Comparative Evaluation of Physical and Antimicrobial Properties of Doxycycline Incorporated Formulation of Mineral Trioxide Aggregate - An In-Vitro Study

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Abstract

Doxycycline is a hydroxyl derivative of tetracycline that has been used as an adjuvant in irrigants, intracanal medicaments. Complete eradication of the causative factors and a sterile environment are essential for assisting in a better prognosis of either apexification or revascularisation method of management. Improving antimicrobial properties can enhance the healing ability thereby faster completion of endodontic treatment with minimal recurrence.

Aim to assess the physical and antimicrobial properties of a doxycycline incorporated formulation of mineral trioxide aggregate.

The antimicrobial properties, compressive strength and final setting time were tested for 4 groups of materials. Group 1 - Mineral Trioxide aggregate - White MTA (Angelus, Londrina, PR, Brazil); Group 2 - Biodentine (Septodont, Saint Maur des Fossés, France); Group 3 - Newer MTA formulation with Doxycycline in powder; and Group 4 - Newer MTA formulation with Doxycycline in liquid.

Material in group 4 had the largest zone of inhibition against E.faecalis while group 3 had the largest zone of inhibition against S.mutans and C.albicans. This difference was found to be statistically significant ($p < 0.05$). Group 2 had the shortest setting time, followed by group 3 and group 4. The highest mean compressive strength was seen in group 2 followed by group 3 and 4 and the least in group 1 for all the time periods.

Under the limitations of the present study, addition of doxycycline, either in the liquid or powder component, can have additive effects on the antimicrobial properties of the modified MTA with minimal to no alteration in its physical properties.

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Introduction

Anaerobic microorganisms play a significant role in cases of non-resolving persistent periapical radiolucencies, which are generally referred to as endodontic failures.[1,2] Necrotic and infected pulp provides a selective habitat for various microorganisms among which anaerobes take up the major part.[3] Immature permanent teeth with pulpal infection, either due to trauma or dental caries, can lead to impairment in the remaining root formation.[4]

Complete eradication of the causative factors and a sterile environment are essential for

assisting in a better prognosis of either apexification or revascularisation method of management.[5]

Such environment can be achieved by proper instrumentation, irrigants, intracanal medicaments and also by the agents used to form calcific barrier in the aforementioned treatment strategies.[6] For decades those agents would be calcium hydroxide, mineral trioxide aggregate or biodentine. Such agents have been periodically tested for its antimicrobial efficacy against the common endodontic pathogens. Previous studies have suggested a nil to minimal amount of antimicrobial activity against anaerobes which were slightly improved by recent modifications.[7,8]

Agents like chlorhexidine gluconate, nitric oxide-releasing compound, fluorohydroxyapatite, zeolite and calcium fluoride were suggested by various authors to increase the antimicrobial activity.[9–13]

There were varied results with integration of such compounds in regards to the physical or the antimicrobial properties. Doxycycline is a hydroxyl derivative of tetracycline that have been used as adjuvant in irrigants, intracanal medicaments over the past decade.[14] On literature search, only one study has been documented to utilise doxycycline along with biodentine and the authors concluded that doxycycline did not potentially increase the antimicrobial activity of the final set cement.[15]

Also a recent study has suggested excluding tricalcium aluminate in MTA due to its cytotoxic nature.[16] A recent study by the authors involved removal of tricalcium aluminate in a modified MTA cement had showed improved compressive strength and better antimicrobial properties [17].

This study's primary objective was to modify the composition of the conventional MTA with the addition of doxycycline either in powder form or liquid form. The secondary objective was to check whether this modification has had any effects on the physical properties of the newly reformulated cement.

Materials and methods

This in-vitro study was conducted at the scientific materials research facility of a private dental institute. The design of the study, experiments done and the composition of the materials used were approved by the members of the institutional ethical committee (Ethical approval number: SRB/SDC/PhD/Pedo/2022/045).

