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In vitro antibacterial effect of fifth generation dentin bonding agent incorporated with nisin on *Streptococcus mutans*

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Abstract

Introduction: Bacterial adherence to restorative materials such as composite resin is one of the aetiology of secondary caries. This study evaluated the antibacterial efficacy of fifth generation bonding agent (BA) modified with nisin, against *Streptococcus mutans* based on its growth, adherence and membrane integrity.

Methods: Adhesive eluents of the experimental bonding agents were obtained using 250 µl Brain Heart Infusion (BHI) broth and the groups were control (BA with 0% Nisin), bonding agent with 1 wt% (NBA 1) and 5 wt% nisin (NBA 5). To this, 10 µl *S. mutans* culture was added and incubated at 37 °C. Bacterial growth was estimated by changes in optical density using spectrophotometer every 20 min for 2 h. The results were statistically analysed using one way ANOVA followed by Tukey Post Hoc test. For adherence and membrane integrity test, 10 µl of BHI supplemented with 1% sucrose and 50 µl of bacterial suspension were inoculated onto the cured specimens, and incubated for 4 h. After rinsing, 1 ml of Live/Dead BacLight bacterial viability stain was added and incubated in the dark for 15 min and observed under confocal laser scanning microscope (CLSM) for intact (green/live) and damaged (red/dead) bacterial membranes.

Results: Mean optical density was significantly higher in control group at all time intervals with maximum value at 2 h (0.83 ± 0.008), while there was a concentration dependant reduction in bacterial growth with the NBA groups (0.50 ± 0.007). Correspondingly, the NBA groups showed higher amount of dead than live bacteria, while live bacteria were predominant in the control group.

Significance: Addition of an antibacterial agent nisin in dentin bonding agent may render the resin dentin interface more resistant to bacterial penetration, and adherence of cariogenic bacteria like *S. mutans*.

Keywords: Antibacterial peptide, Dentin bonding agent, Dental marginal adaptation, Nisin, *Streptococcus mutans*

Introduction

The demand for aesthetic dentistry has resulted in majority of direct anterior and posterior restorations being done with the more favoured material of choice, composite resin. The causes of failure of composite resin restorations is multi-factorial. A systematic review on longevity of posterior restorations done by Demarco et al. shows that

these factors are related to the type and position of the tooth, demographical, socio-economical and behavioural elements [1]. The role of material characteristics is considered minimal and the primary reasons for failure of these restorations are secondary caries, fracture of the restoration or marginal defects [2].

In the recent era of minimal invasive dentistry (MID), the choice of restorative materials is based on their bioactive functions that provide therapeutic effects [3]. With the present concept of minimal invasion, it is expected that more of the saved, affected hard tissue will probably harbour more residual bacteria [4]. Thus the ability of the restorative material to eliminate these bacteria would be advantageous to prevent microleakage and secondary caries. Hence, one of the bioactive functions proposed for these materials is their anti-bacterial activity that may play a major role in restorative treatment of a carious tooth, more so in high caries risk individuals. Though the effect of restorative materials on secondary caries seem limited, the degradation of the hybrid layer may lead to gap formation in the tooth-restorative interface making the tooth more susceptible to secondary caries [5].

Poor marginal adaptation and the resulting secondary caries is the most common reason for failure of adhesive resin based restorations [6, 7]. Advanced analytical techniques to examine the adhesive resin-dentin interfacial region have revealed a number of potentially deleterious phenomena that could interfere with successful dentin bonding [8–10].

Lack of marginal adaptation that eventually leads to microleakage is caused by polymerisation shrinkage of the composite resin that results in gap formation and bacterial invasion into the interface leading to post- operative pain, marginal discolouration and secondary caries [11]. Though some advocated methods such as incremental technique, flowable resin as a liner, or use of bulk-fill composite resins do lower polymerization shrinkage and resultant stress, it is not clinically possible to eliminate shrinkage completely [12]. Bioactivity toward the pulp-dentin complex and prevention of secondary caries were rated as the keys to success and future of restorative dentistry and restorative materials based on the Delphi survey report by Seemann et al. [13].

