Inhibition of bacterial growth around gutta percha cones by different antimicrobial solutions using antibiotic sensitivity test (An in vitro study)

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ABSTRACT

Background: Decontamination of gutta percha cones was important factor for success of root canal treatment. The aim of the present in vitro study was to identify and to compare the antimicrobial effect of following disinfection solutions: 0.2% chlorhexidine gluconate, Iodine, tetracycline hydrochloride solution, EDTA & formocresol mixed with zinc oxide eugenol, on *E faecalis, E coli and Candida albicans* using sensitivity test

Materials and Methods: Three types of microorganisms were isolated from infected root canals *(E faecalis, E coli and Candida albicans*) and cultured on Mueller Hinton agar petri-dishes. Disinfection of gutta percha cones done by immersion in six disinfection solutions (six groups), the groups are: distill water (used as control group), 0.2% chlorhexidine gluconate, Iodine, tetracycline hydrochloride solution, EDTA & formocresol mixed with Zinc oxide eugenol. The immersion times were 5 min. and the incubation was aerobically at 37 °C for 24 hr. for bacterial species and at 28°C for 48 hr. for *candida albicans*. After incubation, zones of inhibition (no growth of bacteria) were examined around the gutta percha cones & diameters of the zones were measured with a boley gauge. The means of inhibition zones for each group were measures and statistically analyzed among groups using ANOVA and LSD tests at 0.05 significance level.

Results: There is highly significant differences (P=0.000) among all the tested groups. 0.2% Chlorhexidine gluconate showed the maximum antibacterial efficacy (broader zones of inhibition) against *E faecalis*. Compound of formocresol + Zinc oxide eugenol showed the maximum antibacterial efficacy against *Candida albicans,* and *E coli*. The minimum antibacterial efficacy against *Candida albicans* occurred with tetracycline group, while against *E faecalis* and *E coli* occurred with EDTA group

Conclusion: All the tested materials had antibacterial efficacy against *Candida albicans, E faecalis* and *E coli*; but chlorhexidine gluconate and compound of formocresol & Zinc oxide eugenol, are more effective agents for a rapid disinfection of gutta-percha cones in five minutes.

Keywords: Anaerobic bacteria, Chlorhexidine, EDTA, Mueller Hinton agar and Formocresol. (J Bagh Coll Dentistry 2013; 25(4):26-32).

INTRODUCTION

There is direct relationship between bacterial infection of dental pulp and periapical lesions formation. Bacterial infection of dental pulp results in pulpal destruction and subsequently stimulates an inflammatory cell response and destruction of bone in the periapical area.[1]

The main goal of endodontic therapy is to eliminate microorganisms from the root canal system and the prevention of subsequent reinfection[2]. Although the majority of bacteria are eliminated by biomechanical preparation of root canal space, a few microorganisms might still survive, thus using of intracanal medication and filling materials with antimicrobial and sealing properties are of essential importance, to avoid the growth of microorganisms[3].

In endodontic practice, the elimination or significant reduction of microorganisms from the root canal by chemo-mechanical preparation is an essential factor in successful treatment. Care must be taken during this procedure to prevent contamination of instruments and filling materials, to avoid root canal cross-infection[4, 5].

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The studies found that gutta-percha cones (gpc) taken directly from the manufacturer's sealed package harbored cultivable microorganisms. While the numbers of these microorganisms were quite low at the time of opening of the package, and clinical use of the packages increased the number of microorganisms contaminating the (gpc), so preventive procedures needed including rapid chair side decontamination of the (gpc) with chemical disinfectants.[5] Several tests were used to observe the antimicrobial activity of chemical decontaminants of gpc, but there is no consensus for the best method. In order to better investigate this matter, we carried out disk diffusion antibiotic sensitivity testing to investigate the antimicrobial activity of several types of medicated solutions.

