minimal setting shrinkage.
7. Should be non-staining on the dentin / tooth structure.
8. It should be ideally bactericidal or at least control the bacterial growth.
9. Should have long working time.
10. Insoluble in tissue fluids.
11. It should be easy to remove if necessary.

12.It should be neither mutagenic nor carcinogenic.

13.It should not provoke an immune reaction.

Grossman's original formula for zinc oxide eugenol based sealers contained ZnO, stabylite resin, bismuth sub carbonate, barium sulphate and sodium borate with eugenol as the liquid component.

Roth's sol sealer (Roth's pharmacy, Chicago, inc.) is essentially the same as Grossman's original formulation with the substitution of bismuth sub-nitrate for bismuth sub-carbonate.

Ricket's formula marketed as Kerr's pulp canal sealer (Sybron endo/ Kerr, Orange, CA) has long been accepted material. But its use has been restricted for staining of tooth structure from the silver used for radio-opacity.

Tubliseal(Sybronendo/ Kerr) is a two paste system contained in two separate tubes developed as non-staining alternative with barium sulphate for radiopacity. It has the disadvantage of rapid setting time.

Wach's cement(Roth international Inc. Chicago) consists of powder of ZnO, bismuth sub-iodide, bismuth sub-nitrate, magnesium oxide and calcium phosphate. It has the advantage of smooth consistency and the Canada balsam make the sealer tachy[14].

Hauman CH et al (2003)[15] reported that there are many formulations and brands of sealer that have zinc oxide as primary ingredient, differing only by other components added to the sealer. Zinc oxide

sealers allow for the addition of other chemicals, such as paraformaldehyde, rosin, Canada balsam all of which may enhance their properties.Endodontic materials can be broadly categorized as those used to maintain pulp vitality and those used in pulp canal therapy for disinfection of the pulp space (irrigants and intra-canal medicaments) and root canal filling (solid materials and sealers). Biocompatibility of these endodontic materials is characterized by many parameters such as genotoxicity, mutagenicity, carcinogenicity, cytotoxicity, histocompatibility or microbial effects. Hence it is impossible to biologically characterize the materials by a single test method alone and their properties need to be investigated by a series of various in vitro and in vivo tests ina structured approach.

Candeiro et al (2012)[16] reported that nowadays various kinds of endodontic sealers are available such as sealers based on glass ionomer, calcium hydroxide, silicone, resin based and bioceramic materials.

Lacey S (2005)[17] stated that as yet no root canal sealers has all the ideal properties and most leak with time, either through poor initial adaptation to the canal walls or due to solubility and disintegration of the sealer. They also stated that successful obturation is achieved by the adhesion and stability of the sealer to the canal walls which, amongst other factors, is affected by its rheological properties. They reported that narrower tubes produced increased velocity, which was significant for all sealers, and reduced volumetric flow, which was

significant for all sealers except Grossman's sealer. Reduction in powder: liquid ratio of Grossman's significantly increased flow in narrow tubes and at higher strain rate.

Eugenol (4- acyl-2-methoxy phenol) is an extract of clove oil widely used in dentistry as a therapeutic agent, most commonly as a component of zinc oxide eugenol cement applied as a base or temporary dressing to dentine, or as a root canal sealer[10].

Lindquist et al (1981)[18] reported that eugenol that leaches out of zinc oxide eugenol based root canal sealers, may participate in the development of periapical inflammation or the continuation of a pre-existing periapical lesion.They conducted a study to check the amount of eugenol released from four different dental materials immersed in phosphate buffer measured by gas liquid chromatography. Maximal release of eugenol from zinc oxide-eugenol cement (ZOE) and IRM was attained within 5hrs and corresponded to 5% and 4%, respectively, of the total amount of eugenol in each material. Eugenol applied to growing cultures of human diploid fibroblasts reduced the number of cells recovered to a value which was 4% of that found for control cultures which grew to form monolayers. Confluent monolayers of H-Uridine labeled cellcultures which were incubated for 1hr in the presence of 4 mm eugenol lost approximately 100% of this cytoplasmic label, indicating total cell death.

Branstetter J (1982)[19] stated that endodontic sealers are used in the obturation of root canal system to achieve a fluid tight or hermetic seal

throughout the canal including the apical foramen and canal irregularities.

Bedoukian (1986)[20] reported that eugenol was first isolated in 1929 and commercial production commenced in the United States in the 1940s. Eugenol can be produced synthetically, the most practical method being the allylation of guaiacol with allyl chloride. However, eugenol is predominantly prepared from natural oil sources by mixing the essential oil with an excess of aqueous sodium (3%) or potassium hydroxide solution and shaking, leading to the formation of a phenolic alkali salt. The insoluble non-phenolic portion is then extracted with a solvent or via steam distillation. The un- dissolved portion is removed, the alkali solution is acidified at low temperatures and the liberated eugenol purified by fractional distillation.

Thompson et al (1990)[21] stated that the eugenol induced cytotoxicity is influenced by the formation of glutathione conjugates during oxidation of eugenol by microsomal fraction of rat liver and lung.

Jeng et al (1994)[22] investigated the effect of eugenol on mouse fibroblast at with a concentration higher than 3 mmol/L, and reported that eugenol was cytotoxic to oral mucosal fibroblasts in a concentration and time-dependent manner. They reported that cell death was associated with intracellular depletion of glutathione. Most of the glutathione was depleted prior to the onset of cell death. At concentrations of 3 mmol/L and 4 mmol/L, eugenol depleted about 45% and 77% of the cells after one-hour incubation. In addition, eugenol

decreased cellular ATP (Adenosine tri-phosphate) level in a concentration and time dependent manner and they concluded that eugenol is cytotoxic to oral mucosal fibroblasts.

Tai et al (2002)[23] stated that zinc oxide eugenol based sealers irritated periapical tissues invivo and exhibited cytotoxic effects exvivo. In this study, tetrazolium bromide reduction assay, DNA precipitation assay, and DNA fragmentation analysis were performed to investigate the cytotoxic and genotoxic effects of four different root canal sealers on cultured V79 cells. These findings suggest that root canal sealers containing formaldehyde and bisphenol-A diglyether proved as not only cytotoxic but also genotoxic. They also stated that mammalian test system might be used routinely for evaluation of the genotoxicity of dental materials in future investigations.

Ito et al (2006)[24] investigated the anti-oxidant activity of eugenol and its isomer isoeugenol using iron-mediated lipid peroxidation and auto-oxidation of Fe 2+. The inhibitory effect of eugenol on lipid peroxidation was less potent with an IC 50 value of ≈80 μm, which was eight-fold the value of isoeugenol. The mechanism of action of the two compounds was investigated and it was suggested that the anti-oxidant capacity of eugenol could be explained by the formation of complexes with reduced metals. The potent inhibitory effect of isoeugenol on lipid peroxidation may be related to the decreased formation of the per-ferryl ion or iron-oxygen chelate complex as the initiating factor of lipid peroxidation.

van Zyl et al (2006)[25] conducted a study in which twenty essential oil constituents were evaluated for their antimalarial activity against the chloroquine-resistant strain *P.falciparum* (FCR-3). Eugenol exhibited some activity with an IC 50 value of 753 µm which was lower than for other constituents such as nerolidol, linalylacetae, α-pinene and pulegone (IC 50 values ranging from 0.9 to 1.4 µm)

Singh et al(2007)[26] investigated the antibacterial effect of eugenol on the growth of Gram-positive (*B.cereus; B.subtilis; S.aureus*) and Gram-negative (*E.coli; S.typhi; P.aeruginosa*) bacteria using the agar well diffusion method. At 1,000 ppm, eugenol inhibited the growth of the bacteria and complete inhibition was obtained against *P.aeruginosa* at a high concentration of 2,000 ppm. This inhibition was high in comparison to ampicillin (1 mg/mL) when used as a positive control.

Tragoolpua et al (2007)[27] tested the in vitro antiviral activity of eugenol against the herpes simplex-1: HSV-1 and HSV-2 viruses. The replication of these viruses was inhibited with IC 50 values of 25.6 µg/mL and 16.2 µg/mL against HSV-1 and HSV-2, respectively. Additional investigations revealed synergistic interactions with a combination of eugenol and acyclovir, a known antiviral drug. Studies have shown that application of eugenol delayed the development of herpes virus-induced keratitis in a mouse model. Eugenol was evaluated for its anti-HSV properties on standard HSV-1(F), standard HSV-2(G) and ten HSV isolates using the plaque reduction assay. Only HSV-1 isolates 1 and 30 were inhibited by eugenol and the inhibition

against these isolates was greater than for the extract obtained from the flower buds of *E. caryophyllata.*

Barceloux et al (2008)[28] reported that Clove has been used in ancient China as a spice and fragrance for more than 2,000 years. Medicinally, the well-known traditional remedy of applying clove oil to treat a toothache was documented for the first time in 1640 in 'Practice of Physic' in France. In Chinese traditional medicine clove oil has been used as carminative, antispasmodic, antibacterial and ant parasitic agent, while the buds were used to treat dyspepsia, acute/chronic gastritis and diarrhoea.

Daniel et al (2009)[29] investigated about Eugenia caryophyllata, which contains eugenol and acetyleugenol as major constituents for potential anti-inflammatory action on COX-2 (cyclooxygenase) and LOX enzymes. The oil exhibited strong inhibitory activity against COX-2 (58.15%) and 15-LOX (86.15%) enzymes at 10µg/mL and 25µg/mL, respectively. The anti-inflammatory activity of eugenol was evaluated by inflammatory exudate volume and leukocyte migration in carrageenan-induced pleurisy and carrageenan-induced paw oedema tests in rats. Eugenol (200 and 400 mg/kg) was found to reduce the volume of pleural exudates without changing the total blood leukocyte count indicating the anti-inflammatory potential of eugenol.

Okamoto et al (2009)[30] evaluated the anaesthetic property of eugenol in juvenile Leporinusmacrocephalus, a fish species also known as "piavuçu". Eugenol had strong anaesthetic effect on *L. macrocephalus*

and a dose of 37.5 mg/L of eugenol was recommended for the fast and safe anaesthesia of piavuçu juveniles (n = 72). In a similar investigation, the efficacy of benzocaine and eugenol as anaesthetics in Trachinotusmarginatus juvenile fish was studied. Benzocaine and eugenol, at 50 ppm, induced fast anaesthesia and recovery (3 min and 5 min, respectively). In European sea bass (Dicentrarchuslabrax) (n = 240), eugenol induced deep anaesthesia without substantially affecting their blood profile as measured by serum cortisol, glucose and haematocrit values. Induction was rapid but recovery was very slow even at low concentrations.

Hussain et al (2011)[31] tested eugenol alone and in combination with a chemotherapeutic drug (gemcitabine) to evaluate its inhibitory effect against cancer cells. Eugenol showed dose-dependent selective cytotoxicity towards HeLa cells in comparison to normal cells. Furthermore, eugenol and gemcitabine in combination induced growth inhibition and apoptosis (programmed cell death) at lower concentrations in comparison to the individual compounds indicating synergistic interactions. Eugenol (150 μm) and gemcitabine (15 μm) resulted in a decrease in cell viability from 84% (eugenol alone) and 51% (gemcitabine alone) to 47% in combination. Treatment of colon cancer cells with eugenol resulted in the reduction of intracellular non-protein thiols and an increase in the earlier lipid layer break. In addition, dissipation of mitochondrial membrane potential and generation of reactive oxygen species accompanied the eugenol-induced apoptosis.