Adhesive materials have decreased antimicrobial activity when compared to silver amalgam and zinc oxide [14]. Composite resin surface favours more plaque accumulation than any other restorative material, due to its intrinsic physico-chemical surface properties, and the passive and active bacterial adhesion mechanism [15]. It has been reported that composite resin and ceramics harbour thicker biofilms than glass ionomers [16]. Several techniques have been employed in order to increase the antimicrobial activity of these materials and to inhibit biofilm formation on composite resin restorative surfaces, mainly by incorporating slow release antibiotics and biocides [17]. However, such attempts proved short term due to its solubility over a period of time, leading to void formation in the composite resin and unfavourable mechanical behaviour of the restoration [18]. Further, such modifications provide antibacterial effect only on the surface of the composite restoration and not at the resin dentin interface, where failure occurs commonly.

Literature shows various attempts at incorporation of antibacterial components such as fluorides, antibiotics, methacrylolyoxydodecyl pyidinium bromide (MDPB) and methacryloxy ethyl cetyl di-methyl ammonium chloride (DMAE-CB) in dentin adhesives [19, 20]. However, fluorides, antibiotics and inorganic agents are dispersed in the

matrix phase; hence it is difficult to strictly control their release kinetics [21]. Also, adhesive bonding may be compromised due to the constant release of these agents. To overcome this, polymerisable cationic monomers such as quaternary ammonium monomers, MDPB and DMAE-CB that can be covalently bound within the polymer matrix, were incorporated in the dental adhesive systems [21]. Since MDPB can polymerise and be immobilised in the polymer, the bonding interface is considered to be stably maintained in contrast to soluble anti-bacterial agents incorporated in the bonding agents. They also showed that MDPB exerts contact inhibition on the growth of *S. mutans* at the bonding interface, that leads to lesser bacterial adherence. Therefore, attempts of functionalizing adhesive system with antibacterial activity was made for proper biological seal without compromising bonding.

This study is one such attempt to incorporate nisin, a polypeptide bacteriocin to fifth generation bonding agent. Nisin, a ribosomally synthesized and post-translationally modified lantibiotic, produced by *Lactococcus lactis subsp* [22, 23]. Lactis, is a food preservative, approved by Food and Drug Administration (FDA) as generally regarded as safe (GRAS) that is incorporated in the binder solutions of acrylic polymer and vinyl acetate co-polymer in food packaging [24]. It has a relatively broad spectrum of antimicrobial activity against various lactic acid bacteria and other gram positive bacteria.

Since nisin has yet to be tried in restorative dentistry, this study has been designed as a preliminary *in-vitro* evaluation of the antibacterial activity of fifth generation bonding agent incorporated with nisin, against *S. mutans*. Confocal laser scanning microscopy (CLSM) in conjunction with fluorescent indicators SYTO-9 and propidium iodide were used for the membrane integrity test.

Materials and methods

The experimental bonding agents were prepared, analysed and compared for growth of *S. mutans*; and their adherence and membrane integrity.

Preparation of the experimental bonding agents

0.01 g and 0.05 g of Nisin (Zhejiang, Silver Elephant Bio-Engineering Co. Ltd., Taizhou, China, Lot: 20130110) were added to 1 ml of fifth generation bonding agent (BA- Adper Single Bond, 3 M ESPE, USA) each to prepare Nisin modified bonding agents NBA1—1wt % and NBA5—5wt % respectively (NBA). The mix was kept in a cyclomixer (Tarsons Spinix Vortex Shaker, LA.SH.CY.1386974) for 1 min for proper mixing.

Grouping

The control group, group 1- BA (bonding agent with 0% nisin) and the two experimental groups, groups 2 and 3- NBA1 and NBA5 (1 wt% and 5 wt% NBA respectively) were evaluated for antibacterial activity against *S. mutans* using the following parameters.

Growth of *S. mutans*

1 ml adhesive of each group was spread onto the bottom of the wells in a 24-well plate and polymerized for 10 s in an anaerobic chamber (Agile Lifescience Technologies India, Ltd., India). Subsequently, each well was rinsed with 1 ml sterile distilled water. Then, 250 µl of brain–heart infusion broth (BHI, Sigma Aldrich Chemicals Private Limited,

Bangalore, India) and 20 µl of distilled water were added directly onto each cured adhesive layer to prepare the adhesive eluent. Addition of distilled water was to compensate for water evaporation during incubation.

