Investigating Ceremagnum Plus as a Potential Bioceramic Substitute for Pulp Capping Agent: Evidence from Zebrafish Larvae Studies

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survival rate, cytotoxicity, breeding time, post fertilisation time, developmental malformations

ABSTRACT

Objectives: Endodontic therapy focuses on preserving or restoring the function and vitality of pulp and periapical tissues. The introduction of calcium silicate-based bioceramics, known for their excellent biological properties, has significantly improved the success rates of endodontic treatments.This study aimed to assess toxicity of indigenously developed ceremagnum plus as a pulp capping agent by investigating its effects on zebrafish larvae survival rate,heart rate and hatching rate by comparing it to calcium silicate based cement MTA.

Materials and Methods: Zebrafish larvae were exposed to different concentrations of Ceremagnum plus and Mineral trioxide aggregate (MTA). Developmental toxicity was assessed by measuring heart rate, survival rate, and hatching rate through microscopy. Statistical analysis was performed using GraphPad Prism software (version 5.03, GraphPad Software, LLC, San Diego, California, USA). A one-way ANOVA followed by Tukey's post-hoc test was conducted to determine significance levels ($p < 0.05$) among the control and experimental groups, based on triplicate means and standard deviation.

Results: Ceremagnum plus showed 100% survival rate at all concentrations (1mg/ml,5mg/ml and 10mg/ml) whereas MTA showed decreased rate of 95% at 10mg/ml conc.In this study,Ceremagnum plus didn't yield a significant change in heart rate at various concentrations (1mg/ml,5mg/ml and 10mg/ml) as compared to decreased rate shown by control group.No significant differences were seen when compared to control group at various concentrations for hatching rate.

Conclusion: Utilising zebra fish experiment ,ceremagnum plus demonstrated favourable attributes, notably enhancing the survival rates of larvae with minimal alterations in heart rates and hatching rates as compared to commercially available MTA, presenting a promising avenue for enhanced biocompatibility.

1. Introduction

Model organisms play a crucial role in biomedical and toxicological research, helping to identify and study various biological phenomena. Over time, different species have been chosen for their unique technical and practical advantages, such as dogs, chimpanzees, pigs, rabbits, mice, rats, birds, *Drosophila* (fruit flies), *Caenorhabditis elegans*, *Arabidopsis*, *E. coli*, and zebrafish. The use of these organisms has increased significantly in many fields of biomedical research, especially with the completion of their genome sequences.

Model organisms are valued for traits like high genetic similarity to humans, rapid development, short life cycles, affordability, and suitability for genetic studies. Despite longer generation times and higher costs, rodents remain the gold standard in biomedical research due to their well-understood biology and strong resemblance to the human genome. However, zebrafish, a small tropical freshwater fish from the Cyprinidae family, has recently gained popularity as a vertebrate model organism in biological research. Native to the northern and northeastern parts of India, Pakistan, and South Asia, adult zebrafish grow to around 3-4 cm in length and feature 7-9 blue and silver stripes along their bodies. They breed best at an optimal temperature of 28.5°C.

First introduced in the 1930s as a model for developmental and embryological studies, zebrafish gained prominence in the 1970s and 1980s as a new genetic model for forward genetic research. By the 1990s, they had become a leading model in developmental biology, largely due to the creation of

thousands of early-stage zebrafish mutants. More recently, zebrafish have become increasingly favored for research into human diseases.

Zebrafish reproduce rapidly, with females typically laying around 200 eggs per week, and they have a short generation time of 3-4 months. Compared to rodents and other mammals, zebrafish are easier to maintain in research labs, and a large number can be housed in small tanks. Since fertilization occurs externally, embryos can be directly manipulated and observed, with no placental barrier to contend with. This makes it simpler to apply toxins or drugs directly into the water, and zebrafish embryos can absorb diluted compounds through their skin and gills. Starting at 7 days post-fertilization (dpf) and continuing into adulthood, drugs can be administered orally.

Zebrafish share 76% of their genes with humans, making them ideal for genetic studies. Their transparent embryos allow researchers to easily observe developmental stages and functions, such as heartbeats. Due to their external and transparent development, zebrafish embryos are suitable for genome-editing techniques. Their rapid development is also advantageous, with gastrulation occurring within 10 hours post-fertilization, heartbeat beginning at 24 hours, and most organs becoming functional by 5 dpf. By 2-3 dpf, zebrafish larvae are inactive, but they start swimming at 4 dpf. They are considered young juveniles between 12 and 24 dpf, and they reach sexual maturity and are classified as adults around three months of age.

