Comparative Fluoride Release and Antimicrobial Analysis of Commercial and Experimental Bioactive Glass/Nano-Oxide-Based Dentifrices

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Abstract

Objectives The objectives were to measure fluoride release and assess the antimicrobial behavior of fluoride-doped nano bioactive glass (F-nBG) and nano zinc oxide (ZnO)-enriched novel dentifrices.

Materials and Methods Experimental dentifrices were synthesized by incorporating ZnO nanoparticles and F-nBG (1.5 wt% and 4 wt%) as active ingredients. The fluoride release behavior of suspensions and elutes of samples were analyzed by ion selective electrode. Antimicrobial activity and minimum bactericidal concentration against Streptococcus mutans and Lactobacillus casei were evaluated. Microbial stability against contamination was also assessed by a challenge test.

Results The fluoride release behavior of experimental dentifrices was higher than that of commercial dentifrices and was dependent on filler loading. The fluoride release was more from suspensions than elutes. Zones of inhibition (ZOIs) and minimum bactericidal concentration values for novel dentifrices showed direct proportionality with filler loading, and effectiveness was exhibited against both strains. Experimental dentifrices exhibited effective antibacterial potential, which could possibly be due to release of sufficient fluoride and zinc ions in aqueous media from F-nBG and ZnO present in their formulations.

Conclusion Combination of F-nBG and ZnO may provide a multi-benefit approach for simultaneously treating early white spot lesions, reducing bacterial growth, and providing core plaque control.

Keywords ► toothpaste ► nanoparticles ► bioactive glass ► zinc oxide ► fluoride release ► antibacterial

Introduction

Brushing teeth with fluoridated toothpaste has been widely practiced worldwide for dental caries prevention, as it removes biofilm mechanically, which adds to the therapeutic effects of fluoride. Other ingredients for functions such as whitening, antiplaque, antibacterial, antitartar, and erosive prevention can be added.1 Availability and stability of fluoride in dentifrices are basic requirements for effectiveness against tooth decay.2,3 Fluoride ions can inhibit
the production of bacterial acids in dental plaque due to the influx of hydrogen fluoride into bacterial cells, and the dissociation to the $\text{H}^+$ and $\text{F}^-$ ions in the cytoplasm.\textsuperscript{4} For caries control, dentifrice formulation should contain a minimum of 1000 parts per million (ppm) fluoride, which must be in a soluble form to impart the anticaries effect.\textsuperscript{5,6} The factors which control the soluble fluoride content in dentifrice are its composition (i.e., fluoride source) and storage conditions (i.e., temperature and aging). It is reported that low-pH toothpastes containing 500 to 550 ppm fluoride showed similar results on enamel demineralization as the one containing 1100 ppm fluoride.\textsuperscript{7}

Dentifrice composition plays a crucial role in disabling free fluoride ions, which may lead to creation of a low-solubility product with a diminished anticaries outcome.\textsuperscript{2,3} Currently, there are no regulations to specify how much of total fluoride should be maintained in a dentifrice formulation.\textsuperscript{7} There has been a growing interest in the use of “smart” bioactive materials (i.e., amorphous calcium phosphate, hydroxyapatite, bioactive glass, etc.) for tooth remineralization. It is reported that the remineralization effect of nano-hydroxyapatite crystals is generally limited to the surface of the initial carious lesion.\textsuperscript{8} Bioactive glass is one such material being considered as a novel dental material with remineralization potential. Some in vitro studies have shown that bioactive glass-containing dentifrices minimized dentin hypersensitivity by dentinal tubule occlusion and apatite formation.\textsuperscript{9,10}

Recently, fluoride-doped bioactive glass nanoparticles (F-nBG) have been synthesized by our group, and the long-term fluoride release behavior of F-nBG and its effect on pH was evaluated.\textsuperscript{11} Zinc oxide (ZnO) powder has been added as a preservative in dentifrices, which, in aqueous suspension, not only inhibits dentin demineralization but also induces antimicrobial action by yielding zinc ions ($\text{Zn}^{2+}$) and reactive oxygen species.\textsuperscript{12-14} The purpose of this study was to assess and compare the antimicrobial efficacy and fluoride release behavior from the suspensions and elutes of experimental dentifrices and a commercial dentifrice.

