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Original Research

In-vitro antibacterial activity of contemporary bioactive dental restorative materials

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Aim: Secondary caries can lead to a failure of composite and glass ionomer cement (GIC) restorations. Bioactive dental restoratives have been introduced to address this problem. However, the evidence supporting the antibacterial efficacy of these materials remains limited. The objective of this study was to compare the antibacterial efficacy of contemporary bioactive and conventional restoratives against *S. mutans*. **Methods**: A conventional composite (Filtek Z350 - FT), a high viscosity GIC (Equia Forte - EQ) and three bioactive restoratives comprising a giomer (Beautifil II - BF), an alkasite (Cention N - CN), and a zirconia reinforced GIC (Zirconomer - ZC) were evaluated. Biofilm quantification was performed using crystal violet (CV) assays and colony forming units (CFU) counts to determine the biofilm biomass and *S. mutans* viability respectively. The surface roughness of the specimens was measured. **Results**: FT had the lowest biofilm biomass (OD570nm) and BF had the lowest *S. mutans* viability (LogCFU/ml). The highest surface roughness was observed for EQ while the lowest surface roughness was observed for FT. Surface roughness after exposure to bacteria suspension was significantly higher in ZC compared to FT and BF. **Conclusion**: GIC-based materials displayed significantly greater biofilm biomass, *S. mutans* viability, and surface roughness compared to resin-based ones. Surface roughness (immediately after fabrication) showed a strong correlation to *S. mutans* viability. Further studies are required to verify if surface roughness is the dominant factor affecting the antibacterial efficacy of dental restoratives.

Keywords: Bioactive glass. Glass ionomer cements. Composite resins. Anti-bacterial agents.

Introduction

Composite resins have been the material of choice for dental restorations due to good aesthetics, reparability and their ability to bond to tooth structure. Despite these qualities, composite resins often fail clinically due to bulk fractures and secondary caries arising from microleakage at the tooth-restoration interface[1]. Another group of commonly used tooth-colored restorative materials is glass ionomer cement (GIC). Unlike composite resins, these bioactive materials release fluorides and enhance remineralization while minimizing demineralization of the tooth structure and impeding bacterial growth. However, clinical surveys still report secondary caries as a common reason for the failure of GIC restorations, suggesting that the level of fluoride released from GICs is insufficient to achieve bacteriostatic and bactericidal effects[2].

To overcome the limitations posed by conventional dental restorative materials, ion-releasing materials (IRMs) have been introduced. These bioactive materials form a calcium phosphate-rich layer at the tooth-restoration interface and reduce microleakage arising from bacterial invasion at the margins. The purported antibacterial mechanisms of these materials are mainly that of (i) elevating pH levels within the biofilm, (ii) raising osmotic pressure, and (iii) inactivating bacteria by damaging their cell walls. The first proposed mechanism occurs through the dissolution of bioactive glass in IRMs, which releases alkali ions such as sodium, calcium and phosphate, resulting in an increased pH. This alteration in the pH gradient influences the cytoplasmic membrane permeability of bacteria, and inhibits the enzymatic activity of enolase, ATPase, and sugar transport, resulting in bacteriostatic and even bactericidal effects[3]. Secondly, this release of ions also causes perturbations of the membrane potential of bacteria via rapid water efflux and a pressure drop across the cell membrane, resulting in altered cell size, cell shape, and membrane stress level[4]. Thirdly, "needle-like" debris found on the surface of Bioglass 45S5 has been found to be capable of destroying the cell walls of bacteria, resulting in bacterial death[5].

Understanding the antibacterial properties of restorative materials allows the clinician to make an informed decision when restoring cavities, especially since biofilm formation at the tooth-restoration interface due to bacterial adhesion is known to be the cause of recurrent caries[6]. Some of the contemporary IRMs claim to have greater ion release[7] and superior mechanical properties compared to the traditional GICs[8,9]. However, the current evidence on the antibacterial properties of IRMs remains limited and inconclusive due to differing testing methodologies and surface treatment of the materials in studies. Materials can also be broadly categorized into two groups, which are either resin-based (e.g. Filtek Z250, Beautifil II and Cention N) or glass-ionomer-based (e.g. Equia forte and Zirconomer). The categorization is largely based on the presence of methacrylate monomers (which are believed to have bacteriostatic properties) in the resin-based group and the absence of these monomers in the glass-ionomer-based group.