MATERIALS AND METHODS

Case selection

This in vitro study examined 20 patients who attended the clinic of college of dentistry, university of Baghdad, conservative department. Their ages ranged from 20 to 40 years with periapical pathology. A detailed medical and dental history was obtained from each patient. None of the patients had received antibiotic treatment during the previous 3 months, and none

had any systemic disease. The lesion diagnosed clinically and confirmed by radiographic examination.

Isolation of bacteria

The whole procedure of culturing was undertaken under strict aseptic conditions. The operating field was isolated using rubber dam and disinfected with 10% povidone iodine solution in order to avoid interference with isolation of bacteria. All coronal restorations and carious lesion were removed from the infected teeth. Then access cavity was prepared with a new sterile carbide fissure bur. New sterile files and barbed broach inserted into the root canals up to the apical foramina (the working length confirmed by diagnostic x-ray) to remove the content of root canals, followed by enlargement of the canals with minimal instrumentation without use of any irrigant. Sterile paper points were introduced in to the full length of the canals and retained in the canals for 1min. for microbiological sampling.[6] The paper points were immediately introduced inside amice transporting media to preserved collected bacteria from dyeing and sealing the bottle tightly until send to the central public health laboratory for isolation of bacterial species within 4 hrs only.

Identification of bacterial species

Broth culture suspensions of bacteria and C. albicans were adjusted to No. 0.5 McFarland standard (approximately 1.5×10^8 cells/mL), and 100 ml aliquots of each microbial suspension were dispersed on the surface of agar plates then transferred to specific fluid growth media before the experiment. Three types of agers used for isolation of bacteria. Blood agar for isolation of Gram +ve bacteria, MacConkey agar for isolation of Gram -ve bacteria (both agers incubated at 37°C for 24 hrs), and Sabouraud agar for *Candida albicans* (*Candida* incubated at 28°C for 48 hrs.)[7] **Fig.1.** From these mixed colonies, single bacterial or fungal species can be identified based on their morphological (size/shape/colour) differences with the help of hand lens. Each type of colony was picked up and subcultured aerobically as well as anaerobically. Only those organisms which failed to grow aerobically were taken as anaerobes. If no growth was obtained then re-incubation was done. Microorganisms were identified by biochemical test using APi. Api 20E for distinguishing of Gram -ve bacteria, Api strep for distinguishing of Gram -ve bacteria and Api candida for distinguishing of candida albican. *Candida albicans* and two types of anaerobic bacterial strains were isolated from the infected canals and pure cultures of them were prepared:

1. *Enterococcus faecalis, E. faecalis* (Gram +ve bacteria).
2. *Escherichia coli, E coli* (Gram-ve bacteria).

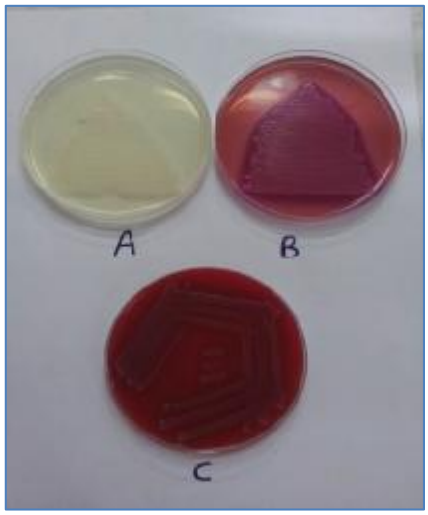

Figure 1: Types of agers used for micobiological A- Candida albicans inoculated on Sabouraud agar B- *E coli* inoculated on MacConkey agar C- *E faecalis* inoculated on Blood agar.

Bacterial Subculturing

After identification of microorganism, each type of microorganism Subcultured onto the following agars: Blood agar for *Enterococcus faecalis*, MacConkey agar for *E coli* (both agers incubated at 37°C for 24 hrs), and Sabouraud agar for *Candida albicans*, incubated at 28°C for 48 hrs inoculated on three types of agers using streaking microbiology technique, the incubator used for performance of the test.