PREPARATION OF TEST MATERIALS

Two commercially available bioactive bioceramics and two newly formulated modified MTA were used in the present study.

The commercially available materials used in the present study were:

Group 1: Mineral Trioxide aggregate was obtained from Angelus (Londrina, PR, Brazil). It is packed as powder-liquid formulation. As recommended by the manufacturer, a 3:1 powder:liquid ratio was dispensed on a pad. The powder was completely hydrated by the liquid until the mix turned to be thick, similar to putty-like consistency. The completely mixed material was then carried using an MTA carrier to the desired experimental design.

Group 2: Biodentine was obtained from Septodont (Saint Maur des Fossés, France). It is also available as a powder-liquid packaging. As recommended by the manufacturer, five drops of the liquid were poured into the capsule containing the powder and mixed using a mechanical triturator (Dentsply Maillefer) for roughly 30 seconds. Once the mix is complete, it would have a thick consistency similar to putty. The material was then carried using a plastic instrument to the desired experimental design.

The newly formulated modified MTA used in the present study were:

Group 3: Newer MTA formulation with Doxycycline in powder - The newly formulated composition of MTA include tricalcium silicate, dicalcium silicate, calcium carbonate, calcium sulphate and calcium fluoride as the base powder components. The core components i.e. tricalcium silicate and dicalcium silicate were manufactured in the lab based on the manufacturing process suggested by Moon HJ et al.[16] Other powder components were procured from TCI Chemicals (India) Pvt. Ltd. Doxycycline hyclate and Calcium chloride were procured in powder form from TCI Chemicals (India) Pvt. Ltd. Doxycycline was added in powder form. Calcium chloride was mixed with 1 ml distilled water to obtain a 20% concentration that comprised the liquid component. The composition is given in Table 1. A 100mg of the proposed powder content and 40µl of the liquid component was dispensed on a pad. After complete hydration of

the powder with the liquid, the mixing procedure was continued until a uniform mix with a moldable consistency was obtained. The material was then carried using a plastic instrument to the desired experimental design.

Group 4: Newer MTA formulation with Doxycycline in liquid - The powder component was similar to the composition as provided in group 3, but without doxycycline. The obtained Doxycycline was added to 1 ml distilled water separately to obtain a 5% concentration. Calcium chloride was mixed with 1 ml distilled water to obtain a 20% concentration. Both the liquids were mixed until a uniform mixture was obtained. The composition is given in Table 1. A 100mg of the proposed powder content and 40µl of the liquid component was dispensed on a pad. After complete hydration of the powder with the liquid, the mixing procedure was continued until a uniform mix with a moldable consistency was obtained. The material was then carried using a plastic instrument to the desired experimental design.

Group 3: Newer MTA formulation with Doxycycline in powder		Group 4: Newer MTA formulation with Doxycycline in liquid	
POWDER	**Weight % for every 100 mg of powder**	**POWDER**	**Weight % for every 100 mg of powder**
Tricalcium silicate	60 wt %	Tricalcium silicate	60 wt %
Dicalcium silicate	20 wt %	Dicalcium silicate	20 wt %
Calcium Fluoride	5 wt %	Calcium Fluoride	5 wt %
Calcium Sulphate	5 wt %	Calcium Sulphate	5 wt %
Calcium Carbonate	4 wt %	Calcium Carbonate	4 wt %
Doxycycline	5 wt %	Zirconium oxide	1 wt %
Zirconium oxide	1 wt %		
LIQUID	**Concentration**	**LIQUID**	**Concentration**
Calcium Chloride	20%	Calcium Chloride	20%
		Doxycycline	5%

Table 1. Composition of newly formulated mineral trioxide aggregate.

