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The antibacterial properties and biocompatibility of carbonated hydroxyapatite as a pulp capping agent

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Abstract

Objective: The present study evaluated the antibacterial efficacy and biocompatibility of carbonated hydroxyapatite (CHA) compared to mineral trioxide aggregate (MTA).

Methods: CHA was synthesized at 0.05, 0.1, and 0.5 M concentrations. The minimal inhibitory concentration (MIC) of CHA and MTA were measured against Streptococcus mutans, Enterococcus faecalis, and Candida albicans. Afterwards, $1x10^7$ CFU/mL of each microorganism was exposed to the concentration corresponding to the MIC of each material and the bacterial colony count was measured. The survival rate, hatching rate, heart rate, and morphology of zebrafish embryos were evaluated when exposed to CHA or MTA (n=20 embryos per group)

Results: For S. mutans, the lowest MIC levels were found in the 0.05 and 0.5 CHA groups. The MIC value of all CHA concentrations against E. faecalis was 25 µg/ml. The lowest MIC against C. albicans was noted in the 0.1 CHA group. At the respective MIC levels, the 0.5 CHA showed the lowest colony count for S. mutans, which was significantly lower than that of MTA and 0.1 CHA groups ($P<0.05$). For E. faecalis, the 0.1 CHA and MTA groups had significantly lower colony count than the 0.05 CHA group ($P<0.05$). Regarding C. albicans, the 0.1 CHA had the lowest colony count, significantly lower than the 0.05 CHA group ($P<0.05$). Zebrafish embryos showed the lowest survival, hatching, and heart rates when exposed to MTA ($P< 0.05$).

Conclusions: The 0.1 and 0.5 CHA concentrations showed a favorable antibacterial efficacy against various oral microorganisms and better biocompatibility than MTA.

Keywords: Biocompatibility, Dental pulp capping, Enterococcus faecalis, Hydroxyapatite, Mineral trioxide aggregate, Streptococcus mutans

Introduction

Maintaining the vitality of the dental pulp is a crucial objective in conservative dentistry. When the pulp is vital, especially in cases of carious pulp exposure, direct pulp capping or partial pulpotomy may be recommended (1). Pulp capping materials aim to support the healing of damaged pulp and encourage the formation of mineralized tissue, leading to the formation of a dentin bridge (2).

Mineral trioxide aggregate (MTA) has been widely used to seal exposed pulp tissue and the root canal system in various applications (3, 4). It exhibits high biocompatibility with pulp cells, reduces inflammation, promotes pulpal healing, and supports cell proliferation and odontoblast-like differentiation. While MTA offers benefits, it has notable drawbacks, including the potential for tooth discoloration, high cost, extended setting time, and difficult handling properties (5).

Previous studies have investigated the role of hydroxyapatite (HA)-based materials, such as carbonated hydroxyapatite (CHA), as pulp capping materials (6, 7). HA is well known for its ability to promote periodontal bone regeneration, decrease dentin hypersensitivity, and aid in remineralizing carious lesions (8). HA can penetrate dentinal tubules and support the integrity and growth of apatite crystals (8). However, HA application is challenging due to its high crystallinity (9).

Carbonate minerals and ions can be added to HA, leading to the formation of CHA. Incorporating carbonate ions decreases the crystallinity and enhances

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solubility, biocompatibility, biomineralization, and bioactivity of HA (10). The efficacy of CHA is enhanced in nanoform due to its larger surface area, a more uniform distribution when added to dental materials, and better interaction with the surrounding environment (10). The incorporation of carbonate has been shown to reduce the bending strength of single-crystal pure HA, consistent with findings that carbonated apatite exhibits increased elasticity (11). CHA can serve as a synthetic scaffold material, providing structural support and maintaining space for cellular ingrowth and tissue regeneration (12, 13). Due to their ability to impede bacterial growth, support pulp regeneration, and modulate inflammatory responses, antimicrobial scaffolds hold significant promise for promoting successful endodontic outcomes (14). CHA is bioresorbable by osteoclasts and soluble in apatite lattices (7). While CHA offers promising scaffold properties for pulp regeneration, its antibacterial activity remains uncertain.

Furthermore, the biocompatibility and toxicity of CHA for fetal development have not yet been evaluated. The zebrafish is a freshwater species and is considered a suitable vertebrate model for investigating developmental biology (15). Zebrafish embryos are transparent, providing a unique in vivo model system for visualizing developmental events and observing functional organ processes, such as heartbeats (16). Furthermore, it is possible to generate thousands of embryos daily in a laboratory and simultaneously utilize them for various experiments (17).