Decontamination of gutta percha and growth inhibition zones measurement

Before the experiment each gutta percha cones **(DiaDent/size 90**) were immersed completely for one hr. in 2 ml of sterile water in a test tube to release for example the free iodine, Because gutta-percha cones remains inert until it comes in contact with tissue fluids[8]. Then gutta-percha cones immersed in six disinfection solutions for 5 min. (types of disinfection solutions and their manufacturer were mentioned in **table 1 & fig.2**).

Pure culture of each microorganism inoculated on Mueller Hinton agar petri-dishes using (Kirby-Bauer antibiotic testing or named disk diffusion antibiotic sensitivity testing) [7,9] and two gpc from the same group placed on agar petri-dishes and incubated aerobically at 37 °C for 24 hr. for bacterial species and at 28°C for 48 hr. for *Candida albicans*. After incubation, zones of inhibition (no growth of bacteria) were examined

around the gpc (**Fig. 3 & 4)**. These appeared as clear, circular halos surrounding the wells.

Diameters of the zones around gutta percha were measured with a boley gauge by one investigator (minus the diameter of the gutta percha). Experiments were repeated (n=10) and the mean value was determined. It should be noted that the inhibition zone size diameters did not necessarily represent absolute inhibitory values of a particular agent/species combination, but rather general indications of the agents' potency or lack thereof in relation to other materials. In addition, slight variations in zone sizes may have resulted from errors made in judgment of well depth and angulations in the agar.[7]

Measurements of inhibitory zone were ranked into five inhibition categories according to the proportional distribution of the data set.[9] **(See table 2)**

Figure 2: The disinfection materials used in study.

Figure 3: Inhibition zones (no growth of *E. faecalis*) around gutta percha cones disinfected by chlorhexidine gluconate 0.2 % on Mueller Hinton agar petri-dishes

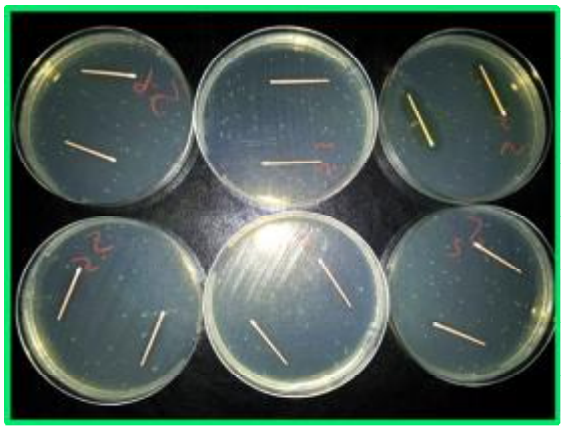

Figure 4: Six groups showed inhibition growth zone of *candida albicans* around gpc.

Table 1: Disinfection solutions used in study and their manufacturers

Materials	Manufacturers
Chlorhexidine gluconate 0.2%	Corsodyl Mint mouth wash, GlaxoSmithKline, UK
Iodine	Media Co., Syria
Tetracycline hydrochloride capsules (50mg/ml)	250 mg, Samacycline S.D.I.-Iraq
EDTA	Glyde syringe kit, A 0901, Dentsply Maillefer , USA
Tricresol & formalin	Tricresol 35%, formaldehyde 40% , PD, Switzerland
Zinc oxide	99.86% zinc oxide, Zomed, Dubai, UAE
Eugenol	Deepak, USA

Table 2: Five inhibition categories according to the proportional distribution of the data set

Rank	Range of Zone Diameters(mm)
No	2
Weak	1.4-6.2
Medium	6.3-10.3
Medium strong	10.4-26.8
Strong antimicrobial activity	> 26.8

Sample grouping

Thirteen gutta percha cones (gpc) disinfected by immersion in each group, then these 30 cones subdivided into 3 subgroups according to types of microorganisms tested in this study **(*Candida albicans, E faecalis and E coli* subgroups)**.

