EVALUATION OF A NEW BIOMIMETIC CEMENT (GEMOSIL) FOR USE IN
ENDODONTIC THERAPY AS COMPARED TO THE WIDELY-USED MINERAL
TRIOXIDE AGGREGATE (MTA)

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ABSTRACT

Hsin Chen: Evaluation of a New Biomimetic Cement (GEMOSIL) for Use in Endodontic
Therapy as Compared to the Widely-Used Mineral Trioxide Aggregate (MTA)
(Under the direction of Ching-chang Ko)

MTA has proven to be an effective material for endodontic therapy. However, due to its

long setting time and dentinal/gingival staining, its use is limited. The aim of this study was to

see if the new biomimetic cement GEMOSIL has comparable properties to MTA, such that it can

be used as an alternative to MTA. Compressive and biaxial strength, discoloration, antimicrobial

effects, cell viability and biocompatibility were analyzed in this study. The compressive and

biaxial strength reached 93 MPa and 59 MPa, respectively, after fully dried. GEMOSIL^{CHX}

demonstrated antibiotic properties against *S. mutans* and *E. faecalis*. GEMOSIL had no

cytotoxicity against human pulp cells and promoted significantly more cell proliferation

($p<0.05$). GEMOSIL demonstrated less discoloration when placed in extracted teeth ($p<0.05$).

GEMOSIL showed biocompatibility with living tissues. From these initial tests, GEMOSIL has

demonstrated better properties compared to MTA, prompting GEMOSIL to be a viable

alternative to MTA.

To my mother, who I dedicate my thesis to.

You have given me endless love and support throughout every minute of my life.

From this point on, I want to provide you everything you have dreamed for in your life.

I love you my dearest Xiao Mi.

Thank you for making me the luckiest child in the world.

ACKNOWLEDGMENTS

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INTRODUCTION

Mineral Trioxide Aggregate (ProRoot MTA; Dentsply Tulsa Dental, Tulsa, OK) is currently the most popular endodontic cement. It was developed by Torabinajed et al in 1993 at Loma Linda University as a root-end filling and perforation material. It consists of tricalcium oxide, silicate oxide, bismuth oxide, tricalcium silicate, and tricalcium aluminate (1). It sets in the presence of moisture and renders a pH of 12.5 (2). The gel solidifies to a hard solid structure in approximately three to four hours in the oral cavity (2). Several studies of MTA have demonstrated that the cement possesses many of the properties sought for in a root-end filling material. The sealing ability of MTA in root-end filling was found to be superior to amalgam, IRM, and Super-EBA (3). An *in vivo* animal study on monkeys demonstrated that MTA caused no periapical inflammation and allowed new bone formation directly against the material when used as a root-end filling (4). Due to its superiority in its biocompatibility and sealing ability, MTA is now also used as pulp capping material (5), and for repair of perforations (6).

Although MTA has demonstrated excellent properties when compared with traditional root-end filling materials such as amalgam or IRM, the cost is expensive. Research has also reported that MTA exhibits some poor handling properties. Fridland et al. (7) found that MTA mixture becomes overly viscous and difficult to deliver even when mixed at the manufacturer's recommended proportion of powder to liquid. Lee (8) stated that MTA has a long setting time which can be easily washed out during procedures. Torabinajed et al (9) also listed several drawbacks for MTA such as slow setting time and permanent dentinal discoloration. Bortoluzzi

et al (10) has reported cases of marginal gingival discoloration when MTA was used for perforation repair.

The shortcoming of MTA had led to the need for the development of an alternative endodontic cement.

GEMOSIL, a new biomimetic cement, was developed for orthopedic applications by Ko et al at University of North Carolina (UNC patent: US 12/685,743 2010 and 61/560,777 2011). It consists of gelatinous hydroxyapatite nanocomposite, calcium silica, and calcium hydroxide. Preliminary studies showed GEMOSIL has a faster setting time, and its chemical composition has the potential for effective osteogenesis and some antimicrobial properties.

In the 1st manuscript, we focused on testing properties of GEMOSIL as an endodontic cement. Mechanical strength, discoloration, antimicrobial effects, cell viability and biocompatibility were tested. During the initial antimicrobial property testing, GEMOSIL formula only showed effective inhibition of S. Mutans but not of E. Faecalis; which is the most common organism cultured from persisted endodontic infections (11).

From literature, chlorhexidine was initially used as a general disinfectant because of its broad antibacterial action (12). It was later shown to inhibit dental caries and reduce the formation of dental plaque (13). In vitro inhibition studies have shown chlorhexidine to be effective against species found in infected root canals such as Enterococcus faecalis (14) and Streptococcus mutans (15), and it consequently was introduced as an endodontic irrigant in the early 1960's (13). Chlorhexidine is increasingly being incorporated into endodontic materials due to its ability to increase antimicrobial properties and improve prognosis.

The 2nd manuscript (article in press) is a study to further investigate the effects of incorporating chlorhexidine into the original GEMOSIL formula to determine if the antimicrobial properties of GEMOSIL can be enhanced against common pathogens found in endodontic infections.

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EVALUATION OF A NEW BIOMIMETIC CEMENT (GEMOSIL) FOR USE IN
ENDODONTIC THERAPY AS COMPARED TO THE WIDELY-USED MINERAL
TRIOXIDE AGGREGATE (MTA)

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ABSTRACT

Mineral trioxide aggregate (MTA) is an effective material for endodontic therapy. However, due to its long setting time and dentinal/gingival discoloration, its use is limited. The objective of this study was to test the hypothesis that a new biomimetic cement, GEMOSIL, has physical/biological properties comparable to MTA in endodontic therapy.

Samples of GEMOSIL were used to measure compressive and biaxial flexure strength with an Instron machine. GEMOSIL and MTA were placed in pulp chambers of extracted human teeth for discoloration testing. GEMOSIL and MTA were spin coated to 35 mm culture dishes, which were then seeded with pulp cells for viability testing. Zones of inhibition against *S. Mutans* and *E. Faecalis* were measured with both materials. GEMOSIL and MTA were also placed as a root-end filling material in rat incisors to assess biocompatibility with living tissues.

The compressive strength of GEMOSIL reached 28 MPa after 2 hours of immersion in PBS and 93 MPa after fully dried. Flexure strength ultimately reached 59 MPa. GEMOSIL

demonstrated less discoloration when placed in pulp chambers of extracted teeth than MTA ($p<0.05$). GEMOSIL presented no cytotoxicity and promoted significantly more proliferation than MTA when in contact with pulp cells ($p<0.05$). *S. Mutans* was susceptible to both GEMOSIL and MTA, but *E. Faecalis* was only susceptible to GEMOSIL[CHX]. Both materials showed biocompatibility as a retro-grade filling material in rats ($p>0.05$). In conclusion, GEMOSIL has similar or better physical and biological properties as compared to MTA; thus GEMOSIL has potential to be a viable endodontic cement.

INTRODUCTION

There are 15 million endodontic therapies done each year in the United States. Although this procedure has a high success rate, amidst ideal circumstances there can still be failures. When there is persistent periapical infection, conventional root canal therapy fails, and non-surgical root canal re-treatment is not feasible, periradicular surgery (commonly known as apicoectomy) is often the preferred approach. This procedure routinely consists of root-end exposure of the involved apex, resection of the apical 3mm of the root, a retro-filling preparation and the placement of a root-end filling material. Various materials have been used as the root-end filling material, such as amalgam, Intermediate Restorative Material (IRM), Super-EBA, glass ionomers and composite resins (1). An ideal root-end filling material should prevent leakage of bacteria into the periapical tissue, it should be non-toxic, non-carcinogenic, biocompatible, insoluble, and have no shrinkage on setting (2). Previous root-end filling materials have had a few drawbacks, such as corrosion in amalgam and higher concentration of eugenol in IRM and Super-EBA, which causes irritations in vital tissues. These drawbacks led to the development of Mineral Trioxide Aggregate (MTA).

