In Vitro Antibacterial Effect of Fifth Generation Dentin Bonding Agent Incorporated With Nisin on Streptococcus Mutans

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Research

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Abstract

Introduction Secondary caries is the most common cause of failure of composite restorations. This study evaluated the antibacterial efficacy of fifth generation bonding agent (BA) modified with Nisin, a polypeptide bacteriocin against Streptococcus mutans based on its growth, adherence and membrane integrity. Methods Adhesive eluents of the three experimental bonding agents, namely control BA only, bonding agent with 1 wt% Nisin (NBA 1) and 5 wt% Nisin (NBA 5) were obtained using 250 μl Brain Heart Infusion (BHI) broth. To this, 10 μl S. mutans culture was added and incubated at 37°C. Bacterial growth was estimated by changes in optical density using spectrophotometry every 20 min for 2 hours. The results were statistically analysed using one way ANOVA followed by Post Hoc test. For adherence and membrane integrity test, 10μl of BHI supplemented with 1% sucrose and 50 μl of bacterial suspension were inoculated onto the cured specimens, and incubated for 4 hours. After rinsing, 1ml of Live/Dead BacLight bacterial viability stain was added and incubated in the dark for 15 min and observed under 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 experimental groups showing concentration dependant reduction in bacterial growth. Correspondingly, the experimental 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 bonding agent may render the resin dentin interface more resistant to bacterial penetration, thus reducing the incidence of secondary caries.

Introduction

In the recent era of minimal invasive dentistry (MID), the choice of restorative materials is based on their bioactive functions that provide therapeutic effects [1]. With the present concept of minimal invasion, it is expected that more of the saved, affected hard tissue will possibly harbour more residual bacteria [2]. Thus the ability to eliminate these bacteria would be advantageous to prevent microleakage and secondary caries. Hence, one of the bioactive functions proposed for restorative materials is their antibacterial activity that may play a major role in restorative treatment of a caries tooth.

The longevity and success of a restoration depends upon a good marginal seal. Poor marginal adaptation is the most common reason for failure of adhesive resin based restorations [3, 4]. 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 [5–7]. 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 discoulouration and secondary caries. Bioactivity toward the pulp-dentin complex and prevention of secondary caries were rated as the key to success and future of restorative dentistry and restorative materials over the next 20 years based on the Delphy survey report by Rainer Seemann et al (2014) [8].
Adhesive materials have decreased antimicrobial activity when compared to amalgam and zinc oxide [9]. Composite resin surface favours more plaque accumulation than any other restorative material. Several techniques have been employed in order to increase the antimicrobial activity of these materials and to inhibit biofilm formation on composite resin, mainly by incorporating slow release antibiotics and biocides [10]. However, such attempts proved short term due to solubility over a period of time, leading to void formation in the composite resin and unfavourable mechanical behaviour of the restoration. Further, such modifications provided 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, methacryloxydodecyl pyidinium bromide (MDPB), methacryloxy ethyl cetyl di-methyl ammonium chloride (DMAEC) in dentin adhesives [11]. However, fluorides, antibiotics and inorganic agents dispersed in the matrix phase; hence it is difficult to strictly control the release kinetics [12]. Also, adhesive bonding may be compromised due to the constant release of these agents. To overcome this, polymerisable cationic monomers such as quaternary ammonium monomer (MDPB), (DMAE-CB) that can be covalently bound within the polymer matrix, were incorporated in the dental adhesive systems [12]. Since MDPB can polymerise and can be immobilised in polymer, the bonding interface is considered to be stably maintained in contrast to soluble anti-bacterial agents incorporated in bonding agents. They also showed that MDPB exerts contact inhibition on the growth of S. mutans at the bonding interface leading to prevention of secondary caries. Therefore, attempts of functionalizing adhesive system with antibacterial activity was made for proper biological seal without compromising bonding.