Limited studies have been done to compare the antibacterial efficacy of IRMs against their conventional counterparts and among themselves. Hence, the objective of this

study was to evaluate the antibacterial activity of IRMs. Additionally, we sought to determine the correlation between surface roughness and antibacterial activity.

Methods and Materials

Selection of materials and determination of sample size

The technical profiles of the materials are listed in Table 1. This study used 3 types of contemporary IRMs namely: (i) a giomer with pre-reacted glass-ionomer fillers (Beautifil II [3M-ESPE, St Paul, MN, USA])(BF); (ii) an alkasite with alkaline fillers (Cention N [Ivoclar Vivadent, Liechtenstein])(CN); and (iii) a zirconia reinforced GIC (Zirconomer [Shofu Inc., Kyoto, Japan](ZC). A conventional composite, Filtek Z250 (3M-ESPE, St Paul, MN, USA)(FT) and a GIC, Equia Forte (GC America INC, Alsip, IL, USA)(EQ) were used as controls. The minimum sample size of 60 (i.e., n=6) was determined using R language program (version 4.0.2; R Foundation for Statistical Computing, Vienna, Austria) based on the assumption of a standard deviation of 0.025 (derived from pilot data) to detect a difference of 0.1 using two-sided two-sample equal variance t-test, with an alpha error of 0.05 (Bonferroni adjusted), and power of 80% for 5 different materials.

Table 1. Technical profiles of the materials chosen for study

Manufacturer	Material (Abbreviation)	Type	Resin (Photo-initiator)	Filler	Filler size (µm)	Filler content % by weight/ % by volume	Curing mechanism
3M-ESPE, St Paul, MN, USA	Filtek Z250 (FT)	Micohybrid composite	Bis-GMA Bis-EMA UDMA TEGDMA (CQ)	Zirconia/ Silica cluster and Silica nanoparticle	Average 0.6 (0.01-3.5)	78.5/63.3	Light cured (20 sec)
GC America INC, Alsip, IL, USA	Equia Forte (EQ)	High viscosity glass-ionomer cement (Glass hybrid)	NIL	Fluoroalumino silicate glass, polyacrylic acid	Average 7	84/70	Light cured (20 sec)
Shofu Inc., Kyoto, Japan	Beautifil II (BF)	Giomer	Bis-GMA TEGDMA (CQ)	S-PRG based on F-Br-Al-Si glass	Average 0.8 (0.01-4)	83.3/68.8	Light cured (10 sec)
Ivoclar Vivadent, Liechtenstein	Cention N (CN)	Alkasite	UDMA DCP PEG-400 DMA	Barium aluminum silicate glass, ytterbium trifluoride	0.1–35	78.4/57.6	Self cured
Shofu Inc., Kyoto, Japan	Zirconomer (ZC)	Zirconia reinforced glass ionomer	NIL	Fluoroalumino silicate glass, zirconium oxide	Average 7 (2-30)	34/64	Self cured

Bis-GMA, Bisphenol-A glycidyl methacrylate; Bis-EMA, Ethoxylated bisphenol-A-glycidyl methacrylate; CQ, Camphorquinone; TEGDMA, Triethylene glycol dimethacrylate; S-PRG, Surface modified pre-reacted glass; F-Br-Al-Si, Fluoroboroaluminosiliciate; UDMA, Urethane dimethacrylate; DCP, dimethanol dimethacrylate; PEG-400 DMA, polyethylene glycol 400 dimethacrylate