ANTIMICROBIAL PROPERTY:

To test the antimicrobial property of the test materials were determined by the Agar Diffusion method. The antimicrobial property was tested against 3 microorganisms, Enterococcus faecalis (ATCC 29212), Candida albicans (ATCC 10231) and Streptococcus mutans (ATCC 35668). Mueller-Hinton agar plates were used. Five plates per material group were used with a total of 20 plates. After inoculation of the plates with the respective microorganisms, three wells of 4 mm deep and 5 mm in diameter were prepared in each agar plate. The test materials were dispensed and manipulated as recommended above. Freshly mixed test materials were filled in the agar plates in the respective wells until the well was completely filled. Once the wells were filled, they were incubated at 37°C and assessed after 24 hours for inhibition zones. A digital calliper was used to measure the diameter of the microbial growth inhibition zones. Data from all the samples were collected and tabulated for further statistical analysis.

COMPRESSIVE STRENGTH:

To measure the compressive strength of the test materials, a stainless steel mould of 4mm diameter and 6mm height was used. The test materials were dispensed and mixed as suggested before. After mixing, all the materials were compacted into each mould using the mixing spatula and dental pluggers were used accordingly to obtain uniformly dense filled samples with minimal to no porosities. The mould was covered using glass slides at either ends until the materials were set completely. The set cements were removed from the mould and transferred into an incubator that maintained the temperature at 37°C until the testing was done.Forty samples were prepared with 10 samples for each cement group. Universal testing machine (Hounsfield Test Equipment, Redhill, Surrey, UK) was used to test the compressive strength of the samples. Force is applied parallel to the long axis of the moulds at a crosshead speed of 1 mm/min until the materials are crushed. The maximum force at which the set cement were fractured were recorded in Megapascals (MPa) which depicts the compressive strength of that specific sample. All the 40 samples were tested individually and the collected data were tabulated for further statistical analysis.

FINAL SETTING TIME:

To measure the final setting time of the test materials, a modified ANSI/ADA Specification Number 9 method was used. A mould of 5mm thickness and 10mm diameter was used to prepare the test samples. A total of 40 samples were prepared i.e. 10 samples for each cement group. The test materials were dispensed and

mixed as suggested before. The materials that underwent testing were mixed under similar conditions of 95% relative humidity and at a room temperature of 37±2°C. Gillmore needle (Humboldt Mfg. Co., Norridge, USA) with a standard weight of 453.6 ± 0.5 g and 1 ± 0.1 mm tip diameter was used to measure the final setting time. A digital timer was used to measure the time duration of the final set cement. The timer was started when the powder was mixed with the liquid and was stopped when the indenter needle barely left a mark in the cement surface. All the 40 samples were tested individually and the collected data were tabulated for further statistical analysis.

STATISTICAL ANALYSIS

The statistical analysis was done using Statistical Package for Social Sciences software version 22 (SPSS Inc., Chicago, IL, USA). Shapiro Wilks test was used to test the normality of the distribution of data. For comparison of zones of inhibition, Kruskal Wallis test was used. For comparison of compressive strength, one-way ANOVA was used. For pairwise comparisons, Tukey's post hoc test was used. A p-value of < 0.05 was considered as statistically significant.

Results

ANTIMICROBIAL PROPERTY:

Table 2 shows comparison of zones of inhibition of all the four tested materials against the three microorganisms. Material in group 3 had the largest zone of inhibition against S.mutans and C.albicans while group 4 had the largest zone of inhibition against E.faecalis. This difference was found to be statistically significant ($p < 0.05$). Among the tested materials, the maximum mean growth inhibition was noticed in group 3 against C.albicans (29.33 ± 0.03 mm) and the least mean growth inhibition was noticed in group 1 against E.faecalis (15.03 ± 0.29 mm). Table 3 shows pairwise comparisons of all the four tested materials against the three microorganisms. Although the difference in antimicrobial activity was highly significant when comparing group 3 against group 1 and group 2 ($p < 0.05$), there was no significant difference when compared between group 3 and group 4 against all the three microorganisms ($p > 0.05$). The zones of inhibition of the different test materials against different organisms are shown in figure 1, 2, 3 & 4.

