A novel zinc oxide eugenol modified by polyhexamethylene biguanide: Physical and antimicrobial properties

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This study was to prepare and screen a novel root canal sealing agent modified by polyhexamethylene biguanide (PHMB) that was in accordance with the ISO 6876:2001 standard and to study its physical and antimicrobial properties. The modified sealers were produced by mixing a certain amount of zinc oxide with eugenol containing different concentrations of PHMB (0.05, 0.1, 0.2, 0.4, 0.6 and 0.8%) at a ratio of 1:1 (w/v). The setting time, flow, film thickness, solubility and dimensional change after solidifying were assessed to screen out the modified sealing agents that the physical properties met the mentioned standards. The modified direct contact test (DCT) was used to evaluate the antimicrobial activity against Enterococcus faecalis. The results suggested that when the concentrations of PHMB were 0.05, 0.1 and 0.2%, the modified root canal sealers showed the best performance in physical and antimicrobial properties.

Keywords: Zinc oxide eugenol, Polyhexamethylene biguanide, Physical properties, Antimicrobial property

INTRODUCTION

The key factors to the success of root canal therapy (RCT) are eradicating microorganisms from the root canal system and preventing infections from reoccurring. Unfortunately, it is hard to eradicate the microorganisms merely by chemo-mechanical preparation because of the complexity of the root canal system and the formation of microbial biofilms. Thus, treated root canals are often re-infected by the remaining microorganisms 1. However, previous studies have demonstrated that the use of sealers with antimicrobial activity is conducive to eradicating microorganisms and avoiding secondary infection in some cases 2,3. The most frequently detected pathogen in re-infected root canals is Enterococcus faecalis 4, which can survive in the airtight root canals and form biofilms in the deep dentinal tubules, resisting conventional endodontic treatments 5. Consequently, improving the antibacterial activity of root canal sealers is beneficial to eliminate infections.

In past studies, the main measure to enhance the antimicrobial activity of endodontic sealers has been adding antimicrobial compounds, such as antibiotics 6, chlorhexidine (CHX) 6, cetrimide (CTR) 6, benzalkonium chloride (BO) 7 and cetylpyridinium chloride (CPC) 9. Modification by adding antibacterial agents improves the antimicrobial activity of the root filling materials; however, the composition could be changed, which degrades the physical properties and long-term chemical stability of these materials 7. For the above reasons, another antibacterial additive was selected in this study.

Polyhexamethylene biguanide (PHMB) is a safe and effective antimicrobial agent with cationic and amphipathic properties 8,9 and has been widely used for wound dressings 10, water treatment, contact lens solutions, mouthwash 12, disinfectants 13 and so on, but it has not been used in root canal sealants. PHMB has good antimicrobial property; even with extensive use for the past 40 years, PHMB-resistant mutants have not been reported 8. PHMB can kill bacteria effectively by binding rapidly to the cell wall and envelope of bacteria, destabilizing the membrane and causing cytoplasmic leakage 6,14 or selectively binding and condensing bacterial chromosomes after entering the bacteria 15. Compared to CHX, BC, CPC and triclosan (TRI), not only does PHMB have better antibacterial properties and is biocompatible 16 but it can also stimulate the proliferation of human fibroblasts in vitro and promote the osteointegration of bone mesenchymal stem cells (BMSCs), which is beneficial to its application as a root canal sealing material 7,18.

AH Plus (Dentsply DeTrey, Konstanz, Germany), the most widespread resin-based root canal sealer, has good sealing ability in clinical practise but cannot adequately release the added antimicrobial agents after being completely solidified due to its strong cross-links in the resin polymers and lower solubility 19 which are unfavourable to maintain long-term antibacterial activity 9. In contrast, zinc oxide eugenol (ZOE) has been widely applied and modified in dentistry for root canal fillers because of its ease of use, low cost, lower porosity 20, excellent physical properties, antibacterial properties, and therapeutic effects, including immunomodulation/anti-inflammation 21. Compared to that of AH Plus, the solubility of ZOE is higher, which implies that ZOE is more conducive to the release of added antimicrobials after solidifying. Therefore, this study used ZOE modified...
Molds (20 mm in diameter, 1.5 mm thickness) were placed on a glass plate, and fresh sealer was filled in them to slight excess. Another glass plate covered with cellophane film was laid on the mold. The glass plate was carefully taken off to ensure that the material was uniformly flat. The assembly was positioned in an incubator (ZDP-2160, Labwit Scientific, ShangHai, China) (37±1°C, 95–100% relative humidity) for a period that was at least 1.5 times that according to the standard setting time. After this period, the samples were removed from the molds and weighed with a precision of 0.001 g. Then, two samples without contact were placed into shallow trays containing 50±1 mL distilled and deionized water. Every tray was sealed and deposited in an incubator (37°C, 95% relative humidity) for 24 h. The samples were removed from the containers and rinsed in distilled and deionized water. If nothing was found in the washed water, the samples were placed in a 110±2°C dehumidifier and desiccated to a constant weight. The samples were then weighed again. The weight loss of every sample, expressed as a percentage of the original mass, was taken as the solubility of the sealers.