Mineral Trioxide Aggregate (ProRoot MTA; Dentsply Tulsa Dental, Tulsa, OK) is currently the most popular endodontic cement. It was developed by Torabinajed et al in 1993 at Loma Linda University as a root-end filling and perforation material. It consists of tricalcium oxide, silicate oxide, bismuth oxide, tricalcium silicate, and tricalcium aluminate (3). It sets in the presence of moisture and renders a pH of 12.5 (4). The gel solidifies to a hard solid structure in approximately three to four hours in the oral cavity (4). Several studies of MTA have demonstrated that the cement possesses many of the properties sought for in a root-end filling material. The sealing ability of MTA in root-end filling was found to be superior to amalgam, IRM, and Super-EBA (5). An *in vivo* animal study with monkeys has shown that when MTA was used for root-end filling, there was no periapical inflammation and new bone formed directly against the material (6). Due to its superiority in its biocompatibility and sealing ability, MTA is now also used as pulp-capping material (7), and for repair of perforations (8).

Although MTA has demonstrated adequate physical properties, superior biocompatibility and sealing ability when compared with traditional root end filling materials such as amalgam or IRM, the cost is expensive. Research has also reported that MTA exhibits some poor handling properties. Fridland et al. (9) concluded that MTA was difficult to handle due to its low viscosity when mixed with the manufacturer's recommended amount of liquid. Lee (10) stated that MTA can be easily washed out during procedures due to its long setting time. Torabinajed et al (11) also listed several drawbacks for MTA such as slow setting time and permanent dentinal discoloration. Bortoluzzi et al (12) has reported cases of marginal gingival discoloration when MTA was used for perforation repair.

The shortcoming of MTA had led to the development of ProRoot MTA White (Dentsply Tulsa Dental, Tulsa, OK, 1998) in which the iron component was removed to improve the color

stability; however, there was little to no improvement in tooth/gingival staining (13). ProRoot MTA White continued to show slow setting time and poor mechanical strength in a moist environment, thus the need for the development of an alternative endodontic cement exists.

Recently, a new biomimetic cement, GEMOSIL, was developed for orthopedic applications by Ko et al at University of North Carolina (UNC patent: US 12/685,743 2010 and 61/560,777 2011). It consists of gelatinous hydroxyapatite nanocomposite, calcium silica, and calcium hydroxide. Preliminary studies showed GEMOSIL has a faster setting time, and its chemical composition has the potential for effective osteogenesis and antimicrobial properties. GEMOSIL has also not demonstrated discoloration.

All preliminary evidence suggests that GEMOSIL can be a new addition to existing endodontic cements including ProRoot MTA and its derivatives. The aim of this study was to compare the physical properties (compressive and biaxial flexure strength), discoloration properties, biological properties (pulp cell proliferation effect and antimicrobial effect) and biocompatibility between MTA and GEMOSIL for endodontic therapy.

MATERIAL AND METHODS

MATERIALS

GEMOSIL

100 milligrams of HAP-Gel powder, 200 milligrams of $Ca(OH)_2$ powder, 300 microliters of enTMOS solution and 40 microliters of PBS were sequentially added and mixed to make GEMOSIL samples.

MTA

The MTA (grey formula, ProRoot MTA; Dentsply, Tulsa, OK) samples were prepared according to the manufacturer's instructions (powder mixed with recommended amount of sterile

MTA water provided by the manufacturer).

Compression Strength

20 cylindrical GEMOSIL samples (4 mm diameter by 8 mm height) were prepared and either immediately placed in PBS for 2 hours or allowed to air dry for 48 hours. Samples were compressed using an Instron 4411 (Instron Co., Norwood, MA) at a crosshead speed of 0.5 mm/min to determine ultimate failure strength σ_{ult}. The stress-strain curve was recorded via the Testworks 4 software (MTS, Eden Prarie, MN) and the highest stress at failure was identified as σ_{ult}.

Biaxial Flexure Strength

The general testing procedure for biaxial flexure strength was performed according to Ban and Anusavice (J Dent Res 69(12):1791-1799, 1990). 10 GEMOSIL disc samples (diameter 12mm by thickness 1mm) were prepared in Teflon molds. The upper and lower surfaces were polished in order to obtain parallel surfaces with no apparent defects. After measuring the sample diameter (d) and thickness (t), the disk was supported on three stainless steel balls (3mm in diameter), which were equally spaced along a 5mm radius (r_s). Prior to testing, a stainless steel piston (radius = r_p=1.5mm) was aligned concentrically with the three balls. A crosshead speed of 0.5mm/min was used, and the maximum force at failure (P) was determined. A Poisson's ratio (ν) of 0.3 was used for all materials unless the exact value was known. The flexure stress at failure (σ in MPa) was calculated using the following expressions: $\sigma = AP/t^2$ and $A = (3/4\pi) [2 (1+\nu) \ln(r_s/r_o) + (1-\nu) (2 r_s^2-r_o^2) / 2 (d/2)^2 + (1+\nu)]$ where $r_o = (1.6 r_p^2 + t^2)^{1/2} - 0.675t$.

Discoloration

Ten extracted human single-rooted teeth per group were used to test dentinal

discoloration of GEMOSIL and MTA. The teeth were collected based on the following criteria:

no caries or restorations present and no previous root canal treatments. Teeth were air dried for

24 hours then mounted with regisil material to standardize angulation, and pre-op photographs

were made (both facial and lingual). Each tooth was then accessed from the lingual surface, and

all coronal pulp tissue (if any) was removed. 3mm of MTA and GEMOSIL were placed in the

pulp chamber. A cotton pellet soaked with chlorohexidine was placed on top of the material and

IRM was used to restore access. Samples were placed in PBS on a shaker for 48 hours. Teeth

were then allowed to air dry for 24 hours, then post-op photographs were made with the same

regisil mount and color change was analyzed using the following described procedure.

The quantification results of discoloration were derived from color differences (δ) by

CIELAB method. Optical color measurement, CIELAB (the Commission Internationale de

I'Eclairage) tristimulus values, is based on the principle of light transmission which is

wavelength-dependent. Given an externally applied illuminating light to the stain, the detectable

color spectrum is used as a signal to differentiate and analyze the degree of discoloration. The

tooth colors of the digital images were converted to CIELAB tristimulus values for each image

pixel. The tristimulus values (L, a*, and b*) of three evenly distributed points from each tooth

for both the pre- and post-op photos were measured using Photoshop software (Adobe System

Inc., CA). For each material group, the formula to calculate the discoloration was a color

difference between the teeth before placement of the material and after placement of the

material, $\delta = ((L-L_o)^2+(a*-a*_o)^2+(b*-b*_o)^2)^{1/2}$ where (L, a*, b*) and (L_o, $a*_o$, $b*_o$) were

tristimulus values for teeth after placement of the material and teeth before placement of the

material, respectively. The mean δ was compared among two groups at different durations using the student t-test.

