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ORIGINAL ARTICLE

Efficacy of Antimicrobial Peptide GH12 on a Multispecies Endodontic Biofilm Model: An *In-vitro* Study

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ABSTRACT

Objective: This study aimed to evaluate the antibacterial efficacy of different concentrations of GH12 on a simulated multispecies biofilm comprising *Enterococcus faecalis*, *Streptococcus mutans, Fusobacterium nucleatum* and *Porphyromonas gingivalis*.

Methods: Single rooted teeth were decoronated, cut into 1.5 mm sections to obtain dentine discs which were randomly allocated into five groups: (n=12 each), Group 1: Phosphate Buffered Solution (PBS) - negative control, Group II: 5% Sodium hypochlorite (NaOCl) - positive control, Group III: Minimum Inhibitory Concentration (MIC) of GH12, Group IV: 2x MIC of GH12, Group V: 4x MIC of GH12. Colony forming units, Crystal violet assay and scanning electron microscopy examinations were performed. One-way ANOVA and Turkey's test were applied for statistical analysis using the SPSS software version 22.0.

Results: Group II (NaOCl) showed maximum reduction in bacterial load followed by Group V (GH12 16mg/mL) with no statistically significant difference (p=1.000). On comparing the mean CFU reduction, the maximum reduction was identified for *S. mutans* and the least was for *P. gingivalis*. There was marked erosion observed in the NaOCl group whereas the GH12 group showed no erosive changes in the morphology and no bacterial colonies was identified.

Conclusion: The findings revealed that GH12 at higher concentrations inhibits and disrupts the growth of multispecies endodontic biofilm comparable to NaOCl but without erosive effects to the dentine, further highlighting its potential to be used as an antimicrobial solution.

Keywords: Antimicrobial action, GH12, multispecies biofilm, peptide, root canal

HIGHLIGHTS

- Antimicrobial efficacy of higher concentration of GH12 peptide was comparable to Sodium hypochlorite in combatting a multispecies biofilm along with no evident erosive changes
- *F. nucleatum* and *P. gingivalis* species showed resistance to elimination compared to *S. mutans* and *E. faecalis*.

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INTRODUCTION

The primary causative factor in pulpal and periapical pathology is a bacterial infection of the root canal system. Dense multi-species biofilm communities are formed when microorganisms infiltrate the anatomic complexities such as accessory canals, canal ramifications, isthmuses and other morphological irregularities (1). These communities persist by embedding in a self-produced matrix making them resistant to antimicrobial agents, thereby posing a significant clinical challenge (2).

Endodontic therapy aims to remove biofilm and eliminate bacteria from the canal space by thorough chemical disinfection using irrigants (3). Sodium Hypochlorite (NaOCl) is the most potent irrigating solution because of its effective

antimicrobial action and excellent organic tissue dissolving ability (4). However, NaOCl is toxic to the host tissue, especially at high concentrations (5). Also, its high surface tension limits its ability to penetrate and disinfect the irregularities of the root canal system (6).

Antimicrobial peptides (AMP) represent a diverse class of biomolecules that offer early-stage defense against encroaching microbes and are suggested as potential substitutes for traditional pharmaceuticals in the fight against multidrug-resistant pathogens and infections caused by biofilms (7). Recently, a cationic peptide GH12 has been developed with broad-spectrum antibiofilm activity. In laboratory study models, it has been demonstrated to have excellent antimicrobial efficacy against cariogenic bacteria and mono-species *Enterococcus faecalis* biofilm (8, 9).

An infected root canal system harbours multiple microbial species. Amongst them, the most common species cultured from persistent endodontic infections is *Enterococcus faecalis* (10). However, considering that endodontic infections are typically polymicrobial in nature, this study developed a multi-species endodontic biofilm comprising *Enterococcus faecalis*, *Streptococcus mutans, Fusobacterium nucleatum* and *Porphyromonas gingivalis* microorganisms.

There is a limited literature on the effectiveness of antimicrobial peptides on endodontic biofilms. To the best of knowledge, there are no research studies analysing the antibiofilm efficacy of different concentrations of GH12 against a multi-species endodontic biofilm. Hence, this study aimed to test the antibacterial efficacy of this peptide in varying concentrations as an antibacterial agent on a simulated *in-vitro* multi-species model.