### Materials and Methods

In this study, all chemicals were analytical grade. Initially, basic ingredients were mixed in optimized ratios and allowed to mix homogeneously. Then 1.5 wt% titanium dioxide (Sigma Aldrich; St. Louis, MO, United States) and 3 wt% zinc oxide nanoparticles were added in increments to avoid agglomeration. The ZnO nanoparticles were synthesized by our group as described previously.\textsuperscript{15} The scanning electron microscopy (SEM) image and X-ray diffraction (XRD) pattern of prepared ZnO nanoparticles are shown in \textit{Fig. 1}. The morphological pattern showing the nanostructure of prepared ZnO and the diffractogram is in accordance with JCPDS N 00-036-1451 (standard X-ray peaks of zinc oxide). F-nBG (5% mol. concentration of F in F-BG) was used in this study and incorporated in experimental dentifrices in two ratios: 1.5% and 4% wt/wt. Colgate Cavity Protection (Colgate-Palmolive, Guildford, UK) was used as the control. The composition of both the control and the experimental dentifrices is given in \textit{Table 1}.

### Fluoride Release Analysis

The fluoride research protocol used in this study was modified from the one suggested by Pearce.\textsuperscript{16} The study was performed in quadruplicates. From each group, a 100 mg dentifrice sample was homogenized in 10 mL deionized water to form dentifrice suspensions, from which half of the suspension was split in two centrifuge tubes, each containing 2.5 mL suspension. The remaining half dentifrice suspension was centrifuged (Hettich EBA 20; Hettich, Tutlingen, Germany) at 6000 rpm for 10 min ($2461 \times g$) to remove the insoluble fluoride bound to filler particles to extract elute into two centrifuge tubes, each containing 2.5 mL of elute. The same procedure was repeated for all dentifrice groups. Then 2.5 mL of 2M HCl (BDH Chemicals; Hull, East Yorkshire, UK) was added to all the centrifuge tubes containing the dentifrice elute and suspension samples, which were then conditioned at 45°C for 1 hour followed by an addition of 5 mL of 1M NaOH (Sigma Aldrich) and 1 mL TISAB III reagent (Hanna Instruments; Woonsocket, Rhode Island, United States). Finally, the samples were analyzed using pre-calibrated ion selective electrode (ISE) potentiometry (Hanna HI3222 pH/ISE meter and fluoride electrode; Hanna Instruments, Woonsocket, Rhode Island, United States).

### Antimicrobial Susceptibility Test

For the antimicrobial susceptibility test, freeze-dried microbial strains \textit{Streptococcus mutans} (ATCC-25175) and \textit{Lactobacillus casei} (ATCC-393) were obtained from American Type Culture Collection (ATCC; Manassas, VA, United States). Dehydrated culture media was obtained from Oxoid (Basingstoke, Hampshire, England) and Merck Millipore (Darmstadt, Germany). Microorganisms were revived from freeze-dried vials in their respective culture media—MRS (de Man Rogosa broth for \textit{L. casei} and BHI (Brain Heart Infusion) broth for \textit{S. mutans}—as per American type culture collection (ATCC) guidelines. For the test procedure, 2 g of selected dentifrice (\textit{Table 1}) was mixed in 2mL of sterile deionized water, and this yielded a 1:1 dilution. The same procedure was applied to prepare three dilutions of each dentifrice in the ratios of 1:1, 1:2, and 1:4, respectively. Dentifrice slurries were vortexed in an electric shaker (Heidolph REAX 2000; Gemini BV Laboratory, DG Apeldoorn, the Netherlands). For inoculum preparation, three to five well-isolated, similar colonies were selected and transferred to tubes containing 5 mL of 0.9% saline. The suspensions were then adjusted to a density equivalent to 0.5 standard of the McFarland Turbidity Scale (DensiCHEK plus; bioMérieux, NC, United States). Within 15 min of the preparation of the adjusted inoculum suspensions, it was swabbed evenly over the entire surface of the respective agar plates of prepared media to create a bacterial lawn.