Pulp Cell Isolation

Dental pulp cells were isolated from non-carious human third molars, which were extracted for clinical reasons. Tooth surfaces were cleaned by submersion in Betadine for one minute and then cleaned with 70% ethanol. Pulp tissue was removed with a broach (Lexicon Barded Broaches, Dentsply Tulsa Dental, Tulsa, OK) after accessing the tooth. After the pulp tissue was removed, cells were isolated by enzyme digestion. The pulp tissue was digested in a solution of collagenase type II (2 mg/ml). The cell suspension was then filtered through a 40 micrometer strainer, centrifuged, and pellets were suspended in Dulbecco's modified Eagle's medium (DMEM). Single-cell suspensions were seeded onto a 100 mm culture plate in DMEM supplemented with 10% Fetal Bovine Serum, and 1% penicillin and streptomycin. Culture plates were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C and medium change was performed every three days. Once a cell colony was formed, cells were trypsinized and redistributed in a new culture plate. When cells reached confluence, they were harvested by trypsinization and sub-cultured. Passage 3 was used for testing.

Cell proliferation: BrdU Assay

MTA and GEMOSIL were prepared and spin-coated onto petri dishes, and then allowed to air dry for 24 hours. All petri dishes were sterilized under UV light overnight before the experiment.

Each coated petri dish was then seeded with pulp cells. Every day the medium was replenished with fresh growth medium. At the end of cultivation (3 days), the dish was rinsed twice with PBS. BrdU stain was applied. Under the fluorescent light, nuclei were counted in 10

regions per dish for both groups and compared by the student t-test between the two groups.

Antimicrobial Testing

Incorporation of Chlorhexidine in $Ca(OH)_2$ for GEMOSIL^{CHX}

$CaCO3$ (Alkaline analysis grade, Aldrich, USA) was calcinated to CaO in a furnace at $1250^{o}C$ for 3 hours. Pure $Ca(OH)_2$ was obtained through the hydration of the calcinated CaO. The hydration was carried out at $300^{o}C$ using 3 times the stoichiometric amount of 0.12% chlorhexidine aqueous solution. The final $Ca(OH)_2^{CHX}$ content was determined by measuring the dry weight [$120^{o}C$ for 3 h].

Disc Sample Preparation

GEMOSIL was prepared and made into 6.2 mm x 0.85 mm discs. A total of eight discs were made in a span of 15 minutes.

MTA was prepared and made into 6.2mm x 0.85mm discs. The MTA was given 30 minutes to set due to its increased inclination to crack and fracture, which would render it impossible to use.

For the control, paper discs were immersed in 0.12% chlorhexidine solution right before placing them on the agar plate.

Microorganism

The microorganisms investigated were *Streptococcus mutans* and *Enterococcus faecalis.* Both organisms were a stock strain in the Oral Microbiology laboratory, University of North Carolina School of Dentistry.

Zone of Inhibition

Bacterial sensitivity of each material was evaluated using the following assay – zone of inhibition of surface growth. The materials were tested against *Streptococcus mutans* and *Enterococcus faecalis* using assays that measured the diffusible inhibition of bacterial growth on a blood-agar surface and estimated the antimicrobial activity. All procedures were performed under aseptic conditions.

Agar Diffusion Assay

Inocula from frozen stock cultures were cultivated in Wilkins-Chalgren (W-C) broth (Oxoid Ltd. Basingstoke, Hampshire, England) at 37°C in ambient atmosphere, after being screened by Gram-staining to confirm purity. Loopful inoculations of *Streptococcus mutans* and *Enterococcus faecalis* were transferred to 10 ml of appropriate broth and incubated at 37 °C under anaerobic conditions. Bacterial suspensions were prepared to 0.5 MacFarland standard and diluted to a 1:10 concentration with W-C broth. Two hundred ml of the 1:10 dilution were then taken and spread-plated using a "hockey stick" on a turntable to ensure confluent bacterial distribution on the plates.

Test specimens were immediately placed on the freshly inoculated agar plates and aerobically incubated for 18-24 hours at 37°C. Each plate contained 7 disc samples - two per material and a paper disc soaked with chlorhexidine at the center as the control. This assay was performed in quadruplets. After 18-24 hours, the diameters of the zone of inhibition of bacterial growth around the discs were measured using a caliper by two independent observers.

Biocompatibility

10 Sprague Dawley male rats were used in this study. Cefazolin 0.06ml and Tetracycline-HCL (30mg/kg) were given before surgery. General anesthesia was induced by intraperitoneal injection of a mixture of Ketamine (80mg/kg) and xylazine (10mg/kg). Eye

ointment was applied. The right and left sides of the mandible were shaved and cleaned with

75% alcohol and β-dine. Under aseptic conditions, an incision was made along the inferior

mandible border and the mandible was exposed by dissecting the subcutaneous tissues and the

masseter muscle. Under irrigation, the buccal plate was perforated with a large round bur in a

slow speed handpiece to expose the incisor apex (oval shape/open apex). Once the apex was

exposed and location of the canal was confirmed with the aid of an endodontic file, placement

was attempted of 3mm of MTA or GEMOSIL at the apex as a root-end fill. 4.0 Vicryl suture

was used to reposition the periostium and muscles and 4.0 Monocryl suture was used to close the

incision. Saline (10ml/kg) was administered subcutaneously after surgery. All animals were

monitored until they recovered from general anesthesia. Cefazolin 0.06ml was administered

intramuscularly once a day and Buprenorphine 0.1ml was administered subcutaneously twice a

day for 3 days. Soft diet was fed to the animals up to 7 days after the surgery. All animals were

euthanized with injection of an overdose of barbiturate on day 21 postoperatively. Both left and

right sides of the mandible were submitted for non-decalcified histological slides to determine

inflammation levels in the PDL, pulp and periapical tissue adjacent to the material for both

materials. Chi-square test was used to compare the two groups.

RESULTS

Mechanical Property

GEMOSIL's compressive strength was 28 MPa after 2 hours setting in PBS and reached

93 MPa after fully dried. Biaxial flexure strength was 59 MPa. The results appear to be better to

those of MTA reported in the literature (1, 14).

	GEMOSIL	MTA
Setting Time	5 min (15)	3-4 hours (1, 14)
Compressive Strength	28 MPa (2 hours) 93 MPa (fully dried)	40 MPa (24 hours) 67.3 MPa (21 days) (1, 14)
Biaxial Strength	59 MPa	No Available Data

Table 1 Comparison between mechanical properties of GEMOSIL and MTA

Discoloration

Figure 1 shows examples of pre-op and post-op photographs for the MTA group. Figure 2 shows examples of pre-op and post-op photographs for the GEMOSIL group. Discoloration can be seen clearly on a few of the MTA samples. The comparisons of the L, a*, and b* values are presented in Figure 3. δ and SD values for each group are listed in Table 2. There was a significant difference in color change between the MTA and GEMOSIL group (p<0.05).

Figure 1 **MTA** (top) Initial, no discoloration (bottom) 3 days after, grey discoloration in cervical third

Figure 2 **GEMOSIL** (top) Initial, no discoloration (bottom) 3 days after, slight/no discoloration in cervical third
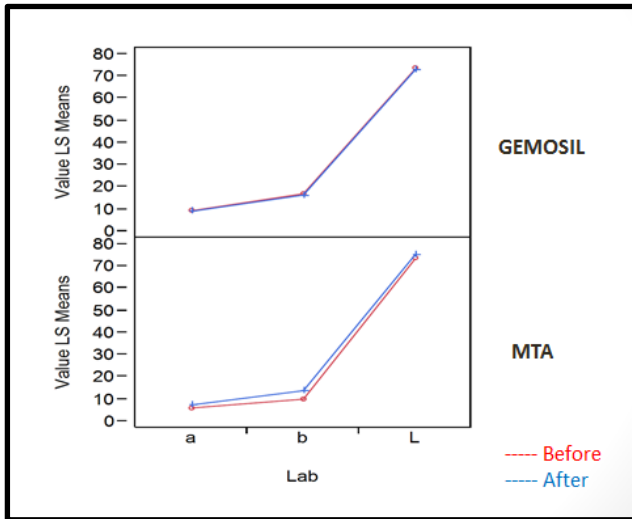