Our 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 [13, 14]. Lactis, is a food preservative, approved by FDA as GRAS (generally regarded as safe) that is incorporated in the binder solutions of acrylic polymer and vinyl acetate co-polymer in food packaging [15]. It has a relatively broad spectrum of anti-microbial 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**

**2.1 Preparation of the experimental bonding agents**

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

2.1.1 Grouping

The three experimental groups, group 1- BA (bonding agent only) groups 2 and 3- NBA 1 and NBA 5 (1 wt% and 5 wt% NBA) were evaluated for antibacterial activity against S. mutans using the following parameters.

2.2 Growth of S. Mutans

1ml adhesive of each group was spread onto the bottom of the wells in a 24-well plate and irradiated for 10 sec in an anaerobic chamber. Subsequently, each well was rinsed with 1 ml sterile distilled water. Then, 250 µl of brain-heart infusion broth (BHI, Sigma Aldrich India, Bangalore) 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 being incubated at room temperature for 24 hours, the BHI broth with the adhesive eluents were transferred to adjacent empty wells. 10 µl S. mutans culture (UA 159) was inoculated into these wells and incubated at 37°C in an anaerobic chamber. Bacterial growth was estimated by changes in the optical density values of each well using spectrophotometer at 600 nm every 20 min for 2 hours. 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 optical density were statistically analysed using one way ANOVA followed by Post Hoc test. Kruskal Wallis non parametric test was used to analyse the growth of S. mutans at different time intervals.

2.3 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 seconds. 10µl of BHI supplemented with 1% sucrose and 50 µl of bacterial suspension were inoculated onto the specimens. After incubation for 4 hours, specimens were rinsed with distilled water to dislodge loosely adherent bacteria. This time of incubation was chosen because initial biofilm formation in oral cavity normally occurs in 2–4 hours. 1ml of Live/Dead BacLight bacterial viability stain (Invitrogen, 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 confocal laser scanning microscope (CLSM) at 40 x magnification and qualitatively analysed for live
(intact membrane) and dead (damaged membrane) bacteria. The bacterial layer was scanned at both the green and red channels (488 and 543nm excitations) for bacteria with integral and damaged membranes respectively.

**Results**

The mean optical density of the three groups and their comparison at different time intervals from 20 minutes to 2 hours are given in Table 1 and Fig. 1 respectively. Mean optical density value in group I (BA only) was significantly higher at all-time intervals with a maximum of 0.83 at 120 min compared to experimental groups, indicating that the growth of *S. mutans* was significantly higher in the control group. Among the experimental groups, NBA 5 (group III) showed the least OD values, indicating significantly lesser growth of *S. mutans* compared to NBA 1 (group II). Confocal images (Fig. 2) reveal 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 5% Nisin group showing the maximum.

| Time/Groups | 20 min   | 40 min   | 60 min   | 80 min   | 100 min  | 120 min  |
|-------------|----------|----------|----------|----------|----------|----------|
| Control     | 0.34 ± 0.04<sup>a</sup> | 0.46 ± 0.04<sup>a</sup> | 0.662 ± 0.008<sup>a</sup> | 0.79 ± 0.008<sup>a</sup> | 0.83 ± 0.004<sup>a</sup> | 0.83 ± 0.008<sup>a</sup> |
| NBA 1       | 0.13 ± 0.005<sup>b</sup> | 0.304 ± 0.015<sup>b</sup> | 0.486 ± 0.005<sup>b</sup> | 0.61 ± 0.008<sup>b</sup> | 0.63 ± 0.0005<sup>b</sup> | 0.62 ± 0.001<sup>b</sup> |
| NBA 2       | 0.029 ± 0.002<sup>c</sup> | 0.158 ± 0.004<sup>c</sup> | 0.30 ± 0.007<sup>c</sup> | 0.47 ± 0.001<sup>c</sup> | 0.50 ± 0.037<sup>c</sup> | 0.50 ± 0.007<sup>c</sup> |

Different superscript letters denote statistical significance.