This study assessed the antimicrobial effects of different CHA concentrations compared to MTA against Streptococcus mutans, Enterococcus faecalis, and Candida albicans. Furthermore, CHA biocompatibility was evaluated by analyzing its effects on the hatching rate, survival rate, heart rate, and morphology of zebrafish embryos compared to MTA.

Materials and methods

Preparation of materials

The present study was conducted following approval by the ethics committee of Saveetha Dental College and Hospitals (ID: SRB/SDC/PhD/ENDO-2309/23/TH-081).

CHA nanoparticles were produced via the precipitation method in three concentrations (0.05, 0.1, and 0.5 M) following our previous research protocol (10). The reaction involved calcium (50 ml) and phosphate (100 ml) precursor solutions in a molar ratio of 1:0.6. To achieve three different levels of CHA, sodium hydrogen carbonate (50 ml) at concentrations of 0.05 M, 0.1 M, and 0.5 M was added to the di-ammonium hydrogen phosphate solution before the reaction. All materials were purchased from Merck Life Science Private Limited in Mumbai, India. The combination resulted in a milky white precipitate, which was subsequently dried in an oven for 24 hours. The synthesized CHA samples were characterized as detailed in our previous publication (18).

MTA (Biostructure MTA, Safe Endo Dental India Pvt. Ltd., Gujarat, India) was prepared following the manufacturer's instructions.

Antimicrobial experiments

The modified broth micro-dilution technique was used to assess the materials' minimum inhibitory concentration (MIC) against S. mutans, E. faecalis, and C. albicans.

S. mutans (MTCC-860 strain) and E. faecalis (MTCC439 strain) were grown in a brain heart infusion (BHI) broth medium (Hi-media, Mumbai, India) in an oxygen-free environment at 37 °C. C. albicans (MTCC-183 strain) was grown on Sabouraud dextrose agar (SDA) (Hi-media, Mumbai, India) and kept at 37 °C. The overnight culture was diluted to obtain a final inoculum of approximately 1×106 colony forming unit (CFU)/mL for bacteria and 1×105 CFU/mL for C. albicans.

CHA was dissolved in sterile distilled water. Stock solutions of CHA at concentrations of 0.5, 1, 2.5, 5, 7.5, 10, 12.5, 15, 25, 50, 100, and 200 µg/mL were prepared. Each concentration was tested in 3 replicates. Serial two-fold dilutions of the CHA stock solution were prepared in BHI broth for S. mutans and E. faecalis or SDA for C. albicans. Each well of the 96-well microtiter plate received 100 µL of each CHA concentration and 100 µL of the microbial inoculum. This resulted in a final volume of 200 µL per well, and the CHA concentration was diluted by half. The microtiter plates were incubated at 37°C for 24 hours. After incubation, the growth of the bacteria was estimated by evaluating its optical density (O.D.) at a wavelength of 600 nm using a spectrophotometer (Shimadzu uv-1601UV, Japan). The MIC was defined as the lowest concentration of CHA that showed no microbial growth after incubation.

Afterwards, 1x107 CFU/mL of each microorganism was exposed to 10 µL aliquots from the concentration corresponding to the MIC. The suspensions were transferred to agar plates and incubated for 24 hours at 37 °C. Subsequently, the number of CFUs was recorded.

Biocompatibility assessment

Inception and maintenance of zebrafish

Zebrafish embryos were chosen as the model to assess CHA's biocompatibility. Sample size calculation was conducted using G*Power software, with an alpha level set at 0.05, a statistical power (β) of 0.9, and an effect size of 0.03. As a result, 20 samples were determined for each group.

The methodology employed in this study was adapted from the work of Makkar et al. (20). Four-month-old, wild-type, AB-strain zebrafish were sourced from an aquarium. They were maintained in a tank system filled with water containing 75 g of sodium bicarbonate (Merck Limited, Mumbai, India), 8.4 g of calcium sulfate (Merck Limited), and 18 g of sea salt per 1000 mL. The zebrafish were fed live brine shrimp three times a day and housed in a 10-liter glass tank maintained at 28.5 °C with a 14:10 hour light-dark cycle. The male and female zebrafish were separated, and after a month of acclimation, they were used for breeding in a designated aquarium. The collected viable eggs were thoroughly rinsed with Holtfreter (HF) 25% medium and subsequently maintained in fresh HF 25% medium, then subjected to microscopic examination.