Dimensional change after solidification
A mold (6 mm inner diameter, 12 mm height) that was overfilled slightly with the mixed sealer was placed on a glass plate covered with polyethylene. Another glass plate also covered with polyethylene, was placed on the mold. The mold, together with the glass plates, was fixed by a C-shaped clamp and positioned in an incubator (ZDP-2160, Labwit Scientific, ShangHai, China) (37°C, 95% relative humidity) for 5 min after the beginning of mixing. When the samples were solidified completely, their ends were flattened using fresh 600 grit wet sandpaper by grinding back and forth. Then, the samples were removed from the mold, and the height of each sample was measured with an accuracy of 10 μm. After all the samples were reserved in distilled water (37±1°C) that was at least 1.5 times that according to the standard within a period of root canal sealers.

**MATERIALS AND METHODS**

**Preparation of ZOE and ZOE modified by PHMB**

PHMB (powder, ≥99.2%) (YanBang Fine Chemical, ChangSha, China) was dissolved in eugenol (ShangHai Medical Instruments, ShangHai, China) to prepare solutions with concentrations of 0.05, 0.1, 0.2, 0.4, 0.6 and 0.8% (w/v). Zinc oxide (powder, ≥99.0%) (ZhiYuan Reagent, TianJin, China) was mixed with the solutions in eugenol (1:1, w/v) to obtain the modified ZOE and ZOE. All operations were performed according to the manufacturer’s instruction.

**Setting time**
The sealers were filled in rings (10 mm internal diameter, 2 mm height) on a glass plate and incubated at 37±1°C and 95% relative humidity. An indenter with a mass of 100±0.5 g and a diameter of 2.0±0.1 mm was pressed vertically onto the horizontal surface of each specimen lightly when the sealers began to solidify. The needle tip was a cleaned, flat-ended cylinder. The operation was repeated at 60-s intervals until the indentations were invisible. The time from the commencement of mixing to this point was recorded as the setting time.

**Flow**
Using a graduated disposable 1-mL syringe, a 0.05 mL volume of cement was transferred to the centre of a glass plate (40×40×5 mm). After 180±5 s of mixing, another plate together with a load weighing 100±2 g was placed onto the materials centrally. Ten minutes after the start of mixing, the load was removed. Then, the maximal and minimal diameters of each compressed specimen were measured using a Digimatic caliper (Terma CD730, GuangDong, China) with an accuracy of 0.005 mm. If the difference between the maximal and minimal diameters was less than 1 mm, the mean of 4 determinations was taken as the flow of each specimen.

**Film thickness**
The thickness of two contact flat glass plates (5 mm thickness, 200±10 mm² contact area) was measured with a resolution of 1 μm (Terma MD710). A 0.05 mL volume of the mixed sealer was laid down centrally on a glass plate. In addition, another glass plate was placed onto the sealer. At 180±10 s from the start of blending, a load of 150±3 N was applied carefully and vertically on top of the plate. When the sealer filled up the interspace of the glass plates, the thickness of the two glass plates and the film was measured with a micrometer. The difference between the plate with and without sealer was calculated as the film thickness of each specimen.

**Solubility**
Molds (20 mm in diameter, 1.5 mm thickness) were placed on a glass plate, and fresh sealer was filled in eugenol (1:1, w/v) to obtain the solubility of the sealers. The original bacterial suspension placed
on the centrifuge tubes without sealers was used for counting the original bacteria. After incubation in 100% humidity at 37°C for 60 min, 240 μL of a sterile lysogeny broth (LB) was added to each centrifuge tube. The bacterial suspension was mixed for 1 min, transferred and diluted serially in LB. The survival of the bacteria was assessed by culturing aliquots of 50 μL on LB agar plates after a 10-fold serial dilution. The plates were incubated (37°C, 95% relative humidity) for 48 h, and the bacterial colonies were counted afterwards. The bacteriostatic rate was calculated for comparing the antimicrobial properties of the sealers. All experiments were performed in triplicate.

Data analysis

The data were analyzed using the statistical software, SPSS v. 19.0 (SPSS for Windows SPSS, Chicago, IL, USA). A one-way analysis of variance (ANOVA), LSD t-test and Dunnett t-test were used for multiple comparisons. The level of significance was set at 95%.