### Agar Well-Diffusion Assay

A sterile cork borer was used to create four wells in each of the agar plates. The punched agar circles were picked with a sterile needle and removed from the plates. The wells were 8 mm in diameter and 4 mm in depth and were placed at distances of 20 mm in each of the plates. Then, 0.2 mL of the three...
Table 1  Grouping and composition of dentifrices used in the study

<table>
<thead>
<tr>
<th>Dentifrice</th>
<th>Abrasive</th>
<th>Other ingredients (% wt)</th>
<th>Fluoride agent Percentage</th>
<th>Group name</th>
</tr>
</thead>
</table>
| Colgate cavity protection (Colgate-Palmolive, Guildford, UK) Lot-number: 10141222 | Calcium carbonate (CaCO₃) | Water
Glycerin
Sodium lauryl sulfate
Sodium saccharin
Cellulose gum
Flavoring agent
Tetrasodium pyrophosphate | SMFP 0.76 w/w (0.15 w/v) | CD         |
| Experimental dentifrices               | Calcium carbonate (CaCO₃) 27% wt | Water ~ 36%
Glycerol ~ 32%
Sodium benzoate ~ 1%
Methylcellulose ~ 1%
Flavoring agent ~ 1%
Sodium lauryl sulfate ~ 2%
Water ~ 36%
Titanium dioxide (TiO₂) 1.5%
Zinc oxide (ZnO) nanoparticles 3% | F-nBG with 5% mol fluoride 1.5% F-nBG (w/w) | ED1 |
|                                        |                       |                                                             | F-nBG with 5% mol fluoride 4% F-nBG (w/w) | ED2         |

Abbreviation: SMFP, sodium monofluorophosphate.

Fig. 1 SEM image and X-ray diffractogram of synthesized zinc oxide. SEM, scanning electron microscope.
dilutions (1:1, 1:2, and 1:4) of each dentifrice and a control containing deionized water were poured into separate wells in each agar plate using a micropipette (Genex Beta; Guangdong China). The pH of prepared, solid agar mediums used for this study was similar to the physiological pH of plaque. 17

All the plates were made in triplicate, and S. mutans was incubated in both aerobic and anaerobic conditions and tested on Mueller–Hinton agar plates. In the case of L. casei, the sets of triplicate were tested in microaerophilic as well as predominant anaerobic conditions on chocolate blood agar plates (Gas-Pak sachet-Oxoid AnaeroGen; Thermo Fisher Scientific, Bartlesville, OK, United States).

**Determination of Minimum Bactericidal Concentration**

Susceptibility break points are expressed as minimum inhibitory concentration (MIC) and/or minimum bactericidal concentration (MBC) using appropriate dilution methods, whereby two-fold dilutions of the experimental and commercial groups were prepared as 1:1, 1:2, 1:4, 1:8, and 1:16 (2 g/2 mL yielded a 1:1 dilution). MBC was determined using modified broth dilution method for dentifrices. 18 Then, 1 mL of each dilution of dentifrice was mixed with 1 mL of respective broth to yield a defined volume of 2 mL in each of the five test tubes. A test tube without dentifrice, containing 2 mL of deionized water for each bacterial strain, was used as control.

All the test tubes, including controls, were inoculated using a sterile wire loop. The test tubes inoculated with S. mutans were incubated aerobically for 24 hours at 37°C, whereas the test tubes inoculated with L. casei were incubated for 48 hours at 37°C. To ensure reproducibility, this step was repeated twice. Following incubation, all test tubes were sub-cultured to Mueller–Hinton agar (S. mutans) and chocolate blood agar plates (L. casei) in duplicate. The plate which showed no visible growth was taken as MBC and reported in g/mL of the corresponding dilution.

**Statistical Analysis**

SPSS version 22 (IBM; Armonk, New York, United States) was used to calculate the means and standard deviations of the groups along with differences within and between the groups by one-way ANOVA, and a posthoc Tukey’s test was used for pairwise comparison between groups.