Three hours post-fertilization (hpf), the fertilized eggs were placed in a six-well plate filled with E3 embryo medium. This medium contained 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, and 0.1% methylene blue.

Evaluating the biocompatibility of CHA in Zebrafish embryos

One gram of each experimental material was placed into separate 50-mL centrifuge tubes. The tubes were incubated for 24 hours at 37°C in a 5% CO2 environment to facilitate a complete material setting. Afterwards, the centrifuge tubes were filled with 50 mL of E3 medium and incubated for 24 hours in a humidified environment containing 5% CO2. Then, 40 mL of conditioned media was taken from each tube and centrifuged at the gravity of 400 g for 1 minute. The supernatant was filtered through a 0.2 µm filter to eliminate residual particles and then diluted twice.

Twenty zebrafish embryos at a critical stage of 3 to 3.5 hpf were exposed to 500 µL of culture media containing each of the following materials in separate wells of a 24-well plate: 0.05 CHA, 0.1 CHA, 0.5 CHA, and MTA. The control group included embryos kept in an E3 medium without the experimental materials. The embryos were maintained under a light-dark cycle of 14:10 hours at 28 ± 1 °C.

The following parameters were assessed in the experimental and control groups:

Hatching rate: The hatching rate was determined as the percentage of embryos that successfully emerged at 48 hpf. This was evaluated by counting the hatched embryos in each well and comparing the count to the total number of embryos in each group (19).

Survival rate: Survival rate was determined as the percentage of viable embryos until 48 hpf.

Heart rate: The heartbeat rate of embryos exposed to each CHA treatment was recorded. Embryos were stabilized with methylcellulose, and their heartbeats were manually recorded under a microscope for 30 seconds. The counts were then converted to beats per minute.

Morphological evaluation: Following treatment, the embryos (now at the larval stage) were subjected to a morphological evaluation via direct observation and stereomicroscopy. Morphological changes included tail bending, a shortened body, spinal curvature, and pericardial edema.

Statistical analysis

Data were analyzed using SPSS software (IBM Corp., Version 23.0, Armonk, NY, USA). One-way ANOVA and Tukey post hoc test were used to assess the differences in O.D. at the corresponding MIC, and heart rate between groups. The Kruskal-Wallis test and Mann-Whitney U-test were used to compare the CFUs between the groups. Survival rate and hatching rate were compared using the chi-square test. The significance level was set at $P<0.05$.

Results

Antimicrobial efficacy of CHA

Figure 1 and Table 1 indicate the MIC values of the experimental materials. For S. mutans, the lowest MIC level was associated with 0.05 and 0.5 CHA groups, which was 50 µg/ml. For E. faecalis, all the CHA groups had MIC values of 25 µg/ml. For C. albicans, the lowest MIC value was observed in the 0.1 CHA group (25 µg/ml). The MIC values of MTA against S. mutans, E. faecalis, and C. albicans were 200 µg/ml, 100 µg/ml, and 100 µg/ml, respectively. Table 1 shows that the mean O.D. values of the tested materials at their corresponding MIC were comparable according to the Kruskal-Wallis test ($P>0.05$).

Table 2 presents the number of CFUs for each microorganism at the corresponding MIC of four materials. The Kruskal-Wallis test indicated significant differences in the number of colonies among the groups for S. mutans ($P=0.015$), E. faecalis ($P=0.045$), and C.