RESULTS

Physical properties tests

The results of the physical properties tests of ZOE modified by PHMB are shown in Fig. 1. Overall, PHMB shortened the setting time ($p<0.05$), but the addition of
0.05% PHMB had no effect on it (p>0.05). When PHMB concentrations were increased from 0.1 to 0.8%, the liquidity of ZOE was reduced (p<0.05). In turn, both the film thickness and the solubility were increased (p<0.05), but similar solubility values were obtained when the PHMB concentrations were increased from 0.05 to 0.4% (p>0.05). Concerning the dimensional change after solidification, the volume showed shrinkage in all the experimental groups (p<0.05).

In addition, all these results were compared with the requirements of ISO 6876:2001\(^\text{22}\)). When the PHMB concentration was more than 0.4%, the film thickness and volume shrinkage of the modified sealers exceeded 50 \(\mu\text{m}\) and 1.0% individually, which unfulfilled the ISO 6876:2001\(^\text{22}\)). When the PHMB concentrations were 0.6 and 0.8%, the diameters of each compressed specimen were less than 20 mm, which did not meet the standard. The samples had a weight loss beyond the 3% threshold established by International Organization for Standardization (ISO) when the PHMB concentration was 0.8%.

In summary, only when the concentrations of PHMB were increased from 0.05 to 0.2% did the physical properties of the modified sealers meet the standards of ISO 6876:2001\(^\text{22}\)).

**Antimicrobial activity test**

The results of the antibacterial effect of the sealers from DCT are presented in Fig. 2. After the PHMB modification, the inhibition rate of the sealers against *Enterococcus faecalis* was significantly increased compared to the control groups (p<0.001). The antimicrobial effect of the modified sealers was improved and the duration of it was also extended with the increasing concentrations of PHMB. In the fresh specimen groups, the bacteriostatic rates of the modified sealers in variation were more than 97% and showed no statistical difference (p>0.05). After 1, 7 and 14 days of setting, the ZOE+0.05% PHMB group showed weaker antimicrobial activity than that of the ZOE+0.1% PHMB and ZOE+0.2% PHMB groups, but there was no statistically significant difference between ZOE+0.1% PHMB and ZOE+0.2% PHMB (p>0.05).

**DISCUSSION**

The purpose of adding antibacterial agents to root canal sealants is to improve their antibacterial property to maintain the sterile environment of the prepared root canals. However, the physical properties of ZOE were changed while adding PHMB. According to the ISO 6876:2001 standards\(^\text{22}\)), the setting time of the sealer must be no longer than 72 h; each disc should have a diameter not less than 20 mm in a flow test; sealers should have a film thickness of not more than 50 \(\mu\text{m}\); solubility should not exceed 3% mass fraction; and the mean dimensional change in the length of the sealer should not exceed 1% in shrinkage or 0.1% in expansion\(^\text{22}\)). The conclusion in this article, which is obtained from these requirements, is that the physical properties of the modified sealers no longer met the ISO 6876:2001 standards\(^\text{22}\) when the PHMB concentrations were higher than 0.4%, which may be closely related to the physical and chemical properties of PHMB.

The setting time is mainly dependent on the constituent components, their particle size, the ambient temperature, and relative humidity\(^\text{23}\)). Incorporating PHMB significantly decreased the setting time, which was still within the standards. This finding may be because PHMB has weak acidity (pH at 4.5–6.5) in a constant humidity environment, which can promote the dissolution of zinc oxide to produce zinc ions that can form an insoluble chelate with eugenol, thus accelerating the solidification process (Fig. 3)\(^\text{24}\)).

Both flow and film thickness reflect the fluidity of the sealents. Suitable fluidity is an important performance, which is related to how easily the sealers will fill the root canals\(^4\)). If the flowability is inadequate, the canal isthmus and lateral root canals cannot be filled firmly and there will be a higher risk of reinfection. In this study, the antibacterial agent was incorporated as a powder,
which increased the viscosity of the modified sealers, reducing the flow and making the films thicker\textsuperscript{23,25}.

The solubility of ZOE was increased by adding PHMB, which was not conducive to reducing the occurrence of apical microleakage. However, it is noted that the antibacterial activity of the sealers is related to high solubilities, meaning that the higher the solubility is, the more antimicrobial agent is released. Therefore, a certain solubility is advantageous for powerful antibacterial root canal sealers\textsuperscript{26,27} when the solubility of under 3% mass is the requirement of ISO 6876:2001\textsuperscript{22}.

