Peer-reviewed paper; submitted October 2017; accepted July 2019

Bacterial adhesion to human enamel after enameloplasty

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Abstract

Background and objective: Enameloplasty is a common clinical procedure that involves removing small amounts of tooth enamel to change a tooth's shape and surface, resulting in an increased surface roughness. The relationship between enamel surface roughness and caries, however, is still a matter of debate. The aim of the study was to assess the adhesion of bacteria to enamel after enameloplasty and the effect of polishing enamel after enameloplasty on bacterial adhesion.

Materials and Methods: Thirty-two human premolar teeth were either subjected to enameloplasty with diamond burs (n=8), diamond discs (n=8), Soflex polishing discs (n=8), or served as controls with no enameloplasty (n=8). The roughness of enamel surfaces was assessed using atomic force microscopy (AFM). *Streptococcus sanguinis* cells were incubated with enamel samples and colony-forming units (CFUs) adhering to the enamel were counted.

Results: The CFUs were highest on the roughest enamel surfaces that were created with the medium bur (CFUs = 12.3 ± 0.5 x 10^5), followed by the mesh disc (CFUs = 4.0 ± 0.5 x 10^5). The control surface had the next highest cell count (CFUs = 1.2 ± 0.1 x 10^5). The smoothest surfaces, created by Soflex polishing discs, had the lowest number of adhering bacteria (CFUs = 0.3 ± 0.05 x 10^5) ($p < 0.001$ for all). A significant positive relationship was found between the enamel surface roughness and number of bacteria adhered ($p < 0.001$).

Conclusions: There was a positive relationship between the adherence of *S. sanguinis* cells to tooth enamel and the enamel surface roughness. Polishing enamel after enameloplasty produced surfaces to which fewer bacteria adhered compared to untreated enamel.

Introduction

Enameloplasty, also called tooth recontouring, reshaping, odontoplasty, stripping, slenderizing or interproximal reduction, is a common clinical procedure that involves removing small amounts of tooth enamel to change a tooth's shape, surface, width or length (Barcoma et al. 2015). A recent study has reported that 66% of orthodontists perform enameloplasty on a routine basis to gain intra-arch space, and 46% of general dentists undertake enameloplasty in their practice (Barcoma et al. 2015). Enameloplasty procedures, however, leave grooves and furrows on enamel that result in significantly increased surface roughness (Piacentini and Sfondrini 1996; Danesh et al. 2007), even after polishing (Gupta et al. 2012).

Bacterial adhesion to enamel surfaces is an early event in oral biofilm formation and caries development (Peterson et al. 2011; Takahashi and Nyvad 2011) that follows the formation of the salivary pellicle. The salivary pellicle is a protein-rich organic film covering the tooth surfaces that can be detected on enamel surfaces within 1 min of exposure to the environment within the oral cavity (Hannig 1999). This film permits the adhesion of pioneer bacteria and subsequent formation of a dental biofilm. The majority of primary colonisers are oral streptococci, such as *Streptococcus sanguinis*, which account for 60-80% of the bacteria in the dental biofilm formed within the first 4-8 hours (Nyvad and Kilian 1987; 1990: Diaz et al. 2006; Dige et al. 2009). If patients cannot remove the bacteria adhered to the enamel, the acid produced by the biofilm can, with time, cause tissue damage including gingival inflammation and dental caries (Nyvad and Kilian 1990; Peterson et al. 2011).

A positive relationship between bacterial adhesion and surface roughness has been shown for a variety of dental materials including composite resin (Carlen et al. 2001), porcelain (Kawai et al. 2000), Co-Cr alloy (Gao et al. 1998), and dental implants (Chin et al. 2007). Whether enameloplasty increases bacterial adhesion to enamel, or not, is still a matter of debate (Rossouw and Tortorella 2003; Zachrisson et al. 2011; Gupta et al. 2012). Previous studies have failed to quantify the bacterial adhesion or measure its strength (Radlanski et al. 1988; Jarjoura et al. 2006; Zachrisson et al. 2011).

The aim of this research was to investigate the influence of enamel roughness after enameloplasty on bacterial adhesion, and to evaluate the effect of enamel polishing on preventing bacterial adhesion.