After incubation at room temperature for 24 h, the BHI broth with the adhesive eluents were transferred to adjacent empty wells. A bacterial suspension having a cell concentration of 50 nephelometric turbidity units (NTU) was prepared in BHI broth. Two µl of fresh broth and 20 µl of cell suspension were added to each well and incubated at 37 °C in the anaerobic chamber. Similarly, multiple such wells were cultured for the groups, each to be used for evaluation at different time intervals. Bacterial growth was estimated by changes in the optical density (OD) values of each well using spectrophotometer (LIM 330, Labard Instruchem Pvt. Ltd., India) at 600 nm every 20 min for 2 h. Each group was tested as a set of five wells, with fresh BHI serving as blank control. All the procedures were performed aseptically.

The results of the OD values were statistically analysed using one-way ANOVA followed by Tukey Post Hoc test. Kruskal Wallis non parametric test was used to analyse the growth of *S. mutans* at different time intervals.

Adherence and membrane integrity of *S. mutans*

Five specimens from each group were prepared by adding one drop of test material on a glass slide and curing for 20 s. 10 µl of BHI supplemented with 1% sucrose and 50 µl of bacterial suspension were inoculated onto the specimens. After incubation for 4 h, specimens were rinsed with distilled water to dislodge loosely adherent bacteria. This time of incubation was chosen because initial biofilm formation in the oral cavity normally occurs in 2–4 h. 1 ml of Live/Dead BacLight bacterial viability stain (Molecular Probes, Eugene, OR, USA) was carefully added to the specimen without disturbing the adherent bacteria. The submerged specimens were incubated in the dark for 15 min at room temperature to allow stain development for image scanning.

After rinsing gently with distilled water, the fluorescence labelled specimens were observed under CLSM, (LSM 700, Carl Zeiss, Germany) at 40 × magnification and qualitatively analysed for live (intact membrane) and dead (damaged membrane) bacteria. The Live/Dead Baclight bacterial viability stain (L13152) consists of a two nucleic acid-binding stains mixture: Syto 9 and propidium iodide. Syto 9 stains all viable bacteria in green, while propidium iodide stains in red the bacteria whose membranes are damaged (non-viable bacteria). The bacterial layer was scanned at both the green and red channels (488 and 543 nm excitations) for bacteria with integral and damaged membranes respectively.

Results

The mean OD of the three groups and their comparison at different time intervals from 20 min to 2 h are given in Table 1 and Fig. 1 respectively. Mean OD value of control group BA (bonding agent with 0% nisin) was significantly higher at all-time intervals with a maximum of 0.83 at 120 min compared to the experimental groups, indicating that the growth of *S. mutans* was significantly higher in the control group. Among the experimental groups, NBA 5 showed lower OD values at each time interval as compared

Table 1 Mean optical density of adhesive eluents at different time intervals

Time/groups	20 min	40 min	60 min	80 min	100 min	120 min
Control	0.34 ± 0.04^{a}	0.46 ± 0.04^{a}	0.662 ± 0.008^{a}	0.79 ± 0.008^{a}	0.83 ± 0.004^{a}	0.83 ± 0.008^{a}
NBA 1	0.13 ± 0.005^{b}	0.304 ± 0.015^{b}	0.486 ± 0.005^{b}	0.61 ± 0.008^{b}	0.63 ± 0.0006^{b}	0.62 ± 0.001^{b}
NBA 5	0.029 ± 0.002^{c}	0.158 ± 0.004^{c}	0.30 ± 0.007^{c}	0.47 ± 0.001^{c}	0.50 ± 0.037^{c}	0.50 ± 0.007^{c}