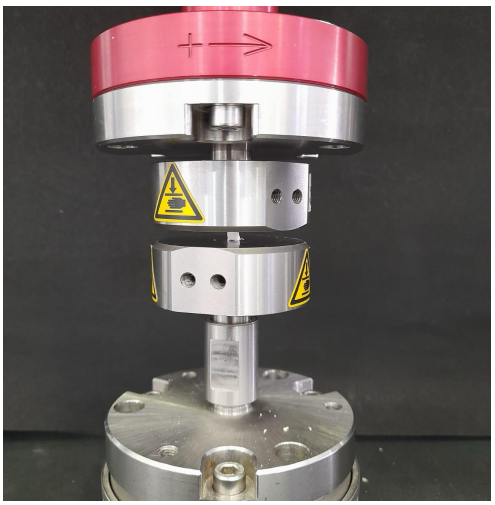

Figure 1. Universal testing machine used in this study with material samples undergoing compressive strength test.

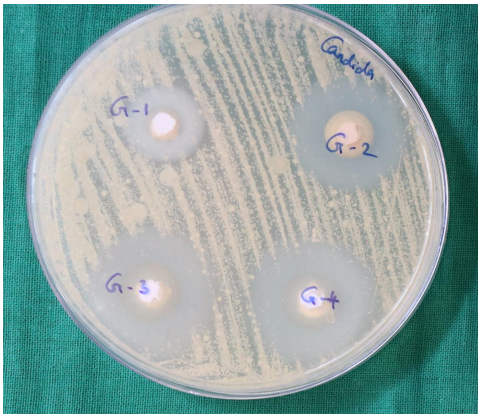

Figure 2. Comparison of Zones of Inhibition of the different test materials against C.albicans.

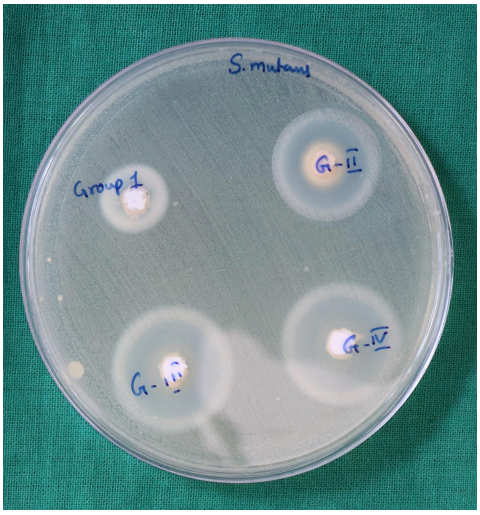

Figure 3: Comparison of Zones of Inhibition of the different test materials against S.mutans.

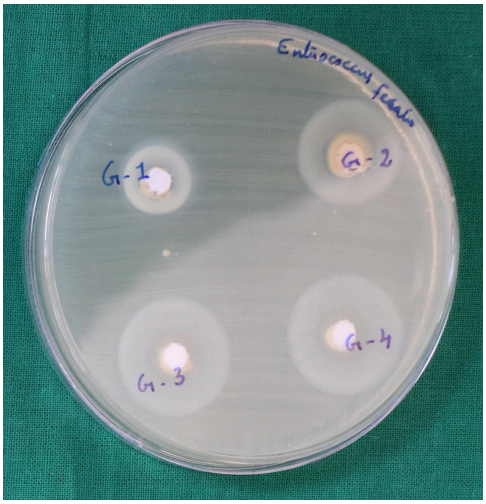

Figure 4: Comparison of Zones of Inhibition of the different test materials against E.faecalis.