**Discussion**

One of the most common reasons for replacement of restorations is microleakage and secondary caries, usually caused by penetration and subsequent propagation of cariogenic bacteria along the micro-gaps present in the tooth- restorative interface [3]. 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 (2008) indicated that bacterial composition in lesions around composite resins differ from that of primary lesions [16]. Beighton (2005) however has suggested that *S. mutans* may be a good marker for secondary caries, though not necessarily being the etiological agent [17].
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 [18, 19]. A study done by Splieth et al (2003) showed upto eight times more microbes beneath composite restorations compared to amalgam [20]. Schmaltz (2004) also showed that the components of dentin bonding agents stimulated the growth of cariogenic microorganisms such as S. sorbinus and Lactobacillus [21].

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 [21]. The micropore 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 [22].

In our study, Nisin was incorporated at two different concentrations 1% and 5% in the fifth generation BA. The concentration of Nisin was chosen in accordance to its minimum inhibitory concentration [23]. The results of our study showed that the growth of S. mutans was significantly higher in group I. The higher growth rate in control group is in accordance with the study done by several authors, Schmaltz et al (2004), Vinay and Vasundhara Shivanna (2010), Hansel et al (1998), 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. [21, 24, 25] 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 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. 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.

Qualitative analysis of adherence of S. mutans revealed the presence of higher live bacteria (green) in all samples of the control group. Both the experimental groups showed lesser live bacteria when compared to the control group (Fig. 2). And as the concentration of Nisin increased from 1–5%, there was a significant qualitative reduction in presence of live bacteria.

Thus, the findings of our study indicate that Nisin incorporated fifth generation BA can exert both contact inhibition as well as leaching out effect after polymerisation. 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, effect of this modification on the degree of conversion, bonding to dentin, bond strength of composite resins, and inhibition of secondary caries has to be further studied.
before further clinical trials. Also, further studies with multispecies biofilm is warranted to evaluate its antibacterial efficacy in clinical conditions.

**Conclusion**

Within the limitations of this *in-vitro* study, it can be concluded that incorporation of Nisin in fifth generation bonding agent exerted a concentration dependant antibacterial effect, both by contact inhibition and leaching out. Incorporation of 5% Nisin (NBA 5) in the adhesive system showed higher antibacterial activity than 1% Nisin (NBA)

**Declarations**

**Availability of Data & Materials**

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

**Competing Interests**

The authors declare that they have no competing interests

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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.

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**References**

1. Imazato S. Bio-active restorative materials with antibacterial effects: new dimension of innovation in restorative dentistry. Dent Mater J. 2009;28(1):11–9.

2. Farrugia C, Camilleri J. Anti-microbial properties of conventional restorative filling materials and advances in antimicrobial properties of composite resins and glass ionomer cements- A literature review. Dent Mater April. 2015;31(4):89–99.
3. Mjör IA, Shen C, Eliasson ST, Richter S. Placement and replacement of restorations in general dental practice in Iceland. Oper Dent. 2002;27(2):117–23.

4. Qvist V, Qvist J, Mjör IA. Placement and longevity of tooth-colored restorations in Denmark. Acta Odontol Scand. 1990;48(5):305–11.

5. Carvalho RM, Manso AP, Geraldeli S, Tay FR, Pashley DH. (2012) Durability of bonds and clinical success of adhesive restorations. Dent Mater. Jan; 28(1).

6. Brackett MG, Dib A, Franco G, Estrada BE, Brackett WW. Two-year clinical performance of Clearfil SE and Clearfil S3 in restoration of unabraded non-carious class V lesions. Oper Dent. 2010;35:273–8.

7. Liu Y, Tjaderhane L, Breschi L, Mazzoni A, Li N, Mao J, Pashley DH, Tay FR. Limitations in bonding to dentin and experimental strategies to prevent bond degradation. J Dent Res. 2011;90:953–68.

8. Seemann R, Flury S, Pfafferko F, Lussi A, Noack MJ. Restorative dentistry and restorative materials over the next 20 years: A Delphi survey. Dent Mater April. 2014;30(4):442–8.