The larger volume shrinkage after solidification means that the filling materials will not contact with the lateral wall of root canals closely. In this study, when the concentrations of PHMB were 0.05, 0.1 and 0.2%, the shrinkage were varying from 0.71 to 0.83%. However, in the dimensional change test, the samples were partially dissolved due to being placed in distilled water for 30 days, which led to a reduction in volume during solidification. Therefore, the real volume change of the samples should be smaller than that shown in the results.

In endodontics, the physical properties of AH Plus cause it to be considered a gold standard. In a previous study, the setting time of AH Plus was approximately 890 min, the value of fluidity was 38.85±0.24 mm, and the solubility (reduction in weight) was 0.197±1.15%\textsuperscript{23}. By comparison, the sealers modified by PHMB had a longer setting time (2862±28.60~3884±68.50 min), lower fluidity (20.625±0.71~21.833±0.66 mm), and higher solubility (1.42±0.05~1.48±0.04%). A low flow can reduce the risk of extravasation of the material to the periapex and consequent damage to the periodontal tissues, but it is not good for lateral root canal filling\textsuperscript{23}. The solubility of the modified sealers showed results below the standardized limit (≤3%)\textsuperscript{22}, which can not only meet the apical sealing capability but is also beneficial for maintaining long-term antimicrobial activity\textsuperscript{26,27}.

DCT is a quantitative and reproducible method and has much more advantages than the agar diffusion test (ADT). It can simulate the contact of \textit{Enterococcus faecalis} with sealers in the root canals, and test the antibacterial effect of the sealants at different stages of the setting reaction\textsuperscript{9}. In DCT, the plating was done immediately after each time of contact, which can show whether all bacteria have been killed. This method also makes it possible to calculate the survival rate of the bacteria. Thus, DCT was used for evaluating the antibacterial effect of the sealers.

The antibacterial effect tests against \textit{Enterococcus faecalis} suggested that newly produced ZOE possesses antibacterial activity\textsuperscript{28}; during the 1-h contact with fresh sealers, 59% of the bacteria were killed. Nevertheless, with the continuous solidification of ZOE, the phenolic hydroxyl of eugenol was chelated with zinc oxide, and the amounts of free zinc ions and eugenol decreased, making the antibacterial activity decline constantly. After 14 days of setting, only 26% of the \textit{Enterococcus faecalis} was eliminated. The continuous decreasing tendency of the antibacterial effect is a common problem in current root canal sealers\textsuperscript{1,9}, which is disadvantageous for maintaining the sterile environment of the prepared root canals. Previous study showed that the antibacterial activity of the sealers (iRoot SP, Apexit Plus, AH Plus, Tubli Seal, Sealapex, Epiphany non–light-cured, Epiphany light-cured, EndoRez non–light-cured and EndoRez light-cured) was relatively stable in 3 days, but most of them lost much of antibacterial effect rapidly after 7 days except for Sealapex and EndoRez\textsuperscript{9}. In contrast, the modified sealers can eradicate microorganisms by releasing PHMB persistently after solidifying, showing its stronger and lasting antibacterial effect. In terms of antibacterial properties, previous studies have reported that only fresh AH Plus possesses antibacterial activity and can kill 60.19% of the \textit{Enterococcus faecalis} after 1 h of contact, whereas 24-h and 7-day-old samples do not show an antibacterial effect against \textit{Enterococcus faecalis}\textsuperscript{28}. The sealers modified by PHMB still showed strong antibacterial activity after 14 days of setting. In ZOE+0.2%PHMB group, the sterilizing rate of it was 85.23% at 14 days of setting, slipping only 14.77 points compared with fresh materials. It is obvious that the antibacterial properties of the modified sealers are superior to those of AH Plus when the PHMB concentrations range from 0.05 to 0.2%. In addition, the antibacterial properties are better than those of iRoot SP, Apexit Plus and Tubli Seal\textsuperscript{9}.

As PHMB has a strong adsorption capacity, it can reach deep dentinal tubules that root canal sealers cannot, binding to the surface of the bacterial biofilm and killing the bacteria in the deep layer of the biofilm after being released by the modified sealers\textsuperscript{29}. In addition, PHMB produces a long-lasting antibacterial effect after adsorbing on the internal surfaces of dentinal tubules, which helps keep a sterile environment in the root canal system and avoid reinfection\textsuperscript{30}.
CONCLUSIONS

In conclusion, the addition of PHMB significantly improved the long-term antibacterial properties of ZOE. Additionally, the physical properties changed but still met the requirements of ISO 6876:2001 by controlling the concentrations of PHMB within a reasonable range (0.05–0.2%). Nevertheless, these are preliminary tests, and further characterization experiments concerning biocompatibility, cytotoxicity and antibiofilm activities should be undertaken for clinical application after the further screening.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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