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RESEARCH ARTICLE

COMPARATIVE EVALUATION OF THE ANTIFUNGAL ACTIVITY OF MINERAL TRIOXIDE AGGREGATE, CALCIUM ENRICHED MIXTURE AND BIODENTINE BY MODIFIED DIRECT CONTACT TEST – AN IN VITRO STUDY

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ABSTRACT

Objective: To evaluate and compare the antifungal efficacy of Mineral trioxide aggregate (MTA), Calcium enriched mixture (CEM) & Biodentine against Candida albicans by modified direct contact test (DCT) at 1, 3 and 7day intervals.
Study design: The study materials selected were MTA (Group1), CEM (Group 2) and Biodentine (Group 3). The cements were mixed in strict compliance with the manufacturer's recommendations. The modified DCT performed was based on turbidometric determination of fungal growth in 96-well microtiter plates. Sidewalls of the microtiter plate wells were coated with freshly mixed test material and a 10 µL fungal suspension was placed. After 1 hr of incubation at 37oC, Sabouraud's dextrose agar (SDA) (245 µL) was added. These were designated as 'Row A' wells. 15 µL was then transferred from row A wells to an adjacent set of 4 wells containing fresh SDA medium (215 µL), designated as 'Row B' wells. Fungal outgrowth was monitored both in the presence (Row A wells) and in the absence (Row B wells) of the test materials. The plates were then placed in the spectrophotometer with optical density measured at 630 nm. The readings were taken hourly for 15 hours on the 1st day, 3rd day and the 7th day. Experiments were triplicated. Data was analysed using the Friedman's test.
Conclusion: All the cements showed antifungal activity against C.albicans on 1st day, which gradually declined over a period of 7 days. MTA exhibited the most efficient antifungal activity as compared to Biodentine and CEM over a period of 7 days.

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INTRODUCTION

Microbes and microbial products are the main etiologic factors of pulpitis and apical periodontitis. The microbial ecosystem of infected root canals may consist of bacteria, including spirochetes and fungi and can rightly be considered a privilegedsanctuary for clusters off bacteria, bacterial by products and degradation products of both microorganisms and pulpal tissue. Fungi have been observed both in primary and refractory endodontic infections (Nair *et al*., 1990; Sen *et al*., 1995; Sundqvist *et al*., 1998; Waltimo *et al*., 1997) with an incidence varying between 7 and 55%.

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The genus Candida is comprised of 150 fungal species, *C. albicans* being the most encountered commensal and pathological yeast in the oral cavity (Fotos and Hellstein, 1992). However, the number of yeast cells in the root canal is usually lower than bacteria (Ruff *et al*., 2006). Also, it has been demonstrated that Candida species are resistant to some medications commonly used in endodontics, including calcium hydroxide mixed with inert vehicles. The presence of *Candida albicans* at the time of obturation can significantly reduce the success rate of root canal therapy. Endodontic therapy aims at complete elimination of microorganisms from the root canal. Instrumentation, irrigation and intracanal medication significantly reduces the number of microorganisms inside the infected root canal (Valera *et al*., 2001). However, it is impossible to completely eliminate the microbes from the root canal system. Consequently, the use of root canal filling

materials with antimicrobial activity is considered to be beneficial in an effort to further reduce the number of remaining microorganisms and to eradicate the infection.

Mineral Trioxide Aggregate (MTA) has emerged as a reliable bioactive material In retreatment, root end resection, internal resorption and in conventional endodontic therapy.However, despite its good biocompatibility and sealing abilitydisadvantages associated with it include its poor handling characteristicsand high cost (Bogen, 2009). Biodentine ™ (Septodont, France) been promoted as a dentin substitute material in the treatment of perforations ofroot canal, floor of pulp chamber, for apexification, and as a retrograde root canal filling material (Colon *et al*., 2010). Recently, abioactive calcium based cement, calcium enriched mixture (CEM) (Bionique Dent, Tehran, Iran) has been introduced.It releases calcium and phosphate ions, forming hydroxyapatite in simulated body tissue fluid and in normal saline (AminiGhazvini *et al*., 2009).