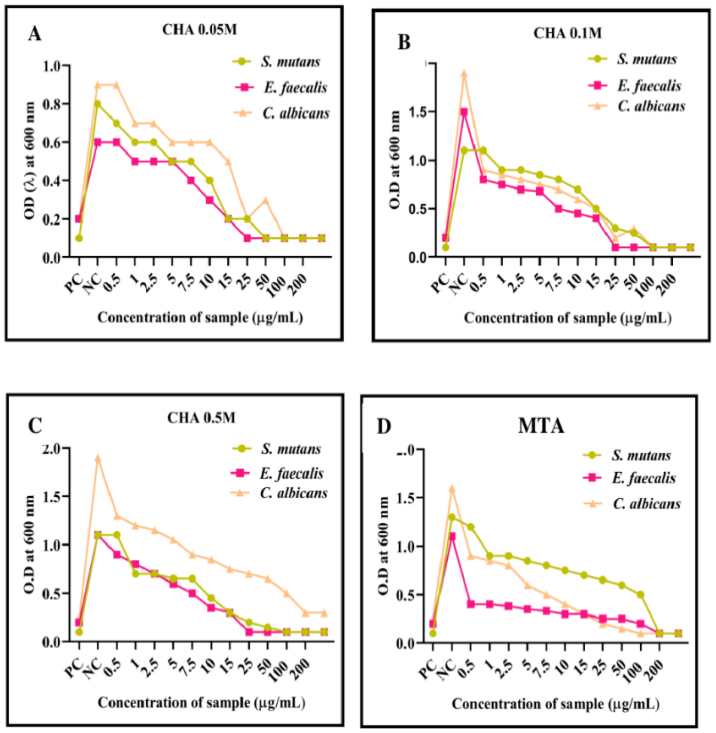

Figure 1. MIC values of different concentrations of CHA and MTA against *S. mutans, E. Faecalis*, and *C. albicans*

albicans (P=0.029). Specifically, the MTA group exhibited a significantly higher number of *S. mutans* colonies (16 ± 1.0 CFU) than the 0.05 CHA group (4.6 ± 1.5 CFU; P = 0.041) and the 0.5 CHA group (1.3 ± 0.5 CFU; P=0.002). Additionally, the 0.1 CHA group had a significantly higher number of S. mutans colonies (8.6 ± 0.5 CFU) than the 0.5 CHA group (P=0.041).

Regarding E. faecalis, the highest number of colonies was observed in the 0.05 CHA group (2.3+ 0.5 CFU), which was significantly higher than that of 0.1 CHA (0.3 ± 0.5 CFUs; P = 0.009) and MTA group (0.6 + 0.5 CFUs; P = 0.032). For C. albicans, the highest number of colonies were observed in the 0.05 CHA groups (7.0 ± 1. CFU), significantly higher than the 0.1 CHA group (0.3 ± 0.5 CFU; P = 0.003).

Biocompatibility test results

Table 3 presents the survival rate, hatching rate, and heart rate in the study groups. The chi-squared test indicated that the survival and hatching rates significantly differed among the groups (P < 0.001). The MTA group displayed the lowest survival and hatching rates compared to the other groups.

At 96 hpf, the average heart rates were 154 ± 0.4 for control, 154 ± 0.4 for 0.05 M CHA, 154 ± 0.3 for 0.1 M CHA, 149 ± 0.4 for 0.5 M CHA, and 99 ± 0.6 beats per minute (BPM) for MTA groups. The statistical analysis

Table 1. MIC values (µg/ml) and the O.D. evalues at the corresponding MIC of the studied materials against *S. mutans, E. Faecalis*, and *C. albicans*

Materials	MIC			O.D at the corresponding MIC		
	S. mutans	*E. faecalis*	*C. albicans*	*S. mutans*	*E. faecalis*	*C. albicans*
0.05 CHA	50	25	100	0.12 ± 0.2	0.11 ± 0.01	0.13 ± 0.3
0.1 CHA	100	25	25	0.26 ± 0.02	0.14 ± 0.03	0.2 ± 0.1
0.5 CHA	50	25	200	0.16 ± 0.1	0.14 ± 0.02	0.4 ± 0.1
MTA	200	100	100	0.10 ± 0.0	0.28 ± 0.05	0.13 ± 0.02
P value	-	-	-	0.150	0.070	0.080

Table 2. Mean± Standard deviation (SD) of colony forming units (CFUs) among the groups against various microorganisms

Materials	Microorganism		
	S. mutans	*E. faecalis*	*C. albicans*
	Mean± SD	Mean± SD	Mean± SD
0.05 CHA	4.6 ± 1.5 ^{a,b}	2.3 ± 0.5 ^{b}	7.0 ± 1.0 ^{b}
0.1 CHA	8.6 ± 0.5 ^{b,c}	0.3 ± 0.5 ^{a}	0.3 ± 0.5 ^{a}
0.5 CHA	1.3 ± 0.5 ^{a}	1.3 ± 0.5 ^{a,b}	1.3 ± 0.3 ^{a,b}
MTA	16 ± 1.0 ^{c}	0.6 ± 0.5 ^{a}	1.6 ± 0.5 ^{a,b}
P value	0.015*	0.045*	0.029*

*Statistically significant differences were noted at P<0.05.
Different lowercase letters indicate a significant difference among the groups at P<0.05.