However, zebrafish lack certain mammalian organs, such as a divided heart, synovial joints, cancellous bone, lungs, prostate, and mammary glands, which limits their use in studying defects of these organs. Additionally, there are differences in organ and body sizes between zebrafish and mammals. Zebrafish also have many gene duplications, and their body temperature is lower than that of mammals. As cold-blooded animals, their body temperature depends on their environment, which presents challenges in certain metabolic pathway studies. Therefore, researchers need to consider these key differences when using zebrafish as a model organism and interpret their findings with caution when applying them to humans.

The periodontium is one of the dental structures absent from zebrafish. However, Porphyromonas gingivalis (P. gingivalis) pathogenicity—the primary pathogen responsible for severe periodontitis—was examined in relation to cardiovascular illnesses by Widziolek et al. using zebrafish. They demonstrated how P. gingivalis can penetrate the vascular endothelium, diffuse into the surrounding tissues, and result in heart damage and pericardial oedemas that can be fatal.

The toxicity of zirconium oxide nanoparticles (ZrONPs), commonly used in biomedical applications such as dental implants, was studied using the zebrafish model. Karthiga et al. exposed zebrafish embryos to varying concentrations of ZrONPs between 24 and 96 hours post-fertilization (hpf) and analyzed the effects on embryonic development using different analytical methods. Doses ranging from 0.5–1 µg/ml of ZrONPs resulted in acute developmental toxicity, including death, malformations, and delayed hatching. At 1 mg/ml, a high mortality rate among unhatched embryos was observed. The study concluded that lower concentrations of ZrONPs tend to be more toxic to zebrafish embryos.

Bioceramics are extensively used in various dental procedures, such as pulp capping, root-end fillings, and perforation repair. Makkar et al. used embryonic zebrafish to assess the biocompatibility of two popular bioceramics: mineral trioxide aggregate (MTA) and biodentine. Zebrafish larvae were exposed to different concentrations of these materials, and toxicity was evaluated .The study found that higher concentrations of both materials led to morphological abnormalities and lower survival rates.

2. Materials And Methods

This study was conducted at Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India. It has been performed with the approval of the Institutional Scientific Review Board (SRB/SDC/ENDO-2103/23/015).

Preparation of Faster Setting Ceremagnum Plus

The cement sample was manufactured in the case where KH2PO4 and calcined MgO mass ratio unchanged in 1:1 ratio(500 mg:500mg) KH2PO4 and MgO in turn into the mortar, first dry mixing 2min, then added water at low speed stirring 1min thereafter high-speed stirring 30s, and the stirred and the specimen was placed at room temperature curing for 24hrs.MgO is calcined to about 1100degrees celsius and then cooled slowly over a period of about 24 hours.

Figure 1

$$MgO + KH2PO4 + 5H2O \rightarrow KMgPO4 \cdot 6H2O$$

According to Equation (1), magnesium potassium phosphate hexahydrate (KMgPO4·6H2O), also known as K-struvite, is the MKPC stoichiometric reaction product. This chemical reaction involves the dissolution of MgO, producing Mg2+ and OH− ions, and then the interaction with KH2PO4 (PO43− and K+ ions), resulting in the precipitation of K-struvite in dry powder form.

On weighing scale 1000mg of KMgPO4,1000mg of CaSiO3,32.4mg of cerium oxide and 65.1mg of ZrO2 was added in 1:2 ratio and 39.45 mg NaF ,these all were weighed separately for the sample group whereas 1000mg for MTA Angelus was weighed.Each of the weighed sample were transferred to eppendrof tubes .

The next step included to grinding the powders of Ca silicate and KMgPO4 into a homogeneous fine powder form to achieve an acid-base reaction by transferring them into a beaker then using two spatulas of the mix each time to the mortar and pestle followed by grinding to a smooth mix for 10mins is recommended .

After all the mixture along with other components is ground it is again transferred to one centrifuge tube and kept aside.Around 200mg of this mixture is taken on a paper pad and 200ul of CaCl2 liquid solution is mixed to a homogeneous paste form and allowed to set.

Method of Concentrating Solutions

Each concentration level must be precisely measured while dissolving indigenously developed bioceramic powder in distilled water: 1 mg, 10 mg, and 20 mg in 1 ml of distilled water. To make sure everything was uniform, the stirring method was used. The utilization of the direct dissolving approach facilitates a precise and regulated preparation procedure, guaranteeing the production of

discrete concentration levels that are essential for the research stages of the study. The selected concentration of periostracum was made in order to look into any possible relationship between elevated toxicity and increased periostracum concentration.