Microorganisms	Material Group	Mean ± Standard Deviation (mm)	p-value
E. faecalis	Group 1	15.03 ± 0.29	0.01
	Group 2	21.35 ± 0.60	
	Group 3	25.81 ± 0.40	
	Group 4	25.01 ± 0.18	
C. albicans	Group 1	17.95 ± 0.18	0.01
	Group 2	25.81 ± 0.77	
	Group 3	29.33 ± 0.03	
	Group 4	28.89 ± 0.29	
S.mutans	Group 1	15.78 ± 0.43	0.01
	Group 2	19.99 ± 0.82	
	Group 3	27.33 ± 0.54	
	Group 4	26.78 ± 0.48	

Table 2. Comparison of Zones of Inhibition of the different test materials against different organisms.

COMPRESSIVE STRENGTH:

Table 4 shows comparison of mean compressive strength of the different test materials at different time periods of 3 hours, 1 day, 3 days, 7 days and 21 days. Among the tested materials, the highest mean compressive strength during all the time periods was seen in group 2 followed by group 3 and 4 and the least in group 1. This difference in compressive strength was statistically significant (p=0.001). Pairwise comparison shows significant differences between all the groups ($p < 0.05$) except between group 3 and 4.

Microorganisms								
E. faecalis			*C. albicans*			*S.mutans*		
Material (I)	Material (J)	Significance	Material (I)	Material (J)	Significance	Material (I)	Material (J)	Significance
Group 1	Group 2	0.00	Group 1	Group 2	0.00	Group 1	Group 2	0.00
	Group 3	0.00		Group 3	0.00		Group 3	0.00
	Group 4	0.00		Group 4	0.00		Group 4	0.00
Group 2	Group 1	0.00	Group 2	Group 1	0.00	Group 2	Group 1	0.00
	Group 3	0.00		Group 3	0.00		Group 3	0.00
	Group 4	0.00		Group 4	0.00		Group 4	0.00
Group 3	Group 1	0.00	Group 3	Group 1	0.00	Group 3	Group 1	0.00
	Group 2	0.00		Group 2	0.00		Group 2	0.00
	Group 4	0.49		Group 4	0.79		Group 4	0.38
Group 4	Group 1	0.00	Group 4	Group 1	0.00	Group 4	Group 1	0.00
	Group 2	0.00		Group 2	0.00		Group 2	0.00
	Group 3	0.49		Group 3	0.79		Group 3	0.38

Table 3. Pairwise comparisons of the different test materials against different organisms.

Time Period	Mean compressive strength ± Standard Deviation (MPa)				p-value
	Group 1	Group 2	Group 3	Group 4	
3 hours	0	155.84 ± 0.03	94.05 ± 0.22	92.74 ± 0.08	0.001
1 day	45.67 ± 0.01	226.33 ± 0.45	135.74 ± 0.43	134.88 ± 0.19	0.001
3 days	58.29 ± 0.88	320.47 ± 0.83	157.27 ± 0.45	155.22 ± 0.85	0.001
7 days	96.89 ± 0.23	368.03 ± 0.28	190.11 ± 0.68	188.04 ± 0.09	0.001
21 days	145.38 ± 0.48	422.06 ± 0.13	206.44 ± 0.90	202.08 ± 0.41	0.001

Table 4. Comparison of mean compressive strength of the different test materials at different time periods.

FINAL SETTING TIME:

Among the tested materials, group 2 had the shortest setting time (78.03 ± 0.45 mins), followed by group 3 (98.11 ± 0.02 mins), followed by group 4 (101.05 ± 0.46 mins) and group 1 had the longest setting time (211.78 ± 0.83 mins). This difference among the groups was statistically significant ($p < 0.05$).