Marcos-Arias et al (2011)[32] evaluated the effect of eugenol against 38 Candida species isolated from denture-wearers and 10 collection Candida strains using the CLSI M27-A3 broth micro dilution method. It was found that eugenol exhibited antifungal activity with the MIC value ranging from 0.06 to 0.25% (v/v), while the minimum concentration of drug that inhibited 50% of the isolates tested (defined as MIC 50) ranged from 0.06 to 0.12% (v/v) . MIC and skin lesion scoring tests were performed in vivo to evaluate the antifungal effect of eugenol in a guinea pig model infected by Microsporum gypseum. Eugenol exhibited promising activity with the MIC value ranging from 0.01 to 0.03%. The skin lesion scoring test showed that eugenol was clinically effective at improving the lesion during the first week of application. However, eugenol did not improve the skin lesions infected by *M. gypseum* as determined in the hair culture test.

The word **ozone (O_3)** is derived from the Greek word ozein (odorant). It is one of the most powerful antimicrobial agents available for use in medicine and dentistry. In 1920, **Dr.Edwin Parr**, a Swiss dentist started the use of O_3 as a part of his disinfection system[33].

Mudd JB et al (1969)[34] conducted a study on the reaction of ozone with amino acids and proteins and stated that ozone acts on bacterial cell membranes by oxidation of their lipid and lipoprotein component.

Roy D et al (1981)[35] conducted a study on the mechanism of enteroviral inactivation with ozone by comparing the sedimentation

profiles of nucleic acids of ozone-exposed viruses with those of control viruses, they observed that viral nucleic acid was damaged as a result of ozonation. It would appear that the major cause of viral inactivation, as determined with poliovirus 1, by ozone using a residual concentration less than 0.3 mg/liter and a contact time up to 2 min is damage of the RNA. They concluded that all viruses are susceptible to ozone; yet differ widely in their susceptibility and also stated that the analysis of viral components showed damage to the polypeptide and envelope proteins impairing viral attachment capability.

Alvarez et al (1982)[36] studied the germicidal properties of the ozonized sunflower oil (OLEOZON), they decided to make an investigation in 80 children between 1 and 14 year old, with the main objective of evaluating the effectiveness of the OLEOZON in primary pyoderma, comparing this with Gentamycin cream. The investigation was carried out in areas of health of the general teaching hospital "Comandante Pinares ", San Cristobal, Pinar del Rio, lasting 6 months. Patients were randomly assigned to both group of treatment; experimental (using OLEOZON) and control (using Gentamycin) .To all patients bacteriological studies were done, at the beginning and after 15 days of treatment. The results demonstrated that the most frequent isolated germ was the Streptococcus haemolytic of group A. Healing effectiveness after 15 days of treatment, was 68 % for the OLEOZON and 28 % in those patients where Gentamycin was used. No side effects were observed.

Morgolis HL et al (1985)[37] reported that ozone had a severely disruptive effect on cariogenic bacteria, resulting in elimination of acidogenic bacteria. The strongest naturally occurring acid, produced by acidogenic bacteria, pyruvic acid is decarboxylated to acetic acid, which in turn encourages remineralisation.

Arsalane K et al (1995)[38] conducted a study on ozone stimulation of inflammatory mediators and reported that ozone behaves as a weak cytokine such as tumour necrosis factor (TNF-α), interleukin (IL-2, IL-6) and transforming growth factor-beta (TGF-β). In the present study, the in vitro effect of low concentrations of O_3 (0.1 to 1 ppm) was evaluated on cell viability and cytokine secretion by alveolar macrophages (AM) from guinea pigs and healthy subjects. Cell injury was estimated immediately after O_3 exposure by evaluation of ATP cell content as measured by bioluminescence and lactic dehydrogenase (LDH) release in the culture medium.

Mudd JB et al (1997)[39] investigated the effect of ozone on blood cells and concluded that ozone reduces or eliminates clumping of red blood cells and its flexibility is restored along with oxygen carrying ability. Ozone was applied to sealed red cell ghost membranes at the rate of 95 mmol/min for periods up to 20 min. Acetylcholine esterase, on the outer face of the membrane, was inhibited up to 20%. Glyceraldehyde-3-phosphate dehydrogenase, on the inner surface of the membrane, was inhibited up to 87%. These differences reflected the inherent susceptibilities of the two enzymes and the presence or absence of the

membrane barrier. They also stated that there was no decline in the amount of cholesterol in the lipids derived from ozone-treated red cell membranes.

Liu Z et al (1998)[40] stated that the application of ozonized oils of vegetal source constitutes an appropriate method for the local therapy of several diseases. The interaction of ozone with these oils produces a mixture of chemical compounds namely ozonoides and peroxides with better germicidal power. The results of topical application of ozonized oil (OLEOZON) in dermatological diseases of viral, fungi and bacterial origin, during 9 years of study, were presented. Among the diseases of bacterial origin (pyoderma), 600 patients were treated, obtaining healing criterion in 87%. In case ofmycotic diseases (epidermophythosis and onychomycosis), 1000 patients received the treatment, with 91% of healing. In viral diseases (herpes simplex), 300 patients were treated and in 74% the relapses disappeared. No side effects were observed in any of the cases after treatment.

Oizumi M et al (1998)[41] reported that oral microorganisms and no viable C.albicans were detected after exposure to flowing ozonated water (2 or 4 mg/L) for 1 min, suggesting the application of ozonated water might be useful in reducing the number of C.albicans on denture bases.

Velano HE et al (2001)[42] conducted invitro studies and reported that ozone was effective over most of the bacteria found in cases of pulp

necrosis. Ozone is effective when it is used in sufficient concentration, for an adequate time and delivered appropriately.

Ebensberger U et al (2002)[43]experimented the use of ozonated water in decontamination of avulsed tooth before replantation and concluded that 2 minute irrigation of avulsed teeth with non-isotonic ozonated water not only provides mechanical cleansing but also decontaminated the root surface with no negative effect on periodontal cells.

Reth et al (2002)[44] reported that ozone reacts with unsaturated fatty acids of the lipid layer in cellular membranes, forming hydrogen peroxide, one of the most significant cytokine inducers. It also plays an important role as a secondary messenger in the initiation and amplification of signaling at the antigen receptor. The antigen receptors themselves are H_2O_2-generating enzymes and that the oxidative burst in macrophages seems to play a role not only in pathogen killing but also in the activation of these cells as well as neighboring cells.

Holmes J (2003)[45] studied the clinical reversal of root caries by ozone using double blinded randomized control study and reported that ozone is capable of clinically reversing the leathery root carious lesion. He assessed the effect of an ozone delivery system, combined with the daily use of a re-mineralizing patient kit, on the clinical severity of non-cavitated leathery primary root carious lesions **(PRCL's)**, in an older population group. He also reported that leathery non-cavitated primary root caries can be arrested non-operatively with ozone and re-

mineralizing products and this treatment regime is an effective alternative to conventional “drilling and filling”.

Walker JT et al (2003)[46] reported that in model dental unit water lines, ozone achieved a 57% reduction in biofilm and a 65% reduction in viable bacteria in spite of being used in a very low dose and with a short time of application. They also concluded that dental manufacturers should be invited to design dental units that incorporate automated devices to disinfect DUWLs.

Nagayoshi M et al (2004)[47] reported that ozonated water had strong bactericidal activity against bacteria in plaque biofilm. It was found that ozonated water (0.5–4mg/L) was highly effective in killing of both gram-positive and gram-negative microorganisms. Gram-negative bacteria, such as *P.gingivalis P.endodontalis* were more sensitive to ozonated water than gram-positive oral streptococci and C.albicans in pure culture.

Baysan A et al (2004)[48] conducted a study on the efficacy of ozone on primary root caries lesion (PRCL) and found that ozone application for either for 10 or 20 s dramatically reduced most of the microorganisms in PRCLs without any side effects recorded at recall intervals between 3 and 5.5 months.

Young SB et al (2004)[49] examined the mechanism of killing *B.subtilus* spores by ozone and reported that it seems to render the spores defective in germination perhaps because of damage to the spore’s inner membrane.

Huth KC et al (2005)[50] conducted in vitro studies with short term follow-up and assessed the effect of ozone on pit and fissure caries and primary root caries with results showing significant reductions in the number of microorganisms in the carious lesions. In this study gaseous ozone (HealOzone) was applied once for 40 seconds to the randomly assigned test molar of each pair without the use of re-mineralizing solutions. Lesion progression or reversal was monitored by the laser fluorescence system DIAGNOdent for up to 3 months and the deterioration or improvement compared between the ozone-treated lesions and the untreated control lesions

Celiberti P et al (2006)[51] stated that ozone had no influence on the physical properties of the enamel to enhance or hinder the sealing ability. Thus, ozone can be applied over intact and prepared enamel during the restoration process. They also stated that the application of gasiform ozone does not affect the modulus of elasticity and the Vicker's hardness of dentin.

Polydorou OPK et al (2006)[52] experimented the antimicrobial efficacy of ozone and reported that long exposure to ozone gas has a strong anti-bactericidal effect on microorganisms within the dentinal tubules of deep cavities, which could result in increasing the clinical success of the restoration. In this study thirty-five human molars were divided into 5 groups. Cavities were then cut into the teeth (n=28 cavities per group). After sterilization, the teeth were left in broth cultures of 106 colony-forming units (CFU) ml) of S. mutans at 36ºC for 48hrs. The

appropriate treatment followed (group A, control; group B, Clearfil SE Bond; group C, Clearfil Protect Bond; group D, 40 s of treatment with ozone; and group E, 80 s of treatment with ozone), and the cavities were then filled with composite resin. After 72hrs, the restorations were removed, dentin chips were collected with an excavator, and the total number of microorganisms was determined. All treatments significantly reduced the number of S.mutans present compared with the control group. The antimicrobial effect of both bonding systems and treatment with 80 s of ozone was significantly higher than the 40 s ozone treatment. They concluded that Heal Ozone and the bonding systems showed striking antimicrobial effects against S.mutans.

Baysan A et al (2007)[53] assessed the effect of the ozone-mediated killing of bacteria in infected dentine associated with non-cavitated occlusal carious lesions and stated that the longer the contact time, the better the microbiological kill rate. It was reported that by increasing the contact time from 10 to 20 s, the bacterial kill rate changed from ozone being a disinfectant to acquiring sterilizing effect.

BaysanA et al (2007)[54] suggested that the reversal of carious lesions after treatment with ozone depend on the size and localization of the carious lesion. Non-cavitated lesions were more likely to reverse than cavitated lesions. The hardness values improved significantly in the ozone-treated test lesions after 4, 6, and 8 months ($P < 0.05$) compared with baseline while the control lesions had no significant change in hardness at any recall interval.