Materials and Methods

Enamel sample preparation

Thirty-two human premolar teeth, removed for orthodontic purposes, were collected at the University of Otago School of Dentistry using the following exclusion criteria: presence of any staining, demineralization, decay, fluorosis, enamel cracks, defects or restorations. Ethical approval for the study was obtained from the University of Otago Ethics Committee (H13/105).

The extracted teeth were immediately cleaned and disinfected using 70% ethanol and stored at 4°C in sterile distilled water for less than 1 week before being used in the experiments (Hosoya et al. 2003). Enamel blocks measuring 3.5 mm (height) x 3.5 mm (width) x 2 mm (depth) were cut from the interproximal surfaces of the teeth. The 2 mm depth was measured from the highest point of the outer enamel towards the dentine. The blocks

were cut using a straight, cylindrical, coarse diamond bur (Meisinger FG 842 012, Hager & Meisinger GmbH, Neuss, Germany) and special care was taken to not damage the outer enamel. The blocks were allocated to one of three dental stripping instrument groups or the control group (n = 8 per group) (Meredith et al. 2017).

Enamel surface preparation

Three commonly used dental stripping instruments were used for the enameloplasty in this study (Lapenaite and Lopatiene 2014) (Fig. 1 and Table 1), including medium diamond bur, mesh disc and Soflex polishing discs. There was also a control group that was not subjected to any enameloplasty procedures.

All the enamel stripping was carried out according to the manufacturers' instructions and performed by one investigator (0.2 mm thickness of enamel was removed). For all groups the sample was held along its axial walls in mosquito forceps whilst the stripping instrument was used on the outer enamel surface. For the bur, the hand-piece was run at 400,000 rpm with water-cooling; and for discs, the handpieces were run at 5,000 rpm. Each stripping instrument (*i.e.* bur or disc) was used for one enamel sample only and then replaced. To ensure equal reduction of all teeth, an enamel reduction of 0.2 mm, measured by vernier calipers, was performed on each enamel surface. For the polishing group, the coarse Soflex disc was used until enamel reduction of 0.2 mm had been reached and then the medium, fine and extra fine Soflex discs were used sequentially for 20 s each to polish the reduced surface (*i.e.* 1 min polishing in total).

Figure 1. Dental instruments used for enameloplasty in the study.

After completion of enameloplasty, the samples were cleaned individually in 100 ml of distilled water with sonication for 2 min (Elmasonic S-30, Elma Schmidbauer GmbH, Singen, Germany). The enamel samples in the control group were only cleaned with sonication for 2 min without any enameloplasty procedures.

Enamel surface roughness

The surface roughness of the prepared enamel samples was assessed using atomic force microscopy (Nanosurf NaioAFM, Liestal, Switzerland), in contact mode with ACLA Probe (Applied NanoStructures Inc., Mountain View, California, USA) at 190 kHz. All enamel blocks from each group were assessed and imaged at three randomly selected areas (50 µm x 50 µm), and surface plots were made to obtain average surface roughness (Ra) values.

Four significantly different roughness scales of enamel surfaces were selected for the study, including Ra = 34 ± 14 nm (Soflex polishing), Ra = 149 ± 39 nm (control), Ra = 307 ± 107 nm (Mesh disc), and Ra 702 ±134 nm (Medium bur) ($p < 0.001$ for multiple comparison among all groups).

Bacterial growth

Streptococcus sanguinis ATCC10556 was plated on Columbia Sheep Blood Agar (Fort Richard Laboratories, Auckland, New Zealand) and incubated in an anaerobic chamber at 37°C for 24 h. For adhesion experiments, bacteria from the blood agar plates were cultured in 10 ml pre-warmed, sterile Tryptic Soy Broth (30 g of Tryptic Soy Broth (Bacto™) Soybean-Casein Digest Medium powder per l distilled water; TSB) in a glass tube statically, at 37°C, for 14 h. The optical density (OD) of a 1 in 10 dilution of this culture in sterile broth was measured in a spectrophotometer (Ultrospec 6300 Pro Spectrophotometer: Biochrom, Cambridge, UK) at a wavelength of 600 nm (OD_{600}). A portion (0.5 ml) of the remaining bacterial broth was used to inoculate 10 ml sterile, pre-warmed TSB (*i.e.* a 1 in 20 dilution). This culture was incubated at 37°C and the OD_{600} measured every hour to find the mid-log phase (in order to best represent the growth stage of bacteria within the mouth).