Preparation of specimens and immersion protocol

There were 6 specimens of each restorative material which were prepared separately for the crystal violet (CV) assay and colony forming units (CFU) assay measurements. Specimens of 8 mm in diameter and 1.5 mm in thickness were fabricated using cylindrical-shaped stainless-steel molds of 12 mm in external diameter, 8mm in internal diameter and 1.5 mm in internal height. All specimens were prepared according to manufacturers' instructions. The lateral walls of the stainless-steel molds were smeared with a thin layer of Vaseline using a microbrush to prevent material adhesion. The various materials were packed into a stainless-steel mold, covered entirely with Mylar strips and compressed between two glass slides using finger pressure to extrude excess material. The specimens were cured with two overlapping irradiations from the top and bottom surfaces using an LED curing light unit (Radii-cal, SDI, Australia). This was done with an 8 mm curing tip diameter with output irradiance of 1200 mW/cm (20s) and 460nm wavelength following the manufacturers' curing times, where applicable. The light curing unit was maintained at full charge before use, and the light intensity and wavelength were monitored after every 5 specimens using a radiometer (LED Radiometer, Kerr, Orange, CA, USA). No finishing or polishing was performed, to maintain a standardized surface smoothness for all the specimens. Additionally, the smooth surface achieved with the Mylar strip is often considered to be a final finish[10]. The specimens of each group were then immersed in 20ml of distilled water in a sterile Falcon tube for seven days in complete darkness at 37°C and 100% humidity[11].

Sterilization

It was important to ensure that the specimens were sterile to avoid contamination by other microorganisms that could interfere with the biofilm culture and ultimately affect the results obtained. Sterilization of the specimens was done through UV irradiation. The UV lamp built within the biosafety cabinet was used at an ambient temperature. The UV lamp emitted UV light with a wavelength of 254 nm at an intensity of 10W. Specimens were irradiated for 15 minutes on each side[12-14].

Biofilm formation

The *S. mutans* biofilm formation protocol was adapted from Ho et al.[15] (2017). *S. mutans* strain ATCC 35668 was stored at 37°C and 5% CO_2 in aerobic conditions and reactivated onto Mitis Salivarius (MS) agar. Single colonies from the MS agar were inoculated in sterile brain heart infusion broth with 2% sucrose supplementation at 37°C at 200rpm for 24 hours. Bacterial cultures were adjusted to the inoculum of 10^8 CFU/ml (equivalent to an optical density(OD)600 of 0.5). The specimens from the different experimental groups were then inoculated with 1.5 mL of the inoculation medium (sterile brain heart infusion broth with 2% sucrose supplementation) in a 24-well plate platform and incubated at 5% CO_2 and 37 °C for 24 hours at 200rpm to allow for biofilm formation[16]. A duration of 24 hours was chosen to allow for *S. mutans* biofilm formation to reach the stationary phase of the *S. mutans* growth curve[17]. Additionally, this is a commonly used duration in many in-vitro biofilm studies as there is no need for a change in the medium, allowing undisrupted

bacteria growth[18]. After 24 hours of exposure to *S. mutans* suspension, the bacteria suspensions were aspirated from each well and biofilms were washed gently with Phosphate Buffered Saline (PBS) to remove the non-adherent cells on one side. The samples were then carefully flipped and transferred to a new well for gentle washing with PBS again.

Biofilm quantification

To provide a complete assessment of the antibacterial effect of the materials tested against *S. mutans*, biofilms were quantified by 2 methods: CV assay and CFU counts. CV assay was used to determine the biofilm biomass by measuring the optical density of the content using a microtiter plate spectrophotometer (μQuant Microplate Spectrophotometer, Biotek, Winooski, VT, USA) at 570 nm. CFU counting method was used to determine *S. mutans* viability, whereby the bacterial colonies were counted and converted to the corresponding log CFU values.

Surface roughness measurements

Surface roughness measurements were determined with a profilometer (Model MR200, Mitech, China) at 2 time points; immediately after fabrication and after exposure to *S. mutans* suspension for 24 hours. Mean surface roughness (Ra) was measured from 3 different points on each specimen before and after 24 hours of exposure to bacteria suspension in the CFU count experiment. An average of 3 readings was recorded for each specimen, and the median was taken from the 6 specimens per group. Change in surface roughness was defined as the difference in surface roughness measured immediately after specimens were fabricated and after 24 hours of exposure to *S. mutans* suspension.