Huth KC et al (2007)[55] stated that Ozonated water has an excellent anti- inflammatory capacity on NF (nuclear factor) -kappa system, a paradigm for inflammation-associated signalling/transcription. Their results showed that NF-kappaB activity in oral cells in periodontal ligament tissue from root surfaces of periodontaly damaged teeth was inhibited following incubation with ozonized medium.

Muller et al(2007)[56]compared the influence of ozone gas with photodynamic therapy (PDT) and known antiseptic agents (2% Chlorhexidine, 0,5 and 5% hypochlorite solutions) on a multispecies oral biofilm in vitro. The following bacteria were studied – *A.naeslundii, F.nucleatum, S.sobrinus, S.oralis and C.albicans*. Gasiform ozone was produced by vacuum ozone delivery system Kavo Heal ozone. They concluded that the matrix-embedded microbial populations in biofilm are well protected towards antimicrobial agents. Only 5 % hypochlorite solution was able to eliminate all bacteria effectively. Usage of gasiform ozone or PDT was notable to reduce significantly or completely eliminate bacteria in the biofilm.

Petrucci MT et al (2007)[57] conducted a study in which they compared the use of ozonated oil in an experimental group to a control group in which antibiotic therapy was used in the treatment of alveolitis, it was found that patients treated with ozonated oil healed more quickly. There was complete healing of the lesions with the disappearance of symptoms.

Nogales CG et al (2008)[58] conducted an experimental study on Ozonated oil and concluded that when applied on herpes labialis and mandibular osteomyelitis demonstrated faster healing times than conventional protocols. Ozone in these cases neutralises herpes virions by direct action, thus inhibiting bactericidal supra-infections and stimulating the healing of tissues through circulatory prompting.

Noguchi et al (2009)[59] reported that irrigation of the root surface of avulsed teeth with ozonated water did not reveal a negative effect on periodontal ligament cell proliferation. In this study after the pre-treatment of LPS (Lipopolysaccharide) with aqueous ozone, effects of LPS and aqueous ozone -treated LPS on cell viability; calcification ability; expression of cyclooxygenase 2 (COX-2), interleukin 6 (IL-6), and tumour necrosis factor α (TNF-α); and activation of p38 of KN-3 cells were examined. Their results suggest that odontoblastic cells exhibit inflammatory responses against LPS and that ozonated water has the ability to improve LPS-induced inflammatory responses and suppression of odontoblastic properties of KN-3 cells through direct inhibition of LPS.

Huth KC et al (2009)[60]demonstratedantimicrobial action against bacterial strains, such as *Mycobacteria, E.faecalis, and C.albicans.* Notably, when the specimen was irrigated with sonication, ozonated water had nearly the same antimicrobial activity as 2.5% NaOCl. This study investigated whether gaseous ozone (4 · 106 l gm.) and aqueous ozone (1.25–20 l gm.) exert any cytotoxic effects on human oral

epithelial cells and gingival fibroblast (HGF-1) cells compared with established antiseptics [chlorhexidine digluconate (CHX) 2%, 0.2%; sodium hypochlorite (NaOCl) 5.25%, 2.25%; hydrogen peroxide (H_2O_2) 3%), over a time of 1 min, and compared with the antibiotic, metronidazole, over 24hrs. Cell counts, metabolic activity, Sp-1 binding, actin levels, and apoptosis were evaluated. Ozone gas was found to have toxic effects on both cell types. Essentially no cytotoxic signs were observed for aqueous ozone suggesting its biocompatibility when compared to other chemicals.

Kim HS et al (2009)[61] has undertaken a study to evaluate the therapeutic effects of topical ozonated olive oil on acute cutaneous wound healing in a guinea pig model and also to elucidate its therapeutic mechanism. After creating full-thickness skin wounds on the backs of guinea pigs by using a 6 mm punch biopsy, we examined the wound healing effect of topically applied ozonated olive oil (ozone group), as compared to the pure olive oil (oil group) and non-treatment (control group). Immunohistochemical staining demonstrated up regulation of platelet derived growth factor (PDGF), transforming growth factor-beta (TGF beta) and vascular endothelial growth factor (VEGF) expressions, but not fibroblast growth factor expression in the ozone group on day 7, as compared with the oil group. He concluded that these results demonstrate that topical application of ozonated olive oil can accelerate acute cutaneous wound repair in a guinea pig in association with the increased expression of PDGF, TGF-beta, and VEGF.

Atabek et al (2011)[62] conducted a study to evaluate the efficiency of ozone alone and with a re-mineralizing solution following application on initial pit and fissure caries lesions in permanent mandibular molars. In their study forty children (9-12 years) having non-cavitated fissure caries lesions on bilateral 40 first permanent mandibular molar teeth were participated in the study. Patients were randomly allocated to 2 experimental groups consisting of 20 subjects. In the first group, ozone was applied once for 40 seconds to the assigned test teeth of each pair. In the second group, ozone was applied once for 40 seconds to the assigned test teeth of each pair with the use of re-mineralizing solution. Progression or improvement of the caries was assessed at baseline, immediately after treatment and at 1-,2-,3-, and 6 month follow-up by comparing the DIAGNOdent values, Clinical Severity Indexes, Oral Hygiene Scores. They concluded that ozone treatment either alone or combined with a re-mineralizing solution was found to be effective for remineralisation of initial fissure caries lesions.

Gerspach et al (2012)[63] investigated the antimicrobial efficacy of gaseous ozone on bacteria adhered to various titanium and zirconia surfaces and to evaluate adhesion of osteoblast like MG-63 cells to ozone-treated surfaces. Saliva-coated titanium (SLA and polished) and zirconia (acid etched and polished) disks served as substrates for the adherence of *S.sanguinis and P.gingivalis*. The test specimens were treated with gaseous ozone (140 ppm; 33 mL/s) for 6 and 24 s. Bacteria were re-suspended using ultrasonication, serially diluted and cultured. MG-63 cell adhesion was analysed with reference to cell attachment,

morphology, spreading, and proliferation. Surface topography as well as cell morphology of the test specimens was inspected by SEM. They concluded that gaseous ozone showed selective efficacy to reduce adherent bacteria on titanium and zirconia without affecting adhesion and proliferation of osteoblastic cells. This in vitro study may provide a solid basis for clinical studies on gaseous ozone treatment of peri-implantitis and revealed an essential base for sufficient tissue regeneration.

Chandra SP et al (2014)[64] conducted a study to evaluate the success rate of mixture of ozonated oil and zinc oxide as a primary teeth root filling material. The study included 60 infected primary mandibular molars which were equally divided into study group (ozonated oil-zinc oxide) and control group (zinc oxide-eugenol). Pulpectomy procedure was performed and the children were followed at regular intervals. All the children were available for evaluation at the end of 12 months. The teeth were evaluated for success or failure based on clinical and radiographic criteria by a blinded investigator. Clinical and radiographic evaluation suggested that teeth obturated with ozonated oil-zinc oxide demonstrated good success rate (93.3 %) as compared to zinc oxide eugenol (63.3 %). However, no statistically significant variation ($p = 0.408$) was observed between the groups. They concluded that Ozonated oil-ZnO demonstrated a good clinical and radiographic success at 12 months follow-up and it can be considered as an alternative obturating material in infected primary teeth.

Noites et al (2014)[65] conducted a study to determine whether irrigation with sodium hypochlorite, chlorhexidine, and ozone gas, alone or in combination, were effective against *E.faecalis and C.albicans* and concluded that only the combined action of 2% CHX and ozone gas for short period promotes the complete elimination of both microorganisms tested in the tooth model. The results of this study could lead to redirect efforts towards new protocols for reducing microbial load in infected root canals looking for synergisms between new modalities (ozone) or already known antimicrobial products.

Anand et al (2015)[66]evaluated the antibacterial property of ozonated water, white wine (14%) and de-alcoholised white wine against *S.mutans* and *E.faecalis* and concluded that ozonated water has the best antibacterial property among the study groups and the antibacterial action of Calcium hydroxide is enhanced when it is mixed with de-alcoholised white wine.

Ahmedi et al (2016)[67]assessed the efficacy of ozone gas (O_3) on the reduction of dry socket (DS) occurrence following surgical extraction of lower jaw third molars and concluded that O_3 gas has a positive effect on reducing the development of DS and pain following third molar surgery. The microbiological and metabolic capabilities of O_3 for promoting haemostasis, increasing the supply of oxygen, and inhibiting bacterial proliferation increase the opportunities for its use in all fields of surgery. These changes on the cellular level explain elevation of functional activity and regeneration potential of tissues

and organs which may account for the increased percentage of viability in mouse fibroblasts when treated with ozonated eugenol. It also activates aerobic processes like glycolysis and Krebs cycle at cellular level to stimulate blood circulation; hence ozone is also used for treatment of circulatory disorders.

MATERIALS AND METHODS

Materials used

1. Zinc oxide powder (Dental Products of India, Mumbai). Batch no: LC 01
2. Eugenol (Dental Products of India, Mumbai). Batch no: LG 01
3. Stainless steel rings (dimensions : height- 1.5 mm; inner diameter- 10mm)
4. Glass slab.
5. Stainless steel spatula.
6. Ozonator machine (Eltech Ozonators, Mumbai). Model no:D-400
7. Humidity chamber (calibrated Labtherm Humidity chamber).
8. Mettler AE 260 Delta range Analytical Balance (Mettler- Toledo Internet INC, Columbus OH).
9. Shore A Hardness Durometer.
10. pH meter (Eutech Instrument, pH tester).
11. Petri dish.
12. Fibroblast culture medium.
13. MTT reagent
14. Spectrophotometer

Methods

This study was conducted to evaluate the physical properties and biocompatibility of zinc oxide – ozonated eugenol and conventional zinc oxide eugenol as an endodontic sealer. Invitro lab testing was conducted to evaluate and compare the hardness, solubility, pH and biocompatibility of zinc oxide-ozonated eugenol and conventional zinc oxide-eugenol.

Eugenol was ozonated using ozonator machine with an output of 400mg/hr. 100ml of eugenol is taken in a glass beaker and subjected to ozonation for a time period of 30 min.

Zinc oxide powder and eugenol were weighed before manipulation using Mettler analytical balance with an accuracy of 0.0001g. Pre-weighed quantities of zinc oxide powder was mixed with ozonated and conventional eugenol in the ratio 6:1 by weight (*P:l ratio*). Manipulation was done as per manufacturer's instructions and following physical properties were evaluated. Samples were divided into two groups Zinc oxide-eugenol (Group-I) and Zinc oxide-ozonated eugenol (Group-II).