Thus if the root canal filling material can offer additional properties that decrease the bacterial survival and promote bioactive mechanisms necessary for regeneration and healing then some of the ideal requirements of the filling material might be viewed less important when the distinct advantages are considered. It is postulated that the antifungal activity of root canal filling materials on the microorganisms may help eliminate residual microorganisms unaffected by effects of both chemo-mechanical preparation and intra canal medicaments. Also, there is little information on the antifungal activity of Biodentine and CEM cement with no comparative study evaluating the antifungal activity of MTA, CEM and Biodentine. Therefore the aim of the study was a comparative evaluation of the antifungal activity of Mineral trioxide aggregate, Calcium enriched mixture & Biodentine by modified direct contact test.

MATERIALS AND METHODOLOGY

This *in vitro* study was conducted in the KLE's Dr. Prabhakar Kore Hospital and Research Centre, Belagavi, with the objective of evaluating the antifungal efficacy of biomimetic materials against *Candida albicans* using the modified direct contact test. Direct contact test is based on determining the turbidity of microbial growth in 96 well microplates. Facultative strain of Candida albicans (ATCC 90028) was grown on Sabourauds Dextrose agar. Microorganisms were sub cultured in appropriate culture media and under gaseous conditions to confirm their purity. Facultative strain was then inoculated individually into tube containing 5ml of sterile saline. The suspension was adjusted to 0.5 McFarland scale =1.5x108 C.F.U spectrophotometrically at 630nm. A 96-well microtiter plate was held vertically, and an area of fixed size on the sidewall of the six wells was coated with an equal amount of each material by using a cavity liner applicator. The materials were mixed in strict compliance with the manufacturers' recommendations.

- Five experimental groups were divided into:
- Group 1 - 6 wells coated with MTA
- Group 2 - 6 wells coated with CEM
- Group 3 - 6 wells coated with BIODENTINE
- Group 4 - 6 wells containing only Candida (positive control)
- Group 5 - 6 wells containing only CEMENT (negative control)

A 10 µL (approx. 10^7) fungal suspension was placed in the coated wells (Row A). After incubation for 1 h in humidity at 37^0 C,the suspension liquid evaporated, ensuring direct contact between Candida and surface of test materials.

Sabaoraud's infusion broth (245 µl) was added to each of the wells and the plates were gently vortex mixed for 2 min.15 µl of ensuing fungal suspension was then transferred from wells into an adjacent set of wells (Row B) containing fresh medium (215 µl) and again mixed for 2 min. The kinetics of fungal outgrowth in each well of row A and B was then measured at 630 nm using a micro plate spectrophotometer (Stat Fax 2100,Awareness Technology INC, 630 nm). Densitometric readings were done hourly for 15 hours and with each set of samples. Experiments were triplicated. Hence sample size for each material was 18 as the experiment was triplicated (n=6*3=18).Similar experimental procedure was carried out in which the tested material was allowed to age for 1,3 and 7 days in phosphate buffered saline.

RESULTS

Biodentine

MTA showed sustained antifungal activity, followed by Biodentine and CEM cements. As the cements were allowed to age for 7 days, the antifungal activity decreased with MTA, it was not statistically significant. CEM and Biodentine showed a statistically significant difference in OD values when comparisons were made on the 1st day and 7th day and the freshly mixed cements of MTA and CEM showed similar antifungal activity and better ability to inhibit the growth of C.Albicans when compared to Biodentine.

DISCUSSION

Endodontic infections are known to be polymicrobial in nature with preponderance toward anaerobic species (Baumgartner *et al*., 2006). Kakehashi *et al*. (1965) in a classic study proved that bacteria caused pulpal disease; however, numerous studies have revealed a possible role of fungi and more recently viruses in the incidence of endodontic infections.

C. Albicans are predominant in persistent or refractory endodontic infections. *Candida albicans* have been considered a dentinophilic microorganism because of its invasive affinity to dentin. Within dentinal tubules the *Candida albicans* is protected from the lethal action of endodontic medications because of the inactivating effects of dentin. It has been demonstrated that *C.albicans* is able to invade dentinal tubules to variable extents (Siquera *et al*., 2003). Because of these unique qualities, Candida, which is an aerobic microorganism, is able to survive in the harsh ecologic environment of the root canal, which primarily favors the growth of anaerobes (Eidelman *et al*., 1978; Sen *et al*., 1997; Heinic *et al*., 1992).