Discussion

The basic properties for materials used in apexification and revascularization would be better compressive strength, complete sealing ability, ability to produce a calcific barrier and also good antimicrobial properties. Calcium hydroxide was the traditional material used in such clinical situations that was available in powder and liquid packaging.[18,19] Due to its various downsides, the material has evolved in multiple different formulations that had entered into the dental market a few decades ago. Torabinejad et al developed the conventional MTA which was approved before the start of the 21st century by the U.S. FDA for endodontic use. A decade later, Biodentine hit the endodontic market. Although Biodentine could overcome the drawbacks of MTA like discoloration potential, longer setting, acceptable compressive strength and higher material cost, it had its own setbacks like reduced flexural strength, diminished wear resistance and decreased radio-opacity.[20,21]

Although the agents that were used in the past few decades for such a treatment strategy tried to fulfil the ideal requirements of such materials, they did not set at a faster rate and also had a compromised antimicrobial activity against anaerobes like E.faecalis. One of the recent studies has also mentioned eliminating tricalcium aluminate due to its cytotoxic nature[16]. So this study was planned to modify the components of the conventional MTA, thereby enhancing the handling and setting properties, for faster setting and elevating its antimicrobial nature. In the present study, tricalcium aluminate was replaced with other calcium rich fillers in the powder component. We added calcium chloride at 20% concentration in the liquid component so that it can act as an accelerator to allow faster binding and set of the cement.

Since the introduction of antibiotics in the 1920s, they have reformed health care by restraining and eradicating bacterial infections.[22] During endodontic treatment, antibiotics are administered either systemically or topically, with the latter being most preferred. The era of topical use of antibiotics began in 1951 when Grossman suggested disinfection of root canal using polymicrobial paste.[23] Since then antimicrobial agents have been used in irrigants, intra canal medicaments, pulp capping agents for various treatments in endodontic practice and has caused a radical change in the endodontic treatment strategies.[23] Topical use of antimicrobial agents can help in creating a completely disinfected canal space that can aid in rapid healing of endodontic infections, which can accelerate a favourable prognosis during endodontic therapy. Enhancement of antibacterial activity was the core reason for the present study. In the present study, an antimicrobial agent was added into the powder and liquid component separately to assess whether such an addition would affect the required physical properties. Doxycycline was employed in this study to enhance the antibacterial action. Although, doxycycline acts as a bacteriostatic agent by allosterically binding to the 30S prokaryotic ribosomal unit during bacterial protein synthesis, additional non-antibacterial properties like removal of free radicals, reducing reactive oxygen species and stimulation of new bone formation can be utilised for faster healing purposes.[24] Previous studies involving doxycycline showed better antibacterial activity when incorporated to glass ionomer cement while minimal activity when incorporated to biodentine.[15,25] We added doxycycline to 5 weight % for every 100mg of the powder component in group 3. For group 4, we added a five percentage doxycycline solution into the liquid component. Adding such components may or may not alter the properties of the final set cement which was assessed in the present study.

Conventional MTA and Biodentine were employed for comparing the newly modified material with the gold standards of Portland or tricalcium silicate cements that have been used for decades. MTA-Angelus was employed in the present study as it had satisfactory sealing ability, better marginal adaptation, favourable antibacterial activity and acceptable compressive strength. Arsenic content was also confined to ISO standardizations. Biodentine from Septodont was preferred for the current study as this was an innovative evolutionary biomimetic material that came to the spotlight since its very introduction due to its handling properties, faster set and exceptionally high compressive strength. Since it is available in pre-packed capsular form, the wastage of the material is high.[26]

Since the introduction of MTA and Biodentine, many antibacterial studies have been performed which shows a diversity of results.