Figure 3 The comparison of L, a* and b* values, pre-op and post-op

Material	δ	SD
MTA	5.0251	3.0980
GEMOSIL	1.7004	0.7079

Table 2 δ and SD for MTA and GEMOSIL groups

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Cell Proliferation

Figure 4 shows the nuclei counts for the three groups, including the control group. Figure 5 shows the BrdU staining of the nuclei for all three groups. There was a significant difference in the nuclei counts between the groups ($p < 0.05$). The GEMOSIL group had significantly more nuclei, indicating more cell proliferation.
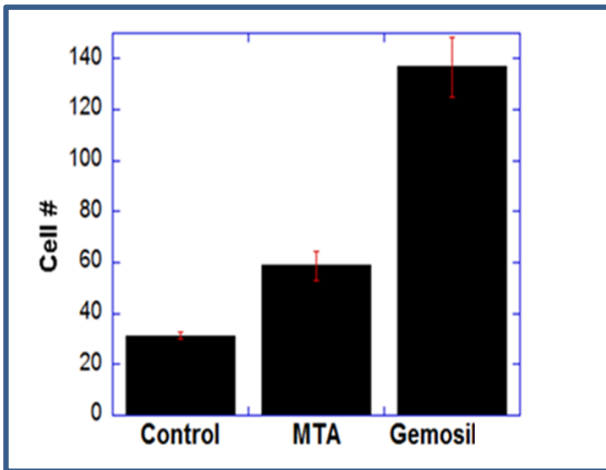

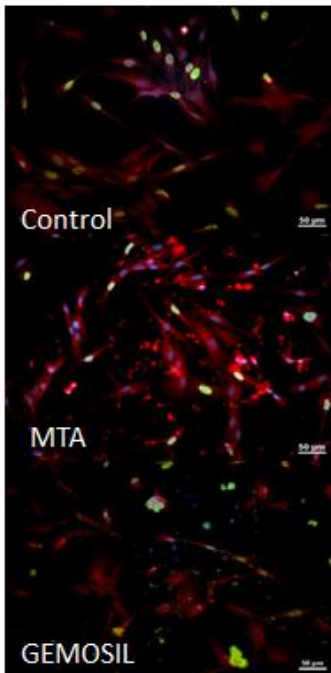Figure 4 Cell count (nuclei) under BrdU staining with 3 day culture

Figure 5 BrdU stain (green) for nuclei under cell proliferation

Antimicrobial Testing

Figures 6 and 7 show photographs of the zones of inhibition for *S. mutans* and E. *faecalis*, respectively. The data for the zones of inhibitions are presented in Tables 3 and 4. Figure 8 plots the mean values of the zones of inhibition for all materials. A greater value implies a better antimicrobial effect. In the control, growth of both microorganisms was inhibited by chlorohexidine. MTA, GEMOSIL, and GEMOSIL^{CHX} are inhibitory to *S. mutans*. In this case, the means of the zones of inhibition were 17.7±0.76 mm for MTA, 15.58±1.41 mm for GEMOSIL, 17.08±0.64 mm for GEMOSIL^{CHX}, and 16.45±1.5mm for Chlorhexidine.

For *E. faecalis*, the means of the zones of inhibition were 0±0.0 mm for MTA, 0±0.0 mm for GEMOSIL, 8.18±0.73 mm for GEMOSIL^{CHX} and 9.85±0.47mm for Chlorhexidine. The data showed no zones of inhibition with MTA and GEMOSIL, compared to GEMOSIL^{CHX} and chlorhexidine, which showed zones of inhibition. The difference among the materials was statistically significant ($p < 0.01$).
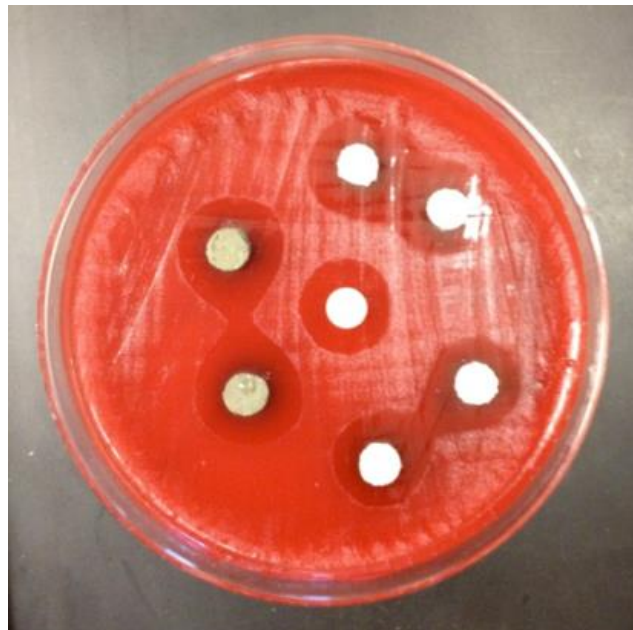

Figure 6 Photograph of the zones of inhibition in *Streptococcus mutans*. In the dish, the upper right: GEMOSIL^{CHX}, lower right: GEMOSIL, left: MTA, and center: Chlorhexidine.

Figure 7 Photograph of the zones of inhibition in *Enterococcus faecalis*. In the dish, the upper right: GEMOSIL^{CHX}, lower right: GEMOSIL, left: MTA, and center: Chlorhexidine.

Material	Plate 1	Plate 2	Plate 3	Plate 4
MTA	17.2±0.01	18.7±0.71	17.7±0.71	17.2±0.01
GEMOSIL	14.2±1.41	16.7±0.71	16.7±0.71	14.7±0.71
GEMOSIL^{CHX}	17.2±0.02	16.7±0.71	17.2±0	17.2±1.41
CHX	18.2±0.28	17.2±0.28	15.2±0.57	15.2±0.28

Table 3 Zones of inhibitions (diameter/mm) in *Streptococcus mutans*

Material	Plate 1	Plate 2	Plate 3	Plate 4
MTA	0	0	0	0
GEMOSIL	0	0	0	0
GEMOSIL^{CHX}	7.9±0.42	9.2±0	7.9±0.42	7.7±0.71
CHX	9.2±0.28	9.8±0.57	10.2±0.28	10.2±0.28

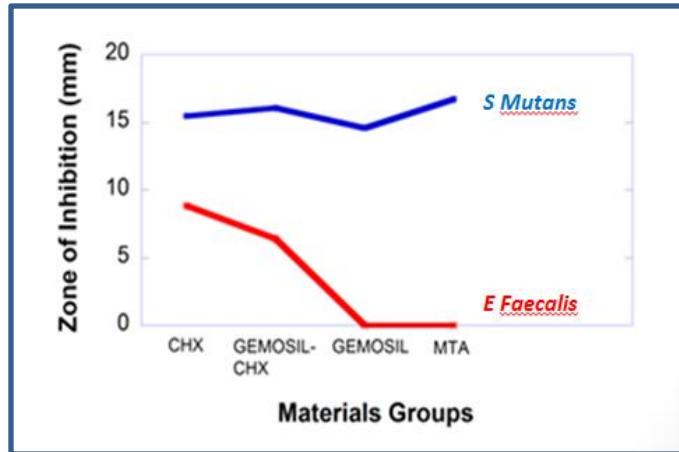Table 4 Zones of inhibitions (diameter/mm) in *Enterococcus faecalis*

Figure 8 Plot of the mean values of the zones of inhibition comparing the four groups, p<0.01

Biocompatibility

There were a total of 18 samples submitted for histology, 9 samples for MTA and 9 samples for GEMOSIL. Half of the samples were horizontally sliced and the other half were vertically sliced at the apex.