Table 3. Comparing the survival rate, hatching rate, and heart rate between groups

Materials	Survival rate (%)	Hatching rate (%)	Heart rate (beats/minute)
0.05 CHA	100	100	154 ± 0.4^{A}
0.1 CHA	92	95	154 ± 0.3^{A}
0.5 CHA	90	86	149 ± 0.4^{A}
MTA	60	58	99 ± 0.6^{B}
Control	100	100	154 ± 0.4^{A}
P value	<0.001*	<0.001*	<0.001*

*Statistically significant differences were noted at P<0.05.
Different lowercase letters indicate a significant difference among the groups at P<0.05.

revealed a significant difference in heart rate among the groups (P < 0.001). The post-hoc analysis revealed that the heart rate in the MTA group was significantly lower than in the other groups (P < 0.05).

Figure 2 displays light-field microscopic observations that reveal notable morphological changes in zebrafish larvae exposed to MTA compared to the control group. Specifically, the larvae in the MTA group showed tail bending and abnormal morphology. In contrast, no such morphological changes were observed in the CHA groups.

Discussion

This study assessed the antimicrobial effects and biocompatibility of CHA as a potential pulp-capping material. The antibacterial properties of the materials were evaluated against various microorganisms. S. mutans was chosen due to its central role in the initiation and progression of dental caries (20). E. faecalis was selected due to its association with endodontic failures and primary root canal infections (21). Research suggests that fungi, notably the oral pathogen C. albicans, may migrate from the mouth into the root canal during endodontic therapy due to coronal leakage or inadequate root canal cleaning (22, 23).

The antimicrobial properties were assessed using a modified broth microdilution method. This technique involves inoculating containers with equal volumes of broth, each with progressively higher concentrations of an antimicrobial agent. Compared to conventional broth microdilution, this technique contains supplemental compounds such as glucose, divalent cations, or specific enhancers to improve bacterial detection (24).

The antimicrobial effects of pulp capping agents are critical to promoting successful pulp healing and preserving tooth vitality, thus reducing invasive treatments like root canal therapy or extractions. In the present study, CHA exhibited antimicrobial efficacy against common oral microorganisms in lower concentrations than MTA. The outcomes of this study agree with those of Calasans-Maia et al. (25), who reported that CHA has antimicrobial and biocompatibility properties as a bone regeneration material. In our previous study, the antimicrobial efficacy of CHA was evaluated using the agar diffusion method (18). It was found that 0.1 M CHA exhibited the highest effectiveness against E. faecalis and C. albicans compared to MTA, 0.05 M CHA, and 0.5 M CHA (18). In contrast to the present study, Mohammad et al. (26) reported that the mesoporous CHA showed low antibacterial activity, with a high MIC of 200 mg/mL against Escherichia coli. This finding suggests that CHA's efficacy might be related to the microorganism type.

The antimicrobial activity of CHA nanoparticles is mainly attributed to the generation of reactive oxygen species (ROS) such as hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2), and superoxide anions (O_2^{-2}) on their surface (27). These species exhibit antibacterial efficacy through four main mechanisms: disrupting bacterial cell

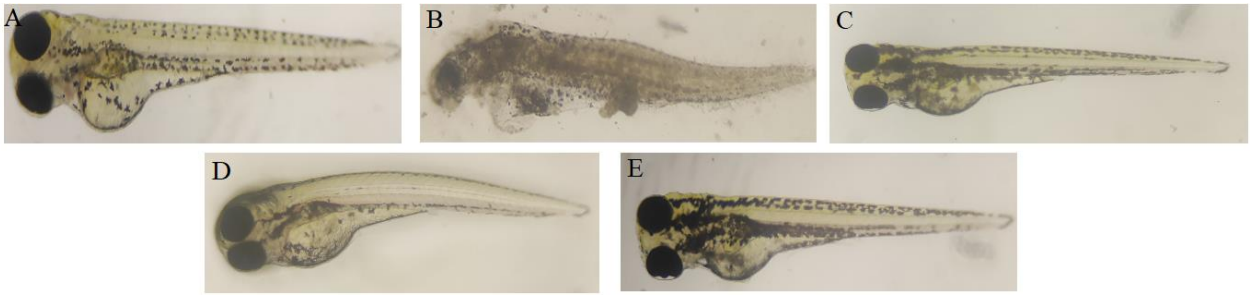

Figure 2. Morphological analysis of 96 hpf zebrafish larvae in the following groups: A) Control, B) MTA, C) 0.05 M CHA, D) 0.1 M CHA, and E) 0.5 M CHA

membranes, inducing oxidative stress, penetrating compromised cell membranes, and interacting intracellularly with biomolecules such as DNA and proteins, resulting in bacterial cell death (27).