Group 1(Control): 30 gpc immersed in distill water.

Group 2(CHX): 30 gpc immersed in chlorhexidine gluconate 0.2% .

Group 3(Iodine): 30 gpc immersed in povidone – iodine.
Group 4(Tetracycline): 30 gpc immersed in tetracycline HCL 250 mg capsule /5ml distill water = 50 mg/ ml.
Group 5(EDTA): 30 gpc immersed in 17% EDTA.
Group 6(FC+ ZOE): 30 gpc immersed in formocresol + Zinc oxide eugenol. Formocresol: ZnO: Eugenol =2 drops: 1 scoop:6 drops = 0.02 cc: 0.2 g:0.06cc.
(See table 1 & fig. 2)

Statistical Analysis

All statistical analyses were carried out using SPSS statistical software (version 19.0, SPSS, Chicago, IL**,** USA). After data collection, mean values and standard deviations were calculated for all groups and subgroups. One way analysis of variance **(ANOVA)** was performed among the experimental groups to determine if there is any statically significant difference among the groups. When a significant difference was found, Least significant difference **(LSD)** test was done to find where is the significance occurs. The mean difference is significant at the 0.05 level.

RESULTS

The inhibitory potential of each material was categorized as strong, medium strong, medium, weak, or non-inhibitory depending on the average size of the zones. Mean of inhibitory zone size for each groups and their categories, standard deviation **(SD)** presented in **Table 3**. The result found that, control group showed no inhibition activity against the tested microorganism, while other groups showed medium strong or medium inhibition activity; and only EDTA materials showed weak effect on *Candida albicans* **(see table 3 & fig 4)**, With highly significant differences **(HS)** among the groups revealed by one way **ANOVA** test **(P = 0.000)** as shown in **tables 4.** Further investigation using **LSD** test showed that a statistically high significant differences **(P<0.05)** among the groups and with three tested microorganism **(Table 5).**

Table 3: Mean of inhibitory zone size, categories & standard deviation for each groups and their subgroups.

Group	Supgroup	Mean	Zone Categories	SD
Control	*E. feacalis*	0.00	No inhibition	0.00
	E. coli	0.00	No inhibition	0.00
	Candida albicans	0.00	No inhibition	0.00
CHX	*E. feacalis*	18.2	Medium strong	0.63
	E. coli	17.2	Medium strong	0.37
	Candida albicans	13.4	Medium strong	0.51
Iodine	*E. feacalis*	17.3	Medium strong	0.49
	E. coli	13.2	Medium strong	0.5
	Candida albicans	14.8	Medium strong	0.5
Tetra.	*E. feacalis*	9.1	Medium	0.42
	E. coli	12.6	Medium strong	0.48
	Candida albicans	11.1	Medium strong	0.69
EDTA	*E. feacalis*	5.8	Weak	0.23
	E. coli	11.4	Medium strong	0.37
	Candida albicans	12.3	Medium Medium	0.32
FC+ ZOE	*E. feacalis*	10.2	Medium	0.27
	E. coli	25.8	Medium strong	1.53
	Candida albicans	27.6	Medium strong	0.38

Table 4: One way ANOVA test among the six groups and for each subgroup.

Subgroup	F- value	P -value
E. feacalis	60.36	0.000
E. coli	93.61	0.000
Candida albicans	81.95	0.000

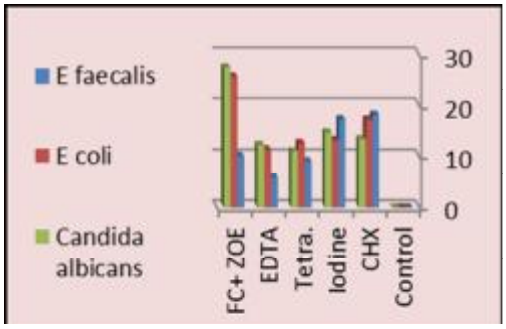

Figure 4: Bar chart shows the differences in mean of inhibitory zone size according to types of disinfection solution used in the study.