The first null hypothesis was that there was no difference in the amount of bacterial reduction in the multi-species endodontic biofilm after irrigating with different concentrations of GH12 and NaOCl peptide solution when compared with Phosphate Buffer Solution (PBS) solution. The second null hypothesis was that there was no difference in the surface morphology of the dentin e pecimens after irrigating with different concentrations of GH12 and NaOCl peptide solution when compared with Phosphate Buffer Solution (PBS) solution. The third null hypothesis tested was that there was no difference in the biofilm biomass after irrigating with different concentrations of GH12 and NaOCl peptide solution when compared with Phosphate Buffer Solution (PBS) solution.

MATERIALS AND METHODS

The study was conducted in the I.T.S Centre for Dental Studies and Research, Ghaziabad in the Department of Conservative Dentistry and Endodontics in collaboration with the Advanced Research Centre after obtaining institutional ethical clearance under the protocol number ITSCDSR/IIEC/2020-23/CONS/01 on October 12, 2021. The study was conducted in accordance with the Declaration of Helsinki.

Sample Size Calculation

It was revealed from the literature that the expected mean±SD of parameters between the two groups were 99.75±0.02 and 100.0±0.12 respectively (9). G*Power version 3.1.9.7 was used as follows, power 95% and α 5% the effect size was 2.90619. The sample size was estimated to be 5 for each group and the actual power was 97.91%. However, in this study, 12 samples were allocated for each group.

Sampling and Specimen Preparation

Single-rooted teeth with one canal from patients requiring extractions for orthodontic purposes were chosen for this study. The freshly extracted teeth were rinsed with the phosphate buffer solution, followed by the removal of gingival or periodontal tissue tags using an ultrasonic scaler. The inclusion criteria were intact root, completely formed apices, unrestored teeth and non-carious teeth. Teeth with open apices, root resorption, calcification, root canal treated, anatomical developmental anomalies and fractured tooth were excluded. The teeth were decoronated and sixty sections of 1.5 mm thickness were obtained from the middle portion of the teeth. The smear layer was removed by placing the dentine blocks in an ultrasonic bath containing 5.25% NaOCl and 17% EDTA (Aarc Dental, India) for 1 min each following which the dentine discs were placed in a Brain Heart Infusion (BHI) broth (HiMedia Lab, Mumbai) and autoclaved for 20 min at 121°C.

Peptide Synthesis and Storage

GH12 peptide was synthesised and purified from Grey Matter Foundation, India. The peptide was stored in an airtight container until usage, as per the recommendations of the manufacturer. A strict aseptic protocol was ensured by wearing Latex free gloves at all times when working with peptides to avoid cross contamination. Also, while handling the peptide, it was ensured that all the instruments are sterilized.

Bacterial Culture

Four bacterial strains of *E. faecalis* ATCC 29212, *F. nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277 and *S. mutans* ATCC 25175 were procured from HiMedia, India. They were culutred on BHI agar plates to form subcultures. A 0.5 McFarland standard suspension was prepared in BHI broth and then diluted 4-fold to obtain an initial bacterial suspension of approximately 1.5×10^4 colony forming units per millilitre (CFU/mL). Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) for each bacterium was obtained using the broth dilution method.

Formation of a Multispecies Biofilm

Bacterial susceptibility assay was performed for all the bacterial strains using the broth microdilution assay and absorbance at 600 nm was recorded using a spectrophotometer (Lab India Analytical, India). To establish the multi-species biofilm, under sterile conditions, the bacterial suspensions were co- cultured in a 1:1:1:1 ratio in a glass jar. 100µL of each of the four bacterial cultures were taken and incubated in BHI media (HiMedia Lab, Mumbai) for 24 hr anaerobically. The prepared bacterial suspension containing the four species was distributed into Eppendorf tubes (HiMedia, Mumbai) with 100µL of the suspension using sterile micro-pipettes. The tooth substrate was inoculated into these Eppendorf tubes containing the bacterial suspension and was incubated under anaerobic conditions