Origin

The genesis and upkeep of zebrafish:

Four-month-old adult wild-type zebrafish (AB strain) were obtained from the NSK Aquarium located in Kolathur, Tamil Nadu, India. When the fish arrived, they were kept apart in our facility in 10-L glass tanks with a 14/10 hour light/dark cycle and a temperature of 28.5℃. Live brine shrimp (Artemia salina) were fed to them three times a day. The fish were used for breeding after a month-long acclimation period, and the resulting larvae were used in further studies . After a microscopic examination, the collected embryos were divided into fertilized and unfertilized groups and placed in a six-well plate.

Developmental Toxicity

Toxicological development in larvae

Zebrafish larvae that are transparent are widely used in research on developmental toxicity because it is easier to see flaws and anomalies during the fast growth of these organisms. In this study, the substances used to assess cytotoxicity included the zebrafish subjects were given these chemical substances to examine. For a set period of 24 hours, zebrafish were usually submerged in solutions containing these chemical agents to expose them . After therapy, possible anomalies are found and examined by looking at the developmental stages of the embryos under an inverted microscope.

Survival Rate

Evaluating the survival rate of zebrafish larvae is a commonly used technique to determine the effects of different chemicals and environmental factors on the growth and viability of the larvae. These larvae's small size, translucent bodies, and quick development make them an important model organism. The method includes exposing zebrafish larvae to varying doses of the drug under test, and then tracking the proportion of embryos that survive at each concentration continuously . Every day, the number of surviving larvae was counted, and any that had died were immediately removed. In order to calculate the survival rate (%) of larvae, important indicators such as egg coagulation, heartbeat, and the lack of spontaneous motility were observed.

Heart Rate

An approach used in heart rate analysis is to measure the heart rates of growing zebrafish larvae. Because of their translucent bodies, which allow for excellent observation of heart development, zebrafish are a commonly used model organism for examining heart development and function. 48 hours post-fertilization (hpf) zebrafish larvae are suitable for recording the whole heartbeat frequency. The embryos were placed under a microscope that had a high-speed camera after being rendered immobile in a tiny agarose volume. The heartbeats were recorded by this camera, and the images were processed using specialized software to ascertain the heart rate.

Hatching Rate

The analysis of the hatching rate in zebrafish larvae involves assessing the proportion of larvae successfully hatching from their chorions and the protective outer shells surrounding developing embryos. Zebrafish larvae usually hatch between 48 and 72 hpf, and a reduction in hatching rate may suggest developmental abnormalities or exposure to toxins or stressors. In this procedure, hatching rates were examined using a subset of larvae from each exposed group, including the control. Larvae's were immersed in an E3 embryonic medium for 24 hours that dissolved the chorion, facilitating hatching. Daily counts were conducted to document the number of hatched larvaes , and

any deceased larvaes were promptly removed. In each treatment group, the number of hatched larvae was recorded, and the hatching rate (%) was calculated using the formula:

hatched numbers/total exposed numbers × 100. The number of hatched larvae is then tallied and compared to the total used to calculate the hatching rate.

3. Results

Statistical analysis was performed using GraphPad Prism software (version 5.03, GraphPad Software, LLC, San Diego, California, USA). A one-way ANOVA followed by Tukey's post-hoc test was conducted to determine significance levels ($p < 0.05$) among the control and experimental groups, based on triplicate means and standard deviation.

Developmental Toxicity in Embryos

In this study, zebrafish larvae were exposed to bioactive bioceramics for assessment. The control group received MTA, while the intervention groups were treated with indigenously developed ceremagnum plus at concentrations of 1 mg/ml, 5 mg/ml, and 10 mg/ml. Exposure to gold standard bioceramic MTA Angelus and indigenously developed bioceramic Ceremagnum plus caused no developmental toxicity in the zebrafish larvae, leading to deformation and the emergence of defective larvae.