Inflammation of the PDL and the pulpal and periapical tissue adjacent to material was analyzed. Almost all samples have mild inflammation, but there was no significant difference between the two materials (p>0.05). Both materials showed new bone formation adjacent to the materials (Figure 9 for MTA and Figure 10 for GEMOSIL).

One GEMOSIL sample showed cartilage formation (Figure 11) and one MTA sample showed formation of apical abscess (Figure 12).
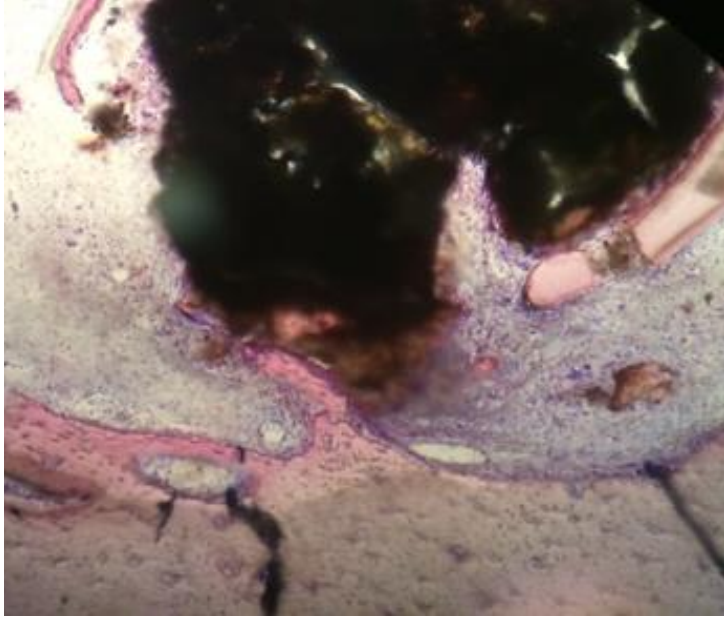
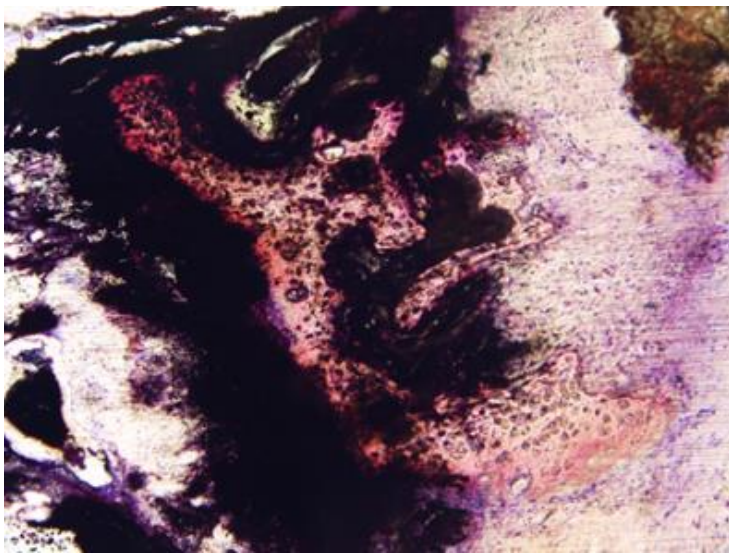Figure 9 New bone formation adjacent to MTA

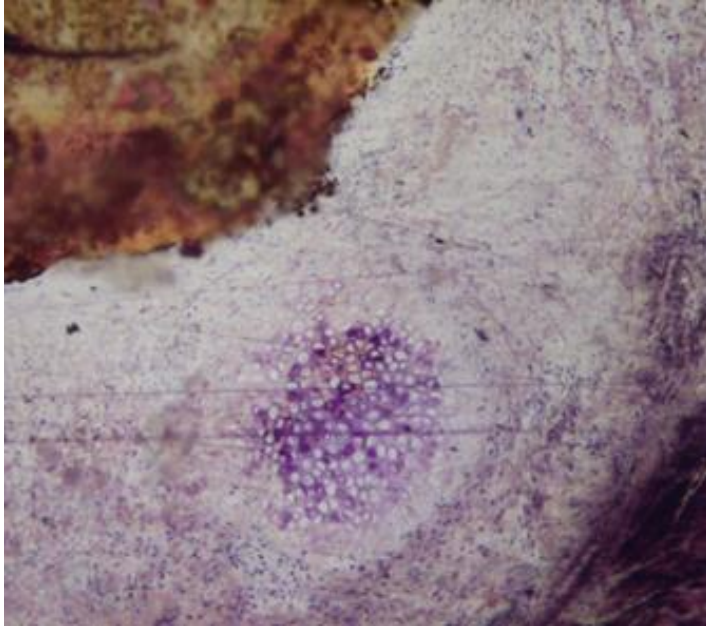

Figure 10 New bone formation found in GEMOSIL material

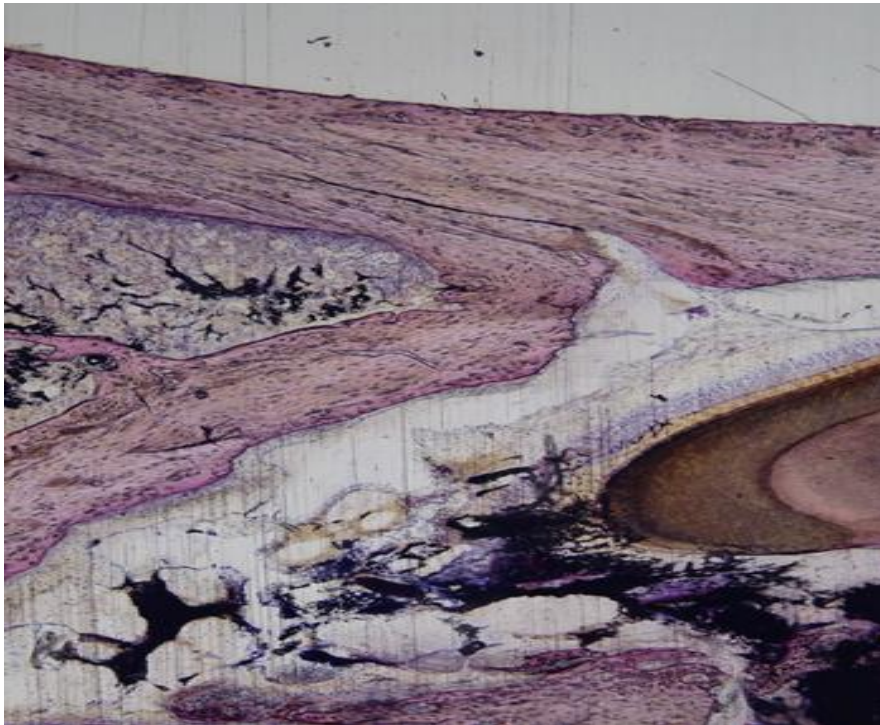Figure 11 Cartilage formation adjacent to GEMOSIL material

Figure 12 Apical abscess formation adjacent to MTA retro fill

DISCUSSION

The mechanical property testing determined the initial setting time of GEMOSIL to be 5

minutes (15) and the mixture can be hardened in an ambient or PBS solution. The faster setting

property and its capability of setting in moist environments make GEMOSIL a good candidate for a retrograde filling material during apicoectomy procedures. The compressive strength (28 MPa after 2 hours setting in water and 93 MPa after fully dried) appears to be superior to that of MTA reported in the literature. Additionally, the formulation of GEMOSIL that incorporated chlorhexidine did not lead to a decrease in compressive strength.