TABLE 1. Intergroup comparison of mean reduction in bacterial load

Subgroup	PBS	NaOCl	4mg/mL GH12	8mg/mL GH12	16mg/mL GH12
E. faecalis	1.42×10^5	1.79×10^6	1.69×10^6	1.70×10^6	1.76×10^6
S. mutans	1.66×10^5	2.07×10^6	2.01×10^6	2.03×10^6	2.05×10^6
F. nucleatum	1.54×10^5	2.12×10^6	1.88×10^6	2.09×10^6	2.10×10^6
P. gingivalis	1.52×10^5	2.21×10^6	2.08×10^6	2.09×10^6	2.20×10^6

PBS: Phosphate Buffered Solution, NaOCl: Sodium hypochlorite

TABLE 2. Pairwise comparison of mean reduction in bacterial load among the five groups

Subgroups	*E. faecalis*	*S. mutans*	*F. nucleatum*	*P. gingivalis*
Gr I vs Gr II	<0.001*	<0.001*	<0.001*	<0.001*
Gr I vs Gr III	<0.001*	<0.001*	<0.001*	<0.001*
Gr I vs Gr IV	<0.001*	<0.001*	<0.001*	<0.001*
Gr I vs Gr V	<0.001*	<0.001*	<0.001*	<0.001*
Gr II vs Gr III	0.010*	0.906	<0.001*	<0.001*
Gr II vs Gr IV	0.022*	0.982	0.988	0.519
Gr II vs Gr V	0.850	0.998	0.994	1.000
Gr III vs Gr IV	0.998	0.998	<0.001*	1.000
Gr III vs Gr V	0.117	0.979	<0.001*	0.515
Gr IV vs Gr V	0.214	0.9999	1.000	0.594

Post-hoc Turkey test. *: Indicates significant difference at p≤0.05

for 21 days. The anaerobic conditions were maintained using an incubator at 37°C. The incubator was rendered anaerobic by infusing with 10% hydrogen, 10% CO_2 and 80% Nitrogen.

Grouping

Sixty samples were randomly divided into five groups (n=12 each) according to the antimicrobial treatment being used. Group 1: PBS solution - negative control, Group 2: 5%NaOCl - positive control, Group 3: MIC of GH12 (4mg/ml), Group 4: 2x MIC of GH12 (8 mg/ml), Group 5: 4x MIC of GH12(16 mg/ml).

The Eppendorf tubes containing the biofilm were taken out after 21 days of contamination in the incubator and pre-quantification of the biofilm was done using a digital colony counter.

Anti-biofilm Treatment

1 mL of the test solution (PBS, NaOCl and GH12) according to the groups were added to the samples and left for 10 minutes. Samples were taken from the dentine surface and plated into specific media plates and were grown anaerobically for 24 hrs.

Bacterial Sampling and Colony Forming Unit Count (CFU)

After incubation, serial dilutions were done and 1µL of the diluted bacterial suspension was placed in specific agar plates to form colonies and the number of colonies was recorded with the help of a digital colony counter.

Scanning Electron Microscopy (SEM)

The dentine samples were fixed with serial dilutions of 2.5% glutaraldehyde and then sputter coated with gold and viewed under SEM (JEOL JSM-6610LV, Tokyo, Japan), to observe the morphological changes.

TABLE 3. Intergroup comparison of mean absorbance based on A_{595} quantitation

Group	Mean reduction	SD	p
Group 1 (PBS)	1.367	0.002	<0.001*
Group 2 (NaOCl)	0.203	0.002	
Group 3 (GH12)	0.546	0.002	
Group 4 (2×GH12)	0.339	0.002	
Group 5 (4×GH12)	0.205	0.003	

One-way ANOVA test. *: Indicates significant difference at p≤0.05. SD: Standard debiation, PBS: Phosphate Buffered Solution, NaOCl: Sodium hypochlorite

Crystal Violet Assay

5mL of the bacterial suspension after treating with the respective test solutions were placed in a test tube. 1mL of 0.2% crystal violet stain (HiMedia, Mumbai, India) was added to the test tubes and left for 5 minutes. The tubes were gently washed and 100% ethanol was added to them. Absorbance was recorded for the samples at 590 nm using a spectrophotometer (Lab India Analytical, India).