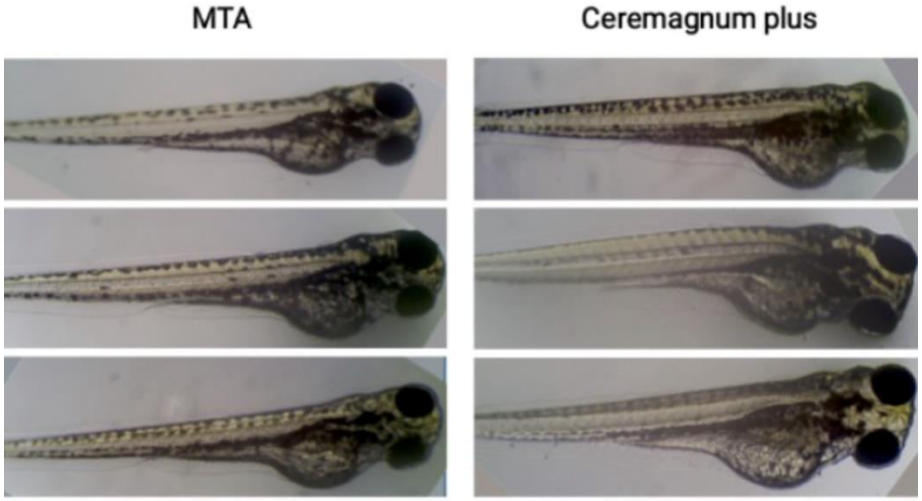

Figure 2

At concentrations of 1mg/ml,5mg/ml and 10mg/ml no significant developmental differences were seen in terms of notochord,tail bending or edema of yolk sac of the set powdered control sample MTA and test sample Ceremagnum plus.

Heart rate in Zebrafish Embryos

The embryonic heart rate was measured for one minute under a microscope, and the average heart rate per minute was then recorded. Ceremagnum plus showed 100% survival rate at all concentrations (1mg/ml,5mg/ml and 10mg/ml) whereas MTA showed decreased rate of 95% at 10mg/ml conc.

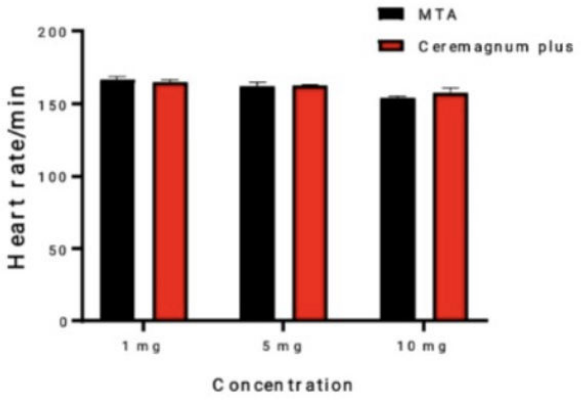

Fig 3:No significant difference between the control and intervention groups.

Hatching Rate in Zebrafish Embryos

Analyzing hatching rates in zebrafish embryos is an essential approach for understanding developmental processes and the effects of environmental stressors. This metric is vital for evaluating the impact of different interventions. Ceremagnum plus didn't yield a significant change in heart rate at various concentrations (1mg/ml,5mg/ml and 10mg/ml) as compared to decreased rate shown by control group.

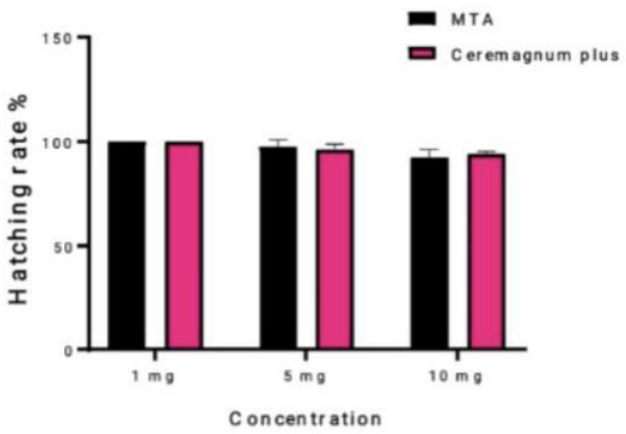

Fig 4:No significant difference between the control and intervention groups.

Survival Rates

The investigation of zebrafish embryo survival rates produced some interesting findings about the effects of several chemicals on the viability of the embryos.In our study Ceremagnum plus showed 100% survival rate at all concentrations (1mg/ml,5mg/ml and 10mg/ml) whereas MTA showed decreased rate of 95% at 10mg/ml conc.

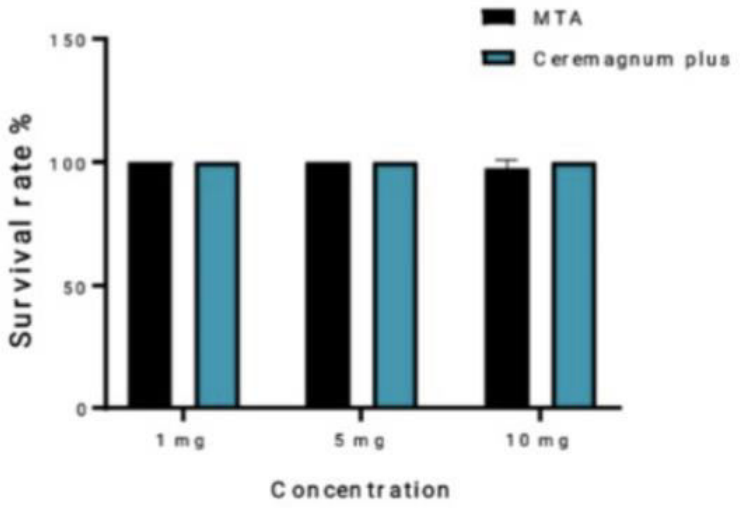

Fig 5:No significant difference between the control and intervention groups.