In each column, different superscript letters denote statistical significance

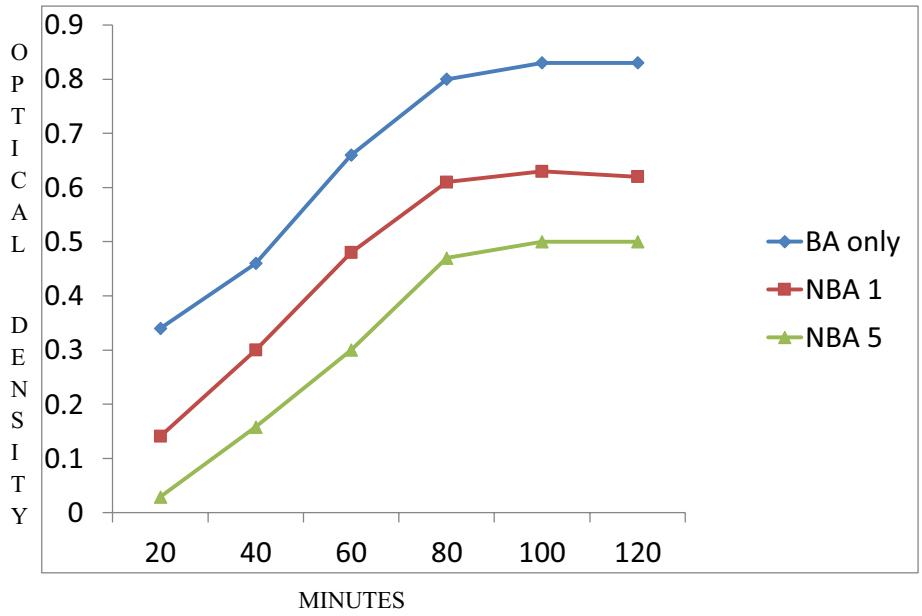

Fig. 1 Comparison of mean optical density values of three groups with respect to time

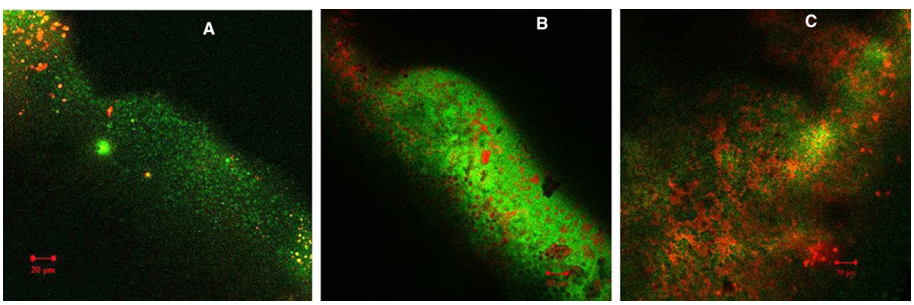

Fig. 2 Confocal images of adherence and membrane integrity of *S. Mutans*. Green emission: Live bacteria (intact membrane). Red emission: Dead bacteria (damaged membrane). A—Group 1: BA B—Group 2: NBA 1 C—Group 3: NBA 5. No contact inhibition Minimum contact inhibition Maximum contact inhibition

to the NBA 1 group with the least OD values (0.029 ± 0.002) at 20 min, indicating significantly lesser growth of *S. mutans* compared to the NBA 1 group.

These results correlated with the findings of CLSM evaluation. Confocal images (Fig. 2) revealed the presence of higher live bacteria (green) in all the samples of the

control group. Addition of nisin resulted in higher percentage of dead bacteria (red) with the NBA 5 group showing the greatest reduction in live bacteria.

Discussion

Some of the most common reasons for replacement of restorations are microleakage and secondary caries, usually caused by penetration and subsequent propagation of cariogenic bacteria along the micro-gaps present in the tooth- restorative interface [6]. The type of restorative material used seems to have an effect on the composition of the micro-flora on the surface of secondary caries. Thomas et al. indicated that bacterial composition in lesions around composite resins differ from that of primary lesions [25]. Beighton however has suggested that *S. mutans* may be a good marker for secondary caries, though not necessarily being the etiological agent [26]. Hence *S. mutans* was evaluated for its adherence and membrane integrity, in the assumption that nisin may inhibit biofilm formation within 2 h of exposure.

Components such as ethylene glycol dimethacrylate (EGDMA) and tri ethylene glycol dimethacrylate (TEGDMA) released from composite resins may enhance the growth of cariogenic bacteria such as *Streptococcus mutans* and *Lactobacilli* [27, 28]. A study done by Splieth et al. showed upto eight times more microbes beneath composite restorations compared to amalgam [29]. Schmaltz also showed that the components of dentin bonding agents stimulated the growth of cariogenic micro-organisms such as *S. sorbinus* and *Lactobacillus* [30].