Statistical Analysis

All the results were calculated and entered in MS Excel. SPSS (Statistical Package for Social Sciences) Version 22.0 (IBM Corp 2013, New York, USA) software was used for the statistical analysis. For comparison of the reduction in colony forming units of specific bacteria and biomass reduction among the groups, one-way ANOVA was applied and Turkey's test for pairwise comparison of means of different groups.

The level of significance and the confidence interval were set at 5% and 95% respectively.

RESULTS

Group II (NaOCl) showed maximum reduction in bacterial load followed by Group V (GH12 16 mg/mL) with no statistically significant difference (p=1.000) (Table 1, Table 2, Fig. 1). On comparing the mean CFU reduction for specific bacteria, a similar pattern was observed in all the five groups, in increasing order: *S. mutans* > *E. faecalis* > *F. nucleatum* > *P. gingivalis*. Maximum reduction was seen for *S. mutans* and the least was seen for *P. gingivalis*.

The SEM images revealed the presence of biofilm colonies in the PBS group (Fig. 2). There was a marked erosion observed in the NaOCl group whereas the GH12 group showed no such erosive changes in the morphology and the presence of any bacterial colonies was also not visible.

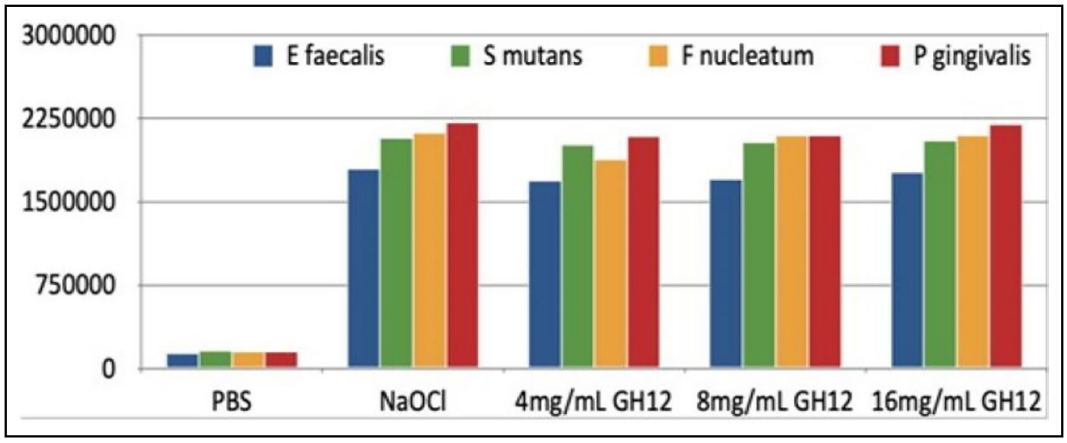

Figure 1. Intergroup comparison of mean reduction in bacterial load

PBS: Phosphate Buffered Solution, NaOCl: Sodium hypochlorite

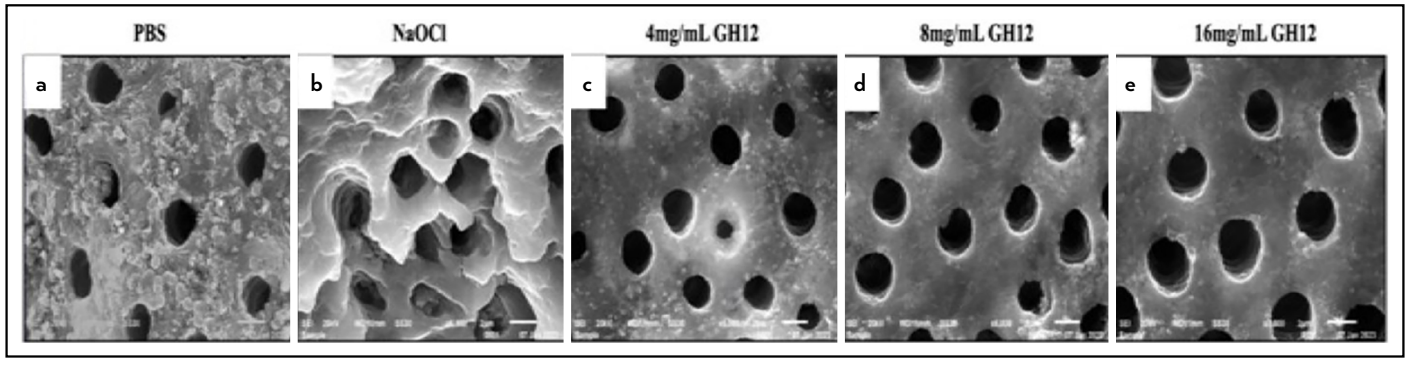

Figure 2. SEM images presenting morphological changes when treated with (a) PBS solution (b) NaOCl (c) 4mg/ml GH 12 (d) 8 mg/ml GH12 (e) 16 mg/ml GH12

SEM: Scanning electron microscope, PBS: Phosphate Buffered Solution, NaOCl: Sodium hypochlorite

The absorbance values revealed that Group II (NaOCl) showed maximum biomass reduction and Group I (PBS) showed the minimum reduction (Table 3, Fig 3).

DISCUSSION