4. Discussion

This report presented a thorough in vivo and analysis of the biocompatibility of two dental bioceramics, proposing a reliable, cost-effective, and comprehensive model for evaluating the toxicity of dental materials. To assess the biocompatibility of dental materials using the zebrafish model as a platform, the toxicity of MTA was compared with that of the indigenously developed Ceremagnum Plus. Karthiga et al. exposed zebrafish embryos to various concentrations of nanoparticles during the 24–96 hours post-fertilization (hpf) period, using different analytical techniques to observe the effects on embryonic development. They found that doses of 0.5–1 µg/ml of ZrO2 nanoparticles induced acute developmental toxicity, death, malformations, and delayed hatching. At a concentration of 1 mg/ml, the mortality of unhatched embryos became a common occurrence. Based on their findings, the researchers concluded that lower concentrations of ZrO2 nanoparticles are more toxic to zebrafish embryos.

Recently, there has been growing interest in utilizing metal oxide nanoparticles, such as zirconia, in biomedical tissue engineering and implants, particularly in orthopedic and dental applications. In vitro studies have demonstrated that ZrO2 nanoparticles exhibit cytotoxic, genotoxic, and neurotoxic effects on PC12 and N2a cells (Asadpour et al., 2014, 2016). Research on Wistar rats has revealed that these nanoparticles can cause cellular toxicity in red blood cells (Kozelskaya et al., 2016). Additionally, exposure to ZrO2 nanoparticles in the Drosophila melanogaster model has been shown to impact phenotype, function, and neuronal development (Mishra et al., 2017). Further studies have indicated that ZrO2 nanoparticles exert toxic effects on growth and reproduction in aquatic crustaceans such as Thamnocephalus platyurus and Daphnia magna (Załeska-Radziwiłł and Doskocz, 2016). However, since the toxicity of ZrO2 nanoparticles has not been studied in non-mammalian lower vertebrates like fish, it is crucial to investigate their toxic effects using Danio rerio as a model organism. Bioceramics are commonly used in various dental clinical procedures, including pulp capping, root-end fillings, and perforation repair. Makkar et al. conducted a study using embryonic zebrafish to assess the biocompatibility of two widely used bioceramics: mineral trioxide aggregate (MTA) and Biodentine. Zebrafish embryos were exposed to varying concentrations of these materials, and toxicity was evaluated by examining apoptosis and ROS induction. The study revealed morphological abnormalities and reduced survival rates with increasing concentrations of the materials, with Biodentine proving to be more biocompatible than MTA.

While a larger particle may obstruct the pore canals, a smaller particle has a greater probability of

passing through the chorion pore canals. Compared to larvae and adults, embryos with a chorion are less vulnerable to poisoning from waterborne contaminants (Osaki et al. 2006). Additionally, it has been discovered that larger-sized NPs exhibit lower toxicity while smaller-sized NPs exhibit higher toxicity (Ganesan et al. 2016). According to Kimmel et al. (1995), the hatching period of typical zebrafish embryos, which lasts from 48 to 72 hours, is regarded as a crucial stage for embryonic development.

In our study as as the ground cement had particle size of ZrO2 more than 20nm ,hence the toxicity levels were very minimal even at higher concentrations at 10mg/ml.

5. Limitations of the Study

Species-Specific Differences: Zebrafish may not fully represent human physiological responses to novel ceremagnum plus due to differences in immune system, metabolism, and tissue structure.

Scale of Application: Bioceramics are primarily used in dental and endodontic applications in humans, whereas zebrafish are small aquatic organisms, making direct translation to human dental contexts challenging.

Ethical Considerations: While zebrafish are widely used due to their small size and rapid reproduction, ethical considerations regarding animal use in research persist.

6. Conclusion

The study presents Ceremagnum plus as a promising bioceramic to be used as pulp capping agent in terms of biocompatibility when compared to conventional MTA.Utilising zebra fish experiment,ceremagnum plus demonstrated favourable attributes, notably enhancing the survival rates of embryos with minimal alterations in heart rates and hatching rates as compared to commercially available MTA, presenting a promising avenue for enhanced biocompatibility.

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