In discoloration testing, the GEMOSIL group demonstrated less color change than the MTA group when placed in pulp chambers of extracted teeth ($p < 0.05$), which implies that GEMOSIL may cause less discoloration when used for vital pulp therapy in the esthetic zone of human dentition. 3 days could be too short for the materials to penetrate through dentinal tubules. A longer storage period is recommended for future studies in order to fully understand the impact of discoloration on the facial side of the tooth. Further testing will be necessary to determine whether the addition of chlorhexidine to the formulation of GEMOSIL impacts discoloration.

In the biological property testing, the GEMOSIL scaffold presented no cytotoxicity when assessed by a viability assay and a 3 day culture with human dental pulp cells. Short term pulp cell cultures show significantly more cell growth in GEMOSIL coated dishes compared to those of MTA ($p < 0.05$). GEMOSIL's non-toxicity means it could be effective at maintaining pulp vitality in vital pulp therapy. Previous GEMOSIL studies have shown higher mineralization rates in human osteoblasts, which combined with GEMOSIL's cell proliferation effect, indicate it could possibly stimulate dentinal bridge formation. Future tests are indicated to determine the optimal concentration of chlorhexidine to be added to the formulation of GEMOSIL[CHX] without adversely affecting cell proliferation.

In testing the antimicrobial effect, *S. Mutans* was found to be susceptible to all three materials- GEMOSIL, GEMOSIL[CHX] and MTA. *E. Faecalis* was only susceptible to GEMOSIL[CHX] and resistant to GEMOSIL and MTA. GEMOSIL[CHX] demonstrates antimicrobial properties that may be highly effective against persistent endodontic infections when used as a root-end filling material. The difference in zone of inhibition needs further investigation in order to determine its clinical significance.

Lastly, in biocompatibility testing, both GEMOSIL and MTA showed biocompatibility with periapical tissues when placed *in vivo* as a root-end filling in rat incisors. There were no adverse allergic reactions or skin irritation noted in the rats. Histologically, all samples showed new bone formation adjacent to both materials. There was no difference in PDL, pulpal or periradicular inflammation levels between the two materials ($p>0.05$). GEMOSIL's biocompatibility with rat tissues indicates promise for biocompatibility with other living tissues, including those of humans. Due to anatomical differences, there are limitations to the rat model. To further understand how the material affects humans, future studies could consider the usage of an animal species more closely related to humans.

In conclusion, GEMOSIL demonstrates similar physical and biological properties compared to MTA. Continued study is necessary; however, initial testing finds that GEMOSIL and GEMOSIL[CHX] could potentially become a viable substitute for MTA in endodontic therapy.

ACKNOWLEDGEMENT

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ANTIMICROBIAL EFFECTS OF FORMABLE GELATINOUS HYDROXYAPATITE-CALCIUM SILICATE NANOCOMPOSITES FOR BIOMEDICAL APPLICATIONS

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ABSTRACT

The recently developed GEMOSIL, a nanocomposite material composed of gelatinous hydroxyapatite (HAP-GEL) and animosaline by pozzolanic crosslinking, provides a potentially improved alternative to current endodontic cements. The mechanical performance of fully dried GEMOSIL approximated that of cortical bone. In its pre-set form, the moldable cement solidified in approximately 5 minutes when submerged in water, primarily due to the pozzolanic crosslinking. The purpose of this study was to test if adding chlorhexidine to $Ca(OH)_2$ during the formation of this material could provide enhanced antimicrobial effects. Four zones of inhibition were formed using GEMOSIL, GEMOSIL[CHX], Mineral Trioxide Aggregate (MTA; Tulsa Dentsply), and Chlorhexidine as a control. Each material was prepared into disc samples (6.2mm x 0.85mm; 8 per material). The *Enterococcus faecalis* and *Streptococcus mutans* strains were cultured at $37^{o}C$ under anaerobic conditions. Bacterial suspensions were evenly distributed on a

blood-agar plate surface. The MTA/GEMOSIL/GEMOSIL[CHX]/Chlorhexidine discs were placed

on the inoculated blood-agar plates which were immediately incubated at 37°C for 18-24 hours

under aerobic conditions. Results showed that the Chlorhexidine and GEMOSIL[CHX] provided a

similar antimicrobial effect to *Enterococcus faecalis,* while GEMOSIL and MTA did not. All

four materials showed similar effects to *Streptococcus mutans*. In combination with previous

reports showing osteogenesis of GEMOSIL, the GEMOSIL[CHX] may be applicable to both

orthopedic and endodontic cements.

INTRODUCTION

The biomimetic cement, GEMOSIL, consisting of gelatinous hydroxyapatite

nanocomposite, calcium silica, and calcium hydroxide particles, was developed for orthopedic

applications by Ko and co-workers (1,2).

The advantages of GEMOSIL include hardening in a moist environment, moderately

strong compressive strength (94 MPa) after dehydration, and biocompatibility with osteoblasts.

Upon investigation, its properties appear to match some demands of an ideal endodontic cement

including 1) good mechanical strength, 2) short setting time in the presence of moisture, 3)

injectability, 4) absence of dentinal discoloration, 5) bioactive dentinogenesis or osteogenesis, 6)

antimicrobial activity and 7) sealability. Currently, mineral trioxide aggregate (MTA) is widely

used as endodontic cement; however, MTA shows tooth/marginal gingiva staining, slow setting

time, and poor mechanical strength in a moist environment (3,4,5,6). This has led to the

development of several new derivatives of MTA (Dentsply Tulsa Dental, Tulsa, OK, 1998),

although none have significantly improved upon these problems.

Our previous studies have shown that the working time of GEMOSIL is approximately 1-

3 minutes, and the compressive strength reaches 28 MPa after two hours of setting in water and 94 MPa after fully dried. GEMOSIL's chemical composition has shown the potential for effective *in vitro* osteogenesis and *in vivo* bone formation (7,8). All evidence suggests that GEMOSIL can be a new addition to existing endodontic cements including MTA and its derivatives. One missing property of GEMOSIL is the antimicrobial effect against common microorganisms found in infected root canals.

Chlorhexidine initially was used as a general disinfectant because of its broad antibacterial action (9). It was later shown to inhibit dental caries and reduce the formation of dental plaque (10). *In vitro* inhibition studies have shown chlorhexidine to be effective against species found in infected root canals such as *Enterococcus faecalis* (11) and *Streptococcus mutans* (12), and because of this, it was introduced as an endodontic irrigant in the early 1960's (10). Chlorhexidine is increasingly being incorporated into endodontic materials due to its ability to increase antimicrobial properties and improve prognosis.
In the present study, we demonstrated that chlorhexidine solution can be incorporated into the synthesis of calcium hydroxide, labeled as $Ca(OH)_2$ creation of a chlorhexidine-impregnated GEMOSIL (GEMOSIL^{CHX}). With this newly formed material, we hypothesized that it can improve the antimicrobial effect of GEMOSIL.

Ultimately, the purpose of this *in vitro* study was to determine if the addition of 0.12% chlorhexidine would enhance the antimicrobial activity of GEMOSIL, and to compare the antimicrobial activity to the widely used material MTA.

MATERIALS AND METHODS

Incorporation Of Chlorhexidine In Ca(OH)$_2$ For GEMOSIL

CaCO3 (Alkaline analysis grade, Aldrich, USA) was calcinated to CaO in a furnace at 1250°C for 3 hours. Pure Ca(OH)$_2$ was obtained through the hydration of the calcinated CaO. The hydration was carried out at 300°C using 3 times the stoichiometric amount of 0.12% chlorhexidine aqueous solution. The final Ca(OH)$_2^{CHX}$ content was determined by measuring the dry weight [120°C for 3 h].