Hardness of the mixed samples was measured using Shore hardness Durometer type A (ADIS Gauges and Tools Manufacturing Company, Mumbai) with an intender tip Hardened steel rod 1.1 mm – 1.4 mm diameter, with a truncated 35° cone, 0.79 mm diameter. The samples were mixed and 10 stainless steel ring molds of inner diameter 10mm and height 1.5mm were used to shape the samples. After predetermined

periods of 12hrs and 24hrs the hardness of the sealer samples were measured using Shore hardness Durometer type A. The load applied was 0.822 Kg with a resulting force of 8.064 N.

pH change of the samples was measured using a previously calibrated digital pH meter (Eutech Instrument, pH tester). The samples mixed immediately after manipulation were denoted as fresh samples, and the samples mixed and stored in the incubator (37°C, >95% relative humidity) for a period of 12hrs and 24hrs were denoted as set samples. Stainless steel rings of inner diameter 10mm and height 1.5mm were used to shape the molds. Each set sample was put in a flask containing 10ml of distilled water and stored at 37°C throughout the study period. For fresh samples, the same volume as the set samples of mixed sealer was placed in a flat bottom container containing 10ml of distilled water at 37°C. After predetermined periods of 12, 24 and 48hrs the pH of the solution was measured using a calibrated digital pH meter.

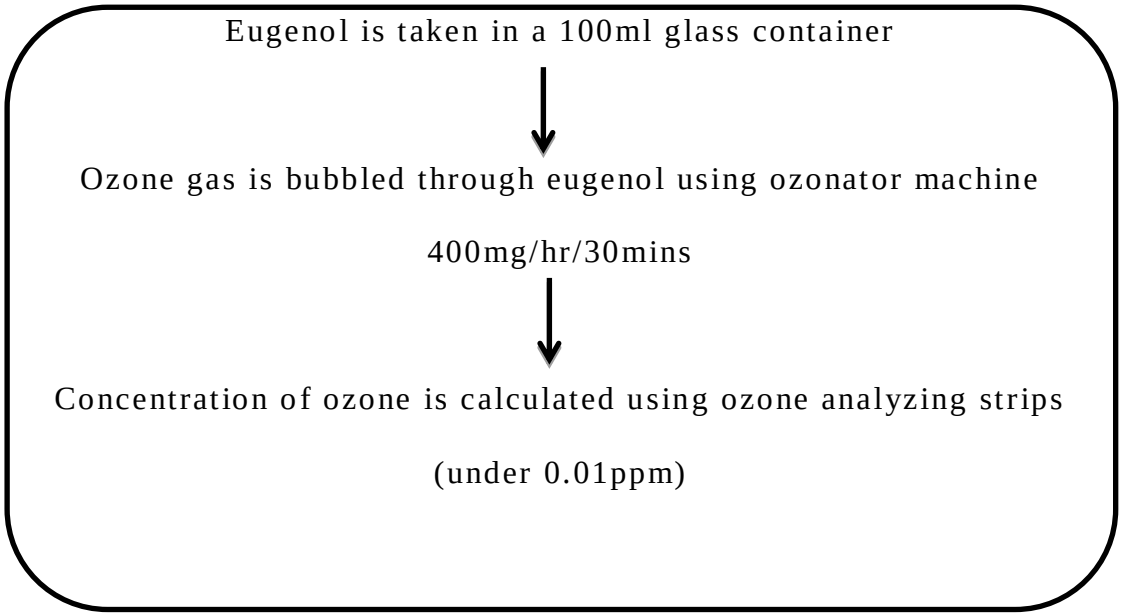
Solubility was tested as a percentage of the mass of specimen removed from the distilled water compared with the original mass of the specimens. Three 1.5 mm thick stainless steel ring of inner diameter 10mm were used for each group. The rings were filled with sealers and supported by a glass plate. The filled molds were placed in an incubator (37°C, >95% relative humidity) for a period of 24 hrs. The samples were removed from the mold and weighed three times each with an accuracy of 0.0001g (Mettler analytical balance). Each sample was put in a petri dish, which was weighed before use with 50ml

distilled water. Then the samples were discarded, and the petri dish were dried in an oven at 110 c, cooled in the desiccator to room temperature, and reweighed. The amount of sealer removed from each specimen was calculated as the difference between the initial mass and final mass of the petri dish.

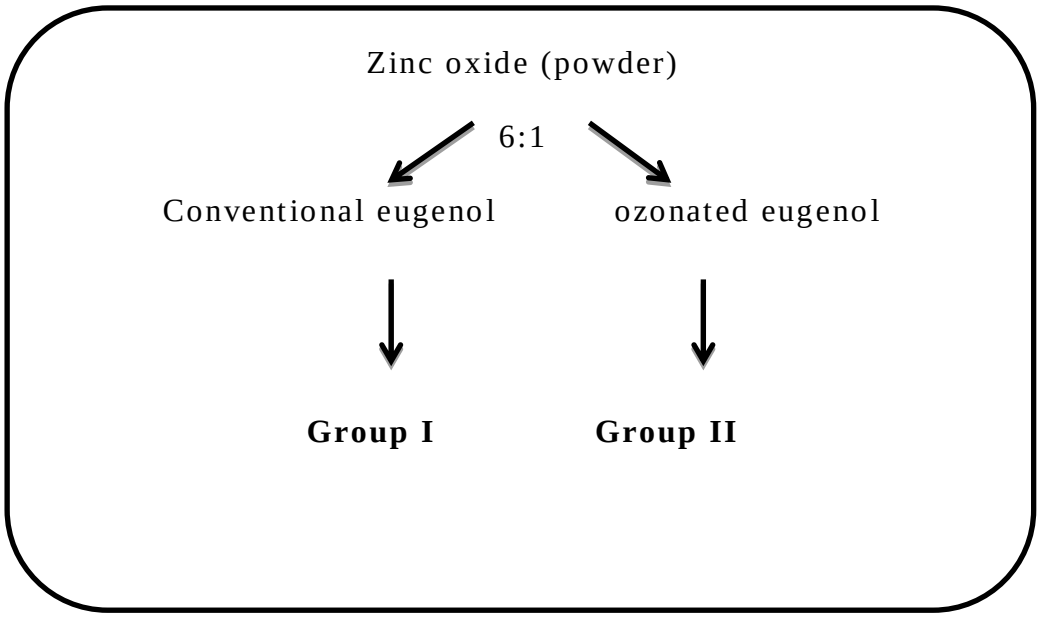
Biocompatibility of the tested samples was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay. It was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS).The MTT substrate was prepared in a physiologically balanced solution, added to cells in culture at a final concentration of 0.2 - 0.5mg/ml, and incubated for 1 to 4 hours. After 24hrs biocompatibility was evaluated as the percentage of viability of mouse fibroblast cell line L929 when test samples were added to it. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer.

Flow chart:

Ozonation



Manipulation



Hardness of the mixed sealers was measured using Shore hardness Durometer type A (ADIS Gauges and Tools Manufacturing Company, Mumbai)

Stainless steel rings of inner diameter 10mm and height 1.5mm- shape the molds.

↓

10 samples for each group were subjected to hardness testing

↓

After 12hrs and 24hrs the hardness of the sealer samples were measured.

pH change of the samples was measured using a previously calibrated digital pH meter (Eutech Instrument, pH tester).

3 samples for each group were subjected to pH testing

Samples mixed immediately → Fresh samples

Samples mixed and stored in the incubator (set samples) → After 12hrs, After 24hrs

↓

Subjected to pH testing

Solubility was tested as a percentage of the mass of specimen removed from the distilled water compared with the original mass of the specimens.

Stainless steel rings of dimensions 1.5x10mm were used to shape the samples.

↓

3 samples for each group were subjected to solubility testing

↓

Samples were removed from the mold

↓

Weighed three times each with an accuracy 0.001g

↓

Each sample was put in a petri dish - weighed before use with 50ml distilled water.

↓

Samples discarded from petri dish,

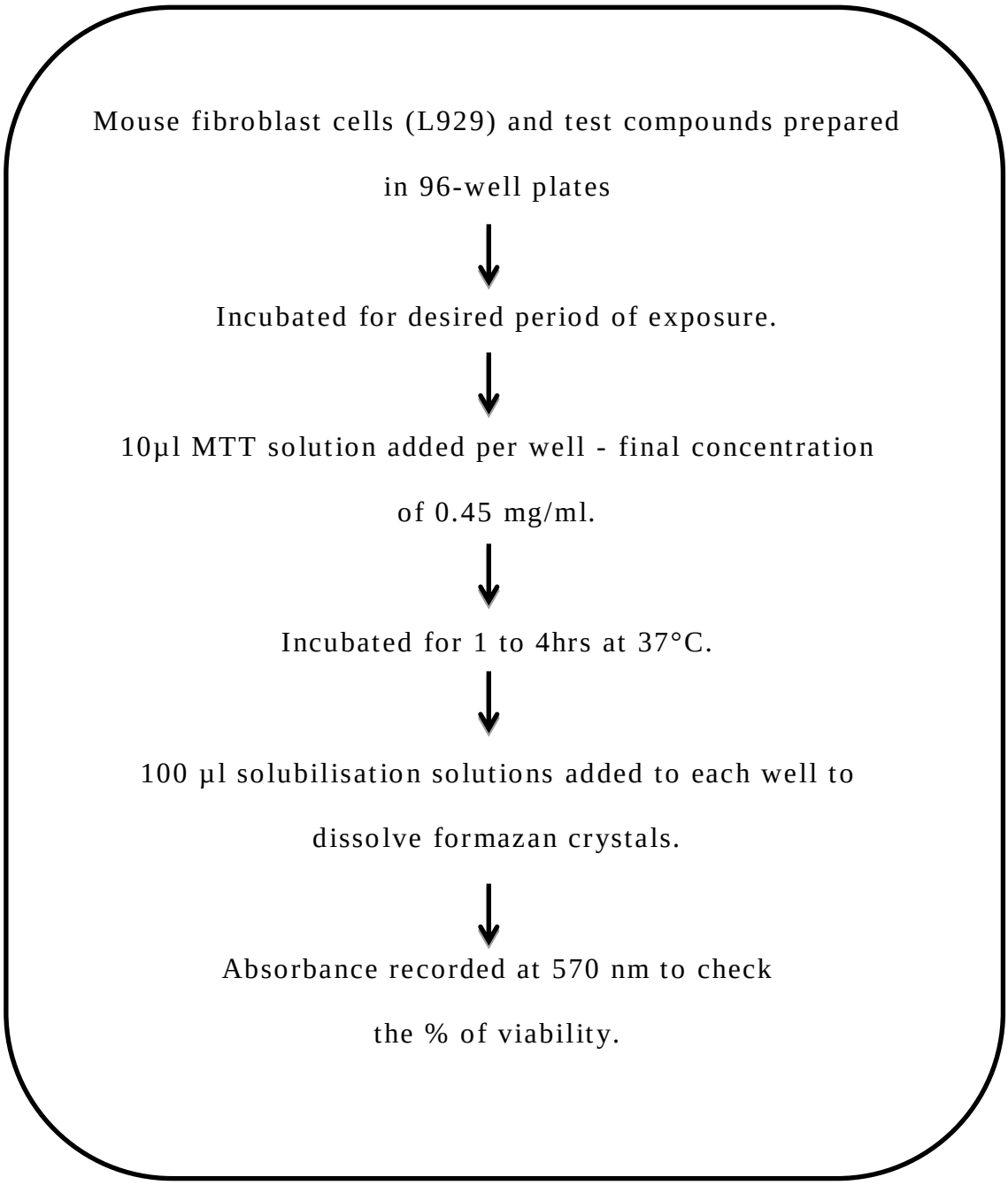
↓

Dried in an oven at 110° C, cooled in the desiccator and re-weighed.