Bhavana et al and Esteki et al concluded that Biodentine was more effective than MTA against S.mutans, E.faecalis and C.albicans.[27,28] Koruyucu et al and Ji et al showed that Biodentine and MTA had similar antibacterial actions against E. faecalis.[29,30] The results of the present study showed that the antimicrobial property of the doxycycline incorporated MTA was better when compared to conventional MTA and Biodentine and also the difference was statistically significant (Table 2 and 3). Addition of doxycycline showed an enhancement in the antimicrobial properties of the set cement. The conventional MTA and Biodentine showed some amount of antimicrobial activity based on the alkaline pH of the calcium hydroxide which was the end product of the hydration of tricalcium silicate present in the cement. The pH of 11-12 at best, was the major reason for the bactericidal activity. And also the addition of Doxycycline, had an influence in the larger mean zone of inhibition against all the three bacterium. Incorporating doxycycline into the powder component had highest antimicrobial activity against S.mutans and C.albicans, while incorporating in the liquid component had improvised antimicrobial activity against E.faecalis. Also the presence of calcium fluoride, which was added as a filler to replace the cytotoxic tricalcium aluminate, would have a supplemental antimicrobial effect. Calcium fluoride interferes with bacterial metabolism by inhibiting glycolytic enzymes causing a bactericidal effect. The result of the present study was contradicting the study done by Nikhil where the addition of 10% doxycycline decreased the antimicrobial activity of Biodentine.[15,24] This reduction in antimicrobial activity could be due to minimal incorporation of the doxycycline powder into the liquid component of Biodentine. While the present study used two different drug delivery methods i.e. the doxycycline was incorporated in both the powder form and the liquid form.

The physical property that determines MTA's ability to withstand stress at the occlusal regions and as well as apical regions would be the compressive strength of the material. This clearly indicates the cements ability to hydrate during the setting reaction in the environment provided. Biodentine had favourable results in regards to compressive strength when compared to MTA.[31,32] The current study showed that both the modified MTAs had improvised compressive strength compared to the conventional MTA, but cannot reach the level of Biodentine. Although this difference was statistically significant, when comparing group 3 and group 4, there was no significant difference ($p>0.05$)(Table 4). This comparative result suggests that addition of doxycycline in either powder or liquid form, did not have any significant influence on the compressive strength of the final set cement. Although not statistically significant, group 4 showed a slight reduction in compressive strength as compared to group 3. The reason for this change could be due to the addition in the liquid component that could have caused some interference in the hydration reaction thereby causing a slight compromise in the setting mechanism of the modified MTA.

Setting time would determine and influence the handling properties of the material. As per manufacturer instructions, Biodentine sets by 12 minutes. In the current study, 78.03 ± 0.45 mins was noticed for the final set of Biodentine. Contradicting results exist with its setting time as one of the previous studies concluded the completion of setting around 45 mins[33] while another study suggested an 85 mins time period.[34,35] The current study shows that MTA sets by 211.78 ± 0.83 mins while other studies have shown a wide variation in setting time period ranging from 40 mins to 225 mins.[34–37] This variation could be due to the different ISO standardisations of the manufactured material available in different countries. Doxycycline incorporation in the powder component had a faster setting time compared to its incorporation in the liquid component. This could be due to the similar reason mentioned above where there could be an interference in the hydration reaction. Although there is a compromise, the compressive strength was higher and the material sets faster than the conventional MTA.

The results of the present in-vitro study showed that the newly formulated MTA with addition of 5% doxycycline has better antimicrobial properties than conventional MTA and Biodentine, regardless of the powder or liquid form. The results were also promising in terms of compressive strength, with values higher than conventional MTA but lower than Biodentine. Anyhow, the final setting time and the compressive strength was not critically affected by the solid or liquid medium of drug delivery. The strengths of this study were that modifications have been done to the composition

of conventional MTA by removing cytotoxic components and adding an antimicrobial agent to improvise the properties of the cement. Also the core components i.e. tricalcium silicate and dicalcium silicate were manufactured in-house to maintain the quality of the material. Further studies on cell cytotoxicity, calcium ion release, mineralizing ability, push-out bond strength, sealing ability, in-vivo analysis, and long term evaluation on animal models followed by human clinical trials are yet to be done which could further justify the clinical application of the newly formulated material. As from the literature search, this is the only study to assess a modified MTA with a combination of doxycycline in both the liquid and solid form.

Conclusions

Under the limitations of the present study, addition of doxycycline, either in the liquid or powder component, can have additive effects on the antimicrobial properties of the modified MTA with no alteration in its physical properties. Although further clinical and cytotoxic studies are required to justify its clinical application.

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Declaration of Interest

The authors report no conflict of interest.

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