Biofilm is a microbial community adhered to a solid surface and embedded with a polysaccharide or a glycocalyx matrix which exhibits increased antibiotic resistance and the ability to evade host immune defense mechanism by showing unique traits in terms of gene expression, protein synthesis, growth rate, and metabolic processes (11, 12). The present study investigated the antibiofilm activity of 5% Sodium Hypochlorite (NaOCl) and different concentrations of GH12 antimicrobial peptide on a multispecies biofilm model.

A significant reduction in bacterial load was observed after irrigation with NaOCl and different concentrations of GH12 when compared to PBS solution thereby rejecting the first null hypothesis. The antibacterial activity of NaOCl may be attributed to the release of chlorine ion which interferes with the cellular metabolism and causes inhibition of essential bacterial enzymes (4).

GH12 when used at a higher concentration of 16 mg/mL, showed lower but statistically comparable results to NaOCl. The design of GH12 is such that it is positively charged and has a high propensity to fold into an amphipathic alpha-helical structure in hydrophobic environments. Such a structure of

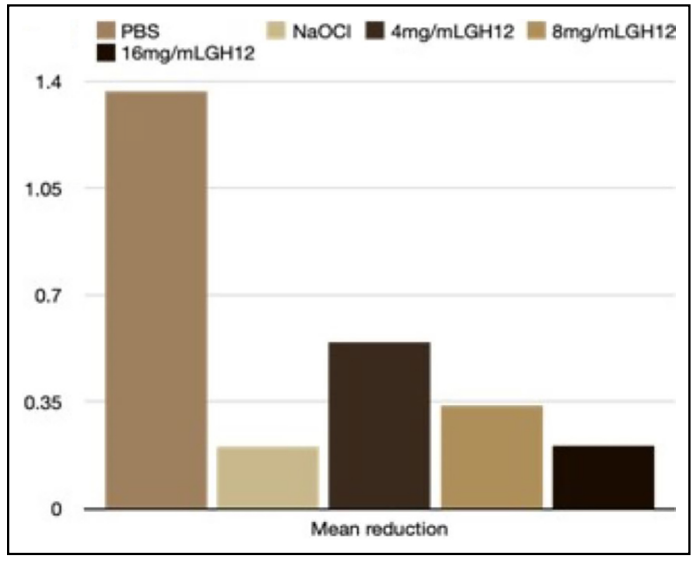

Figure 3. Intergroup comparison of absorbance

PBS: Phosphate Buffered Solution, NaOCl: Sodium hypochlorite

GH12 makes it a broad-spectrum antimicrobial agent (13). In the first step, the positively charged peptide accumulates on the negatively charged bacterial membrane by means of electrostatic interactions. After which, the hydrophobic groups of the antimicrobial peptide insert into the lipid bilayers and

cause direct disruption of the bacterial membrane via several mechanisms hypothetically proposed in the literature such as barrel-stave, toroidal pore, aggregate, and carpet models. This eventually results in the leakage of intracellular metabolites and essential ions causing subsequent cell death (14).