↓

Amount of sealer removed from each specimen (w1 – w2 of the petri dish)

Biocompatibility of the tested samples were evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay.



Armamentarium

Figure 1: Zinc oxide powder, Eugenol, Glass slab and stainless steel spatula.

Figure 2:Stainless steel ring.

Dimension : 1.5 x 10 mm

Figure 3: Ozonator D-400

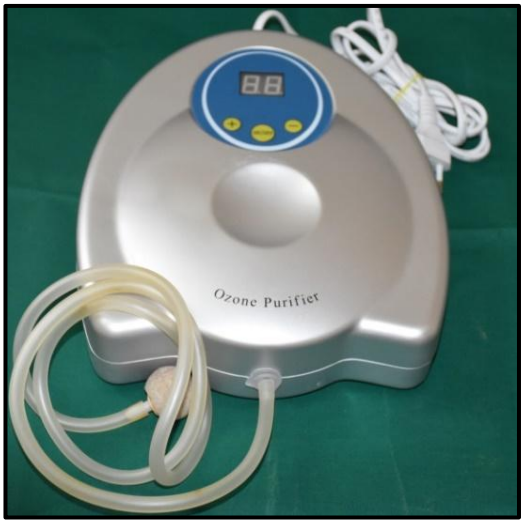


Figure 4: Eugenol ozonated by bubbling gases through it.

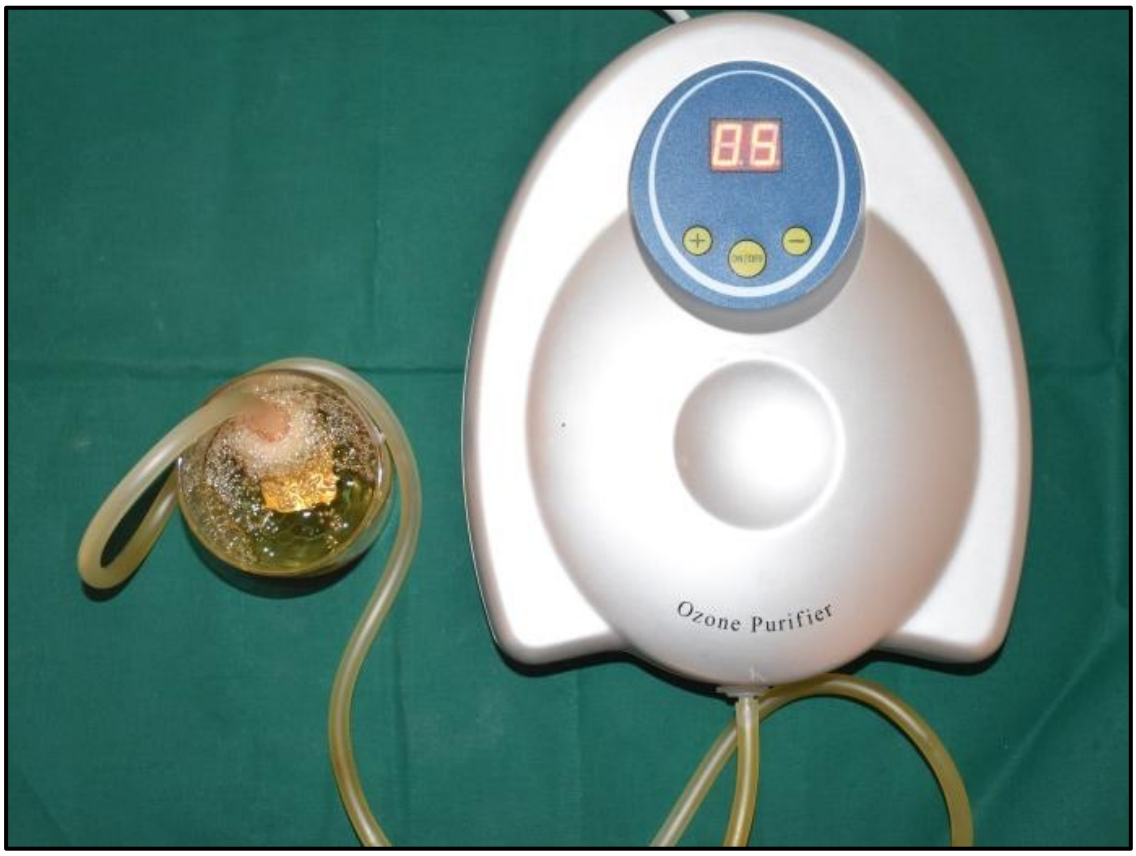


Figure 5: Shore hardness Durometer type A (ADIS Gauges and Tools Manufacturing Company, Mumbai)

Figure 6: Calibrated digital pH meter (Eutech Instrument, pH tester).

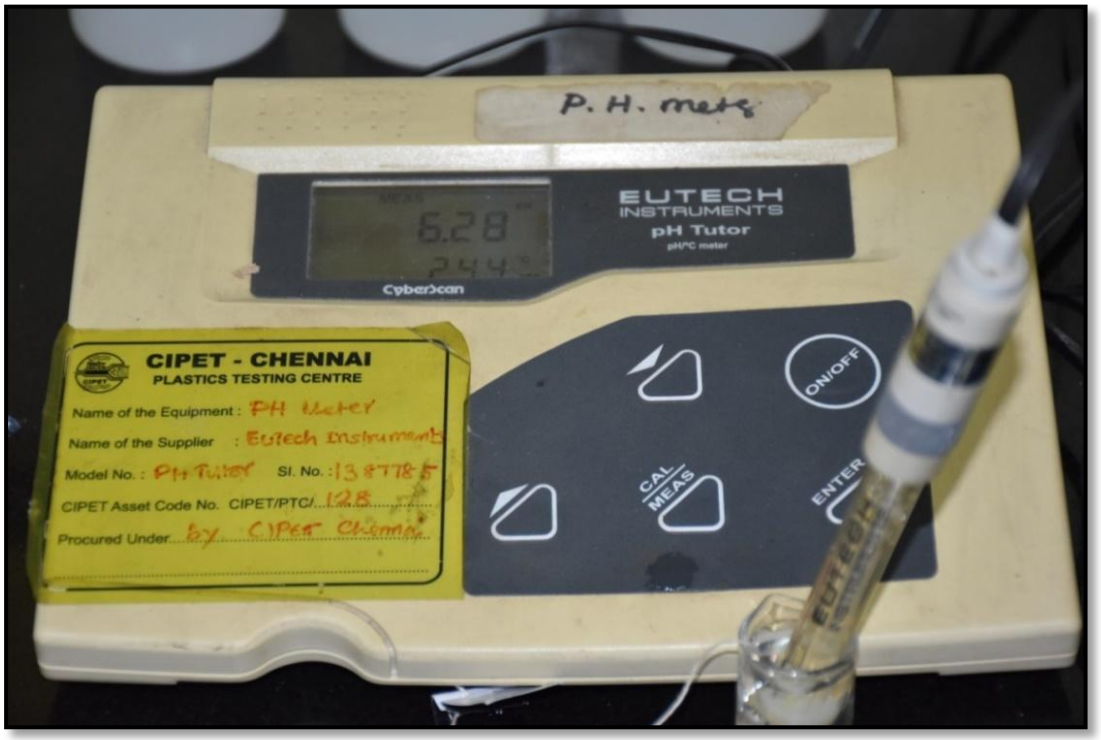


Figure 7: Samples shaped using stainless steel moulds

Figure 8: Mettler AE 260 Delta range Analytical Balance

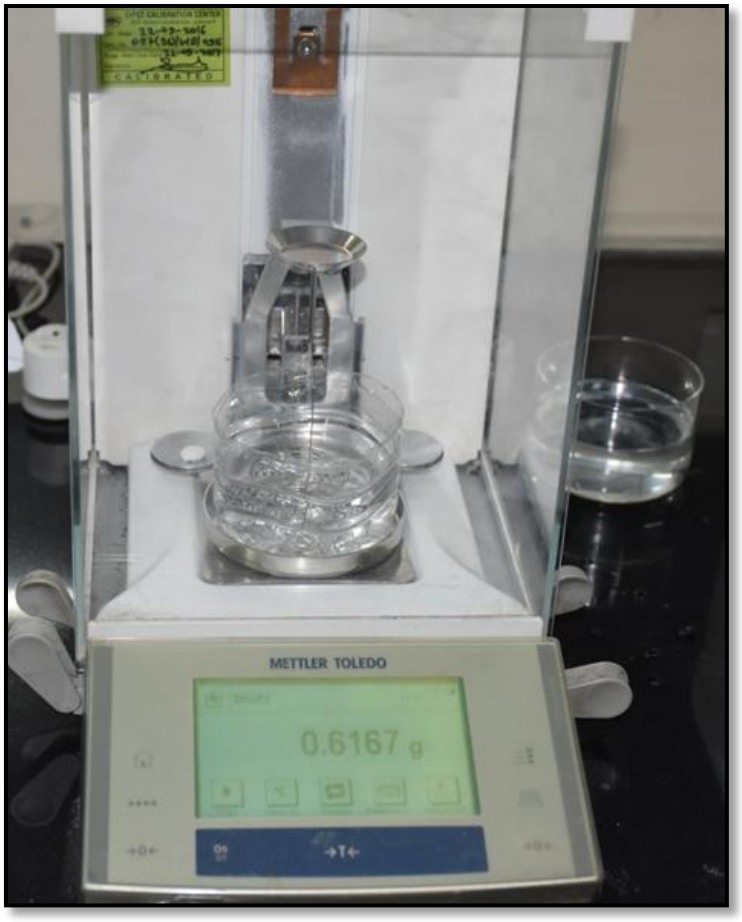


Figure 9: MTT Reagent

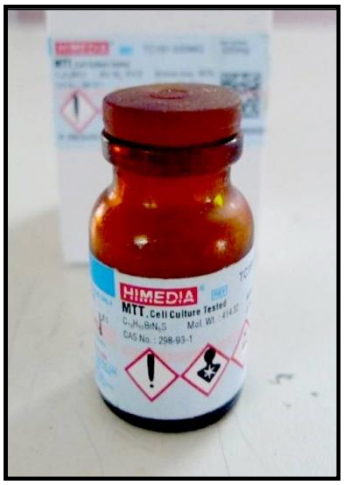


Figure 10: Micro plate reader

Figure 11:Biocompatibility Group I

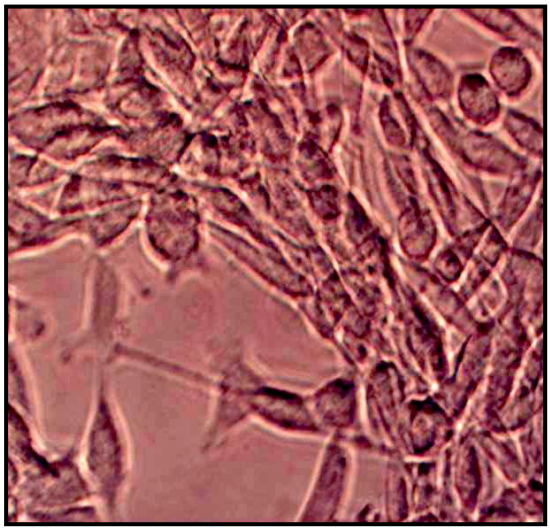
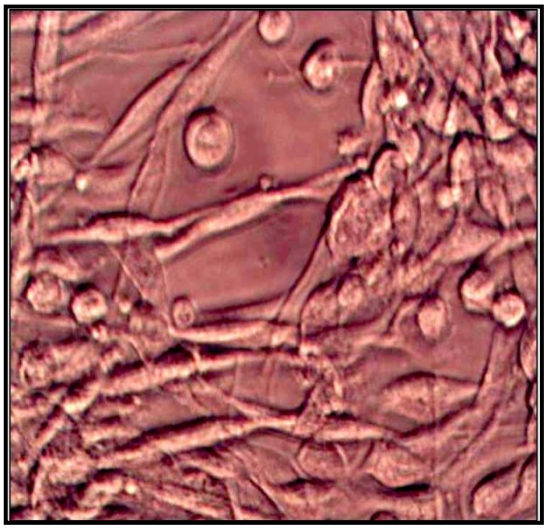