**Results**

**Fluoride Release Analysis**

The fluoride release in ppm for both the elutes and the suspensions of all the study groups are presented in **Fig. 2**. Among elutes of the dentifrices, maximum mean fluoride release (± standard deviation) was detected in ED2 (13.22 ± 0.09 ppm), followed by ED1 (9.74 ± 0.05 ppm), and the control dentifrice (6.28 ± 0.05 ppm). Among suspensions of the study dentifrices, the maximum mean fluoride release was exhibited by ED2 (14.20 ± 0.09 ppm), followed by ED1 (10.07 ± 0.09 ppm), and the control group (6.63 ± 0.05 ppm). In both elutes and suspensions of experimental dentifrices, a rise in mean fluoride release was observed with a rise in F-nBG filler content. The posthoc Tukey’s test showed a statistically significant difference

![Fig. 2 Mean fluoride elusion values with standard deviations (error bars) in the elutes and suspensions of the experimental dentifrices.](image-url)
between the mean fluoride release of elutes of all the study dentifrice groups and between the mean fluoride release of suspensions of all study dentifrice groups (p = 0.000).

**Antimicrobial Analysis**

**Antimicrobial Susceptibility Test: Agar Well Diffusion Assay**

Following incubation, resultant zones of inhibition were observed on the agar plates. The clear and circular halos surrounding the wells were observed. The zone of inhibition against *S. mutans* appeared after 24 hours, and the inhibition zone against *L. casei* appeared after 48 hours. The inhibition zones increased in a dose-dependent manner for the dilutions 1:1, 1:2, and 1:4 of dentifrice slurries. The negative control wells containing deionized water produced no observable inhibitory effect for any of the bacteria in each of the triplicate sets of agar plates. The assessment of antibacterial potential against caries-causing odonto-pathogens was done by recording the diameters of zones of inhibition (ZOIs) (mm) using a Vernier caliper (Table 2). One-way ANOVA (analysis of variance) revealed that only ED1 and CD indicated statistical significance (p < 0.05). Pair-wise comparisons using a posthoc Tukey’s test revealed differences between the concentration groups. ED1 and ED2 were statistically insignificant (p > 0.05) for both bacterial strains under all conditions.

**Determination of Minimum Bactericidal Concentration**

ED2 showed increased effectiveness and was bactericidal (S. mutans) even at the dilution of 1:16, similar to CD. In the case of L. casei, ED1 was effective up to a dilution 1:4 only and growth was observed on plates at a dilution of 1:8. This finding also corresponded with the smallest mean ZOIs observed for ED1 against *L. casei* using agar well-diffusion assay (Table 2). The remaining dentifrices were bactericidal (L. casei) up to a dilution of 1:8. For both bacterial strains, marked growth was observed on the plated controls.

**Discussion**

In the current study, evaluation of the proportions of F-nBG fillers in dentifrices that yield maximum fluoride elusion among novel dentifrices was performed. Upon increasing the F-nBG filler content in experimental dentifrice groups, an increase in fluoride release rates in both elutes and suspensions of dentifrices was observed, whereas a direct proportionality of fluoride release rate with the F-nBG filler loading in novel dentifrices was observed.

Dentifrices are used in suspension form during tooth brushing procedures; therefore, the fluoride elusion in dentifrice suspensions is of greater clinical significance than the fluoride elusion in the dentifrice elutes. However, no remarkable difference was observed in fluoride release values of elutes and suspensions of the same group. This may possibly be due to instant release of fluoride ions upon dissolution of fluoride compounds used in experimental dentifrices in aqueous media. Regarding the bactericidal effect of fluoride, it is reported that fluoride release of 20 ppm exhibits direct bactericidal action. However, due to the buffering action of saliva, this amount drops and may not be as relevant clinically. The elutes and suspension of ED2 showed 13.72 ppm and 14.20 ppm and close to the required amount.

In a previous study conducted by our group, F-nBG particles with 5 wt% fluoride showed burst fluoride release for the initial 9 days. As dentifrices are used to brush teeth for only a few minutes, therefore, the burst fluoride release by F-nBG filler particles with 5 wt% fluoride is of clinical significance in dentifrices. Addition of fluoride to bioactive glass fillers in dentifrices that yield maximum fluoride elusion is of clinical significance in dentifrices.