Statistical analysis

All statistical analyses were conducted using the R language program (version 4.0.2; R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at $p<0.05$. As the results obtained from CV assay and CFU counts did not follow a normal distribution based on QQ plots, non-parametric tests were employed to compare the median of the biofilm biomass, *S. mutans* viability and change in surface roughness among the groups. The Kruskal-Wallis test was employed to compare the biofilm biomass, *S. mutans* viability, and change in surface roughness (immediately after fabrication and after 24 hours of exposure to *S. mutans*) among the groups. Post-hoc Kruskal-Nemenyi tests were performed to compare individual materials in terms of the biofilm biomass, *S. mutans* viability and change in surface roughness.

Univariate and multivariate linear regression were performed to test the association between surface roughness immediately after fabrication, surface roughness after 24 hours of exposure to bacteria suspension and type of material with *S. mutans* viability. The Kruskal-Wallis test was performed to determine the change in surface roughness after 24 hours of exposure to bacteria suspension among different groups. Wilcoxon signed ranked test was then performed to compare the change in surface roughness within each of the 5 groups tested.

Results

Comparison of biofilm biomass, *S. mutans* viability, and surface roughness before and after 24 hours exposure to bacteria suspension

The median and interquartile range of each group are presented in Table 2. The biofilm biomass (p<0.001), *S. mutans* viability (p<0.001), surface roughness before 24 hours of exposure to bacteria suspension (p<0.001), and surface roughness after exposure to bacteria suspension (p<0.001) were significantly different between test groups. The amount of biofilm biomass measured from CV assay was significantly higher for ZC compared to FT (p<0.001) and BF (p=0.004). EQ reported significantly higher biofilm biomass compared to FT (p=0.004). *S. mutans* viability was significantly higher in ZC compared to FT (p=0.004) and BF (p<0.001). EQ reported significantly higher *S. mutans* viability compared to BF (p=0.004).

Surface roughness before exposure to bacteria suspension was significantly higher in ZC compared to FT (p<0.001) and BF (p=0.001). EQ reported significantly higher surface roughness values compared to FT (p=0.012). Surface roughness after 24 hours of exposure to bacteria suspension was significantly higher in ZC compared to FT (p<0.001) and BF (p=0.001).

Table 2. Comparison of biofilm biomass (CV assay), S. mutans viability (CFU) and surface roughness before and after 24 hours exposure to bacteria suspension.

	Median (IQR)					p value	Significant *post hoc* pairwise comparison
	FT	BF	CN	EQ	ZC		
Biofilm biomass (OD570nm)	0.19 (0.18, 0.20)	0.25 (0.23, 0.26)	0.47 (0.44, 0.55)	0.74 (0.73, 0.76)	0.89 (0.87, 0.92)	<0.001	ZC, EQ > FT ZC > BF
S. mutans viability (LogCFU/ml)	2.38 (2.35, 2.40)	2.13 (2.08, 2.18)	2.84 (2.83, 2.88)	2.98 (2.94, 3.01)	3.53 (3.50, 3.57)	<0.001	ZC, EQ > BF ZC> FT
Mean Surface roughness (Before) (µm)	0.076 (0.075, 0.078)	0.078 (0.077, 0.08)	0.170 (0.164, 0.172)	0.282 (0.279, 0.284)	0.381 (0.374, 0.387)	<0.001	ZC, EQ >FT ZC> BF
Mean Surface roughness (After) (µm)	0.079 (0.077, 0.08)	0.081 (0.079, 0.082)	0.172 (0.165, 0.175)	0.287 (0.281, 0.298)	0.388 (0.386, 0.389)	<0.001	ZC> FT, BF

FT, Filtek Z250; BF, Beautifil II; CN, Cention N; EQ, Equia forte; ZC, Zirconomer.

Association between surface roughness and S. mutans viability

On univariate analysis, a significant positive association was detected between surface roughness immediately after fabrication with *S. mutans* viability (p<0.001), and surface roughness after exposure to bacteria suspension with *S. mutans* viability (p<0.001). Immediately after fabrication, for every unit increase in surface roughness, CFU was found to have increased by 3.971 units (95% CI=3.488-4.453). After 24 hours

of exposure to bacteria suspension, for every unit increase in surface roughness, CFU was found to have increased by 3.861 units (95% CI=3.372-4.349). On multivariate analysis, there were no significant associations between surface roughness immediately after fabrication (p=0.212), surface roughness after exposure (p=0.897) and type of material (p=0.581) with *S. mutans* viability.