Figure 12: Biocompatibility Group II



RESULTS

This study was conducted to evaluate the physical properties and biocompatibility of zinc oxide – ozonated eugenol and to compare it with conventional zinc oxide eugenol as an endodontic sealer. Eugenol was ozonated using ozonator machine by bubbling ozone through it. The powder and liquid were mixed in the ratio of 6:1 by weight and the mixed samples were divided into two groups: Group I (zinc oxide eugenol) and Group II (zinc oxide – ozonated eugenol). Physical properties such as hardness, pH, solubility and biocompatibility of the samples were evaluated and compared. The obtained results were tabulated and the data collected were statistically analysed using unpaired T test with P value significant at 5% level ($p<0.05$).

Hardness of the mixed samples was measured using Shore A hardness Durometer type A (ADIS Gauges and Tools Manufacturing Company, Mumbai) with an intender tip Hardened steel rod 1.1 mm – 1.4 mm diameter. A total of 10samples for each group were mixed and shaped using stainless steel ring molds of inner diameter 10mm and height 1.5mm. After 12hrs the mean hardness values of the samples in Group I and Group II was 55.9100 and 57.3600 respectively. After 24hrs the mean hardness values of the samples in Group I and Group II was 73.1700and 73.5000 respectively. On comparison there was no statistically significant difference between the groups with p values 0.064 and 0.545 after 12hrs and 24hrs respectively.

Table 1:

Group I : zinc oxide eugenol Group II: zinc oxide – ozonated eugenol

Shore A Hardness		**No.of samples**	**Mean**	**Std. Deviation**	**P-Value**
After 12hrs	Group I	10	55.9	1.95587	P=.064 NS
	Group II	10	57.3	1.24829	
After 24hrs	Group I	10	73.1	.90068	P=.545 NS
	Group II	10	73.5	1.43295	

Significant at the 5% level. ($p<0.05$).Statistically no significant difference using unpaired t'test

pH change of the samples was measured using a previously calibrated digital pH meter (Eutech Instrument, pH tester). A total of three samples from each group were subjected to pH testing after predetermined time periods: freshly mixed, 12hrs and 24hrs. The mean pH values of the freshly mixed samples in Group I and Group II was 6.5667and 7.5667 respectively with statistically significant difference between them (P=.013). After 12hrs the mean pH values of the samples in Group I and Group II was 6.1000 and 6.7667 respectively with statistically significant difference between them (P=.007). After 24hrs the mean pH values of the samples in Group I and Group II was 5.7667and 6.2667 respectively with no statistically significant difference between them (P=.123).

Table 2:

Group I : zinc oxide eugenol Group II: zinc oxide – ozonated eugenol

pH of the test samples		No of samples	Mean	Std. Deviation	P-Value
Freshly mixed	Group I	3	6.5667	.20817	P=.013 Sig
	Group II	3	7.5667	.35119	
After 12hrs	Group I	3	6.1000	.17321	P=.007 Sig
	Group II	3	6.7667	.15275	
After 24hrs	Group I	3	5.7667	.30551	P=.123 NS
	Group II	3	6.2667	.32146	

Significant at the 5% level.(p<0.05).Statistically significant difference using unpaired t'test in freshly mixed samples and after 12 hrs.Statistically no significant difference after 24 hrs.

Solubility was tested as a percentage of the mass of specimen removed from the distilled water compared with the original mass of the specimens. 1.5 mm thick stainless steel ring of inner diameter 10mm and were used to shape the samples. A total of three samples from each group was subjected to pH testing after 12hrs and 24hrs. After 12hrs the mean solubility values of the samples in Group I and Group II was 0.6233and 0.6100 respectively. After 24hrs the mean hardness values of the samples in Group I and Group II was 0.5500 and 0.5400 respectively. On comparison there was no statistically significant difference between the groups with p values 0.612 and 0.513 after 12hrs and 24hrs respectively.

Table 3:

Group I : zinc oxide eugenol Group II: zinc oxide – ozonated eugenol

Solubility of the test samples (wt%)		**No of samples**	**Mean**	**Std. Deviation**	**P-Value**
After 12hrs	Group I	3	.6233	.05033	P=.612 NS
	Group II	3	.6100	.02000	
After 24hrs	Group I	3	.5500	.02000	P=.513 NS
	Group II	3	.5400	.02000	

Significant at the 5% level.(p<0.05). Statistically no significant difference using unpaired t'test

Biocompatibility of the tested samples were evaluated using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay. A total of three samples from each group were subjected to biocompatibility testing after 24hrs. After 24hrsbiocompatibility was evaluated as the percentage of viability of mouse fibroblast cell line L929 when test samples were added to it. After 24hrs the mean biocompatibility values of the samples in Group I and Group II was 52.6000 and 68.4333 respectively. On comparison there was statistically significant difference between the groups with p value set at level 5% after 24hrs (p=0.002).

Table 4:

Group I : zinc oxide eugenol Group II: zinc oxide – ozonated eugenol

Biocompatibility (% of viability)		**No of samples**	**Mean**	**Std. Deviation**	**P-Value**
After 12hrs	Group I	3	52.6000	1.00167	P=.002 Sig
	Group II	3	68.4333	1.15326	

Significant at the 5% level (p<0.05). Statistically significant difference using unpaired t'test