Moreover, since most restorations are done on carious tooth structure prepared conservatively retaining affected dentin, some micro-organisms may still be present in the cavity walls, left behind intentionally or otherwise [30]. The microspace between the restoration and the cavity margin can provide a favourable environment for the cariogenic *S. mutans* and *lactobacilli* to demineralize the tooth structure. Since tooth- restoration interfaces do not provide a hermetic seal against diffusion of micro-organisms and / or their by-products, it could be beneficial if the restorative material and or the bonding agents could exert some anti-bacterial activity post insertion [31]. The discrepancy in the depth of demineralisation and resin infiltrated zone with separate etching and bonding protocols will further provide the suitable environment for growth of cariogenic bacteria. Hence fifth generation bonding agent was used in this study.

Nisin was incorporated at two different concentrations of 1% and 5% in the fifth generation BA. The concentration of nisin was chosen in accordance to its minimum inhibitory concentration [32]. The results of this study showed that the growth of *S. mutans* was significantly higher in the control group. The higher growth rate in control group is in accordance with the study done by several authors, Schmaltz et al., Vinay and Shivanna, Hansel et al. [27], who reported that the components of dentin bonding agents such as HEMA (hydroxyl ethylene methacrylate) or TEGDMA, do not inhibit the growth of cariogenic micro-organisms such as *S. sorbinus* and *Lactobacillus acidophilus*. [30, 33, 34] In our study, incorporation of nisin in the BA resulted in significantly lower growth rate of *S. Mutans* with the action being concentration dependent (Fig. 1). Since the experiment was performed with polymerized blocks of the BA, it can also be surmised that nisin leaches out to exert its antimicrobial action.

The experimental time interval of 20 min for a period of 2 h was chosen to evaluate the inhibitory effect of nisin on *S. mutans* biofilm formation. Both concentrations of nisin showed antibacterial effect from 20 to 80 min time period after which, the values become constant, suggesting the possibility of inhibiting adhesion of the microorganisms on the surface of the tooth-restorative material interface. Further anti-biofilm activity of nisin incorporated adhesives is warranted in future studies to understand the duration of this action.

The antibacterial action of lantibiotic nisin is based on its interaction with the target microorganism's cell membrane. It interacts with the cell wall precursor lipid II in the membrane forming pores, thereby inhibiting cell wall biosynthesis. It binds to the lipid II forming pyrophosphates via hydrogen bonds, rendering the bacteria susceptible to nisin [35]. Nisin has more effect on gram positive bacteria since these microorganisms have relatively higher concentrations of anionic lipid for interaction with nisin, in their cytoplasmic membrane, as compared to gram- negative species [36]. This was evidenced in the significant qualitative reduction of live bacteria in the present study, that increased as the concentration of Nisin increased from 1 to 5%. Whether this effect is effective over greater time period, against the other cariogenic microorganisms and help prevent secondary caries is the scope of future research.

However, incorporation of any new additive to the bonding agent must not compromise their degree of conversion, mechanical properties or bond strength to composite resin. Su et al. reported that the microtensile bond strength of nisin incorporated BA is not affected when the concentration of nisin is 1%. The bond strength decreased as the concentration of nisin increased [37]. Further, the interaction of nisin with the components of the BA needs to be investigated.

In today's general issues of conventional antibiotic resistance, bacteriocins may be safely considered. However, since this is a preliminary study involving addition of nisin to the BA, further studies incorporating nisin in self etch adhesives and with multispecies biofilm is warranted to evaluate its antibacterial efficacy in clinical conditions. Future studies will also concentrate on the effect of the addition of nisin on the degree of conversion, secondary caries formation and bond strength evaluation over a period of time, before clinical trials are attempted.

Conclusion

Within the limitations of this *in-vitro* study, it can be concluded that incorporation of Nisin in fifth generation bonding agent exerts a concentration dependant antibacterial effect, both by contact inhibition and leaching out.

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Authors' contributions
GK carried out the study, collected the data and contributed to manuscript writing. NR analysed the data and helped with the manuscript writing. SM conceptualised and supervised the study, and contributed to manuscript writing. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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