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In-Vivo Comparison of Antimicrobial Efficacy of ZincumOxydatum with Chlorhexidine: RTPCR Evaluation

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ABSTRACT
Endodontic infections are known to be multi-microbial and hence need broad-spectrum antimicrobial agents. Chlorhexidine is well established as an antimicrobial agent but has drawbacks of cross reactivity. Thus, a new alternative like ZincumOxydatum a Homeopathic medicine has been tested for its antimicrobial property. The aim was to compare the antimicrobial efficacy of ZincumOxydatum with Chlorhexidine in the IN-Vivo conditions using the RTPCR technique. 40 patients were selected on the bases of inclusion criteria. Informed consent was taken and assigned into 2 groups based on the medicaments used. Root canal therapy was performed on the indicated tooth. Preoperative samples for bacterial load were collected using paper points, cleaning and shaping was done followed by placement of medicaments inside the root canal for 7 days. Post-operative samples were then collected after 7 days and root canal treatment was completed. Both the preoperative and post-operative samples were subjected to the DNA isolation procedure using HipurA®Multisample DNA purification kit (HiMedia Laboratories Pvt. Ltd. Mumbai, India) followed by PCR amplification using 16s rRNA gene sequence and SYBR green markerHi- SYBr Master Mix (with Taq Polymerase) kit (HiMedia Laboratories Pvt. Ltd. Mumbai, India). The results were obtained in terms of cycle threshold (Ct) values. Statistical analysis showed that Zincumoxydatum(30.2160± 0.02437) and (30.4385± 0.03117) respectively; had better efficacy for the decrease in the bacterial load as compared to the chlorhexidine group (30.1010± 0.01210) and (30.1500± 0.01974) respectively. It was concluded that the homeopathic medicine ZincumOxydatum can be used as an endodontic medicament.
KEYWORDS: *Endodontic medicaments, Chlorhexidine, Homeopathy, ZincumOxydatum, Real-Time Polymerase Chain Reaction*

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INTRODUCTION

Multiple species of organisms along with the surface topography of the pulpal chamber make endodontic infection unique [1]. The endodontic treatments aim in overcoming these with the use of mechanical instruments to clean and shape the canal space. They are accompanied by the use of endodontic irrigants to achieve the goal [2]. But even with the use of these aids the complete disinfection of the pulpal space is not possible [3]. Especially in cases where the infection has reached the periapex or in chronic cases, it is not possible to completely disinfect the canals with mechanical cleaning and irrigation alone. The organisms are surviving in the infected canals by multiple factors and of all; biofilm formation is the most prominent feature of their survival [4].

An endodontic medicament is a drug of choice that serves the purpose of complete disinfection and renders the canals free of microbes for the obturation [5]. The medicament should be non-irritant, broad spectrum, and inert in association with the other materials used during the cleaning and shaping procedure [6]. currently chlorhexidine has been the most widely accepted and used endodontic medicament [7]. It is used both as gel and liquid in 2% concentration [8]. Its use is advocated owing to its property of being long-term substantivity and the broad spectrum of activity [9]. It works both on gram-negative and gram-positive microbes. [10] It acts by attacking the microbial cytoplasm [11].

Although chlorhexidine is a potent antimicrobial agent it does have drug reaction complications [12]. It reacts with sodium hypochlorite; a potent endodontic irrigant to yield unwanted product para-chlorophphenyl isocyanate. This, later on, degrades slowly to para-chloro-aniline (PCA). [13] The main effect of PCA is cyanosis [14]. Also the formation of precipitate occludes the dentinal tubules leading to potential bond failure between the sealer and the canal wall [15].

Many others drugs both natural the laboratory-made; have been used to replace chlorhexidine. Out of all the most untouched are the homeopathic drugs. Homeopathy is a field of medicine widely accepted to be the second largest field by the World Health Organization [16]. It works on the "Stimulation Therapy" concept [17]. Many homeopathic drugs are well known to be potential antimicrobial agents [18]. Among all ZincumOxyadtum is a potent agent which contains nanoparticles of Zinc Oxide [19]. Zincumoxidatum has been found effective against infections of *Staphylococcus aureus*, *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* origin [20]. ZincumOxydatum is a drug manufactured from Zinc Oxide. Zinc has been widely used since ancient times by Egyptians and Romans and is well considered the "calcium of the twenty-first century" [21]. It works by oxidative stress-induced cell lysis and also cell permeability is altered causing lysis [22].