When comparing the mean CFU reduction, none of the bacterial groups were completely eradicated, although all the bacteria were significantly reduced. *S. mutans* is a facultative gram-positive bacterium, with a semi-permeable lipid bilayer membrane. Upon interaction with NaOCl, it causes the oxidation of outer membrane proteins and inner with subsequent cell death, thereby significantly reducing the bacterial colony count. These results are in accordance with two studies (15, 16). GH12 directly affects the activity of F1F0-ATPase located on the cell wall of *S. Mutans* and inhibits the synthesis of water-soluble EPS which in turn causes cytoplasmic acidity inhibiting the normal process of glycolysis and further triggers a cascade of molecular reactions which leads to disruption of microbial cells. These results are in accordance with the study by Jiang et al. (8), wherein GH12 was reported to be effective against the cariogenic virulence factor of *S. mutans* (8).

For *S. mutans*, NaOCl caused the oxidation of the outer membrane. The lipid bilayer membrane composed of proteins undergoes oxidation causing subsequent cell death and a significant reduction in bacterial colony count. The incomplete eradication of NaOCl could be attributed to the invasion of *E. faecalis* into the dentinal tubules because of its small size (4). Also, *E. faecalis* has adaptive capabilities and display increased resistance to antimicrobial action (17). These results are in accordance with two studies (18, 19). In the current study, *E. faecalis* was co- cultured with four other bacterial species, allowing for intricate bacterial interactions that possibly resulted in increased antimicrobial resistance in the community.

Studies have shown that *E. faecalis* has specific genes which facilitate primary attachment and biofilm formation. Gelatinase, an extracellular zinc-containing metalloproteinase that is encoded by these genes, facilitates hydrolysis of bioactive compounds such as gelatin and collagen, which makes *E. faecalis* more immune and thereby harder to eradicate by endodontic medicaments. GH12 acts against *E. faecalis*, making it susceptible to endodontic treatment by suppressing these pathogenic genes (9).

The antibacterial efficacy of GH12 against *F. nucleatum* and *P. gingivalis* showed inferior results when compared with NaOCl, though it was non-significant. The long rod shape of *F. nucleatum* makes it a bridge organism and enables its interaction with many other microbial cells. It has the ability to co-aggregate with P ginigvalis which enhances further colonization and biofilm advancement, which makes them resistant to complete elimination by antimicrobial treatment, this synergistic effect between the two bacterial species has been emphasized in previous studies (20, 21). According to Altman et al. (22), the virulence factors that allow them to survive in hostile environments could be the most probable reason for the resistance to elimination of these two bacterial species after treatment with NaOCl. These findings are in accordance with two studies that reported similar resistance of these microbial species to hypochlorite treatment (23, 24). Wang et al. (25) stated that *P. gingivalis* has a strong proteolytic action which has caused the degradation of the AMP, thereby affecting the antimicrobial efficacy of the peptide (25).

The bacterial reduction achieved with lower concentrations (4 mg/mL and 8 mg/mL) of GH12 was less than that with NaOCl in all the bacterial subgroups. The lower antibacterial action of 4 mg/mL GH12 when compared to NaOCl could be attributed to the fact that 4 mg/mL of GH12 is the minimum inhibitory concentration (MIC) of the peptide against single-species planktonic bacteria. When the individual bacterial species are combined to form a multispecies biofilm, the microorganisms coaggregates and undergo complex interactions that play a key role in their increased pathogenicity and virulence (26). Studies have reported that these coaggregated cells may have a combined metabolic advantage over single cells, and hence, the cells show resistance to elimination with reduced concentration of antimicrobial agents (24, 27).

There was a significant difference in the biofilm biomass after irrigating with different concentrations of GH12 peptide solution when compared with NaOCl and PBS solution, thus rejecting the second null hypothesis. The reason for the superior biomass reduction ability of NaOCl can be attributed to the bactericidal activity against microbial cells as well as its ability to act on the extracellular matrix of proteins and polysaccharides (28).