The time taken for *S. sanguinis* ATCC10556 to reach mid-log phase (OD_{600} ~0.8) was found to be 5 h. At this time point, the OD_{600} of a 1 in 10 dilution of the culture was measured and bacteria in the remaining culture were harvested by centrifugation at 8,228 x g for 10 min. The bacteria were washed in 1 ml phosphate buffered saline (PBS) and centrifuged again at 8,228 x g for 3 min. The supernatant was poured off and bacteria were resuspended in 1 ml PBS. The bacterial suspension was subjected to sonication at 25% power (Branson Digital Sonifier, Emerson, Danbury, USA) with a probe for 10 s to separate the cells prior to being used in the adhesion assays. The OD_{600} of a 1 in 20 dilution of the bacterial suspension was measured and the cells were stored on ice until needed.

Dental instruments	Model	Manufacturer	Grit	Hand-piece
Medium bur	Safe-tipped medium diamond bur	Dentsply, York, USA	Medium (100-120 mm)	High speed (400,000 rpm) with water cooling
Mesh disc	Flexview Mesh disc	Dentsply, York, USA	Medium (100-120 mm)	Slow speed (5000 rpm)
Polishing	Soflex system kit	3M ESPE, Irvine, USA	Variable	Slow speed (5000 rpm)
None (control)	N/A	N/A	N/A	N/A

Table 1. Dental instruments used for enameloplasty in the study.

Measurement of bacterial adhesion

Bacterial adhesion experiments used a modification of a previously published method (Hosoya et al. 2003). Whole saliva was collected on ice from three subjects and an equal volume from each was pooled. Dithiothreitol (125 mM) was added to the saliva to give a final concentration of 2.5 mM. Saliva was clarified by centrifugation at 40,000 x g for 30 min (Sweet et al. 1990). Following its preparation, saliva was transferred to microfuge tubes in 1 ml portions and stored at -20°C until needed. Saliva samples were thawed at room temperature for 30 min before being used. Unused saliva samples were not re-frozen. Enamel blocks were incubated in the wells of a sterile 24-well microtitre plate in 1 ml clarified human saliva statically at room temperature (one block per well) for 30 min to allow a salivary pellicle to form. Each block was washed by dipping it in 1 ml sterile PBS 3 times, and placed into an unused well of the sterile 24-well microtitre plate. The sonicated bacteria in PBS (1 ml) at an OD_{600} of 1.0 were added to each well containing an enamel block. The microtitre plate was then gently shaken at 180 rpm for 30 min to mimic the intra-oral flow of saliva across the enamel surfaces. After this incubation, each enamel block was held along its axial walls and washed once by dipping it in 1 ml sterile PBS to remove any non-adherent bacteria. Extreme care was taken to ensure that the roughened upper surface of the enamel block with attached bacteria was not disturbed. A sterile cotton swab, pre-moistened with PBS, was used to remove bacteria from the roughened surface. The swab tip was broken off and placed into a sterile microfuge tube containing 1 ml PBS. The solution was vortexed for 1 min to disperse the bacteria and the swab was removed. The bacterial suspension was diluted 1 in 100 with PBS and then pipetted onto Columbia sheep blood agar plates (Fort Richard Laboratories) which had been warmed to room temperature for 30 min prior to use. Three separate portions (50 µl) of the diluted suspension were placed on each agar plate at least 1 cm apart thereby giving three readings per enamel block. The agar plates were incubated anaerobically at 37°C for 24 h. Colony forming units (CFUs) from each droplet (three per sample) were counted and averaged to calculate the number of bacteria adhering to each enamel block. The experiment was repeated three times.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 (SPSS Inc, Chicago, IL, USA). The data were presented as mean ± SD and compared using a one-way analysis of variance (ANOVA). Type I error was set at 0.05. Bonferroni correction was used for multiple testing.