9. Sevinc BA, Hanley L. Antibacterial activity of dental composites containing zinc oxide nanoparticles. J Biomed Mater Res B Appl Biomater. 2010;94(1):22–31.

10. Imazato S, Ebi N, Takahashi Y, Kaneko T, Ebisu S, Russell RRB. Antibacterial activity of bacteriocide-immobilised filler for resin based restoratives. Biomater. 2003;24:3605–9.

11. Imazato S, Kinomoto Y, Tarumi H, Torii M, Russell RRB, McCabe JF. Incorporation of Antibacterial Monomer MDPB into Dentin Primer. J Dent Res. 1997;76(3):768–72.

12. Li F, Chen J, Chai Z, Zhang L, Xiao Y, Fang M, Ma S. Effects of a dental adhesive incorporating antibacterial monomer on the growth, adherence and membrane integrity of Streptococcus mutans. J Endod. 2009;37:289–96.

13. Daniela D, Amato, Sinigaglia M. (2010) Antimicrobial agents of microbial origin: Nisin. Application of alternative food preservative technologies. 83–91.

14. Delves-Broughton J. Nisin and its uses. Food technol. 1990;44:100–17.

15. Kim YM, An DS, Park HJ, Park JM, Lee DS. Properties of Nisin incorporated polymer coatings as antimicrobial packaging materials. Packaging technology science. 2002;15:247–54.

16. Thomas RZ, Van der Mei HC, Van der Veen MH, de Soet JJ, Huysman MC. Bacterial composition and red fluorescence of plaque in relation to primary and secondary caries next to composite: an in situ study. Oral Microbiol Immunol Feb. 2008;23(1):7–13.

17. Beighton D. The complex oral microflora of high-risk individuals and groups and its role in the caries process. Community Dent Oral Epidemiol Aug. 2005;33(4):248–55.

18. Hansel C, Leyhausen G, Mai UE, Geurtsen W. Effects of Various Resin Composite (Co)monomers and Extracts on Two Caries-Associated Micro-Organisms in Vitro. J Dent Res. 1998;77(1):60–76.

19. Gupta SK, Saxena P, Pant VA, Pant AB. (2012) Release and toxicity of dental resin composite Toxicol Int. 19(3): 225–234.

20. Splieth C, Bernhardt O, Heinrich A, Bernhardt H, Meyer G. Anaerobic microflora under Class I and Class II composite and amalgam restorations. Quintess Inter. 2003;34(7):497–503.
21. Schmalz G, Ergucu Z, Hiller KA. Effect of Dentin on the Antibacterial Activity of Dentin Bonding Agents. J Endod. 2004;30(5):352–8.

22. Duque C, Negrini TDC, Spolidorio DMP, Hebling J. Effect of light activation on the antibacterial activity of dentin bonding agents. Braz J Oral Sci. 2009;8(4):175–80.

23. Tong Z, Huang L, Ling J, Mao X, Ning Y, Deng D. Effects of intracanal irrigant MTAD combined with Nisin at sub-minimum inhibitory concentration levels on Enterococcus faecalis growth and the expression of pathogenic genes. plus one. 2014;9(3):1–6.

24. Shivagange V, Shivanna V. Comparative evaluation of micro-leakage of fifth, sixth, and seventh generation dentin bonding agents: An in vitro study. J Cons Dent. 2010;13(3):136–40.

25. Imazato S, Kinomoto Y, Tarumi H, Ebisu S, Tay FR. Antibacterial activity and bonding characteristics of an adhesive resin containing antibacterial monomer MDPB. Dent Mater Jun. 2003;19(4):313–9.

Figures

![Graph showing comparison of mean optical density values of three groups with respect to time.]

**Figure 1**

Comparison Of Mean Optical Density Values Of Three Groups With Respect To Time
Figure 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 only B - Group 2: NBA 1 C - Group 3: NBA 5 NNo contact inhibition Minimum contact inhibition Maximum contact inhibition