Table 5: LSD test among the groups and for each tested microorganism.

Groups	*P. value*		
	E. feacalis	***E. coli***	***Candida albicans***
Control **vs.** CHX, Iodine, Tetra., EDTA and FC+ZOE			
CHX **vs.** Iodine, Tetra., EDTA and FC+ ZOE	**0.000**	**0.000**	**0.000**
Iodine **vs**. Tetra, EDTA and FC+ZOE.	**HS**	**HS**	**HS**
Tetra. **vs.** EDTA and FC+ ZOE.			
EDTA **vs.** FC+ZOE			

Significant at the 0.05 level.

DISCUSSION

The major causes of pulpal and periapical diseases are living and nonliving irritants. The nonliving group includes mechanical, thermal and chemical irritants. The living irritants include various microorganisms including bacteria, yeasts and viruses. When pathological changes occur in the dental pulp, the root canal space acquires the ability to harbor various species of bacteria, along with their toxins and by products.[8]

Two of the main goals of endodontic therapy are: the elimination of microorganisms from the root canal system and the prevention of subsequent reinfection. The most common types of root canal filling materials used were gutta percha. Commercially available gpc come in pre-sterilized packages. However, some studies have shown that 5-8% of the cones from sealed packages can be contaminated with bacteria [5-10]. Also, gpc can be contaminated by handling, when exposed to the dental operatory environment and during storage [11]. So for preventing cross-contamination of the root canal during endodontic treatment, it has been recommended that gutta-percha cones be sterilized prior to obturation.

Gutta-percha cones cannot be sterilized by conventional autoclaving; different chemicals have been suggested for use in decontamination of cones. In this study five types of solutions used for decontamination of gutta percha against three bacterial strains: ***E. faecalis, E. coli*** and ***candida albicans***; which are frequently isolated during routine endodontic treatment of an infected root or from teeth with periapical pathology.[8-12] In particular, *E. faecalis* is Gram-positive facultative anaerobic coccus that are a normal part of human intestinal flora. *E. faecalis* cells are ovoid in appearance and can grow in single cells and in chains. They have been implicated in persistent root canal infections included failed cases and has been used in several previous studies on the efficacy of endodontic irrigants, its characterized by high resistance to a wide range of antimicrobial agents and causes reinfection of root canal .[13-14] While *E. coli* is Gram-negative, facultative anaerobic and non-sporulating, rod-shaped cells.[15] *Candida albicans* is a diploid fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infections in humans.[16] In this study the results found that distill water showed no antimicrobial activity against *E. faecalis, E. coli* and *Candida albicans*, no inhibition of growth occurred with distill water **(control group)**. **(Table 3 & Fig 4).**

Chlorhexidine has broad spectrum of antimicrobial activity, kills vegetative bacteria by disrupting the membrane integrity and inducing the precipitation of the cytoplasm. **CHX** used either as irrigant solution or as an intracanal medication and it's found to be effective at 15 sec. to 2 hrs in direct contact with infected gpc[17]. Resent form of gutta percha matrix embedded with 5% chlorhexidine diacetate. Chlorhexidine gutta percha showed strong antimicrobial activity on *E.faecalis*.[10] **(Table 3)**

In this study 0.2 % chlorhexidine gluconate solution **(CHX)** used for measurement of **CHX** effect on bacteria by using antibiotic sensitivity test, the result found medium strong inhibition effect on the three tested microorganism. This result agree with **Gomes et al** [10] they found that chlorhexidine in liquid form was most effected in disinfection of gpc contaminated by (*E. faecalis, Streptococcus sanguis, aerobes Staphylococcus aureus and Candida albicans*) than gel form. Also they found that **CHX**, at all concentrations and presentation forms tested, was not effective in eliminating *Bacillus subtilis* spores on gpc, even after 72 hours of contact.