Antimicrobial property is best analyzed by the process that involves the analysis of both the dead and vital organisms after the drug action.[23] Real-time reverse transcription–polymerase chain reaction (RT-PCR) is an efficient tool that is rapid and sensitive [24]. It calibrates the target molecule during the amplification process rather than at the end [25].

Thus the study aimed to compare the antimicrobial efficacy of ZincumOxydatum with chlorhexidine when used as an intracanal endodontic medicament in invivo conditions using the RTPCR technique.

MATERIAL AND METHODS

The current single-blinded study was approved by the ethical committee of the SGT Dental College, SGT University, Haryana, and carried out at the Department of Conservative Dentistry and Endodontics in association with the Department of Oral Pathology and Microbiology at Himachal Institute of Dental Sciences, Paonta Sahib.

40 Patients who had open carious lesions with exposure to the pulp clinically and or inter radicular/periapical radiolucencies in radiograph and patients with no history of previous endodontic treatment in the selected tooth were elected for the research after obtaining informed consent.

Rubber dam isolation was done and access opening was carried out using slow-speed round bur. The establishment of working length was done using an apex locator (Root ZX; J Morita, Japan) and reconfirmed using a Radio Visio Graphy (RVG 5200, Carestream Dental, Japan). Initial cleaning and shaping was carried to ISO size 25 Hand K file with 2ml of saline as irrigant. 5 paper points were then placed consecutively inside the canals for 10 seconds each to collect the pre-op sample (S1). The paper point was transferred immediately to vials containing 1ml of Tris EDTA buffer.

Cleaning and shaping was then completed till F3 using the Protaper Universal system using the modified crown down technique. Endodontic irrigant 5ml of 3% Sodium Hypochlorite (CanalPro, coltenewhaledent Pvt. Ltd., India) was used to flush the canals during the cleaning and shaping process. Smear layer removal was done with 2ml of 17% EDTA (PyraxPolymars, India) for 2 min. The patients were then divided based on the endodontic medicaments used.

Group I - 2% Chlorhexidine Gluconate (Dentochlor, Ammdent, India)

Group II - ZincumOxydatum (200 CH, Dr. WillmerSchwabe India Pvt. Ltd.)

For all the groups the treated canals were rinsed with 2ml of the tested drug solution and the access cavity was covered with a cotton pellet and sealed with Cavit G (3M™ Cavit™G, 3M India Ltd, India) a temporary restorative material.

All the subjects were recalled after 7 days of the time interval for re-evaluation. A post-operative second sample S2 was obtained similarly. This was followed by obturation using Sealapex; a calcium hydroxide-based resin sealer and gutta-percha points and permanent post-endodontic restoration by dental composite.

For all the samples collected DNA isolation was carried out and a Reverse transcription-polymerase chain reaction (RT-**PCR**) procedure was carried out. The results were obtained in terms of cycle threshold (Ct) values.

HipurA®Multisample DNA purification kit (HiMedia Laboratories Pvt. Ltd. Mumbai, India) was used for DNA extraction.[26] 1.5 ml of collected sample was taken in a 2.0 ml capped collection tube. The sample was subjected to 2 min centrifugation at 12000g. After discarding the supernatant pellet was resuspended in 200µl of lysozyme solution and incubated at 37°C for 30 minutes. The addition of 20µl of proteinase K solution (20 mg/ml) was done to the sample followed by the Addition of 20µl of Rnase A mix and incubated for 5 min at room temperature. This was followed by the addition of 200µl of lysis solution.

Vortexing of the mix was done and incubated at 55°C for 10 min. lastly, 200µl of ethanol (96-100%) was added to the sample. Lysate obtained was loaded in Hi elute Mini prep spin columns & centrifuge at 10000 rpm for 1 min. The liquid was discarded through the flow. 100µl of the elution buffer was directly pipetted onto the column without spilling to the sides and incubated for room temperature and centrifuged at 10000rpm for 1 minute to elute the DNA.