Group I: Zinc oxide eugenol **Group II**: Zinc oxide – ozonated eugenol

Chart 1: Hardness values of tested samples after 12hrs

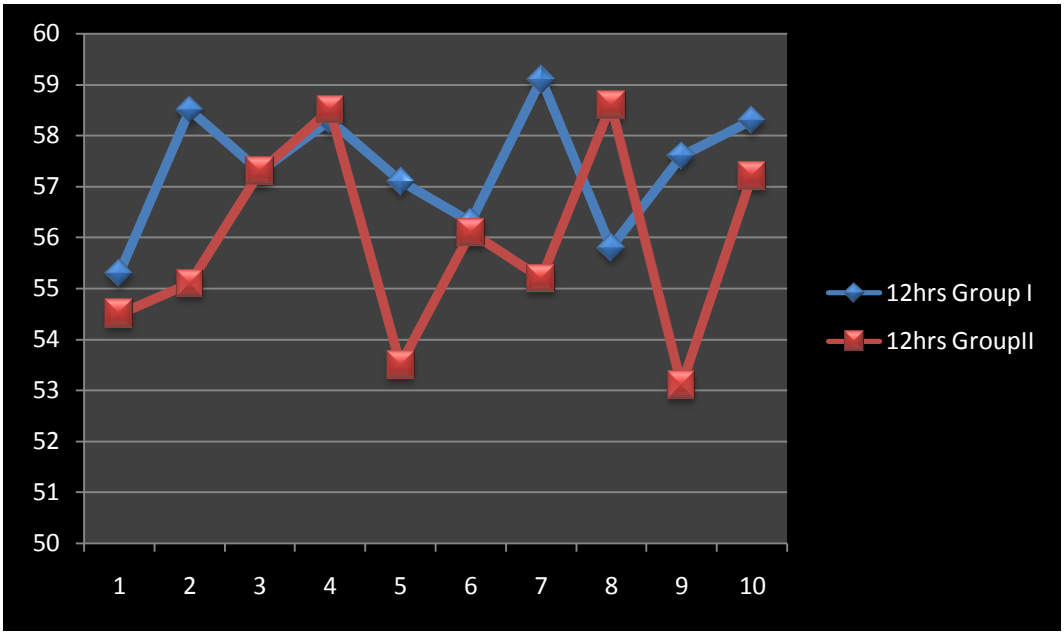


Chart 2: Hardness values of tested samples after 24hrs

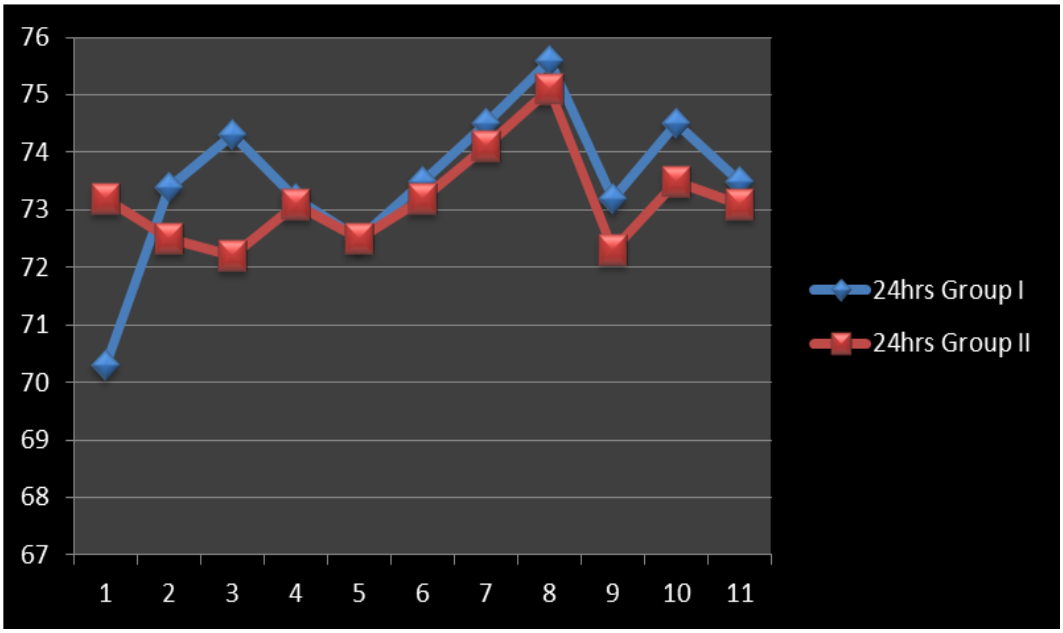


Chart 3: Mean hardness values of tested samples

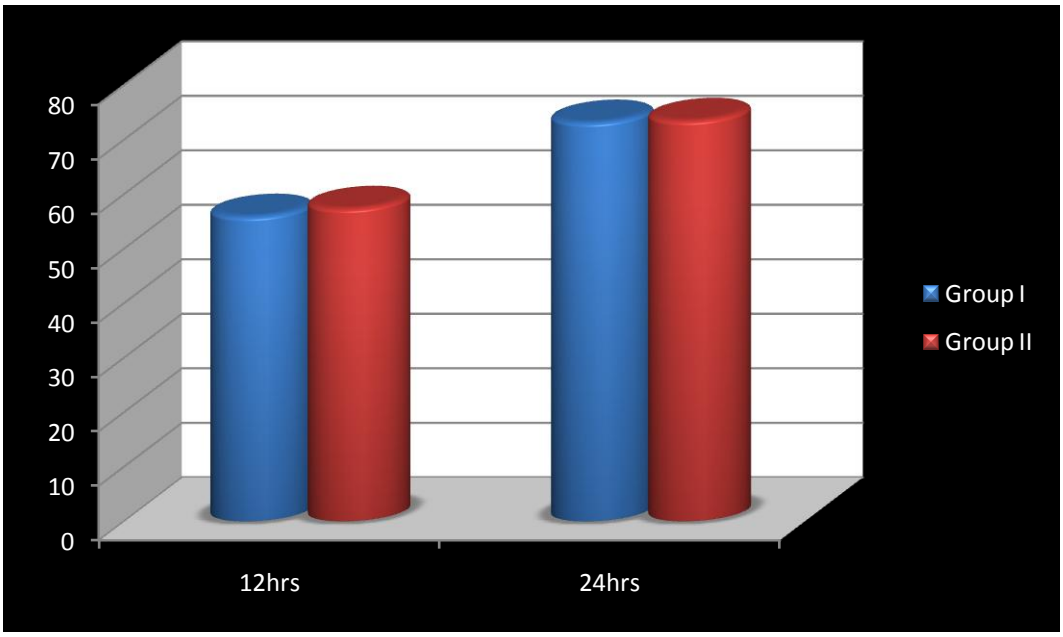


Chart 4: Mean pH values of tested samples

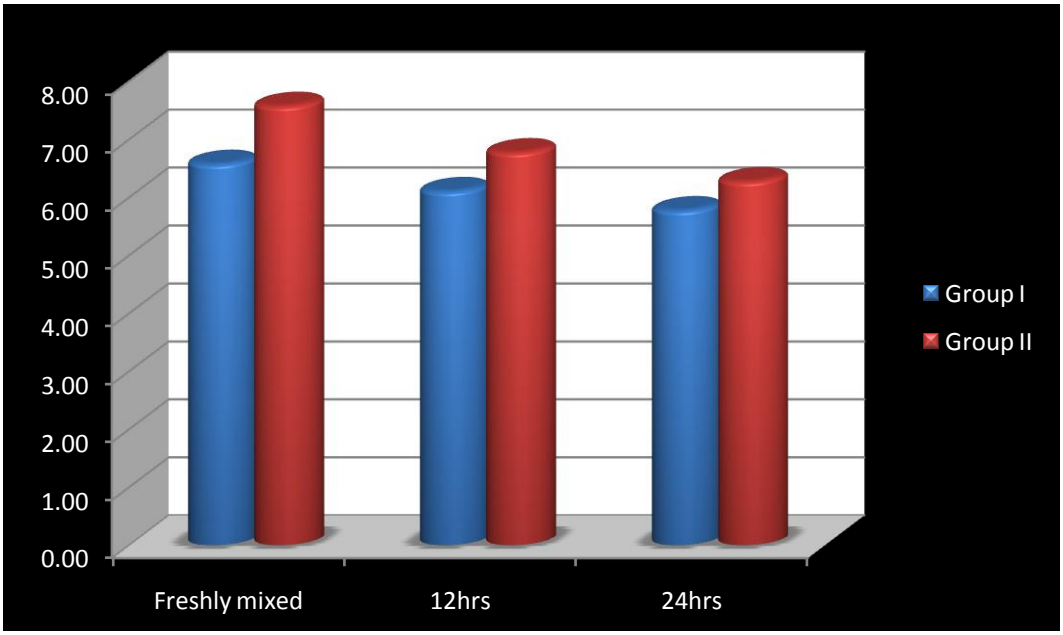


Chart 5: Mean solubility values of tested samples

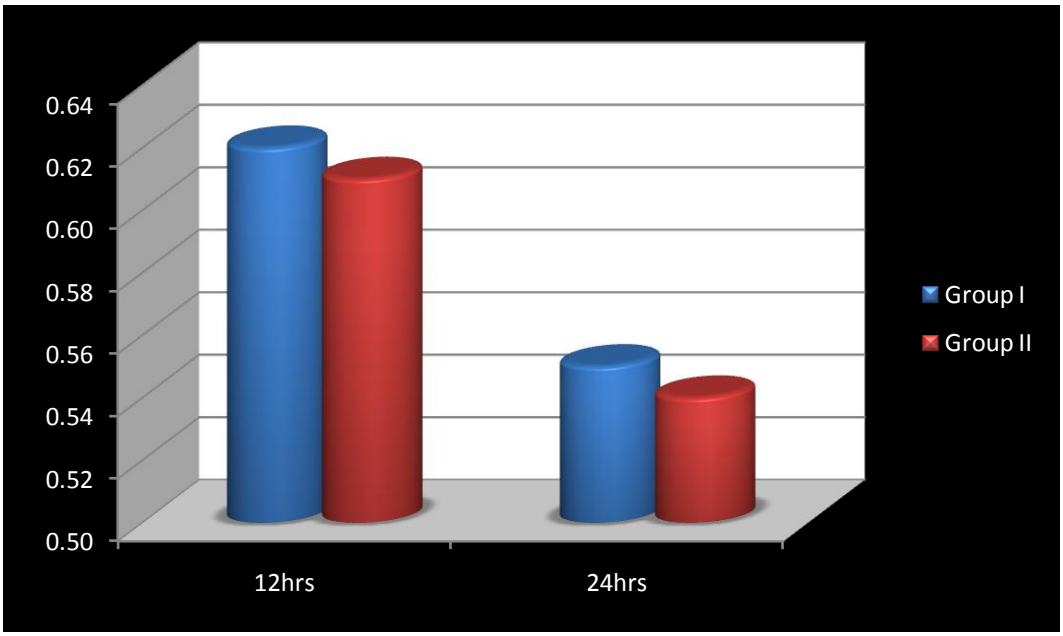
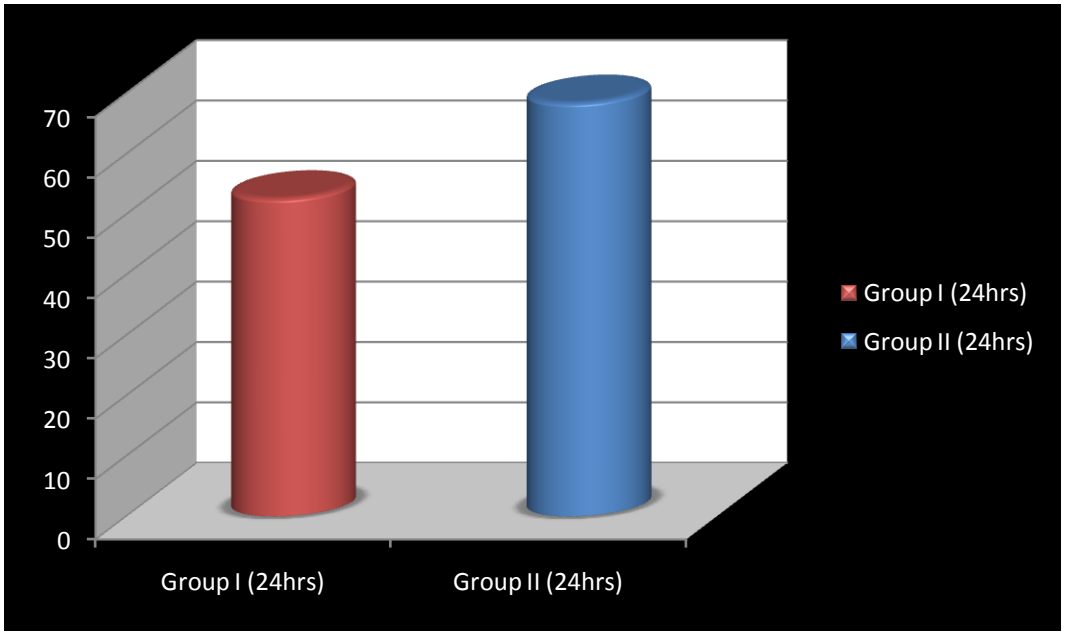


Chart 6: Mean biocompatibility values of tested samples



DISCUSSION

Root canal sealers along with solid core material plays a vital role in achieving the three dimensional sealing of the root canal system. These sealers are binding agents which are used to bind the rigid gutta-percha to the canal walls and to fill up the voids, accessory canals and irregularities in the canal[1]. Endodontic sealers, although used only as adjunctive materials in the obturation of root canals, they also have shown to influence the outcome of endodontic treatment. A perfect combination of sealing ability, antimicrobial activity and biocompatibility is what an ideal root canal sealer should possess.

An ideal root canal sealer should possess following properties[14].

1. It should be tacky when mixed, to be able to provide adhesion/sealing between the canal walls and the core obturating material.
2. Flowability in its non-set state to promote luting.
3. It should be non-irritating to the periapical tissues.
4. Should possess a hermetic sealing ability.
5. It should be radiopaque.
6. Minimal setting shrinkage.
7. Should be non-staining on the dentin / tooth structure.
8. It should be ideally Bactericidal or at-least control Bacterial Growth.
9. Should have Long Working Time.
10. Insoluble in Tissue Fluids.
11. It should be easy to remove if necessary.

12.It should be neither Mutagenic nor Carcinogenic.

13.It should not provoke an Immune reaction.

ZOE sealers have a history of successful use over long period of time. It has both anaesthetic and antimicrobial properties. The quality of the seal achieved with gutta-percha (GP) and conventional zinc oxide eugenol (ZOE) sealers is not quite perfect[68]. Despite of its advantages, GP and conventional sealer combination still has its own drawbacks, like its inability to strengthen the root, as it does not adhere to dentin, inability to control micro leakage, and the solubility of sealer makes the prognosis questionable[69]. Also, the eugenol released from ZOE is reported to be an irritant to periapical tissues and has cytotoxic properties with limited antimicrobial activity.

In search of modern endodontic therapeutic modalities with efficient and broad antimicrobial activity, ozone has proved to elicit superior action in various fields of dentistry. Ozone (O_3) is an energised form of oxygen and dissociates readily back to oxygen, liberating a singlet oxygen which is responsible for its so called oxidising potential. Ozone is 1.6-fold denser and 10-folds more soluble in water (49.0 mL in 100 mL water at 0° C) when compared to that of oxygen. Though ozone is not a radical molecule, it is the third most potent oxidant (E_ 5 12.076 V) after fluorine and per sulphate. Ozone is an unstable gas that cannot be stored and should be used immediately because it has a half-life of 40 min at 20 °C[33]. In the clinical setting, an oxygen/ozone generator simulates lightning through

an electrical discharge field to generate ozone. Ozone gas has a high oxidising potential and it is 1.5 times greater than chloride when used as an antimicrobial agent against bacteria, viruses, fungi, and protozoa. It also has the ability to stimulate blood circulation and the immune response. These features justify the current interest in its application in medicine and dentistry and have been suggested for the treatment of 260 different pathologies.