Change in surface roughness

The change in surface roughness after 24 hours of exposure to bacteria suspension was not significantly different among different groups (p=0.072). When comparing the change in surface roughness within each of the 5 groups, 3 out of the 5 materials, namely EQ (p=0.036), BF (p=0.034) and CN (p=0.036), reported significant changes in surface roughness (Table 3).

Table 3. Change in surface roughness within each group

Group	Median surface roughness (IQR)			p value
	Surface roughness (µm)(before)	Surface roughness (µm)(after)	Change in surface roughness (µm)	
FT	0.076 (0.075, 0.080)	0.079 (0.079, 0.081)	0.003 (0.002-0.003)	0.053
BF	0.078 (0.077, 0.080)	0.081 (0.078, 0.083)	0.003 (0.002, 0.003)	**0.034**
CN	0.170 (0.161, 0.175)	0.172 (0.163, 0.179)	0.003 (0.003, 0.006)	**0.036**
EQ	0.282 (0.274, 0.288)	0.287 (0.276, 0.309)	0.005 (0.003, 0.015)	**0.036**
ZC	0.381 (0.373, 0.391)	0.388 (0.383, 0.394)	0.008 (0.008, 0.012)	0.059

FT, Filtek Z250; BF, Beautifil II; CN, Cention N; EQ, Equia forte; ZC, Zirconomer.

Discussion

FT showed significantly lower *S. mutans* viability compared to EQ and ZC. This is a surprising finding since composite restorations are believed to be more prone to secondary caries development. Reasons for this belief include the absence of antibacterial properties in composites, and the promotion of growth of *S. mutans* and other cariogenic bacteria due to the release of methacrylate monomers, which result from incomplete polymerization and resin biodegradation of composites[19]. However, the leachable components in methacrylate-based dental resins have since been proven to be bacteriostatic[20]. Consequently, this resin-rich layer resulting from the adaptation of the Mylar strip during fabrication (which comprises poorly polymerized resin monomers) could have led to an increased elution of unbound monomers. This is a possible explanation for the low biofilm biomass and *S. mutans* viability observed in FT. Similarly, BF also reported significantly lower biofilm biomass compared to ZC, while *S. mutans* viability from FT and BF were both significantly lower than ZC.

The glass-ionomer-based groups exhibited lower antibacterial activity than the resin-based groups. *S. mutans* viability levels were found to be significantly higher in ZC compared to FT and BF. EQ also displayed significantly higher *S. mutans* viability than BF. The weak antibacterial activity of glass-ionomer-based groups observed in this study contrasts with other studies that report a significant antibacterial effect of GICs against *S. mutans*[21,22]. The antibacterial effect of GICs is believed to be due to their ability to release fluoride ions, which prevents bacterial growth. GICs have been reported to be capable of achieving a fluoride concentration of 7 ppm or more after 14 days[23], with in-vitro studies suggesting that only small amounts of fluoride (approximately 0.03–0.08 ppm) are required to shift the equilibrium from demineralization to remineralization[24]. A possible explanation for the poor performance of the glass-ionomer-based materials in this study was that the dissolution of fluoride ions from these materials during the 7-day water-aging process did not result in an ion concentration higher than the minimum inhibitory concentration required for *S. mutans* during the 24 hours biofilm formation[25], although we did not measure fluoride concentrations for the specimens used. The short duration of exposure of specimens to the bacterial suspension could also have dampened the antibacterial effects of fluoride on the specimens. However, an in-vitro study suggested that even a continuous supply of fluorides at therapeutic levels had minimal inhibitory effect on the aggregation of *S. mutans*[26]. A correlation between fluoride-releasing potential and antibacterial properties in fluoride-releasing composites, compomer and resin-modified GICs has also not been shown[27]. In addition, it has been proposed that surface roughness plays a more important role in *S. mutans* viability compared to fluoride release in GICs[28]. Hence, further studies of the fluoride release capacity of GICs are needed to understand this relationship better.