As the concentration of GH12 increased, a reduction in biomass was observed. This could be attributed to the reduction of bacterial load because of its antibacterial activity which has been previously explained and also to its ability to degrade the polysaccharide and biofilm matrix (25). It may be speculated that lower concentrations of GH12 peptide might have been insufficient to penetrate the EPS matrix. Also, the contact time in this current study was 10 minutes, which might not be enough to allow sufficient interaction to eliminate the biomass (29). This is in accordance with the study by Li et al (9), which showed that as the contact time was increased, greater antimicrobial efficacy was observed (9).

The dentine samples treated with 5% NaOCl showed erosive changes. Higher concentrations of NaOCl have a significantly deeper penetration (up to 300 µm) into the dentinal tubules and thereby leading to irregular erosive changes in the peritubular and intertubular dentine resulting from the loss of organic matrix. These erosive changes have also been reported by two studies (29–31).

The SEM images of the GH12 peptide, even when used at higher concentrations (16mg/mL), revealed no erosive changes with no evident bacterial aggregation on the dentine surface. These results can be corroborated with the study by Li et al. (9), which reported no apparent damage to the root canal dentine and further highlighted the advantage and potential of using GH12 as a root canal irrigating solution (9). Since there was a significant difference in the surface morphology of the dentine specimens after irrigating with different concentrations of GH- 12 peptide solution when compared with NaOCl and PBS solution, thus rejecting the third null hypothesis.

The high production cost has been recognised as one of the limitations of peptides especially in clinical settings. Another limitation of this study design could be that endodontic biofilm consists of more than 1000 bacterial species and the interaction of only four bacterial strains was considered in our study. Though the current study was *in-vitro*, it allowed standardization, which is not possible with *in-vivo* models. In a clinical scenario, other factors such as contamination of GH12 while using it as an irrigation solution or the time of contact may also occur. Any possible synergistic or antagonistic relation between the other bacterial species might change the results further. In this study, the effect of the time of exposure to different antibacterial agents was not considered, which might affect the antibacterial efficacy of the peptide. Any interaction like synergism or antagonism with other antibiotics was also not considered, which is the future

scope of this study. Further studies should also be done by using the peptide in adjunct with other irrigation devices. Only CFU and crystal violet assay were used as quantitative parameters to evaluate the antibacterial efficacy. Supplementing GH 12 as a final irrigant after NaOCl irrigation may be explored in future studies. Also, the antimicrobial efficacy of the antimicrobial peptide may be affected by the concentration and contact time which needs to be further explored. Moreover, the peptide could be mixed with an aqueous or viscous vehicle and its antimicrobial efficacy may be evaluated as an intracanal medicament. Further investigations using polymerase chain reaction and confocal laser scanning microscope may be considered as additional strategies to provide more evidence on multispecies biofilm disruption at biological levels.

CONCLUSION

Within the limitations of the study, it can be concluded that higher concentrations of GH12 showed comparable results with NaOCl against multispecies endodontic biofilm. *S. mutans* were susceptible to all three concentrations, including the lowest concentration used (4 mg/mL). *F. nucleatum* and *P. gingivalis* showed significant reduction but were relatively resistant to GH12 and NaOCl compared to *E. faecalis* and *S. mutans*. No evident erosive changes were identified in any of the groups treated with GH12 peptide. Considering the fact that NaOCl is the gold standard irrigant of choice for endodontic therapy, the potential of GH12 antimicrobial peptide as a root canal irrigant seems promising.

Disclosures

Note: This study was conducted at I.T.S Centre for Dental Studies and Research, Ghaziabad in the Department Of Conservative Dentistry and Endodontics. All the required aids and support for the study was provided by the department and the materials were funded by Dr Aishi Sinha.

Ethics Committee Approval: The study was approved by the I.T.S Centre for Dental Studies and Research, Ghaziabad in the Department of Conservative Dentistry and Endodontics Ethics Committee (no: ITSCDSR/IIEC/2020-23/CONS/01, date: 12/10/2021).

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