Disc Sample Preparation

For each GEMOSIL sample, 100 mg of hydroxyapatite-gelatin powder and 200 mg of Ca(OH)$_2$ were ground and mixed with 300 ul of 95% enTMOS and 40 ul of PBS. The mixture paste was then pressed into 6.2 mm x 0.85 mm disc samples. A total of eight discs were made in about 15 minutes prior to testing.

For each MTA sample, two 200 mg packages of grey ProRoot MTA (Tulsa, Dentsply) were mixed with sterile water into paste form. The resultant mixture was made into 6.2mm x 0.85mm disc samples. The MTA was given 30 minutes to set due to its increased inclination to crack and fracture, which would render it impossible to use.

For the control, paper discs were immersed in 0.12% chlorhexidine solution right before placing them on the agar plate.

Microorganism

Microorganisms investigated were *Streptococcus mutans* and *Enterococcus faecalis.* Both organisms were a stock strain in the Oral Microbiology laboratory, University of North Carolina School of Dentistry.

Antimicrobial Assay

Bacterial sensitivity of each material was evaluated using the following assay – zone of inhibition of surface growth. The materials were tested against *Streptococcus mutans* and *Enterococcus faecalis* using assays that measured the diffusible inhibition of bacterial growth on a blood-agar surface and estimated the antimicrobial activity. All procedures were performed under aseptic conditions.

Agar Diffusion Assay

Inocula from frozen stock cultures was cultivated in Wilkins-Chalgren (W-C) broth (Oxoid Ltd. Basingstoke, Hampshire, England) at 37°C in ambient atmosphere, after being screened by Gram-staining to confirm purity. Loopful inoculations of *Streptococcus mutans and Enterococcus faecalis* were transferred to 10 ml of appropriate broth and incubated at 37 °C under anaerobic conditions. Bacterial suspensions were prepared to 0.5 MacFarland standard and diluted to a 1:10 concentration with W-C broth. Two hundred ml of the 1:10 dilution were then taken and spread-plated using a "hockey stick" on a turntable to ensure confluent bacterial distribution on the plates.

Test specimens were immediately placed on the freshly inoculated agar plates and aerobically incubated for 18-24 hours at 37°C. Each plate contained 7 disc samples - two per material and a paper disc soaked with chlorhexidine at the center as the control. This assay was performed in quadruplets. After 18-24 hours, the diameters of the zone of inhibition of bacterial growth around the discs were measured using a caliper by two independent observers.

The data for each material were subjected to one-way ANOVA to determine if significant differences in zones of inhibition occurred between different materials. Confidence level was set at $p < 0.05$.

In-Vitro Cell Cytotoxicity Testing Through MTS

Since chlorhexidine has an antimicrobial effect, it may also affect normal cell growth. MC3T3E1 preosteoblasts were cultured to test *in vitro* cytotoxicity for the GEMOSIL^{CHX} coated on the 35 mm culture dishes. The proliferation of the MC3T3-E1 cells on the coated dishes was conducted using MTS assay. The MTS absorbance of each group was measured on day 1, 3, 5, 7, and 9 respectively at 490 nm using a Plate reader (Biorad, Hercules, CA USA). The control used GEMOSIL. The higher the absorbance, the more cell growth.

RESULTS AND DISCUSSION

Figure 1 shows photographs of the zones of inhibition for all testing samples. The data for the zones of inhibitions are presented in Table 1 and Table 2. Figure 2 plots the mean values of the zones of inhibition for all materials. A greater value implies a better antimicrobial effect. In control, growth of both microorganisms was inhibited by chlorohexidine. MTA, GEMOSIL, and GEMOSIL^{CHX} are inhibitory to *Streptococcus mutans*. In this case, the means of the zones of inhibition were 17.70±0.76 mm for MTA, 15.58±1.41 mm for GEMOSIL, 17.08±0.64 mm for GEMOSIL^{CHX}, and 16.45±1.50 mm for CHX.
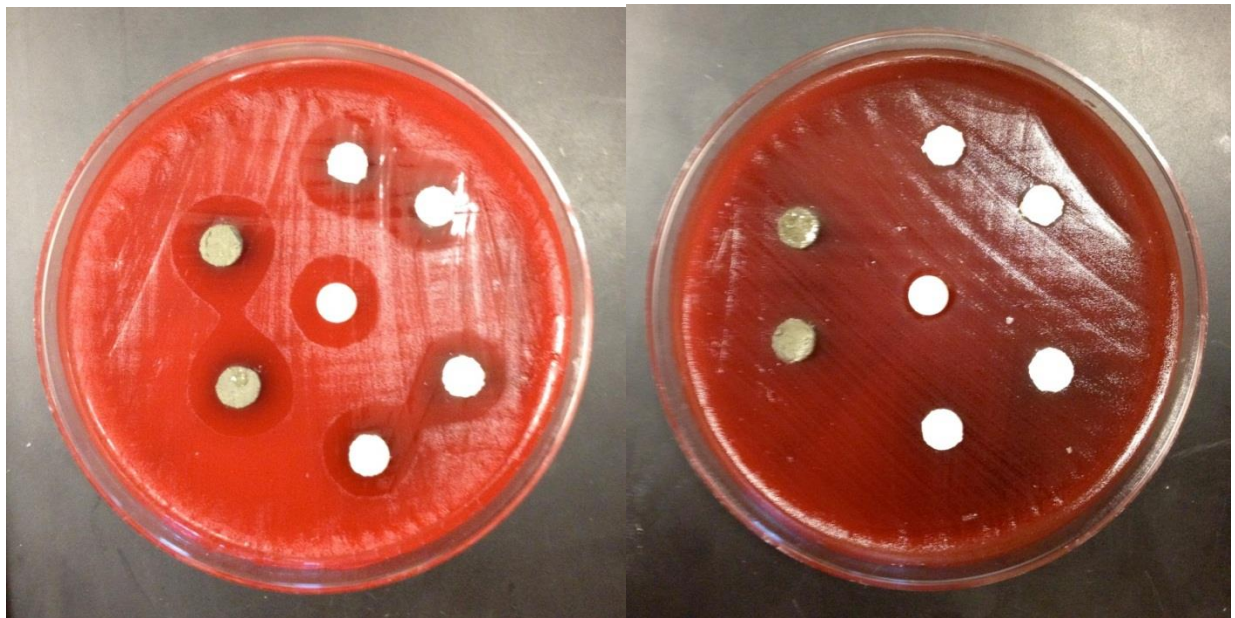
For *Enterococcus faecalis*, the means of the zones of inhibition were 0.00±0.00 mm for MTA, 0.00±0.00 mm for GEMOSIL, 8.18±0.73 mm for GEMOSIL^{CHX}, and 9.85±0.47 mm for CHX. The data showed no zones of inhibition with MTA and GEMOSIL, compared to GEMOSIL^{CHX} and chlorohexidine, which showed zones of inhibition. The difference among the materials was statistically significant ($p<0.05$).

The size of the zone of inhibition of an antibacterial substance depends on a couple factors: the toxicity of the substance for the particular microorganism and the diffusibility of the substance in the test agar being used. The diffusibility of the substance is based on whether it is hydrophilic or hydrophobic, molecular size and its rate of release from the insoluble matrix in

which it is bound. Therefore the size of the zone of inhibition may not be entirely due to its toxicity to the microorganism.