The glass-ionomer-based group was found to exhibit greater surface roughness than the resin-based group. FT displayed the lowest surface roughness values, followed by BF, CN, EQ and ZC. A similar study also found that FT exhibits the lowest surface roughness values of 0.260μm followed by CN (0.378μm)[29]. The surface roughness of these materials may be attributed to filler particle size. Multiple studies have shown a positive association between surface roughness and filler particle size[30,31]. The average filler particle size of EQ and ZC are both 7μm, which is much larger than that of FT (0.6μm) and BF (0.8μm). Hence, the study results are not unexpected.

The study found a significant positive association between the amount of viable *S. mutans* and surface roughness on univariate analysis. This finding is congruent with reports that rougher surfaces promoted dental plaque formation[32]. It has been demonstrated that surface roughness influences the early attachment of *S. mutans* to the surfaces of nanofilled and nanohybrid resin-based composites surface[33]. This is important, as the proliferation of the initial adhering microorganisms accounts for a major part of microorganism mass increase during plaque formation[34].

Surface roughness of 0.2μm has long been considered the critical surface roughness for bacterial adhesion and retention on dental materials[35], with values surpassing 0.2μm resulting in a higher volume of bacterial attachment, and a higher caries risk.

Out of all the materials in this study, only EQ and ZC were found to have surface roughness values greater than 0.2µm. This may explain why the amount of biofilm biomass and viable *S. mutans* measured were significantly higher in these two materials. A significant association was also found between surface roughness (before 24 hours of exposure to *S. mutans* suspension) and *S. mutans* viability in our study, which supports the view that surface roughness is a crucial factor in influencing bacterial adhesion on dental materials.

After 24 hours of exposure to *S. mutans* culture, a change in surface roughness was observed in all groups. However, this change in surface roughness was only significant in BF, CN and EQ, with the change in surface roughness in FT and ZC approaching statistical significance. This increase in surface roughness may be due to biodegradation of the material from the acids produced by cariogenic bacteria in the biofilm layer, which lowers the pH of the environment, and degrades the monomers in the resin matrix[36]. This leads to an increase in the surface roughness of the dental material, thereby encouraging further bacterial accumulation.

Several limitations of our study are worth highlighting. Firstly, we used a monospecies *S. mutans* biofilm, which cannot replicate the in-vivo biofilm model which can investigate the complex interactions between multiple species of bacteria. However, the monospecies biofilm model can still help to investigate how a chosen cariogenic microorganism would respond in an in-vitro setup and can improve our understanding as to how it may play a role in complex biofilm models. Secondly, a 2-dimensional contact profilometer was used in this study. However, contact profilometry may underestimate the surface roughness of dental composites[37], and there is a possibility that the surface roughness values reported in this study may be higher if atomic force microscopy was used. Thirdly, confocal laser microscopy was not performed to confirm the proportion of live and dead bacteria on the dental materials. Fourthly, we did not measure the fluoride concentration of the specimens used, which may have a biological effect on inhibiting bacterial growth and biofilm formation. Lastly, although materials are usually polished clinically, we opted for a Mylar finish in this study to minimize the confounders affecting the surface roughness of the materials studied.

In conclusion, we found that glass-ionomer-based groups displayed greater biofilm biomass, *S. mutans* viability and surface roughness compared to the resin-based groups. A significant positive association was detected between *S. mutans* viability and surface roughness. Future in-vitro studies are required to investigate other factors apart from surface roughness that could affect bacterial adhesion in IRMs, such as the extent of residual monomers and fluoride ion release.

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None.

Conflicts of interest

None.

Data Availability

Datasets related to this article will be available upon request to the corresponding author.

Author Contribution

Rui Ling Kong: Study conception and design, Data collection, Analysis and interpretation of results, manuscript drafting and preparation. **Adrian Ujin Yap**: Study conception and design, Analysis and interpretation of results, manuscript drafting and preparation. **Chaminda Jayampath Seneviratne**: Study conception and design, Data collection, Analysis and interpretation of results, manuscript drafting and preparation. **Keson Beng Choon Tan**: Analysis and interpretation of results, manuscript drafting and preparation. **Christina Poh Choo Sim**: Analysis and interpretation of results, manuscript drafting and preparation. All authors actively participated in the discussion of the manuscript's findings, and have revised and approved the final version of the manuscript.

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