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**Table 2** Mean diameter and SDs of ZOI against microbial strains

<table>
<thead>
<tr>
<th>ZOIs against <em>S. mutans</em></th>
<th>Experimental</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dilution 1:1</td>
<td>Dilution 1:2</td>
<td>Dilution 1:4</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>ED1</td>
<td>F-BG 1.5%</td>
<td>22.43 ± 0.61</td>
<td>21.8 ± 1.29</td>
<td>20.07 ± 1.46</td>
</tr>
<tr>
<td>ED2</td>
<td>F-BG 4%</td>
<td>25.10 ± 0.69</td>
<td>23.23 ± 1.45</td>
<td>21.90 ± 1.51</td>
</tr>
<tr>
<td>CD</td>
<td>SMFP 0.76%</td>
<td>29.83 ± 0.23</td>
<td>29.83 ± 1.31</td>
<td>25.83 ± 1.31</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ZOIs against <em>L. casei</em></th>
<th>Experimental</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microaerophila</td>
<td>Anaerobic</td>
<td>Microaerophila</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>ED1</td>
<td>F-BG 1.5%</td>
<td>10.5 ± 0.41</td>
<td>11.17 ± 1.02</td>
<td>8.17 ± 0.62</td>
</tr>
<tr>
<td>ED2</td>
<td>F-BG 4%</td>
<td>13.1 ± 0.54</td>
<td>15.33 ± 0.85</td>
<td>10.17 ± 1.03</td>
</tr>
<tr>
<td>CD</td>
<td>SMFP 0.76%</td>
<td>16.33 ± 1.25</td>
<td>17.67 ± 0.47</td>
<td>11.33 ± 0.62</td>
</tr>
</tbody>
</table>

Abbreviation: ZOI, zone of inhibition.
formulation can enhance its potential for converting tooth surface hydroxyapatite to more acid-resistant fluoro-
hydroxyapatite and its ability to inhibit bacterial enzymes.20,21
For apatite formation in teeth, the F-nBG particles must
stay on the teeth and their surroundings for sufficient time
to induce remineralization without undergoing dilution by
saliva and beverage intake. As salivary flow is lowest at night
during sleep, the desired time for apatite formation should be
less than 8 hours22 following the use of dentifrices enriched
with F-nBG fillers.

For efficacy of fluoride dentifrice, free available fluoride
in an appropriate concentration is required. This means that
the fluoride agent in dentifrice should not chemically react
with any other dentifrice ingredients.23 Fluoride compounds
generating fluoride ions like sodium fluoride, stannous fluo-
ride, and ammonium fluoride are not compatible with chalk-
based abrasives as ionic fluoride reacts with ionic calcium
forming insoluble calcium fluoride.23,24 By contrast, sodium
monofluorophosphate [SMFP (Na3PO3F)] displays greater
compatibility with calcium-based abrasives, as the mono-
fluorophosphate anion is stable and therefore cannot bind
to ionic calcium.23 In the mouth, protective action in SMFP
dentifrices results either by the direct effect of the monoflu-
orphosphate anions or by the fluoride ions released due to
hydrolytic degradation of SMFP in the mouth.25,26

Studies have shown that SMFP dentifrices with calci-
um-based abrasives lose an average of 25% to 35% ionic
fluoride upon aging at 22°C and 29°C, respectively, within
1 year.27–29 Although the commercial dentifrices used in this
study were within 3 years of expiration, high-temperature
storage conditions (before coming to laboratory) could be
blamed for the presence of smaller amounts of free fluoride
available in the elutes and suspensions of the control group.

The literature mostly focuses on the total fluoride con-
tent of dentifrices and does not deliver sufficient informa-
tion about the effective concentrations of free available
fluoride in toothpaste to yield best anticaries effectiveness.
The guidelines for cosmetic dentifrices mostly focus on total
fluoride content only and state that this should not surpass
1500 ppm fluoride. The US Food and Drug Administration
states that NaF/SMFP dentifrices comprising total fluoride
of 850 to 1150 ppm should hold ≥ 65 ppm and ≥ 800 ppm
free fluoride, respectively. The justification for these values
remains unclear. ISO Standard 1160920 at present mentions
requirements for the physical and chemical properties of
dentifrices, provides guidelines for suitable test methods
including total fluoride content of dentifrice, and does not
report the necessity to specify the amount of free fluoride
in dentifrice, although this is a vital condition for anticaries
efficiency. ISO7405[1]31 specifies and quantitative require-
ments for freedom from biological and toxicological hazards;
however, no specific ISO guidelines for microbiological stud-
ies available. In addition, not a single study was found cov-
ering fluoride release of dentifrice containing F-nBG fillers.
As all the novel dentifrices contain calcium carbonate as an
abrasive, therefore, the effect of ionic calcium on free avail-
able fluoride in elutes and suspensions of novel dentifrices
cannot be established. This study not only highlights the
fluoride release behavior of the novel dentifrices enriched
with F-nBGs but also covers the antimicrobial analysis of
such dentifrices.