There is an evidence of use of ozone as a disinfectant from 1881, which was mentioned in a book on Diphtheria. In 1893, the world's first water treatment plant utilizing ozone was installed in Ousbaden, Holland. During World War 1, ozone was used medically for treating wounds and other infections. Medical grade Ozone is a combination of pure O2 and pure O3 in the ratio of 0.1% to 5% of O_3 and 95%-99.5% of O_2[70].

Interestingly enough, in 1930, a German dentist, **Dr. E.A. Fisch**, treated Austrian surgeon **Ernst Payr** for a gangrenous pulpitis using ozone and thereafter inspired him to begin a new line of researches dedicated to ozone use in health care. However, ozone seems to have disappeared from practice in dental care until 2001 when the first scientific study was published examining the biomolecules present in dental caries, before and after treating with ozone[71]. As dental demographics changed and in the era of minimally invasive therapy, the new challenges of managing and treating an aged population those who have retained rather than lost their teeth needed to be addressed.

This was of particular concern in regard to elderly patients with the increased susceptibility of root surfaces caries. In dentistry, the applications of ozone are as follows[57]:

1. Reversal of incipient caries.
2. Prevention of pulpal infection in deep caries affected dentin.
3. Disinfection during root canal treatment.
4. Disinfection of cavity preparations prior to restorations.
5. Treatment of cervical sensitivity.
6. Prior to placement of all fissure sealant.
7. For bleaching teeth.
8. Combined with other approaches to manage caries such as ART.
9. For treating apthus ulcer and herpes simplex lesions.

Ozone (O_3) can be used in gaseous, aqueous form or as ozonated oils. In order to control the decomposition of O_3 into oxygen it can be combined with a vehicle with aqueous properties to improve the conversion more quickly or with a vehicle with relatively more viscous properties to retard the conversion. In a study conducted to evaluate the biocompatibility of ozone, the aqueous form of Ozone was found to be less cytotoxic than gaseous Ozone. Due to the unstable nature of ozone in gaseous and aqueous form, we opted to use ozone in the form of oils.

Estrela C et al used ozonated oils to sterilize the root canal systems and to clear the canals of necrotic debris by virtue of ozone's bactericidal and effervescent properties. In their study they used

ozonated oils for disinfection and concluded that irrigation with ozonated oil is more quick and efficient in canal sterilization than that of conventional irrigation by the sodium hypochlorite solution[72].

Chandra SP et al conducted a study to evaluate the success rate of mixture of ozonated sesame oil and zinc oxide as a primary teeth root filling material. They concluded that Ozonated sesame oil-ZnO demonstrated a good clinical and radiographic success rate at 12 months follow-up and it can be considered as an alternative obturating material in infected primary teeth[7].

Eugenol that has been used in dentistry for a long time due to its analgesic and antiseptic property is a colourless to pale yellow oily liquid extracted from certain essential oils especially from clove oil, nutmeg, cinnamon, basil and bay leaf. In Endodontics studies have focused on the use of ozonated water and oils as an intra-canal medicament and as an irrigant. But none of the studies have conducted by ozonating eugenol for being used as an endodontic sealer.

In the present study, ozone was used to overcome the drawbacks of ZOE sealer by bubbling ozone gas through eugenol using an ozonator machine which has an output of 400mg/hr. The concentration of ozone in eugenol was checked using ozone analysing strips and was kept under 0.05ppm (safety dose of ozone as per OSHA: less than 0.1ppm)[73]. Invitro studies were conducted to evaluate and compare the hardness, solubility, pH and biocompatibility of conventional zinc oxide-eugenol (Group I) and zinc oxide-ozonated eugenol (Group II).

Hardness of the tested samples was evaluated using Shore A hardness Durometer type A (ADIS Gauges and Tools Manufacturing Company, Mumbai). The samples were mixed as per manufacturer's instructions and subjected to hardness testing at the end of 24hrs and 48hrs. The hardness values obtained were almost similar for both the groups with no statistically significant difference between them. ($p>0.05$)

pH of the mixed samples was evaluated using calibrated pH meter after predetermined time intervals (freshly mixed, 24hrs and 48hrs). In Group II where eugenol is ozonated the pH of freshly mixed samples was relatively higher than that of the samples in Group I. This may be due to formation of hydroxyl ions readily due to the unstable nature of ozone. This transient radical anion rapidly becomes protonated, generating hydrogen trioxide (HO_3), which, in turn, decomposes to an even more powerful oxidant, the hydroxyl radical (OH)[57]. There was statistically significant difference between the two groups ($p < 0.05$) in the freshly mixed samples and set samples after 12hrs. However there was no statistically significant difference between the two groups in the set samples after 24hrs which may be due to the depletion of singlet oxygen with time.

Solubility of the tested samples was evaluated by the difference in the percentage of the mass of the specimen before and after subjecting to solubility testing. Solubility of the tested samples after 12hrs and 24hrs were almost similar in both the groups with no

statistically significant difference between them ($p>0.05$). In this study, the samples in both the groups demonstrated good results, none of the sealers exceeded 3% mass fraction after immersion in water for 24 hours, in accordance with ISO 6876 and ANSI/ADA Specifications No. 57 and No. 30 [13].

Biocompatibility of the tested samples was evaluated using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay. In the present study biocompatibility was evaluated as the percentage of viability of mouse fibroblast cell line L929 when test samples were added to it. After 24hrs, the percentage of viability was measured by recording the changes in absorbance at 570 nm using a plate reading spectrophotometer.

In Group II where eugenol is ozonated, there was higher percentage of viability when compared to conventional eugenol with statistically significant difference between them ($p<0.05$). These findings were similar to the studies of **Ebensberger U** and **Huth KC** in which a high level of biocompatibility of aqueous ozone has been found on human oral epithelial cells, gingival fibroblast cells, and periodontal cells[43]. The metabolic activity of L-929 mouse fibroblasts was high when the cells were treated with ozonated water, whereas that of significantly decreased when the cells were treated with 2.5% NaOCl[59].

As a response to inflammatory process, Ozone stimulates the release of interleukins, leukotriene and prostaglandins, to promote

wound healing. It activates mechanisms of protein synthesis, increases amount of ribosomes and mitochondria in cells[74]. These changes on the cellular level explain elevation of functional activity and regeneration potential of tissues and organs which may account for the increased percentage of viability in mouse fibroblasts when treated with ozonated eugenol. It also activates aerobic processes like glycolysis and Krebs cycle at cellular level to stimulate blood circulation; hence ozone is also used for treatment of circulatory disorders.

Ishizaki K et al exploring the effect of ozone on *E. coli*, they stated that ozone penetrated through cell membranes, reacting with cytoplasmic contents, cleaving the circular plasmid DNA, thus impairing bacterial procreation. They also stated that higher organisms have developed mechanisms for protecting DNA and repairing them when disrupted while subjecting to ozone therapy[75]. This could provide a partial explanation for why ozone at doses prescribed, is toxic to pathogens and not to the healthy cells and these features justify the current interest in its application in dentistry.

SUMMARY AND CONCLUSION

Dentistry is changing, as we are now using modern science to practice dentistry. In Endodontics, the sealers are responsible for the principal functions of the final root filling: sealing off of the root canal system, entombment of remaining bacteria and the filling of irregularities in the prepared canal. The Zinc Oxide Eugenol (ZOE) based sealers were introduced in Endodontics by **Grossman** in 1936. ZOE sealers have a history of successful use over extended period of time. However its use has been restricted in recent years due to its few drawbacks including the cytotoxic and irritating property of eugenol[3]. Eugenol (4- acyl-2-methoxy phenol) is an extract of clove (Eugenia caryophyllata) commonly used as an anaesthetic and antimicrobial agent in the field of medicine and dentistry.

Several attempts that have been made to overcome these disadvantages have failed and there is continued research for chemical compounds with better physical properties and biocompatibility. The evolution of sealers is from the conventional ZOE to the contemporary ones like epoxy-based resin and methacrylate- resin based sealers, and to the most recent MTA sealer and bioceramic sealer. However till date, none of the sealers has proved to be the ideal except a few which can come closer to being one.

Considering the beneficial effects of eugenol with few drawbacks, we decided to improve its properties by other modalities. In

search of other treatment modalities, ozone therapy has a long history of research and clinical applications with humans because of its interesting biological properties. In Endodontics studies have focused on the use of ozonated water and oils as an intra-canal medicament and as an irrigant. But none of the studies have conducted by ozonating eugenol for being used as an endodontic sealer. In the present study, ozone was used to overcome the drawbacks of ZOE sealer by bubbling ozone gas through eugenol using an ozonator machine.

In vitro lab testing was done to evaluate and compare the physical properties and biocompatibility of zinc oxide eugenol (Group I) and zinc oxide- ozonated eugenol (Group II). The proportional values obtained were tabulated and statistically analysed by unpaired t test. Solubility and hardness of the tested samples were almost similar in both the groups with no statistical difference between them. pH of freshly mixed samples in Group II was relatively higher than the samples in Group I which may due to the increased formation of hydroxyl ions.

Biocompatibility of the tested samples in Group II was comparatively more than that of the samples in Group I. The increased percentage of viability may be due to the activation of protein synthesis, increased amount of ribosomes and mitochondria synthesis in cells. These changes on the cellular level explain the elevation of functional activity and regeneration potential of tissues and organs. Ozone brings about the rise of pO_2 in tissues and improves

transportation of oxygen in blood, which results in change of cellular metabolism – activation of aerobic processes (glycolysis, β-oxidation of fatty acids and Krebs cycle) and use of energetic resources.

Within the limitations of the study, it can be concluded that considering the beneficial properties of eugenol with few drawbacks: ozone with interesting biological properties seems to be a better treatment modality in improving the properties of eugenol. However further clinical trials and randomized control studies are required in this field to use ozonated eugenol in combination with zinc oxide powder to use it as an endodontic sealer invivo.

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This ethical committee has undergone the research protocol submitted by **DR.C.RAVI VARMAN**, Post Graduate Student, Department of Conservative Dentistry and Endodontics under the title **"COMPARATIVE EVALUATION OF THE PHYSICAL PROPERTIES AND BIOCOMPATIBILITY OF ZINC OXIDE-OZONATED EUGENOL AND CONVENTIONAL ZINC OXIDE EUGENOL AS AN ENDODONTIC SEALER – AN INVITRO STUDY"** Reference No: 2014-MD-BrIV-SAT-09 under the guidance of **DR.S.THILLAINAYAGAM MDS.,** for consideration of approval to proceed with the study.

This committee has discussed about the material being involved with the study, the qualification of the investigator, the present norms and recommendation from the Clinical Research scientific body and comes to a conclusion that this research protocol fulfils the specific requirements and the committee authorizes the proposal.

Date: **Member secretary**