It had been shown that MTA has antimicrobial and antifungal effect (13, 14). Several investigation reports that MTA has limited antimicrobial effect against some microorganisms (facultative/strict anaerobes) (15). Al-Hezaimi et al evaluated the antimicrobial effect against two different kinds of MTA and found that grey MTA demonstrated significantly more antimicrobial effects than white MTA when present in low concentrations (16). Also, when it was compared between *Enterococcus faecalis* and *Streptococcus sanguis*, *Enterococcus faecalis* requires significant higher MTA concentration for growth inhibition (16). In our study MTA only demonstrated growth inhibition to *Streptococcus mutans* but not *Enterococcus faecalis*.

Figure 1. Photographs of the zones of inhibition in *Streptococcus mutans* (left) and in *Enterococcus faecalis* (right). In each dish, the upper right: GEMOSIL[CHX], lower right: GEMOSIL, left: MTA, and center: chlorhexidine.

Table 1. Zones of inhibitions (diameter/mm) in *Streptococcus mutans*

Material	Plate 1	Plate 2	Plate 3	Plate 4
MTA	17.20±0.01	18.70±0.71	17.70±0.71	17.2±0.01
GEMOSIL	14.20±1.41	16.70±0.71	16.70±0.71	14.70±0.71
GEMOSIL^{CHX}	17.20±0.02	16.70±0.71	17.20±0.00	17.20±1.41
CHX	18.20±0.28	17.20±0.28	15.20±0.57	15.20±0.28

Table 2. Zones of inhibitions (diameter/mm) in *Enterococcus faecalis*

| Material	Plate 1	Plate 2	Plate 3	Plate 4
MTA	0	0	0	0
GEMOSIL	0	0	0	0
GEMOSIL^{CHX}	7.90±0.42	9.20±0.00	7.90±0.42	7.70±0.71
CHX	9.20±0.28	9.80±0.57	10.20±0.28	10.20±0.28

In this present study, GEMOSIL demonstrated antimicrobial activity against *Streptococcus mutans* but failed to demonstrate antimicrobial activity against *Enterococcus faecalis* (Table 2). This study also evaluated the effect of antimicrobial activity when chlorhexidine was added to the formulation of GEMOSIL (GEMOSIL$^{CHX)}$. In the case of GEMOSIL^{CHX}, the antimicrobial efficiency against *Enterococcus faecalis* increased significantly ($p<0.05$). In comparison with MTA, which is the most widely used endodontic cement, GEMOSIL^{CHX} demonstrated significantly more antimicrobial effects against *Enterococcus faecalis*. Because *Enterococcus faecalis* is the most common microorganism cultured from persisted endodontic infections (17), the growth inhibition demonstrated by GEMOSIL^{CHX} suggests that this newly developed cement may lend itself to certain endodontic treatment modalities.
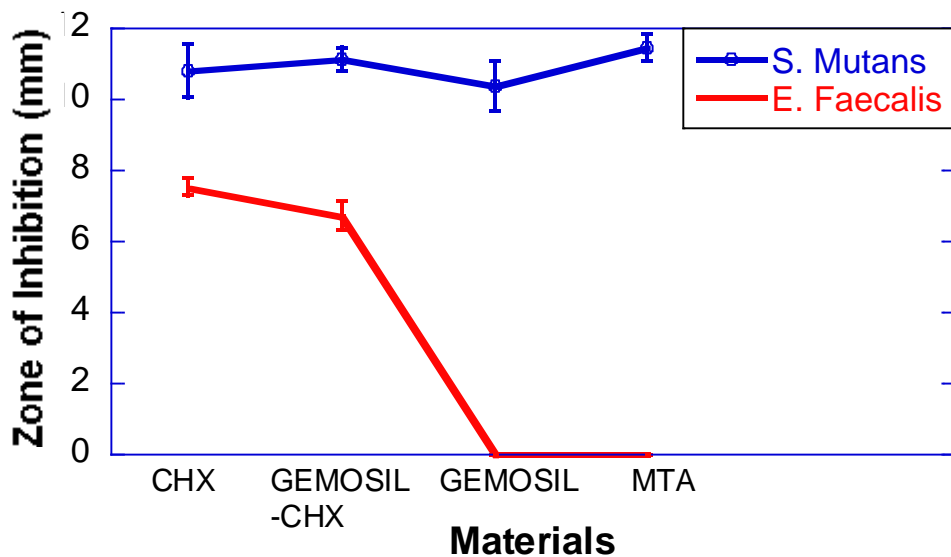
Figure 2. Plot of the mean values of the zones of inhibition comparing the four groups. The greater the value, the better the antimicrobial effect. S. Mutan and E.F. stand for *Streptococcus mutans* and *Enterococcus faecalis*, respectively.

In vitro cytotoxicity testing showed GEMOSIL^{CHX} did affect normal cell growth (Figure 3) which is proportional to the increased level of MTS absorbance. Two-way ANOVA showed that the difference was significant for both factors, Day and Material. At the beginning of the culture, the chlorhexidine increased cell growth approximately 25% but then decreased around 25% after day 7.

When testing the viability of pulp cells in the presence of the new cement, the preosteoblast culture showed that GEMOSIL^{CHX} increased cell growth 25% from day 1 to day 5 but decreased cell growth 25% after the day 5. It was not clear by what mechanisms the material affects cell cycles and whether the cells in the preosteoblast cell culture underwent apoptosis in the presence of chlorhexdine. Future investigations on dose effect and cellular mechanisms are necessary in order to determine the applicability of this new cement to endodontic treatment.
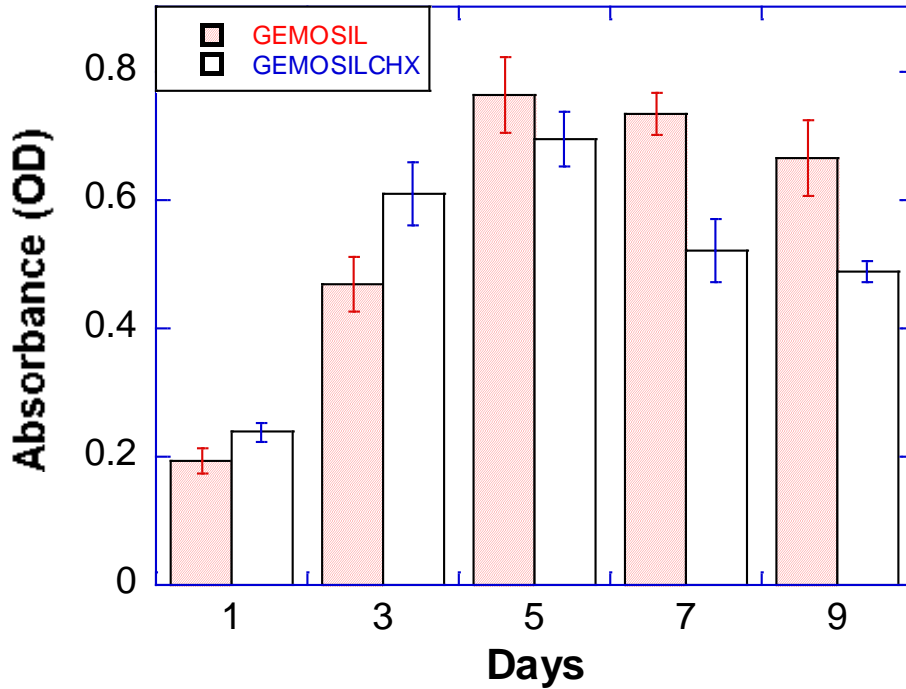
Figure 3. MTS absorbance for GEMOSIL and GEMOSIL[CHX], measured on day 1, 3, 5, 7, and 9 respectively.

CONCLUSION

Chlorhexidine-impregnated GEMOSIL appears to provide superior mechanical strength, faster setting time, and greater antimicrobial properties than MTA and therefore, may offer a better future therapeutic alternative for endodontic cement.

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