After 48 hours of incubation PCR was carried out using a thermal cycler (BIO-RAD CFX96™ Real-Time System) with SYBR greenHi- SYBr Master Mix (with Taq Polymerase) kit (HiMedia Laboratories Pvt. Ltd. Mumbai, India) [27].16S rRNA gene sequence-based nested species-specific Primers (forward primer **5'CGCTTCTTTCTCCCGAGT3'** and reverse primer **5' GCCATGCGGCATAAACTG 3'**) (Eurofins Genomics, India) were used [18]. thermocycling was carried out in a DNA thermocycler (C1000 Touch TM Thermal Cycler, BIO-RAD) with one cycle of Initial denaturation done at 94 or 95°C for 5-10 mins followed by a Denaturation cycle at 94°C for 10 seconds. 40 cycles of Annealing were performed at 55 to 60°C for 45 seconds each. An Extension (Plate Read) cycle was performed at 72°C for 30 seconds. For final analysis consecutive cycles were performed at:

- 95°C : 15 seconds
- 60°C : 1 minute
- 95°C : 15 seconds
- Increment : 0.5°
- On Hold : 10 seconds

RESULTS

Paired t-test was used for comparison. The analysis showed that there was a statistically significant difference in pre-and-post values of chlorhexidine (30.1010± 0.01210) and (30.1500± 0.01974) respectively (Table 1) (P<0.001) and zincumoxydatum (30.2160± 0.02437) and (30.4385± 0.03117) respectively (P<0.001).

Table 1: Pre-and-post values of chlorhexidine and zincumoxydatum respectively

	Mean	N	Std. Deviation	P value
Chlorhexidine (pre)	30.1010	20	.01210	<0.001*
Chlorhexidine (post)	30.1500	20	.01974	
ZincumOxydatum (pre)	30.2160	20	.02437	<0.001*
ZincumOxydatum (post)	30.4385	20	.03117	

Comparison of pre-operative and post-operative value using Paired t-test *Statistically significant (P<0.05, Paired t-test)

DISCUSSION

The endodontic procedure comprises complete disinfection of root canal space followed by proper sealing. This is carried out with mechanical instruments to clean and shape along with the use of chemicals to flush the canal debris and or infected pulpal remnants [28]. It has been proven that even with a variety of available instrument techniques and irrigant solutions these procedures are insufficient due to anatomical complexities and limitations in access by therapeutic agents [29]. Thus the use of an endodontic medicament becomes an invariably important and unavoidable step.

Endodontic medicaments are required to be germicidal, non-irritant to vital tissues, stable in solution, active in presence of body fluids, and non-staining [30]. Of all the Endodontic medicaments available chlorhexidine has been established as a potent antimicrobial agent and is widely used because of its properties like substantivity and wide spectrum of action [31]. But it still possesses drawbacks of cross-reaction, especially with Sodium Hypochlorite leading to the formation of chlorophenyl isocyanate para-chloro-aniline; a carcinogenic product [13].

Homeopathic medicines are products that are obtained from consecutive dilution of a substrate in alcohol or distilled water. This is termed potentisation [32]. As the substances are diluted they trigger the body's natural system of healing. Immunization is based on the law of similarity, thus induces the same symptoms in a healthy person [33]. In dentistry homeopathic medicines have been proven to treat oral abscesses and gingivitis [34].

In the present study both the dugs proved to be an efficient antimicrobial agent with a marked fall in the bacterial count. Tested drug ZincumOxydatum performance can be explained on the bases of its enhanced ability to penetrate biofilm through Extracellular polymeric substances and water channels [35]. Cellular interaction leads to local stress rupture. Nanoparticles of zinc oxide are hydrophobically leading to better

penetration in complex structures and enhanced morphological interactions [36]. Liberation of highly reactive free radicals of oxygen species [37].

The antimicrobial efficacy of chlorhexidine is based on cell rupture ability. Its positively charged molecules interact with negatively charged phosphate ions of the cell membrane causing lysis. chlorhexidine also leads to the precipitation of the cytoplasmic components resulting in cell death.[38]

Chlorhexidine showed a marked drop in the microbial count but when compared with the Zincum Oxydatum group the Values were statistically lower. The results can be explained on the bases as chlorhexidine has been proven to be an excellent membrane-active agent but not effective in germination and on bacterial spores.[39] Apart from this chlorhexidine act by adsorption to the cell membrane by electrostatic interactions.[40] But, it cannot transpose into the molecules composing the biofilm outer matrix to reach the bacterial wall.[41]

The present study concludes that the alternative therapies of medicine do have a scope and can be used as an effective tool in improving patients' health and reducing the complexities of the treatment. The results have also favored the use of homeopathic medicine ZincumOxydatum as an antimicrobial agent and support its use as endodontic intracanal medicament.

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