Results

Choice of bacterial strain

The objective of the study was to measure the adhesion of a bacterial species that is an initial coloniser of enamel. In order to select a suitable bacterial strain, the growth of the following bacterial strains was investigated: *Streptococcus gordonii* ATCC 10558 and DL1, *Streptococcus oralis* J22, *Streptococcus mitis* ATCC 9811 and ATCC 6249, and *S. sanguinis* ATCC 10556. Several of these strains have been used in previous bacterial adhesion studies (Carlen et al. 2001; Yang et al. 2001; Mei et al. 2011; Eick et al. 2013; Schweikl et al. 2013). *S. sanguinis* ATCC 10556 was selected for use in adhesion assays because it could be pre-cultured overnight and then sub-cultured and used in experiments the next day. This removed the need to store bacterial cells on ice for extended periods before use in adhesion assays.

Optical Density (OD_{600}) readings were taken following overnight growth of bacteria as well as at hourly intervals for the sub-culture so that the mid-log phase could be identified. The mid-log phase for *S. sanguinis* ATCC 10556 was reached approximately 5 h following sub-culture of the overnight culture and the mean OD_{600} of cultures used in adhesion assays was 0.79 ± 0.10.

Bacterial adhesion

More *S. sanguinis* ATCC 10556 bacteria adhered to the rougher enamel surfaces ($p < 0.001$, Figs. 2 and 3). The roughest surface, prepared with the medium diamond bur (Ra = 702.4 ± 134.4 nm) gave the highest number of colony forming units (CFUs) per enamel block ($12.3 \times 10^5 \pm 0.5 \times 10^5$), followed by the next roughest surface, prepared with the mesh disc (Ra = 307.1 ± 106.9 nm) which had a CFU count of $4.0 \times 10^5 \pm 0.5 \times 10^5$ ($p < 0.001$). The smoothest surface, prepared with the Soflex polishing discs (Ra = 36.7 ± 13.7 nm) had the lowest number of CFUs per enamel block ($0.3 \times 10^5 \pm 0.05 \times 10^5$)($p < 0.001$). The polished enamel was smoother and had a lower CFU count than the control surface (Ra = 148.6 ± 38.5 nm), which had CFU of $1.2 \times 10^5 \pm 0.1 \times 10^5$ per enamel block ($p < 0.001$).

Linear and logistic regression analysis showed that there was a significant positive relationship between the enamel surface roughness and the number of bacteria adhering (the coefficients of determination were >0.98 for both models, Figure 3).

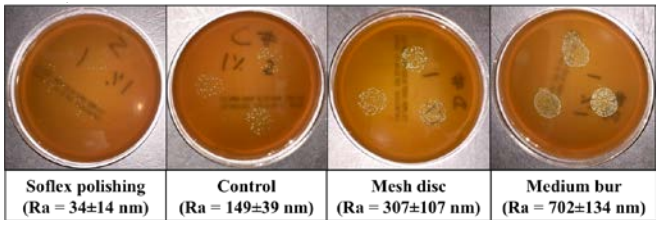

Figure 2. Representative examples of CFUs from enamel blocks with different surface roughness (Ra). Soflex polishing: Ra 34 ± 14 nm, $3 \pm 0.5 \times 10^4$ CFU; Control: Ra 149 ± 39 nm, $1.2 \pm 0.1 \times 10^5$ CFU; Mesh disc: Ra 307 ± 107 nm, $4.0 \pm 0.5 \times 10^5$ CFU; , Medium bur; Ra 702 ± 134 nm, $12.3 \pm 0.5 \times 10^5$ CFU.

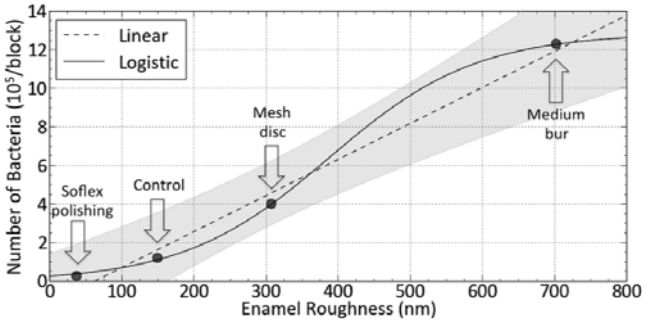

Figure 3. Linear and logistic regression plots for surface roughness and bacterial adhesion. Dark area represents the 95% confidence region for linear regression. The coefficients of determination were >0.98 for both models.