Table 1. Comparison of optical density evaluated at different time intervals in each of the study groups using Friedman test

DAYS	✧ MTA	✧ CEM	✧ BIODENTINE
Day 1	.162(.037)	.160(.114)	.646(.303)
Day 3	.196(.093)	.113(.037)	.590(.126)
Day 7	.225(.088)	.328(.148)	.747(.117)
Chi square Value (df)	4.750(2)	30.33(2)	9.25(2)
p-value	0.093(NS)	<0.001*	0.01*

✧#Friedman test
✧* p-value <0.05 statistically significant.

Table 2. Comparision of antifungal activity of freshly mixed MTA, CEM and Biodentine

	FRESHLY MIXED MTA	FRESHLY MIXED CEM	FRESHLY MIXED BIODENTINE
ANTIFUNGAL ACTIVITY	+++	+++	++

Elimination of microorganisms from the root canal has always been an important part of endodontic therapy. Chemomechanical preparation, irrigation and intracanal medication may reduce significantly the number of microorganisms in an infected root canal (Cobankara *et al*., 2004). Love *et al* reported the presence of microorganisms in areas such as isthmuses, ramifications, deltas, irregularities and dentinal tubules even after thorough chemomechanical preparation of the root canal system. It has also been postulated by Bystrom and Sjogren *et al* that if these microorganisms persist in the root canal at the time of root filling or if they penetrate into the canal after filling, there is a higher risk that the treatment will fail (Sjögren *et al*., 1997; Bystrom *et al*., 1987). Because of the increase in incidence of oral fungal infections and particularly the reported detection of fungi in infected root canals, a considerable interest has been generated regarding the antifungal activity of root canal filling material in endodontic infection. Fundamental research catalyzed the evolution of new dental materials, which could not be envisioned in the past. The grey and white variations of both MTA Angelus and ProRoot MTA have been tested and found to be similar in most characteristics. The cementogenic activity of MTA is because of its release of an abundance of calcium ions, which interact with phosphate groups in the surrounding tissue fluid to form hydroxyapatite on its surface (Sarkar *et al*., 2005). A new bioactive material, which was extensively studied, as "new experimental cement (NEC) consisting of different calcium compounds was later termed as Calcium Enriched Mixture (CEM) by its developers. This material can be handled and sets in an aqueous environment (Asgary *et al*., 2008).Clinical uses of CEM cement are therefore similar to that of MTA. It has a setting time of less than 1 hour, more flow and less film thickness compared to MTA (Asgary *et al*., 2009).Due to better handling properties and less time for setting Calcium Enriched Mixture (CEM) can be handled and sets in an aqueous environment (Asgary *et al*., 2008). Over the years Agar Diffusion Test (ADT) has been used to evaluate the antimicrobial activity of materials. The Direct Contact Test (DCT) has many advantages over Agar Diffusion Test (ADT) and has been studied previously by Weiss *et al* and Fuss *et al* (1996)(Fuss *et al*., 1997). It is a quantitative assay, which allows water insoluble materials to be tested. It relies on direct and close contact between the test microorganism and the tested material and is virtually independent of the diffusion properties of both the tested material and the media.

In addition to its reproducible and quantitative nature, the results of DCT unlike those of the Agar diffusion test (ADT) are not affected by the size of the inoculum and are relatively insensitive to the size of the inoculum brought in contact with the tested material. It facilitates standardized measurements of a large number of specimens and their respective control simultaneously on the same microtitre plate and has the ability to monitor the bacterial growth, both in the presence and in the absence of the tested material. Hence, Direct contact test was used to evaluate the antifungal efficacy. It is essential to test the materials immediately after mixing and also after a period of time when it has assumed its final chemical structure due to release of various transitory and permanent products. The difference in antimicrobial patterns of various materials is related to the degree of setting. Hence the antifungal efficacy was evaluated at 1st, 3rd and 7th day after mixing the cements. Thus, the objective of this *invitro* study was to evaluate and compare the antifungal activity of Mineral trioxide aggregate, Calcium enriched mixture & Biodentine by modified direct contact test at 1, 3 and 7-day intervals.