The current study revealed that CHA showed good biocompatibility. CHA groups did not negatively affect the survival rate, hatching rate, heart rate, and morphology of zebrafish embryos. However, MTA caused significantly lower hatching, survival, and heart rates than the CHA and control groups. Furthermore, MTA caused tail bending and abnormal morphology in zebrafish larvae.

In this study, zebrafish were chosen for toxicity testing due to their over 80% similarity to the human genome, the transparency of their embryos that enables easy observation, and their rapid reproductive cycle (28). zebrafish embryos are susceptible to toxic substances, making them an excellent in vivo model for evaluating the biocompatibility of nanoparticles (29). Zebrafish serve as a cost-effective model for assessing the biocompatibility of pulp capping materials. In recent decades, Zebrafish have become a widely utilized model organism for researching and understanding human diseases (30). In a study by Makkar et al. (31), the molecular toxicity of MTA and Biodentine was evaluated using a zebrafish model. The results revealed that Biodentine exhibited better biocompatibility compared to MTA. They reported that ROS production and apoptosis induction significantly increased in MTA-exposed embryos compared to those exposed to Biodentine.

The different results in hatching rate and survivability among the study groups may be attributed to the internalization of the exposed materials from the chorion, which contains pores ranging from 0.5 to 0.7 µm in diameter. Chemicals smaller than these pores can pass through the chorion, which might explain why MTA, with its smaller particle size, could have had a greater impact on the hatching rate. The size of MTA particles could facilitate easier internalization through the chorion, leading to a stronger interaction with metabolic proteins and the HeLa enzyme. This enzyme is important in embryo hatching, as it digests glycoproteins that harden the chorion during fertilization. Hatching is a crucial part of the zebrafish life cycle linked to a cascade of biochemical and physical systems. During hatching, the proteolytic hatching enzyme digests the chorion, and the viable embryo ruptures the chorion with mechanical force to release itself (32). Karahan et al. (33) also reported that traditional calcium silicate materials (MTA Angelus and Biodentine) significantly decreased the survival rate and hatching rate of zebrafish compared to resin-modified calcium silicate materials (Oxford and Harvard).

The heart rate in the control and CHA groups ranged from 149 to 154. The heart rate of 140–160 beats per minute is considered a normal heart rate for zebrafish embryos (34). Unlike CHA, MTA exposure caused notochord and tail bending in zebrafish larvae. These abnormal morphological signs may result from metabolic disruptions caused by the passage of MTA elutes through the chorion's surface (31).

The outcomes of this study are in agreement with the results of Pratama et al. (35), who reported that different concentrations of CHA (15 µg/mL, 125 µg/mL, 500 µg/mL, and 2000 µg/mL) had no negative effect on the hatching rate, survival rate, and embryo morphology.

This study's limitations included using in vitro models, which may not accurately reflect in vivo conditions. One limitation of zebrafish embryos models is their physiological differences compared to mammals. Zebrafish embryos develop externally and directly absorb chemicals from their surrounding environment. However, mammalian fetuses are protected by the placenta. Therefore, caution must be exercised when extrapolating the results of this study to mammals (36).

Conclusions

Within the limitations of the present study, the following statements are concluded:

- MTA showed a higher MIC against *S. mutans* and *E. faecalis* than 0.5 M and 0.05 M CHA.
- The 0.5 M CHA group exhibited the lowest bacterial colony count for *S. mutans*, while the 0.1 M CHA group showed the lowest counts for *E. faecalis* and *C. albicans*.
- None of the concentrations of CHA had a negative effect on the survival rate, hatching rate, heart rate, or morphology of the zebrafish larvae.
- MTA decreased the zebrafish larvae's survival, hatching, and heart rates compared to CHA and the control group.
- The 0.1 and 0.5 CHA concentrations showed a favorable antibacterial efficacy against various oral microorganisms and better biocompatibility than MTA.

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The authors have no acknowledgements to declare.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

S.P. contributed to the research design and implementation. C.R. and S.P. contributed to the research implementation and writing the manuscript. A.S. contributed to the supervision of the research and writing of the manuscript. All authors read and approved the final manuscript.

Ethical approval

The protocol of the present in vitro study was approved by the ethics committee of Saveetha Dental College and Hospitals (ID:SRB/SDC/PhD/ENDO-2309/23/TH-081).

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