Discussion

The changes in enamel surface roughness following enameloplasty found in this study are consistent with previous research (Joseph et al. 1992; Piacentini and Sfondrini 1996; Radlanski et al. 1988; Lucchese et al. 2001). In this study, it was also shown that the altered enamel surface (whether made rougher or smoother) had a significant effect on the number of bacteria adhering to the enamel. The rougher surfaces had increased numbers of bacteria, which is in agreement with other studies showing increased plaque accumulation at sites with rougher surfaces (Radlanski et al. 1988; Gao et al. 1998; Kawai et al. 2000; Carlen et al. 2001; Chin et al. 2007).

Despite experimental evaluation of several bacterial species during pilot studies (*i.e. S. gordonii*, *S. oralis,* and *S. mitis*), in this study, only *S. sanguinis* ATCC10556 was used for the measurement of bacterial adhesion to roughened enamel because of its optimal growth kinetics for the planned experiments. S. *sanguinis* belongs to the indigenous microbiota and is generally associated with oral health rather than disease (Caufield et al. 2000; Becker et al. 2002). However, as a pioneer species in initial enamel colonisation, it allows subsequent adhesion of disease causing bacteria, which is why its adhesion is of interest. It is presumed that *S. sanguinis* ATCC10556 is representative of other strains of the same species, and that increased adherence of these bacteria will lead to an increase in overall plaque levels and potentially increase the risk of dental decay.

The positive relationship between the enamel surface roughness and the number of bacteria that adhered may indicate an increased potential for future dental caries and calculus on rougher enamel surfaces. However, previous long-term studies investigating roughened enamel have shown that increased roughness does not necessarily equate to an increased amount of decay clinically (Radlanski et al. 1988; Crain and Sheridan 1990). Despite the fact that a potential caries risk clearly exists, an increase in the actual incidence of caries on interproximal surfaces that have undergone enameloplasty versus those that have not, has not been demonstrated (Crain and Sheridan 1990). This may be due to the multifactorial nature of dental decay, where increased bacterial adhesion in a non-susceptible host may not necessarily have a clinically significant effect.

The process of enameloplasty (interproximal reduction), used commonly in orthodontic patients, is one that may result in a roughened enamel surface and may therefore increase bacterial adhesion to this surface. It is still unclear whether this abraded enamel causes bacterial adhesion in the mouth (Rossouw and Tortorella 2003; Zachrisson et al. 2011; Gupta et al. 2012). Previous studies have not quantified the bacterial adhesion or measured the strength of adhesion to roughened enamel (Radlanski et al. 1988; Jarjoura et al. 2006; Zachrisson et al. 2011). However, initial colonizing bacteria have previously been shown to firstly adhere to the cracks and pits on the enamel surface (Nyvad and Fejerskov 1987).

Dental caries is a multifactorial disease in which the host, diet and bacterial flora play a role. In orthodontic patients who wear fixed appliances, there is an increase in the number of plaque retentive sites within the mouth and an increased risk for enamel surface demineralization (Gorelick et al. 1982; Mizrahi 1982), which is of particular concern when present in combination with a roughened enamel surface after a procedure such as enameloplasty. The multifactorial aetiology of dental caries, however, makes it particularly hard to conclude which particular aspect of the patients' history (including previous enameloplasty), has contributed to increased, or lack of, disease.

Enamel surfaces have been shown to be significantly smoother after polishing with Soflex discs (Meredith et al. 2017), and in the present study we found fewer bacteria adhered to the polished enamel surfaces. Routine standardised polishing of the enamel after enameloplasty is therefore recommended to ensure the tooth surfaces are as smooth as possible in order to minimize bacterial adhesion. One of the limitations of the study is that only one bacterial strain (i.e. *S. sanguinis* ATCC10556) was used. Future studies will investigate the adhesion of other bacterial species such as *S. gordonii*, *S. mitis* and *S. mutans.*

Conclusion

Larger numbers of *S. sanguinis* adhered to the rougher enamel, showing that increased enamel surface roughness promoted its adhesion. Polishing enamel after enameloplasty produced surfaces to which fewer bacteria adhered compared to untreated enamel.

Acknowledgments

This study was funded by a University of Otago Research Grant (UORG) and the Foundation for Research and Education, New Zealand Association of Orthodontics (FORENZAO)

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