There was a need to incorporate a bactericidal agent in the
dentifrice. The partial dissolution of ZnO particles releases
Zn2+ ions in an aqueous suspension.14 Zinc ions (Zn2+) display
antimicrobial action against many bacterial strains through
direct contact with cell walls.14 For this purpose, ZnO nano-
powder was incorporated. In this study, the methods include
the content and intent of the recommended methodology
of the American Microbiological Society (ASM). For antimi-

crobial analysis, slurries of all the included dentifrices were
made in various dilutions, as the level at which antimicrobial
properties are buffered or lost in dilution by saliva is import-
ant. Agar well diffusion tests revealed that inhibition zones
decreased in a dose-dependent manner for the dilutions 1:1,
1:2, and 1:4 of dentifrice slurries, further supporting the
direct proportionality of filler loading of F-nBG. No increase
in zone size was observed after 48 hours. All tested dentifrices
exhibited greater antibacterial activity against the major car-
 iogenic pathogen, that is S. mutans, than L. casei. With regard
to the evaluation of the facultative nature of S. mutans,
the recorded diameters of inhibition zones in both aerobic
and anaerobic conditions were similar, whereas the inhibition
zones recorded for L. casei were larger in the case of anaero-
bic conditions when compared with the CO2 jar.

The inhibition zones observed for ED2 were greater than
ED1 because of greater fluoride content along with ZnO and
consequent fluoride release. The ZOIs for CD were slightly
elevated when compared with the inhibition zone for ED2, most
likely due to incorporation of anticalculus agent—tetrasondium
pyrophosphate in the commercial dentifrice—for enhanced
cavity protection. According to previous studies, some denti-
"frices containing such agents may provide reduction in bio-
film formation by approximately 15%.22,33

MBC values of tested dentifrices revealed that all the test
dentifrices had greater effectiveness against S. mutans com-
pared with L. casei. It also revealed that 3 wt% ZnO and an
increased concentration of F-nBG directly corresponded to
increased bactericidal potential of experimental dentifrices.
MBC values for ED2 (4 wt% F-nBG) were same as that for CD
(Colgate Cavity protection). It is important to consider that MIC
values are bacteriostatic,34 whereas MBC values are bactericial
as they corresponded to subcultured plates which showed
absence of bacterial growth.35 Further, in vitro studies need to
be performed to assess the tooth remineralization potentials
of these dentifrices to gauge their efficacy in relieving enamel
white spot lesions and dentin hypersensitivity. Finally, cyto-

Importantly, testing and in vivo usage tests should be performed to
introduce the most suitable composition commercially.

Conclusion
It is concluded that in dentifrices, mainly the composition
of the fluoride source, governs fluoride release behavior.
Ionic fluoride in elutes and suspensions of both novel den-

tifrices was directly proportional to F-nBG filler loading.
Among novel dentifrices, maximum mean fluoride release
was detected in elutes and suspensions of group ED2, which contained the highest filler loading, that is, 4 wt% F-nBG. Filler loading matched the fluoride release of the commercial dentifrice.

Statistical analysis indicated that ED2 (F-nBG 4 wt.%) demonstrated similar antimicrobial potential to that of an ordinary, non-tetra sodium pyrophosphate-containing fluoride dentifrice in terms of ZOIs for both bacterial strains under all growth conditions. The MBC obtained for ED2 was also similar to obtained MBC values for CD. Incorporation of F-nBG and ZnO provide a multi-benefit approach to simultaneously treating early white spot lesions, reducing bacterial growth, and providing core plaque control.

Conflict of Interest
None declared.

References