Yeasts can be best isolated from endodontic samples by using selective media for the primary isolation. Yeasts tolerate a much wider pH range than bacteria, and therefore several selective media are available for the isolation of yeasts. Sabouraud agar is a commonly used medium for the isolation of oral yeasts. The pH of the medium is quite acidic (usually 5.6), allowing the growth of yeasts and aciduric organisms, whereas most bacteria are inhibited. The pH can be further reduced by hydrochloric acid to 3.0–4.0 to prevent the growth of aciduric bacteria. However, it is important that as much of the undiluted sample is plated as possible to ensure detection of yeasts in cases where their total CFU is low (25). Sabouraud dextrose agar medium was preferred in this study as it is nutritious and well buffered to support the growth of wide variety of fungus including *Candida albicans.* The endodontic root canal filling materials have shown to give the greatest antimicrobial effects immediately after spatulation, following which there is a gradual loss of antibacterial effect at a greater or lesser speed over time (Fuss *et al*., 1997; Kaplan *et al*., 1999; Cobankara *et al*., 2004). Because of release of various transitory and permanent products, it is essential to test the materials immediately after mixing and also after a period of time when it is assumed that it has reached its final chemical structure. Root-canal bioactive materials are inserted into the

mouth in a freshly mixed, incompletely set stage and thus it is likely that during this period after clinical application of the material, local responses are provoked by components only partially reacted or unreacted. After the setting period, it is still possible that toxic ingredients may release from the materials. The difference in antimicrobial patterns of various materials also may be related to the degree of setting. Hence the antifungal efficacy was evaluated at 1st, 3rd and 7th day after mixing the cements.

All the cements MTA, Biodentine and CEM showed antifungal activity against C. Albicans with MTA cement showing sustained antifungal activity. Similarly, Stow *et al*. reported that the most antimicrobial activity of MTA was against C. Albicans. The sustained antifungal activity of MTA can be attributed to the Calcium hydroxide, produced by a hydration reaction from the mixing of MTA with water, can inactivate cell membrane enzymes in micro organisms thereby inhibiting their biological activity. Many researchers reported that *C. Albicans* is highly resistant to calcium hydroxide *in-vitro* while others have shown that saturated solution of calcium hydroxide can effectively reduce the presence of *Candida* after a 3-min incubation and completely eliminate them from bovine dentin after 7 days (Waltimo *et al*., 1999); concurring with the results of the present study. CEM cement consists of alkaline earth metal oxides/hydroxides (*e.g.* calcium oxide and calcium hydroxide), calcium phosphate and calcium silicate (Asgary *et al*., 2008). Calcium hydroxide is present in the material itself as well as produced through the hydration reaction during and after mixing. When CEM cement is placed within the wells in contact with the medium, calcium hydroxide dissociates into Ca^{2+} and OH^- and causes an increase of pH. These mechanisms may partially explain the antifungal activity of CEM cement, similar to MTA. An alternative explanation may be that the antibacterial/antifungal components of CEM cement have ample diffusion properties (Asgary *et al*., 2007; Asgary and Kamrani, 2008). The effective antifungal activity of CEM cement; comparable with MTA, indicates the good antibacterial potential of CEM cement.

Laurent and associates (2008) found Biodentine to be biocompatible after evaluating its genotoxicity, cytotoxicity, and effects on the target cell specific functions (Laurent *et al*., 2008). As Biodentine is recently introduced the antimicrobial activity has not been reported. In this study the antimicrobial efficacy of Biodentine can be assumed to be the result of hydration of tricalcium silicate resulting in formation of colloidal gel and release of calcium hydroxide. Biodentine shares both its indications and mode of action with calcium hydroxide, but does not have its disadvantages. Three major disadvantages of calcium hydroxide, higher material resorption rate, mechanical instability, and failure to prevent microleakages are therefore avoided. Biodentine is mechanically stronger, less soluble, and gives a tighter seal. Compared to other materials such as MTA, Biodentine handles easily and needs much less time for setting (Nowicka *et al*., 2013). Hence, in this study, Biodentine had a significantly pronounced antibacterial effect. Within the limitations of this in vitro study, based on the results the null hypothesis was rejected and the following conclusions were drawn All the test materials exhibited antifungal activity against *C. albicans*, but to a varying degree. MTA was the most effective as compared to CEM and Biodentine at the end of 7 days. The antifungal efficacy decreased over 3rd and 7th day. Antifungal activity of tested endodontic cements against *C.albicans* in an ascending order was as follows CEM< Biodentine< MTA.

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