Iodine as antimicrobial agent showed growth inhibition lower than **CHX** and higher than tetracycline solution with higher effect on *E. Faecalis*, and lower effect on *E. coli* **(Table 3),** this agree with **Bodrumlu and Alaçam** [7]; they found that gpc impregnated with povidone–iodine inhibited all bacterial strains (*E. faecalis, Pseudomonas aeruginosa. Staph. aureus, E. coli and Candida albicans*) for up to 72 hours and when compared with regular gpc, no inhibition zones were seen around. Also they found that gpc medicated with tetracycline inhibited the growth

of all bacteria over 24 hours, but in some cases these effects did not continue over longer periods, specifically against ***E. coli*** and ***P. aeruginosa***, its disappeared by 48 and 72 hours.

Tetracycline, a broad spectrum antibiotic has been widely used in the treatment of various endodontic infections. The gpc impregnated with tetracycline acts as an antimicrobial reservoir that is capable of diffusing onto the surface of the gutta percha, thereby inhibiting colonization of bacteria on the gpc and within the root canals. The important fact is that tetracycline can become incorporated into calcified structures due to its ability to bind to mineralized dentinal matrices and its slow release from dentin makes its antimicrobial effect substantive. Moreover tetracycline is stable in an acid environment thereby making it effective in the inflamed area of the root canal periapex, where most alkaline type antimicrobials agents neutralize, this a distinct advantage for the tetracyclines[18]. In this study Tetracycline solution (250mg/ 5ml distill water) used as antimicrobial agent for rapid disinfection of gpc by immersion the cones in tetracycline solution for 5 min. then the cones incubated in agers containing the tested microorganisms and the results found, tetracycline has medium strong disinfection effect on *Candida albicans* and *E.coli* but medium effect on *E. faecalis*. **(Table 3)**. **Vijay & Shashikala** [19] found that tetracycline impregnated gpc showed the maximum antimicrobial efficacy on *E.faecalis* compared to chlorhexidine impregnated gutta percha when used as an intracanal medicament. But in this study the effect is lower than chlorhexidine solution; this may be related to lower concentration of tetracycline solution used.

EDTA is organic acid (ethylene diaminetetraacetic acid) which eliminates the mineral part of pulp tissue [4, 20]. It is advised to use of **EDTA** from the beginning of the preparation in order to eliminate the mineral layer before its thickening and condensing it inside the canal systems which will close the entrances of lateral and accessory canals and dentinal tubules, and facilitates the flow of the different irrigants such as NaOCl or chlorhexidine in the lateral canals permitting a chemical preparation of all the endodontic system.[21] **EDTA** showed lower growth inhibition compared with other tested group against all tested microorganism. Also, its only group that causes weak growth inhibition of *E. faecalis*, followed by *E. coli* and higher effect on *candida albicans*. **(Table 3).** In agreement with Fidalgo et al [22] found that 17% **EDTA** was more effective than 0.5% NaOCl against *Candida albicans* and *Staphylococcus aureus*

In group 6 **(FC+ ZOE)**, the mean of inhibiton zones showed that, it's the most efficient material in growth inhibition of *Candida albicans* and *E.coli* (medium strong effect) **(Table 3)**, but their effect on *E. faecalis* was medium. **Tchaou et al.** [9] used this mixture as root canal filling material and they tested its antimicrobial activity against 21 microbial species including anaerobic gram +ve, anaerobic gram -ve, facultative anaerobic gram +ve and facultative anaerobic gram -ve. They found that **FC+ZOE** mixture inhibited all tested strain. So using this mixture was highly effected on disinfection of tested bacteria but further investigation needed to measure its effect on physical properties of gutta percha and on sealer material, when used as rapid agent for decontamination of gutta perch.

However, in vitro tests such as the ones performed here can only indicate the potential of some materials to inhibit microbial growth and metabolism in the local microenvironment of the root canal; but further studies in vivo needed, with change of the experimental methods.

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